UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Control de la recombinación meiótica durante la diploidización de autopoliploides en Arabidopsis

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MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Pablo Parra Núñez

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Publications

During the development of this thesis, I have contributed in the following publications:

1 **Parra-Nunez, P.**, Pradillo, M., & Santos, J. L. (2019). Competition for chiasma formation between identical and homologous (but not identical) chromosomes in synthetic autotetraploids of *Arabidopsis thaliana*. *Frontiers in plant science*, *9*, 1924. https://doi.org/10.3389/fpls.2018.01924

2 **Parra-Nunez, P.**, Pradillo, M., & Santos, J. L. (2020). How to perform an accurate analysis of metaphase I chromosome configurations in autopolyploids of *Arabidopsis thaliana*. In: *Plant Meiosis* (pp. 25–36). *Humana*. https://doi.org/10.1007/978-1-4939-9818-0_3

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Articles 1 and 2 are an integral part of this thesis, whereas article 3 is the result of a collaboration with other members of the COMREC network.

Main contributions

1 In this article, we conducted a cytological analysis of the multivalent and chiasma frequencies in synthetic autotetraploids of *Arabidopsis thaliana* including the accessions Col, L*er*, and the Col/L*er* hybrid. I was involved in all the experimental procedures described in the article and data analyses.

2 In this chapter of methods in molecular biology, we described an improved cytological protocol, including fluorescence in situ hybridization, to obtain high quality spreads of metaphase I chromosomes from *Arabidopsis thaliana* autotetraploids. This method allows an accurate analysis of the different meiotic configurations and enables the assessment of the number of chiasmata formed by each tetrasome. I contributed to the optimization of the protocol and also captured the images shown in the different figures.

3 In this manuscript, we revealed that the cyclin-dependent kinase A;1 (CDKA;1), the homolog of human Cdk1 and Cdk2, is a major regulator of meiotic recombination in *Arabidopsis*. *Arabidopsis* plants with reduced CDKA;1 activity present a decrease of class I crossovers, especially lowering recombination rates in centromere-proximal regions, without affecting crossover assurance. Conversely, an increase of CDKA;1 activity results in elevated recombination frequencies. Thus, modulation of CDKA;1 kinase activity affects the number and placement of chiasmata along the chromosome axis in a dose-dependent manner. My contribution to this study was related to the analysis of the class I crossover frequency in the mutant *cdka;1* by the immunolocalization of the protein MLH1.

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Al igual que los directores de tesis son indispensables, los compañeros de laboratorio los son casi en igual medida. He aprendido de todo el mundo que ha pasado por laboratorio durante estos años, ya fuesen estudiantes de grado, máster, técnicos o compañeros que estaban haciendo el doctorado como yo. En cuanto a la gente que pasó por allí durante estos años me gustaría agradecer a Cristina, Gonzalo, Miguel H., Miguel P., Fernando, Daniel, Javi, Ceci, Diego, Eugenio, Iván y muchos otros que seguro me estaré olvidando. Todos ellos, pero en especial los “Migueles”, han ayudado en esta tesis, no solamente desde el punto de visto científico, sino también por los muy buenos ratos que he

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Abbreviations

Arabidopsis Biological Resource Center	ABRC
Axial element	AE
Border primer	BP
Central element	CE
Chromosome	Chr
Crossover	CO
Displacement-loop	D-loop
Double Holliday junction	dHj
Double strand break	DSB
Holliday junction	Hj
Homozygous	HM
Homologous recombination	HR
Heterozygous	HZ
Initial Training Network	ITN
Lateral element	LE
Forward primer	LP
Non-crossover	NCO
Nottingham Arabidopsis Stock Centre	NASC
Nucleolar organizing region	NOR
Overnight	ON
Pollen mother cell	PMC
Reverse primer	RP

Room temperature	RT
Real time quantitative PCR	RT-qPCR
Synaptonemal complex	SC
Synthesis-dependent strand annealing	SDSA
Sterilized deionized water	SDW
The Salk Institute Genome Analysis Laboratory	SIGnAL
Synaptic partner switch	SPS
Single-stranded DNA	ssDNA
The Arabidopsis Information Resource	TAIR
Universal Probe Library	UPL
Whole genome duplication	WGD
Wild-type	WT
Univalents	I
Bivalents	II
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Abstract

Polyploids are organisms whose genomes consist of more than two complete sets of chromosomes. Polyploidization is a prominent process in the diversification of plant species and domestication of crops. Following polyploidy, one of the most important drawbacks is the impact of chromosome doubling on meiosis. Polyploids must develop strategies to control pairing preferences that result in disomic inheritance and proper segregation of genetic material during meiosis. Otherwise, the coexistence of more than two copies of the same (autopolyploids) or similar (allopolyploids) chromosome sets may lead to multivalent formation during the first meiotic division and subsequent fertility problems. The process by which polyploid lineages evolve into a diploid-like state where there is a prevention of multivalent associations favoring the formation of bivalents is known as cytological diploidization. In this context, the difference in genome composition in auto- and allopolyploids has important consequences, being more complex in the former ones. The genetic control of the diploidization process in autopolyploids is poorly known and no major locus involved in this process has been identified so far.

The genus *Arabidopsis* constitutes a great model system for the analysis of both long- and short-term consequences of polyploidy. Natural autopolyploids are present within this genus but also can be easily obtained by a colchicine treatment. In this thesis, several approaches have been conducted to shed some light on the meiotic process in autopolyploids of *Arabidopsis thaliana*. The study has been possible after the development of an improved cytological protocol, including fluorescence *in situ* hybridization, to obtain high quality spreads of metaphase I chromosomes.

Firstly, the study has been focused on the analysis of tetraploid lines that *a priori* are at different stages of the diploidization process. For this purpose, synthetic, established, and natural autotetraploid accessions were assessed. The analyses conducted revealed that different genetic backgrounds show different responses to polyploidy in terms of chiasma formation. In addition, it was observed that after genome duplication, the contribution of each chromosome to the total chiasma frequency changes, since we observed a more uniform contribution in synthetic autotetraploids than in their diploid counterparts. Moreover, the assessment of the different chromosome associations at metaphase I

displayed again differences among the different genetic backgrounds analyzed. However, it was not possible to establish a clear correlation between chiasma and bivalent frequencies, but it is worth to mention that the shortest chromosomes displayed higher bivalent rates than the longest ones, suggesting different diploidization speeds depending on chromosome size and/or morphology. Besides, it was observed, as well as in previous studies, that established autotetraploid lines (with just a few generations after the whole genome duplication) show chiasma numbers significantly lower than their *de novo* autotetraploid controls. Regarding gene expression analyses, the results revealed that the genetic background, rather than genome duplication, might have a prevalent influence in determining transcription levels of genes involved in meiotic recombination.

Secondly, we analyzed the impact of intraspecific differences in the cytological diploidization of autotetraploids. The characterization of a synthetic autotetraploid hybrid (obtained by crossing the accessions Col and L*er*) revealed that heterozygosity could play an important role during diploidization, since the hybrid showed a higher bivalent frequency than the synthetic autotetraploids obtained from the parental accessions. Furthermore, the assessment of preferences in chiasma formation between homologous or identical chromosomes highlighted that different chromosomes present dissimilar preference patterns in their associations.

Thirdly, the consequences of polyploidy in mutants defective in meiotic recombination were analyzed in order to find out if the duplication of the entire genome could compensate to some extent the recombination problems that mutants present by increasing the chances of finding homologous sequences to recombine. For this purpose, the phenotypes in terms of chiasma formation, chromosome associations, and gene expression were assessed in nine autotetraploid mutant lines. The analyses revealed that mutants defective for different ZMM proteins, which are essential for the formation of most chiasmata, have similar mean cell chiasma frequencies at the diploid level, whereas they can show different chiasma frequencies at the tetraploid level. Interestingly, the autotetraploid *fancm* presented an unexpected behavior. The protein FANCM has anti-recombination activity during meiosis and the corresponding mutant displays an increased chiasma frequency. However, in a tetraploid situation, this mutant showed lower levels of quadrivalents, an unexpected significant decrease in the mean number of chiasmata per cell

and a significant increase in the frequency of trivalents and univalents compared to those of the control. In contrast, we found that in the absence of either the axis-related protein ASY1 or the protein CAND1, that is essential for ubiquitination, the duplication of the genome increases the capacity to form chiasmata.

Besides, the analysis of the interference-sensitive crossovers levels (class I crossovers) by MLH1 immunolocalization revealed that the mutant *fancm* and the control have a similar behavior at both diploid and tetraploid backgrounds. Nevertheless, it is important to point out that in both genetic backgrounds, the increase in class I crossovers (MLH1 foci) as a response to polyploidy was not proportional to the increase in chiasma frequency. In relation to gene expression analyses, the most remarkable results were an increase in the expression of *SPO11* (essential for the formation of double-strand breaks) and the gene of the recombinase RAD51 in the tetraploid mutant *hei10* (deficient for a ZMM protein); and a reduced expression of *HEI10* in a *fancm* background compared with the control, regardless the ploidy level.

In summary, in this thesis, we have tried to understand the changes in meiotic recombination that emerge with chromosome doubling. Further studies will be required in order to determine the mechanism by which polyploid plants ensure diploid-like chromosome pairing. Nevertheless, the results obtained show that diploidization is an extremely complex process in which the genetic background has a great influence, as well as the degree of heterozygosity. On the other hand, the results highlight that polyploidy can be a very useful tool to understand the functionality of some key proteins involved in meiotic recombination.

Resumen

Los organismos poliploides albergan más de dos juegos completos de cromosomas. La poliploidización es un proceso que ha tenido un papel fundamental en la diversificación de las especies de plantas, así como en la domesticación de especies de interés agronómico. Uno de los problemas derivados de la poliploidía surge como consecuencia del efecto de la duplicación del número de cromosomas en la meiosis. Los organismos poliploides deben desarrollar estrategias para controlar las preferencias de apareamiento de los cromosomas, para favorecer un modelo de herencia disómica y una correcta segregación del material genético durante la meiosis. Estas estrategias surgen porque la coexistencia de más de dos juegos iguales (en el caso de los autopoliploides) o similares (alopoliploides) de cromosomas puede llevar a la formación de multivalentes durante la primera división meiótica, dando lugar a problemas de fertilidad. El proceso por el cual los organismos poliploides evolucionan hacia un comportamiento meiótico similar al que muestran los organismos diploides es conocido como diploidización citológica. A través de este proceso se favorece la formación de bivalentes, mientras que se evita la formación de multivalentes. En este contexto, las diferencias entre los organismos auto- y alopoliploides en cuanto a la composición del genoma conlleva importantes consecuencias en el proceso de diploidización, el cual resulta más complejo en los primeros. Se sabe muy poco acerca del control genético del proceso de diploidización en autopoliploides y todavía no ha sido identificado ningún locus que tenga una función relevante en este proceso.

El género *Arabidopsis* es muy apropiado para llevar a cabo el análisis de las consecuencias de la poliploidía, tanto a corto como a largo plazo. Es posible encontrar poliploides naturales en este género, pero también se puede duplicar el número cromosómico de manera artificial mediante un tratamiento con colchicina. En esta tesis, se han llevado a cabo experimentos desde diferentes perspectivas para tratar de profundizar en el conocimiento sobre el proceso de la meiosis en autopoliploides de *Arabidopsis thaliana*. Este estudio ha sido posible gracias a la adaptación y perfeccionamiento del protocolo de citología, incluyendo la hibridación *in situ* con fluorescencia, lo que nos ha permitido obtener preparaciones de cromosomas en metafase I de alta calidad.

En primer lugar, el estudio se ha centrado en el análisis de líneas tetraploides, que *a priori* se encuentran en diferentes etapas del proceso de diploidización. Para ello, se analizaron líneas autotetraploides sintéticas, establecidas y naturales. Los resultados de dichos análisis revelaron que los distintos fondos genéticos analizados muestran diferentes respuestas frente a la poliploidía en relación con la frecuencia de quiasmas. Además, en estos análisis se observó que, tras la duplicación del genoma, hay cambios en las contribuciones de los diferentes cromosomas a la frecuencia total de quiasmas, de manera que hay una mayor uniformidad entre los cromosomas en los autotetraploides sintéticos que en los diploides del mismo fondo genético. También se detectaron diferencias entre los distintos fondos genéticos cuando se estudiaron las asociaciones entre cromosomas en metafase I. Sin embargo, no se pudo establecer una clara correlación entre la frecuencia de quiasmas y de bivalentes, aunque cabe destacar que los cromosomas más cortos mostraron las frecuencias más altas de bivalentes, lo que sugiere que la diploidización se puede dar a diferentes velocidades dependiendo del tamaño y/o la morfología de los cromosomas. Asimismo, se observó, al igual que en anteriores estudios, que los autotetraploides establecidos (con tan sólo unas pocas generaciones tras la duplicación del genoma completo) muestran frecuencias de quiasmas significativamente menores cuando se comparan con los autotetraploides *de novo* equivalentes. En cuanto a los análisis de expresión, los resultados revelan que el fondo genético, en mayor medida que la duplicación del genoma, puede influir de manera importante en los niveles de transcripción de genes involucrados en la recombinación meiótica.

En segundo lugar, se ha analizado el impacto que pudieran tener las diferencias intraespecíficas en la diploidización citológica de los autotetraploides. La caracterización de un autotetraploide híbrido (obtenido del cruce entre los ecotipos Col y L*er*) reveló que la heterocigosidad podría jugar un papel importante durante la diploidización, ya que el híbrido mostró una frecuencia más alta de bivalentes que los autotetraploides sintéticos obtenidos de los ecotipos parentales. Además, el análisis de las preferencias en la formación de quiasmas entre los cromosomas homólogos e idénticos puso de manifiesto que los diferentes cromosomas pueden presentar distintos patrones de preferencia.

En tercer lugar, se analizaron las consecuencias que la poliploidía puede tener en mutantes para genes implicados en la recombinación meiótica. El objetivo era determinar si

la duplicación del genoma completo puede compensar en cierta medida los problemas en recombinación que estos mutantes presentan, gracias al incremento de la probabilidad que tienen los cromosomas de encontrar secuencias homólogas con las que recombinar. Para este propósito, se analizaron los fenotipos correspondientes a las frecuencias de quiasmas, asociaciones cromosómicas y expresión de genes en nueve líneas mutantes autotetraploides. El estudio mostró que los mutantes con defectos en diferentes proteínas ZMM, las cuales son esenciales para la formación de la mayoría de los quiasmas, tienen frecuencias de quiasmas por célula similares en las líneas diploides, mientras que las frecuencias se diferencian en condición tetraploide. Entre los resultados obtenidos cabe destacar que el mutante autotetraploide *fancm* presentó un comportamiento inesperado. La proteína FANCM posee actividad anti-recombinasa durante la meiosis y el correspondiente mutante diploide muestra un incremento en la frecuencia de quiasmas. Sin embargo, a nivel tetraploide, este mutante presentó una frecuencia menor de cuadrivalentes y una reducción en la media de quiasmas por célula, así como una subida significativa de la frecuencia de trivalentes y univalentes en comparación con el control tetraploide. Por otro lado, se observó que tanto en ausencia de la proteína asociada al eje ASY1 como en ausencia de la proteína CAND1, la cual es esencial en ubiquitinación, la duplicación del genoma incrementa la capacidad para formar quiasmas.

Además, el análisis del número de sobrecruzamientos que son sensibles a interferencia (sobrecruzamientos de clase I) mediante la inmunolocalización de MLH1 mostró que tanto el mutante *fancm* como el control tienen niveles similares tanto en los fondos diploides como en los tetraploides. Sin embargo, es importante destacar que en ambos fondos genéticos, el incremento de los sobrecruzamientos de clase I (señales de MLH1) no fue proporcional al incremento de la frecuencia de quiasmas en respuesta a la poliploidía. En relación con los análisis de la expresión génica, los resultados más destacables fueron el incremento de la expresión de *SPO11* (esencial para la formación de roturas de doble cadena) y del gen de la recombinasa RAD51 en el mutante tetraploide *hei10* (deficiente para una proteína ZMM); y una expresión disminuida del gen *HEI10* en el fondo mutante *fancm* comparado con el control en ambos niveles de ploidía.

En resumen, en esta tesis, se ha intentado profundizar en la comprensión de los cambios en la recombinación meiótica que surgen con la duplicación del número de

cromosomas. No obstante, se requieren más estudios para poder determinar los mecanismos por los cuales las plantas poliploides acaban presentando un emparejamiento de los cromosomas (formación exclusiva de bivalentes) igual al que sucede en las plantas diploides. Los resultados que se han obtenido han revelado que la diploidización es un proceso extremadamente complejo en el cual tanto el fondo genético como el grado de heterocigosidad tienen una gran influencia. Por otra parte, los resultados mostrados sugieren que la poliploidía puede ser una herramienta muy útil para entender la funcionalidad de algunas de las proteínas clave para la recombinación meiótica.

1. Introduction

1.1. Polyploidy and types of polyploids

Etymologically, the word **polyploidy** is composed by the Greek word *poly*, which means many, and the word *ploideus*, a cytological term that refers to the number of sets of chromosomes and is derived from the Greek word *idios* that means individual or peculiar (Dar and Rehman, 2017a). Specifically, the term polyploidy refers to the presence of more than two sets of chromosomes. Polyploid organisms have multiple sets of chromosomes derived from a single diploid species (2*n* = 2x) or a combination of chromosome sets from different but related species. Thus, according to the number of chromosome sets, polyploids are named as triploids (2*n* = 3x), tetraploids (2*n* = 4x), pentaploids (2*n* = 5x), etc. Polyploids can arise spontaneously by different mechanisms but also can be artificially induced using drugs, e.g. colchicine. The relatively high occurrence of polyploids in nature suggests that polyploidy must confer, under determined circumstances, important advantages compared to diploid organisms. In fact, it is thought that **whole genome duplication** (WGD) is one of the fastest mechanisms for speciation with a great impact in evolution. It mainly occurs in plants but also in fungi and different animal species; especially in fishes, amphibians, and insects (Otto and Whitton, 2000). In humans, polyploidy occurs in 1 - 3 % of conceptions and usually results in spontaneous abortion (Eiben *et al.*, 1990).

From a historical perspective, polyploidy has a great importance since it was one of the first features cytogenetically studied in detail (Stebbins, 1950). Probably, the first organism identified as a polyploid was *Oenothera gigas* which was reported by Lutz in 1907. This species is a tetraploid mutant derived from *O. lamarckiana*. However, the term polyploidy was not introduced until 1917, when H. Winkler presumably obtained the first artificial tetraploid in *Solanum nigrum*.

There is a wide variety of mechanisms to form polyploids (see more in section 1.2, Figure 2) and different **types of polyploidy**. It is possible to differentiate between two main types of polyploids: autopolyploids and allopolyploids (Stebbins, 1947). **Autopolyploids** result from the duplication of the entire genome within one species or by the production and merge of unreduced (diploid) gametes from genetically similar individuals (for example a

diploid species with the genome AA doubles to become the autotetraploid AAAA). **Allopolyploids** are usually formed from the hybridization of genetically distinct genomes followed by a WGD event (for example two diploid species AA and BB hybridize and the hybrid AB generates the allotetraploid AABB). These categories are represented in Figure 1. However, there are other kinds of polyploids that cannot be grouped in these two main categories because they have arisen via an array of different processes (Stebbins, 1947). This is the example of the **autoallopolyploids**, which can be hexaploids or polyploids with higher ploidies that combine the processes of auto- and allopolyploid formation. For instance, this situation appears when a triploid is formed by hybridization (AAB) and subsequently undergoes WGD to form a hexaploid (AAAABB). In addition, some autopolyploids can be subcategorized as **segmental allopolyploids**. They result from a WGD event after the hybridization between genetically distinct races or populations of a single species whose genomes differ in many genes or chromosomal segments (for example, the tetraploid AAA´A´ from the diploid AA´).

The differentiation between auto- and allopolyploids is very important since their behavior is very different, especially in meiosis. Autopolyploids contain three or more copies of each chromosome (**homologous chromosomes**), whereas allotetraploids present chromosomes derived from two different species (**homeologous chromosomes**). In autopolyploids, which harbor more than two sets of homologous chromosomes, each chromosome may pair randomly with any of its homologous partners, thus forming either bivalents or multivalents during meiosis. In the case of autotetraploids, this situation leads to **tetrasomic inheritance** (Muller, 1914), in which all the possible allelic combinations are produced in equal frequencies. On the other hand, in allopolyploids, differences between the chromosomes of the parental species favor the pairing between homologous chromosomes (*vs.* homeologous chromosomes) driving to **disomic inheritance**. Alternatively, inheritance patterns may differ among different loci (e.g. in allotetraploids formed through hybridization of close relatives), with some loci exhibiting polysomic inheritance and others a disomic one (Tate *et al*., 2005).

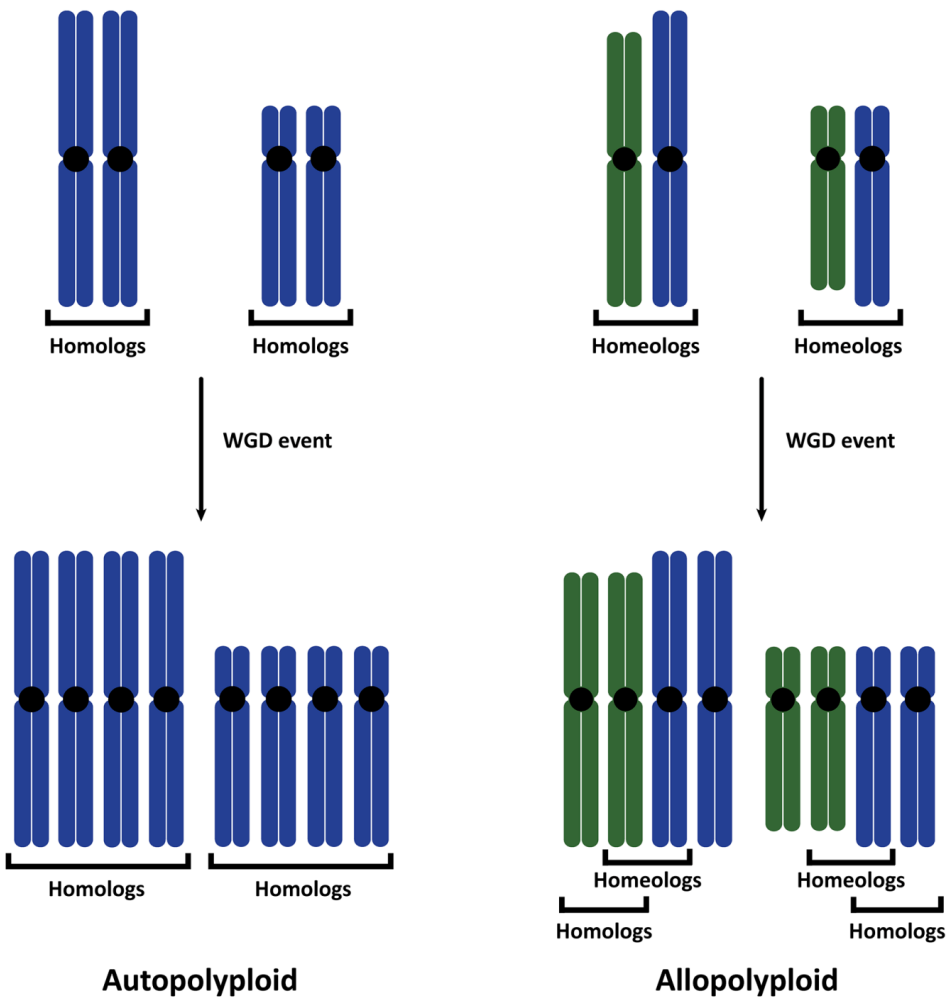


Figure 1. Schematic representation of homologous chromosomes in an autopolyploid and homologous and homeologous chromosomes in an allopolyploid. Chromosomes from different species are represented with different colors. After a WGD event, newly formed autopolyploids have more than two homologous chromosomes, whereas in allopolyploids, WGD leads to the presence of homologous and homeologous chromosomes.

Traditionally, autopolyploids have been considered rare in natural populations (Stebbins, 1971). For a long time, they were associated to problems in fertility resulting from the formation of multivalents during meiosis. However, some nascent autopolyploids show only a slight reduction in fertility. Furthermore, empirical evidences, obtained from genetic and cytological data currently available, indicate that autopolyploids are widely represented in some groups, and probably their prevalence had been underestimated in the past (Ramsey and Schemske, 2002). Autopolyploids are morphologically similar to their diploid progenitors and the taxonomic species concept might have contributed to the thought that they were not as abundant as they really are (Soltis *et al*., 2007). In order to shed some light

into this controversy about the prevalence of autopolyploids, Barker *et al*. (2016) analyzed data obtained from phylogenetic, genetic, and cytological approaches corresponding to 47 plant genera, including 4,003 species. They concluded that 24 % of the species were polyploids, being 13 % autopolyploids and 11 % allopolyploids. However, it is important to consider that, despite the similar numbers of prevalence, autopolyploid plants are produced in a much higher rate (up to 20-fold). This means that, in terms of evolutionary success, allopolyploids seem to have an advantage (Barker *et al.*, 2016). This circumstance is probably owing to the presence of two distinct genomes which leads to an immediate niche differentiation between the neoallopolyploid and its progenitors. This fact increases opportunities of mating with other neopolyploids, which is a key factor in polyploid establishment (Fowler and Levin, 1984; Rodriguez, 1996; Levin, 2000; Oswald and Nuismer, 2011). In addition, allopolyploids show an increased ability to exploit new habitats (e.g. deglaciated regions) that cannot be colonized by the parental diploid populations (Brochmann *et al.*, 2004; Te Beest *et al.*, 2012).

1.2. Mechanisms of polyploid formation

Most of the information about the formation of polyploids has been obtained from plants due to the high prevalence of WGD events in this kingdom. Figure 2 represents the different natural pathways leading to polyploidy. Spontaneous **somatic chromosome doubling** is assumed to be very rare. It can occur in meristematic cells and may result in the formation of mixoploid individuals (Jorgensen, 1928; Newton and Pellew, 1929; Skalińska, 1947; Ahloowalia and Garber, 1961). One example is the allopolyploid *Primula kewensis*, which was originated from fertile tetraploid shoots of a diploid F_1 hybrid between *P. floribunda* and *P. verticellata* (Newton and Darlington, 1929). Polyploid shoots can also emerge from wounds and tumors (Jorgensen, 1928; Lewis, 1980). Additionally, somatic polyploidy also takes place in zygotes and young embryos, thus leading to the formation of completely polyploid sporophytes. This has been well described in cereals when young embryos are exposed to high temperatures (Randolph, 1932; Dorsey, 1936).

One of the most common method of polyploidization is through the **formation and fusion of unreduced gametes** (reviewed in Harlan and De Wet, 1975; Bretagnolle and Thompson, 1995; Brownfield and Köhler, 2011; Mason and Pires, 2015). Alternatively, a polyploid embryo can be also formed by the fertilization of an ovule by more than one sperm nucleus. This phenomenon is called **polyspermy** and has been described in many plant species such as orchids (Hagerup, 1947).

Somatic doubling or the fusion of two unreduced gametes are **"one-step" pathways** for polyploid formation (Figure 2) (Ramsey and Schemske, 1998). In the case of autotetraploids, unreduced gametes come from individuals within the same species. For example, in a very low ratio, autotetraploid seedlings of different open-pollinated varieties of apple where found when the progeny of diploid individuals was checked (Einset, 1952). Autopolyploids were also obtained after crossing clones that produce unreduced pollen grains in the medicinal plant *Costus speciosus* (Tyagi, 1988). On the other hand, in allopolyploids, unreduced gametes come from diploid progenitors corresponding to an F_1 or F_2 generation obtained from an interspecific cross. Levan (1941) reported that around 50 % of the progeny obtained from a hybrid between *Allium cepa* and *A. fistolium* was tetraploid.

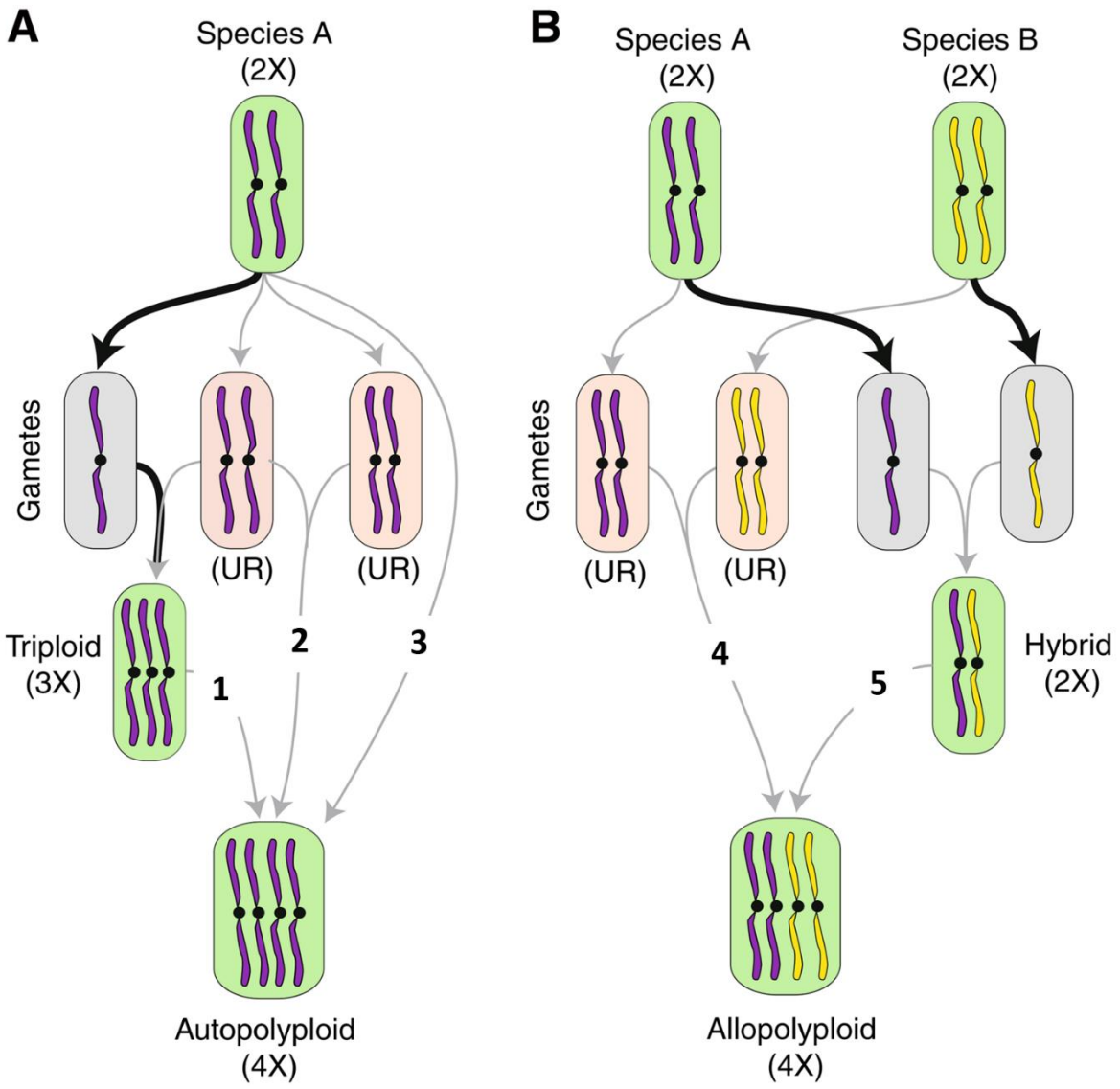


Figure 2. Major pathways in the formation of polyploids. Possible scenarios for the formation of autopolyploid **(A)** or allopolyploid **(B)** individuals. Diploid species generally produce haploid gametes (black paths and grey boxes), yet unreduced gametes (UR) are also formed with low frequency (grey paths and pink boxes). The possible pathways are: Formation of an autopolyploid by the "triploid-bridge" pathway **(1)**, by the fusion of two UR **(2)**, and by a somatic WGD event **(3)**. Formation of an allopolyploid by the fusion of two UR from different species **(4)** or by the formation of a diploid hybrid and a subsequent somatic WGD event **(5)**. Modified from Bomblies and Madlung (2014).

Polyploids can also be formed through the **"triploid-bridge" pathway**. In this pathway, firstly triploid individuals arise from the fusion of unreduced gametes with haploid gametes. The cross between triploids and diploids that produce unreduced gametes or between triploids (in this case it can also be self-fertilization) finally leads to the formation of tetraploids (Ramsey and Schemske, 1998). An example of autopolyploids obtained by the "triploid-bridge" pathway was described by Bergström in 1940. She reported the presence of

some tetraploid individuals (1 %) in the progeny obtained by the cross of triploids with diploids of *Populus tremula*. There are many examples of autotetraploids obtained from crossing triploids with diploids (Dermen, 1931; Johnsson, 1940; Warmke and Blakeslee, 1940; Zohary and Nur, 1959; Dujardin and Hanna, 1988). As far as allopolyploids are concerned, there are also some examples of their formation by the "triploid-bridge" pathway, as it was reported in the genera *Galeopsis* (Müntzing, 1932) and *Aquilegia* (Skalińska, 1945). On the other hand, ploidy levels higher than 4X can also be obtained by the fusion of normal gametes with unreduced gametes of polyploid populations. For instance, 1 % of the tetraploid alfalfa offspring is hexaploid (Bingham, 1968).

The frequency of unreduced gametes is the main factor that determines the rate of polyploid formation. Several defects in meiosis could lead to the formation of these gametes, such as problems in spindle formation or function, and defects in cytokinesis. Besides, the frequency of diploid gametes is much higher in hybrids (27.52 %) than in non-hybrids (0.56 %) (Ramsey and Schemske, 1998). In this case, failures in chromosome pairing and non-disjunction are the common aberrations that lead to the production of unreduced gametes (Mason and Pires, 2015). The natural incidence of unreduced pollen and unreduced ovules is similar. A study conducted by Maceira *et al.* (1992) in *Dactylis glomerata* showed a similar frequency of diploid pollen (0.98 %) and diploid ovules (0.49 %). Similar results were previously found in maize (Alexander and Beckett, 1963) and in *Trifolium pratense* (Parrott *et al*., 1985).

The underlying mechanisms of unreduced gamete formation are not well understood, but it seems that this process is genetically regulated. The formation of diploid gametes can be increased by selection, as it has been demonstrated in red clover (Parrott and Smith, 1986). Indeed, in some species, it seems to be mainly controlled by a single locus (Satina and Blakeslee, 1935; Rhoades and Dempsey, 1966; Sora *et al*., 2016). In potato, the premature cytokinesis presented by the mutants *pc-1* and *pc-2* after the first meiotic division, and the formation of parallel spindles at anaphase II displayed by the *ps* mutant were reported to lead to the formation of unreduced gametes (Mok and Peloquin, 1975). In addition, environmental factors such as temperature, wounding, herbivory, water levels or nutrient stress may modulate the frequency of diploid gametes (Ramsey and Schemske, 1998; De Storme and Geelen, 2013). Temperature variations have been shown to be one of

the major factors (Sax, 1936; Pécrix *et al.*, 2011; Wang *et al.*, 2017). For instance, in *Lotus tenuis*, an increase of diploid gametes has been observed under temperature stresses but mainly due to high temperatures (Negri and Lemmi, 1998). In *Brassica* hybrids, cold temperatures induce the production of unreduced gametes (Mason *et al.*, 2011). Similar results were obtained when a cold stress was applied to *Arabidopsis* species (De Storme *et al*., 2012). In addition, there are wide evidences of increased productions of diploid gametes in high altitudes and latitudes as well as in locations that were glaciated during the last glacial maximum (Sax, 1936; Novikova *et al*., 2018).

1.3. Importance of polyploidy in plant evolution

Polyploidy has been an essential factor during the evolution of eukaryote organisms (Otto, 2007) (Figure 3). As it has been mentioned above, although there is a wide number of polyploid insects, fishes, amphibians, and reptiles (Otto and Whitton, 2000; Mable *et al*., 2011), polyploidy is more common in plants (Stebbins, 1947, 1950; Soltis and Soltis 2000, 2009; Soltis *et al*., 1993; Otto and Whitton, 2000). In angiosperms (flowering plants), it has been estimated that the incidence of polyploidy is between 35 % (Stebbins, 1971) and 70 % (Masterson, 1994). In bryophytes, polyploidy comprises about 80 % of all species (Kuta, 1997), whereas in the case of pteridophytes estimations are around 95 % (Otto and Whitton, 2000). In gymnosperms, only 5 % of the species are polyploids (Khoshoo, 1959; Ahuja and Neale, 2005).

There have been several important WGD events during the evolution of plants. It is thought that gymnosperms emerged from a common ancestor that underwent a WGD event called **"ζ"**, taking place around 390 million years ago, during the Devonian period (Jiao *et al.*, 2011; Li *et al.*, 2015; Clark and Donoghue, 2017; Murat *et al.*, 2017) (Figure 3). In the case of angiosperms, which is the most abundant group of plants nowadays (Chartier *et al.*, 2017), after sequencing a wide number of species, several studies claimed that 300 million years ago, during the Carboniferous period, the first flowering plant emerged from a WGD event of a gymnosperm (this event is called **"ε"**) (Jiao *et al.*, 2011; Clark and Donoghue, 2017; Murat *et al.*, 2017) (Figure 3). Then, it seems that thanks to the impact of polyploidy in evolution, gymnosperms, and especially angiosperms, emerged producing diversity.

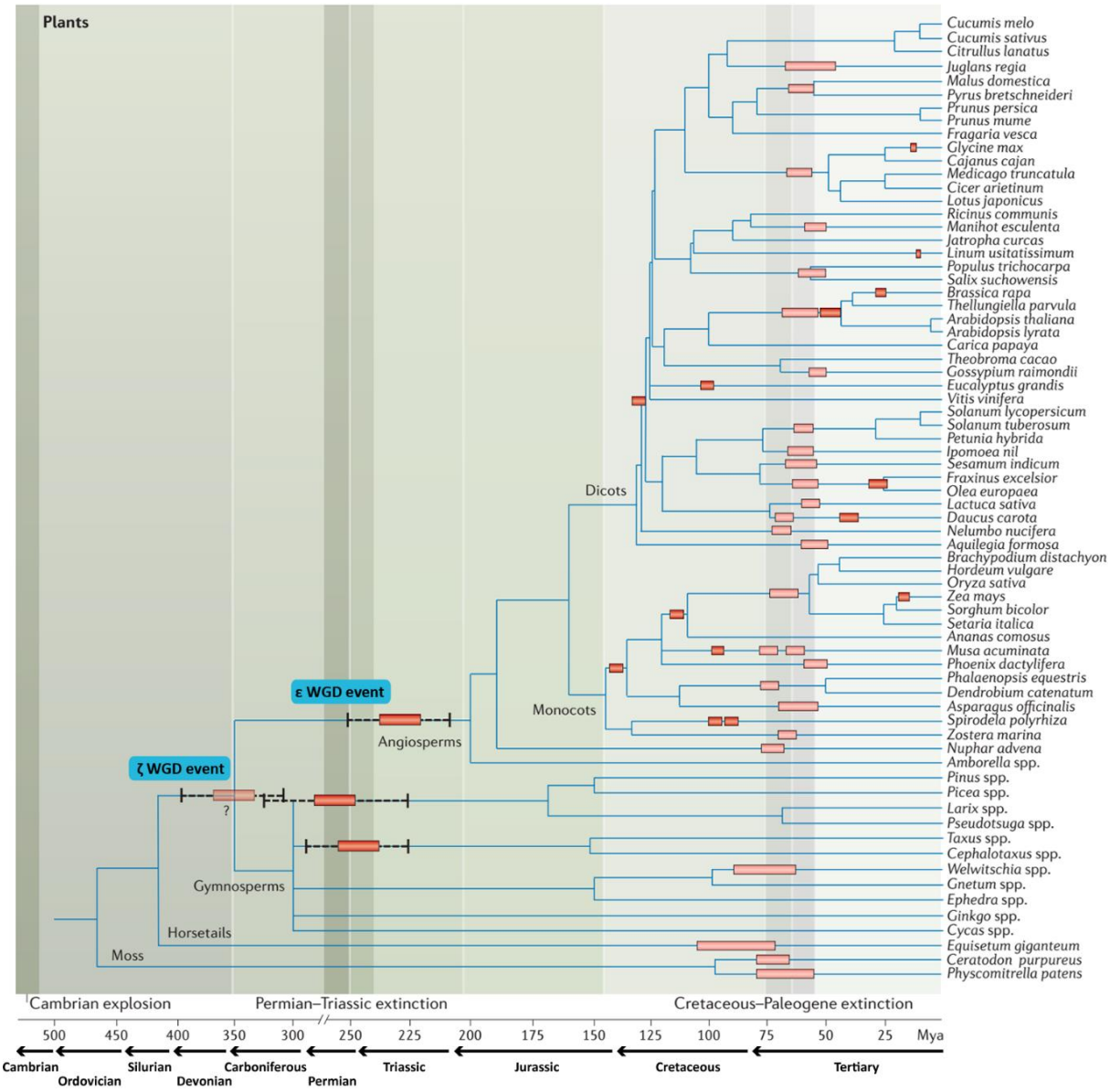


Figure 3. Phylogenetic tree showing the known WGD events during the evolution of plants. Plant species are included in a pruned tree that represents the evolutionary relationship among species for which the transcriptomic data and/or genome sequence is available. WGD events are depicted by orange rectangles (ζ and ε events are also marked in blue). Bold black dashed lines represent uncertainty in the date of the events. Mass extinction events are indicated by shaded areas. Mya, million years ago. Modified from Van de Peer *et al*. (2017).

In the **short term**, changes in gene expression and epigenetic remodeling due to WGD events could underlie variations in the physiology, ecology and morphology of the organisms (Doyle *et al.*, 2008; Parisod *et al.*, 2010; Lavania *et al.*, 2012; Schoenfelder and Fox, 2015; Shi *et al.*, 2015; Song and Chen, 2015; Zhang *et al.*, 2016). These changes can affect interspecies interactions and contribute to the adaptive potential of polyploid individuals (Te Beest *et al.*, 2012; McCarthy *et al.*, 2016). For instance, it has been reported

that polyploidy can produce divergence in traits that are attractive to pollinators, facilitating the reproductive isolation and establishment of polyploid populations (Segraves and Anneberg, 2016). In addition, after WGD events, changes in the production of secondary metabolites might allow plants to escape from herbivory (Lavania *et al.*, 2012; Vergara *et al*., 2016). Differences in the concentration of these metabolites might also explain the increased resistance (or sensitivity on some occasions) to different pathogens (such as fungi, nematodes or insects) (Oswald and Nuismer, 2007; Te Beest *et al*., 2012). Another immediate advantage conferred by the duplication of the entire set of chromosomes is the higher plasticity and the consequent increase in the tolerance to a wide range of environmental conditions, nonetheless this theory is still controversial (Van de Peer *et al*., 2009; Mable *et al*, 2011; Hahn *et al*., 2012; Te Beest *et al.*, 2012). In this context, several studies have been conducted to compare the resistance of polyploid individuals and their diploid progenitors to different abiotic stresses. Tetraploid *A. thaliana* plants present increased drought tolerance (Del Pozo and Ramirez-Parra, 2014) and higher salt tolerance (Chao *et al*., 2013) compared to their diploid counterparts. These features have also been reported in other tetraploid plants such as citrus and rice and related to variations in the expression levels of genes involved in stress and hormonal responses (Yang *et al.*, 2014; Ruiz *et al.*, 2016).

The increased mutational and environmental robustness of polyploids and their key innovations might reduce the chances of extinction during time periods with extreme and unstable conditions (Crow and Wagner, 2006; Van de Peer *et al*., 2009). However, genetic redundancy leads to genome instability in the **long term**, and structural and molecular changes are required after chromosome doubling to gradually restore a diploid-like behavior in polyploid genomes. In addition, specific adaptations during the formation of gametes (meiosis) might be necessary for polyploids to become successful in the longer term. For these reasons, polyploidy seems to be a transient state and WGD might only be successful at very specific times during major ecological or environmental changes and/or periods of extinction. In fact, only few polyploids survive and get established after the short-term adaptation (Van de Peer *et al*., 2017). One of the most important factors favoring the long-term retention and persistence of polyploids is genetic redundancy. The relaxed selection on the copies of duplicated genes allow them to acquire new functions

(**neo-functionalization**) that contribute to increase the biological complexity. This can result in adaptive diversity, leading to specialization, and ultimately to speciation (De Smet and Van de Peer, 2012; Zhou *et al.*, 2016). For instance, the appearance of flowers in plants is an example of specialization after polyploidy (Jiao *et al.*, 2011; Soltis and Soltis, 2016). It has been hypothesized that a WGD event in the common ancestor of the angiosperms produced an array of duplicated genes that finally led to the origin of flowers. Specifically, the duplication of the *MADS-box* genes provided the conditions for the origin of the transcription factors that regulate the formation of the different floral organs (Buzgo *et al.*, 2005; De Bodt *et al*., 2005; Zahn *et al.*, 2005).

1.4. Polyploidy, crop domestication and plant breeding

In addition to the importance of polyploidy in the evolution of plants, this circumstance is also associated with **agriculturally favorable traits**, being widespread among crops (Hancock, 2005; Zeder *et al.*, 2006). There is a wide number of examples of both allopolyploid and autopolyploid crop species such as potato, tobacco, sugar cane, rapeseed, oat, banana, peanut, and cotton among others (Sattler *et al*., 2016). Probably, the most famous allopolyploid crops are the bread wheat (*Triticum aestivum*, 6x = 42) and the durum wheat (*Triticum turgidum*, 4x = 28) (Kerby and Kuspira, 1987).

In 2016, Salman-Minkov *et al*. reported that the percentage of polyploid species is higher among crops than among wild species (30 % versus 24 %, respectively). This difference is even higher in monocots (54 % of polyploid species in crops versus 40 % in wild species). The same authors proposed that the coincidence of polyploidy with the domestication process might be the result of two different scenarios: polyploidy is followed by the domestication process (called **"polyploidy first"**), and polyploidy appears as a consequence of domestication (called **"domestication first"**). Their results demonstrated that, although with several notable exceptions, polyploidy more frequently occurs before domestication. They concluded that polyploidy accelerates domestication and the process of selecting favorable new traits.

The fact that many crop species are polyploids has attracted the attention of plant breeders, since polyploidy could be a potential path to future crop improvement. The most common consequence of a WGD event in plants is the increase in cell size known as the **"gigas" effect** (Sattler *et al*., 2016). Polyploid plants can present larger organs such as roots, tubercles, leaves, flowers, seeds, and fruits compared to their diploid counterparts (Stebbins, 1950). However, a cell size increase does not always produce larger plant organs due to a reduction in the number of cell divisions (Stebbins, 1971). Nevertheless, lower growth rates and a delayed flowering time are also interesting features in polyploid ornamental plants (Levin, 2002). Another consequence of WGD with interest for plant breeders is the reduction in fertility to obtain **seedless plants**. Triploid individuals are obtained to get very low fertility rates, as in the cases of watermelon or banana, since meiotic chromosome segregation is highly compromised in these situations (Crow, 1994). Alternatively, polyploidy (specifically allopolyploidy) can **restore fertility in sterile hybrids**

and this can be used as a bridge for genetic transfers that are not possible between two diploid species (Dewey, 1980).

Interestingly, some autopolyploid crops have been obtained inducing WGD and are called **synthetic autopolyploids**. Autotetraploid rye plants (*Secale cereal*e, 4x = 28; Müntzing, 1951) have been produced since 1930s. They provide better baking and stiff-straw qualities than their diploid relatives, although their yield is frequently lower (Sattler *et al*., 2016). Other interesting synthetic tetraploid crop is the tetraploid red clover (*Trifolium pratense*, 4x = 28; Taylor and Quesenberry, 1996). Several tetraploid varieties with commercial interest of this crop have been obtained since colchicine was used for the first time as a drug to induce WGD (Hancock, 1997). This crop shows several advantages after the duplication of its chromosome complement such as an increased disease resistance, winter hardiness, improved forage dry matter yield, and persistence (Sattler *et al*., 2016). The tetraploid ryegrass is another synthetic autotetraploid crop with a high commercial interest. Polyploid varieties have been obtained since 1939, also using colchicine (Myers, 1939). These varieties are commonly used in Europe and provide an increase in water and soluble carbohydrate content, disease resistance, drought tolerance and palatability compared with those from the diploid varieties (Nair, 2004).

1.5. Genomic and cellular impacts of polyploidy

Even though polyploidy has been described as a major force in evolution, it entails problems at different levels, which are more prominent immediately after the WGD event (Comai, 2005; Van de Peer *et al*., 2017). One of the challenges that neopolyploids must face is the **regulation of gene expression**. Although it might be expected that polyploidy affects equally all genes, some genes do not respond proportionally to the increase in the number of copies. This might produce changes in the stoichiometric relationships among proteins and, subsequently, in the proportion of certain cellular components (Comai, 2005). This effect is even more drastic in the case of odd-numbered ploidies as it was demonstrated in maize plants. Tetraploid individuals showed an increased expression in most of the genes compared with those of the diploid individuals, whereas in haploid and triploid individuals the expression levels of some genes exhibited unexpected deviations (Guo *et al*., 1996). Conversely, Albertin *et al.* (2005) made a proteomic comparison between autotetraploid and diploid individuals of *Brassica oleracea* finding no significant differences.

Epigenetic instability is claimed to be another effect that might affect organisms immediately after the duplication of the entire genome (Adams and Wendel, 2005). An interesting example was found in *A. thaliana*, in a study in which the epigenetic effect of the WGD on a specific transgenic locus was analyzed (Mittelsten Scheid *et al.*, 1996; Mittelsten Scheid *et al*., 2003). The transgene was silenced more frequently in autotetraploids than in diploids. The segregation of this epigenetic change followed the Mendelian rules in diploids, whereas heterozygous tetraploids produced more gametes with the inactivated allele than what was expected. The presence of the strong 35S promoter in the transgene could have influenced this phenomenon. Examples of epigenetic instability are more abundant in allopolyploids. Changes in DNA methylation have been reported in allopolyploid plants of *Arabidopsis* (Comai *et al.*, 2000; Madlung *et al.*, 2002; Wang *et al.*, 2004; Madlung *et al.*, 2005) and wheat (Shaked *et al.*, 2001), although in these situations the epigenetic instability may also be attributed to the divergence between the parental species (Comai, 2005).

Another challenge produced by the WGD is the **change in cellular architecture**. The increase in the DNA content frequently leads to an increase in cell volume that subsequently alters the interactions among the components of the cell (Olmo, 1983; Melaragno *et al*., 1993). While in a tetraploid the number of chromosomes is doubled, theoretically the

surface of the nuclear envelope only increases 1.6 times its original size (reviewed in Comai, 2005). This difference could affect the relationships that components of the nuclear envelope and chromatin have, altering the normal peripheral localization of some sequences such as telomeric and centromeric regions (Fransz *et al.*, 2002; Jasencakova *et al.*, 2003; Corredor *et al.*, 2005).

Cellular division might also be affected after the duplication of the sets of chromosomes. For example, in polyploid yeasts, there is a rapid loss of chromosomes which leads to the formation of aneuploid cells (Mayer and Aguilera, 1990). In animals, it has been reported that polyploidy triggers a G1 checkpoint mediated by p53 (Andreassen *et al.*, 2001). If this checkpoint is bypassed, it can result in multipolar spindles producing a chaotic segregation of sister chromatids (Borel *et al.*, 2002). In plants, although the knowledge about the organization of the mitotic spindle has been increased during the last years (Schmit, 2002; Chan *et al*., 2005), little is still known about the consequences of polyploidy in this structure.

One of the most important drawbacks for polyploid organisms is the impact of chromosome doubling on **meiosis** (more information in Gillies, 1989; Zickler and Kleckner, 1999). In this special cell division; pairing, synapsis, and chiasma formation between homologous chromosomes are essential processes to guarantee their correct segregation at anaphase I (Osman *et al.*, 2011). In polyploids, the presence of more than two potential partners for each chromosome (homologous in the case of autopolyploids and homeologous in the case of allopolyploids) leads to complex scenarios. **Multivalent associations** are observed at prophase I due to synaptic interactions involving three or more chromosomes, forming **synaptic partner switches** (SPSs) (Sybenga, 1975). In some occasions, even multiple synaptonemal complexes (SCs) can be observed. In most species, multivalent associations decrease in number during prophase I, but a proportion of multivalents can persist until later stages.

1.6. Diploidization of polyploids

As it was mentioned before, polyploidy has been a major force in eukaryote evolution, especially in plants. Problems derived from the duplication of the entire genome must be overcome and defects resulting from meiotic instability must be resolved to avoid reduced fitness. Natural selection should favor both a reduction in the level of polysomic inheritance and a diploid-like meiosis to avoid aneuploidies. The process by which polyploid lineages evolve into a diploid-like state reducing the level of polysomic inheritance and increasing bivalent chromosome configurations in meiosis is known as **diploidization**. It is possible to differentiate two types of diploidization, cytological and genetic (Ma and Gustafson, 2005). **Cytological diploidization** consists in the prevention of multiple chromosome associations favoring the formation of bivalents. The elimination of certain sequences, chromosome rearrangements, and dysploidy seem to contribute to the cytological diploidization (Mandáková and Lysak, 2018). Since it is a long process, intermediate situations with different chromosomes showing different rates of bivalent formation (tetrasomic inheritance for some chromosomes and disomic inheritance for others) are possible (Santos *et al.*, 2003). **Genetic diploidization** refers to the adjustment of the gene expression levels to make them comparable to those of their diploid counterparts (Soltis *et al*., 1993). The main factors that promote this phenomenon are gene non-functionalization, sub-functionalization, neo-functionalization, as well as other kind of epigenetic modifications.

The cytological diploidization is faster in allopolyploids than in autopolyploids. In the former ones, more-related chromosome copies (homologous chromosomes) may pair and recombine with a higher frequency than less-related chromosome copies (homeologous chromosomes) (Glover *et al*., 2016). During this process, different levels of similarity between the parental genomes result in different levels of homeologous chiasma suppression (Le Comber *et al.*, 2010). However, synaptic interactions involving homeologous chromosomes are usually observed at prophase I in allopolyploids displaying complete absence of chiasmata between these chromosomes at metaphase I. Some examples of organisms in which this phenomenon has been observed are hexaploid wheat (Hobolth, 1981; Holm, 1986; Martinez *et al.*, 2001), *Scilla autumnalis* (Jenkins *et al*., 1988), and tetraploid *Lolium* hybrids (Jenkins, 1986). In the case of the allopolyploids *Avena sativa*,

A. maroccana (Jones *et al*., 1989), and *Allium montanum* (Loidl, 1988), they do not even show synaptic multivalents. In autopolyploids the formation of multivalents is more common. Interestingly, there are exceptions such as the autotetraploid *Arabidopsis arenosa*. This species, despite random recombination of homologous chromosomes, rarely shows multivalent associations at metaphase I. Cytological diploidization is observed at that stage, but homologous chromosomes recombine with no partner preferences, and hence *A. arenosa* shows tetrasomic inheritance (Hollister *et al.*, 2012). The formation of bivalents instead of multivalents most likely is the result of a reduction in chiasma frequency or an increased interference (the phenomenon by which the formation of one crossover, CO, represses the formation of another CO nearby) among chiasmata (reviewed in Bomblies *et al*. 2016). Indeed, a reduction in chiasma frequency to one chiasma per pair of chromosomes has also been reported in other established autotetraploid species such as *Physaria vitulifera* and *Lotus corniculatus* (Mulligan, 1967; Davies *et al*., 1990).

Since meiotic stability is one of the hallmarks of an adapted polyploid, genes controlling meiotic recombination are strongly implicated in adaptation to WGD (Hollister *et al*., 2012; Yant *et al*., 2013). The study of the genetic factors that underlie the diploidization process have been more frequent and successful in allopolyploids than in autopolyploids. In wheat, the ***Ph1* locus** ensures diploid-like meiosis avoiding promiscuous recombination between homeologous chromosomes. It also avoids chiasma formation between wheat chromosomes and chromosomes from other related species in hybrids (Sears, 1976). This locus is situated on the long arm of chromosome 5B and it has been defined as a region containing a cluster of *CDK2-like* genes, S-adenosyl methionine-dependent methyltransferase (SAM-MTases) genes, and a gene that was firstly named as hypothetical 3 (*Hyp3*) (Griffiths *et al.*, 2006; Al-Kaff *et al.*, 2008; Martín *et al.*, 2017). Interestingly, the *Hyp3* gene has recently been reannotated as *TaZIP4-B2*. The corresponding orthologous genes in *A. thaliana* (*AtZIP4*) and *Oryza sativa* (*OsZIP4*) have an essential role in the formation of COs, although they are dispensable for normal synapsis (Chelysheva *et al.*, 2007; Shen *et al.*, 2012; Martín *et al.*, 2017; Rey *et al.*, 2017). Molecular and cytological studies have revealed that the absence of the *Ph1* locus leads to altered chromatin states during early meiosis (Mikhailova *et al.*, 1998; Greer *et al.*, 2012). It has also been shown that in the absence of *Ph1*, transcription of *TaASY1* (a gene that encodes a protein associated to

the meiotic chromosome axis) is dramatically increased (Boden *et al*., 2009). These changes are correlated with the increased pairing and recombination between homeologous chromosomes showed by the *ph1* mutants. However, even with the presence of a functional *Ph1* locus, multivalents are formed during prophase I (Martinez, *et al.*, 2001). This fact suggests that the *Ph1* locus has a role not only preventing but also correcting associations between homeologous chromosomes (Hobolth, 1981; Jenkins, 1983; Holm, 1988). Another locus that controls recombination between homeologous chromosomes in wheat is ***Ph2***, which is located on the short arm of chromosome 3D. The absence of this locus has a slight effect on homeologous recombination, although it produces a reduction or a delay in synapsis (Martinez *et al.*, 2001). However, the *ph2* mutation has a strong effect on homeologous recombination in wheat allohaploids, pentaploids and hybrids between wheat and rye (Grandont *et al*., 2013). In the allotetraploid species *Brassica napus*, it has been reported that the locus ***PrBn*** plays an important role in the suppression of homoeologous recombination (Jenczewski *et al*., 2003). Moreover, in this species, it has been recently reported that *MSH4* (an essential gene for the main CO pathway, see more in section 1.8) could play an important role reducing the formation of COs between homeologous chromosomes (Gonzalo *et al*., 2019). They showed that the absence of this gene prevents the formation of non-homologous COs in the allotetraploid *B. napus* and their number decrease when *MSH4* returns to single copy. In the allotetraploid *Arabidopsis suecica*, Henry *et al.* (2014) identified a genomic region limiting homeologous recombination, although the identity of the gene or genes as well as the mechanisms involved in this regulation are still unknown. The genetic control of the diploidization process in autopolyploids is even less known and so far no gene involved in this process has been identified (Grandont *et al*., 2013). Nevertheless, some meiotic genes seem to have undergone strong ploidy-specific selection (Hollister *et al*., 2012, Yant *et al*., 2013). These genes have been identified in the autotetraploid *Arabidopsis arenosa* and are involved in processes such as sister chromatid cohesion, meiotic axis formation and synapsis (Hollister *et al.*, 2012; Wright *et al.*, 2015).

In order to understand the formation of multivalents in polyploids, several models have been proposed. The simplest one is called **"random-end pairing" model** and predicts, in the absence of any pairing preferences among homologous chromosomes, a multivalent

frequency of 2/3 and 1/3 of bivalents (Sybenga, 1975; Jackson and Casey, 1982). This prediction is based on a situation in which the synapsis initiation would only occur at the chromosome ends. Therefore, each chromosome can pair and synapse with a homologous chromosome at each end with the same probability. The expected frequency (66 %) has been found in a range of synthetic and natural autopolyploids, while some species show lower and higher rates of multivalents. These deviations may be explained by various factors including the establishment of a genetic control or epigenetic alterations (Gillies, 1989; Sybenga, 1996; Santos *et al.*, 2003). In fact, in induced autotetraploid lines of *Aegilops longissima*, the multivalent frequency changes depending on the genetic background (Avivi, 1976). Additionally, first generations of induced autotetraploid lines of *Arabidopsis thaliana* display a high multivalent frequency which is reduced in the following generations (Santos *et al*., 2003).

1.7. Polyploidy in *Arabidopsis*

Arabidopsis constitutes a great model system for the analysis of both long- and short-term consequences of polyploidy. This genus has suffered several ancient polyploidy events, estimated to coincide with the glacial maxima, and has several recent polyploids (Novikova *et al*., 2018). Hence, it can provide important insights into molecular and cytological aspects of polyploid formation, establishment, and genome evolution. Natural autopolyploid and allopolyploid species are present within the genus, and several species occur both as diploids and tetraploids (Bomblies and Madlung, 2014). In addition, autopolyploids can be easily obtained by a colchicine treatment (Yu *et al*. 2009) or after regeneration from in vitro culture (Morris and Altmann, 1994).

The most famous species within the genus *Arabidopsis* is the self-compatible species ***A. thaliana*** (Figure 4), one of the most important model plant organisms for the study of biological processes (Somerville and Koornneef, 2002). It was the first plant genome to be fully sequenced and there is a massive number of mutant lines available that can be screened for phenotypes of interest (reviewed in Page and Grossniklaus, 2002). Moreover, there are more than a thousand accessions from different geographic locations almost all around the world (1001 Genomes Consortium, 2016). Although natural autopolyploid and allopolyploid species are present within the *Arabidopsis* genus, polyploid *A. thaliana* plants are extremely rare (Bomblies and Madlung, 2014). Only a handful of accessions of *A. thaliana* available in stock-centers are polyploids such as Wa-1, Bla-5, and M3385S (Schmuths *et al.*, 2004; Henry *et al.*, 2005; Chao *et al.*, 2013). Therefore, many studies that focus on the effects of polyploidization in *A. thaliana* have made use of artificial polyploid lines, created through treatments with colchicine. Researchers have analyzed them for several generations after the WGD (Santos *et al.*, 2003; Chen *et al.*, 2004; Yu *et al.*, 2009; Hegarty *et al.*, 2013). Diploid and newly induced tetraploid lines have been compared to infer the effects of polyploidization on transcriptome and gene expression patterns (Yu *et al*., 2010), epigenetics (Mittelsten Scheid *et al.*, 1996), abiotic stresses (Chao *et al*., 2013), and phenotypic traits (Miller *et al*., 2012; Fort *et al*., 2015).

On the other hand, ***A. arenosa*** is an obligate outcrossing plant with both diploid and polyploid populations (Schmickl *et al.*, 2012) (Figure 4). In this species, Jørgensen *et al.* (2011) reported the existence of gene flow between diploid and autotetraploid populations,

and this could be the reason of an increased genetic diversity in comparison with other species (Hazzouri *et al.*, 2008; Ross-Ibarra *et al.*, 2008; Hollister *et al.*, 2012). Natural autotetraploid individuals of *A. arenosa* present frequently 16 bivalents at metaphase I (Carvalho *et al.*, 2010; Yant *et al.*, 2013; Higgins *et al.*, 2014), although homologous chromosomes pair randomly at prophase I (Carvalho *et al.*, 2010). However, in *A. arenosa* autotetraploids generated by a colchicine treatment, an extensive multivalent formation is observed at metaphase I, suggesting that the autotetraploid populations have persisted in nature for a long period of time (Yant *et al*., 2013).

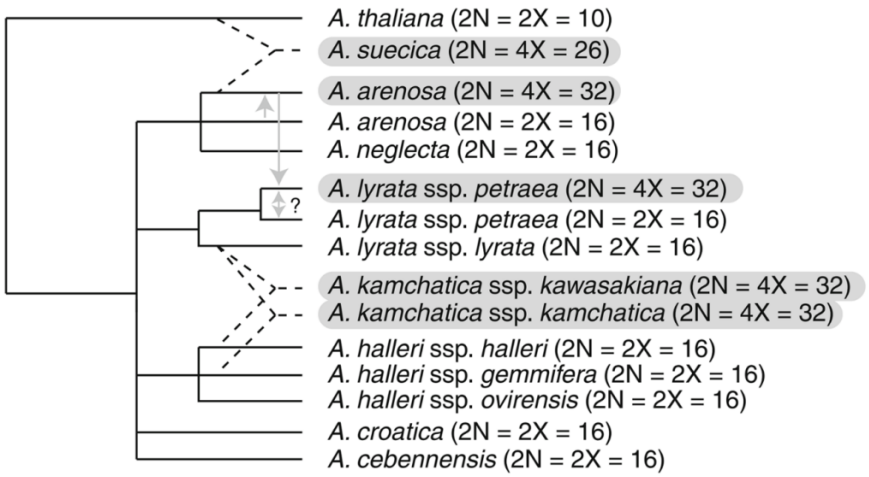


Figure 4. Phylogenetic tree of *Arabidopsis* species. Polyploid species are represented with gray shading. Dotted lines indicate parental species of the allopolyploids. A potential gene flow among species is represented with grey arrows. Modified from Bomblies and Madlung (2014).

A. lyrata is another member of this genus that presents autotetraploid populations (2*n* = 4*x* = 32) (Figure 4), although its distribution is narrower than those of *A. arenosa* (Dart *et al*. 2004). Autotetraploid plants of *A. lyrata* do not show aneuploidies in somatic cells, however cytological studies assessing meiotic chromosome configurations are missing. As it happens in *A. arenosa*, gene flow is evident between diploid and polyploid populations (Jørgensen *et al.*, 2011). Furthermore, in central Austria, there are reported cases of hybridization events between autotetraploid populations of *A. arenosa* and *A. lyrata* (Schmickl and Koch, 2011).

Naturally evolved allotetraploids can also be found in the *Arabidopsis* genus, with both ***A. suecica*** **and** ***A. kamchatica*** presenting a diploid-like behavior at metaphase I (Comai *et al*., 2003, Shimizu *et al*., 2005) (Figure 4). *A. suecica* has been probably formed from a single polyploidization event of a hybrid between *A. arenosa* and *A. thaliana* (Mummenhoff and Hurka, 1995; O'Kane *et al*., 1996; Jakobsson *et al.*, 2006). In addition to the natural populations, synthetic allotetraploids have been obtained by pollinating *de novo* autotetraploid individuals of *A. thaliana* with pollen from tetraploid plants of *A. arenosa* to study molecular events following allopolyploidization (reviewed in Chen, 2007; Madlung and Wendel, 2013). In the case of ***A. kamchatica***, there are two subspecies: *A. kamchatica* ssp. *kamchatica* and *A. kamchatica* ssp. *kawasakiana* (Shimizu-Inatsugi *et al.*, 2009; Higashi *et al*., 2012) (Figure 4). Both were probably originated from several allopolyploidization events between *A. lyrata* and *A. halleri* (Shimizu-Inatsugi *et al.*, 2009; Higashi *et al*., 2012; Schmickl *et al.*, 2012).

1.8. Meiosis and homologous recombination in *Arabidopsis*

Meiosis is a highly conserved and specialized cell division that is essential in organisms with sexual reproduction. This division takes place in the germ line and consists of a single round of DNA replication followed by two rounds of nuclear division, finally leading to the formation of gametes in which the chromosome number has been halved. The original chromosomal number is restored when gametes fuse together during sexual reproduction. At the first division, homologous chromosomes segregate towards opposite poles. For this purpose, they previously undergo pairing, synapsis, and **homologous recombination** (HR) to form COs, reciprocal exchanges of genetic information. These exchanges, together with the cohesion between sister chromatids, give rise to physical connections between the homologous chromosomes that can be cytologically visualized as **chiasmata**. Chiasma formation is required for a correct segregation of the homologous chromosomes at anaphase I but also to create genetic diversity within offspring.

Prophase I is the longest stage during meiosis, and it can be further divided into five substages: leptotene, zygotene, pachytene, diplotene, and diakinesis (Figure 5). These substages are cytologically distinguished based upon the level of chromatin condensation and the appearance of the chromosomes. At the **leptotene stage**, the pericentromeric heterochromatin and the nucleolar organizing regions (NORs) get more condensed. During this stage meiotic recombination is initiated by the formation of programmed **DNA double-strand breaks** (DSBs). The formation of these breaks is catalyzed by a complex that contains the protein **SPO11** (Keeney *et al*., 1997; Keeney, 2001) (Figure 6). In *A. thaliana*, there are three paralogous genes of *SPO11*: *SPO11-1, SPO11-2* and *SPO11-3* (Hartung and Puchta, 2000; Grelon *et al.*, 2001). Only the proteins SPO11-1 and SPO11-2 are involved in meiosis (Grelon *et al.*, 2001; Stacey *et al.*, 2006), whereas SPO11-3 seems to have a role in DNA endoreduplication (Sugimoto-Shirasu and Roberts, 2003). Subsequently, the 5’ ends of the DSBs are resected generating 3´-ended single-stranded DNA (ssDNA), then starting the **HR** process. In addition, the formation of the meiotic axis (axial elements, **AEs**) along the entire length of the chromosomes is completed (López *et al.*, 2008).

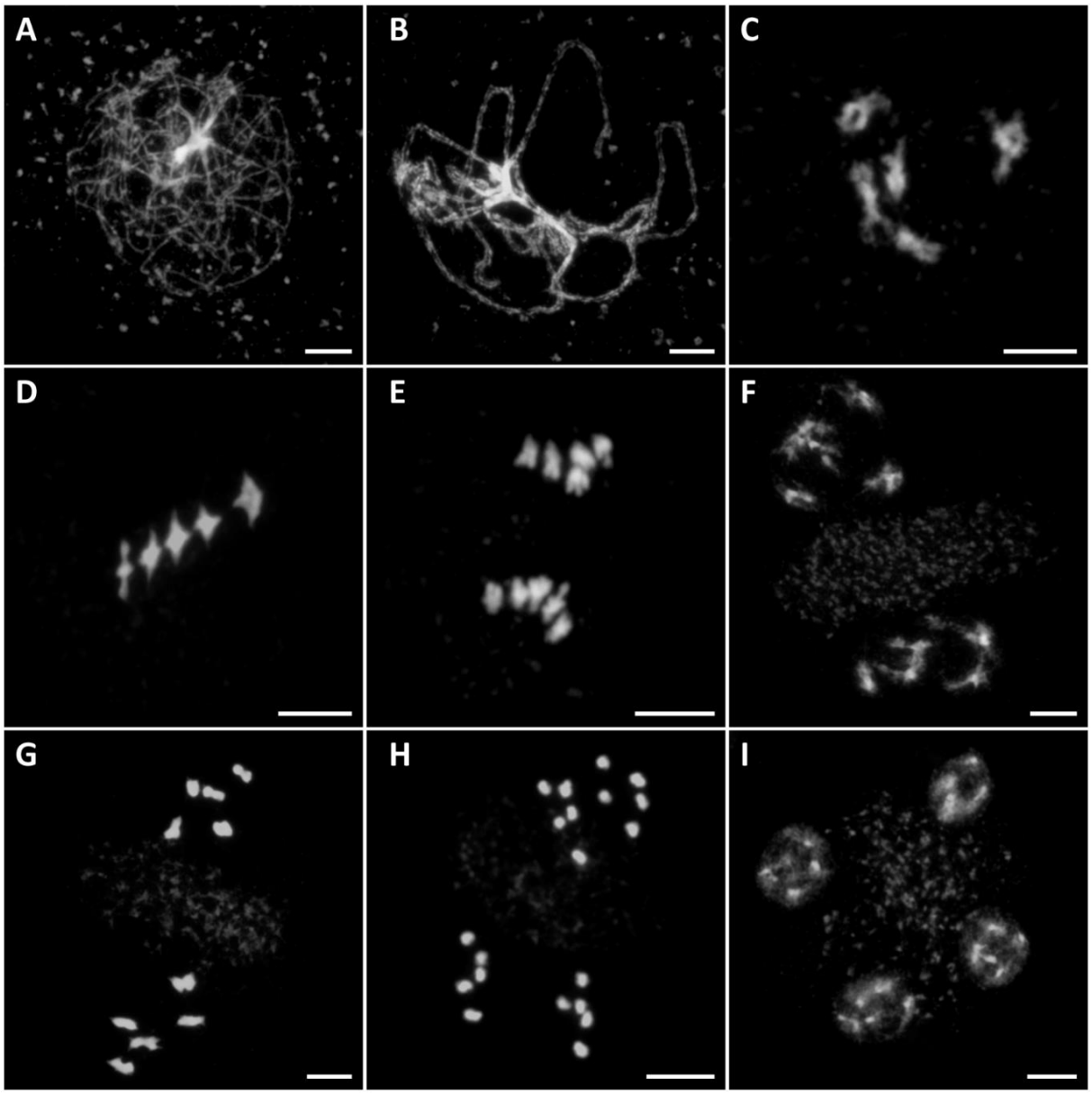


Figure 5. Meiosis overview in Pollen Mother Cells (PMCs) of *Arabidopsis thaliana* (2n=10). PMCs at different meiotic stages of a diploid *A. thaliana*. Zygonema **(A)**. Pachynema **(B)**. Diakinesis **(C)**. Metaphase I **(D)**. Anaphase I **(E)**. Prophase II **(F)**. Metaphase II **(G)**. Anaphase II **(H)**. Tetrad **(I)**. Scale bars represent 5µm.

During the **zygotene stage** (Figure 5A), the AEs of the homologous chromosomes are brought into a close juxtaposition via the formation of the **SC** (Page and Hawley, 2004). This is a tripartite structure consisting of a central element (**CE**) and two AEs, which are henceforth called lateral elements (**LEs**) (Fawcett, 1956; Moses, 1956). The SC is gradually formed between the homologous chromosomes until they are completely bound along their entire length at the **pachytene stage** (Figure 5B). Prior to the formation of the SC, there are two events: i) **alignment**, a DSB-independent event by which the homologous chromosomes

get closer along their entire length; and ii) **pairing**, which is a more stable and DSB-dependent association between the homologous chromosomes (Zickler and Kleckner, 1998). In plants, extension of the SC between homologs is required to enable stable CO formation (Higgins *et al.*, 2005). In *A. thaliana*, **ASY1**, a protein associated to the AEs, is essential for synapsis and CO formation (Ross *et al.*, 1997; Armstrong *et al*., 2001; Sanchez Moran *et al.*, 2001; Ferdous *et al.*, 2012). In this species, the CE of the SC is formed by **ZYP1A** and **ZYP1B.** In lines defective for these proteins, bivalents between non-homologous chromosomes, as well as multivalents, are observed (Higgins *et al.*, 2005).

The formation of 3´-ended ssDNA allows the loading of the recombinases **RAD51** and **DMC1**, which are essential proteins to promote the HR between homologous chromosomes (Klimyuk and Jones, 1997; Doutriaux *et al.*, 1998; Li *et al.*, 2004). At each DSB, one of the nucleofilaments invades the homologous DNA duplex of one non-sister chromatid allowing the occurrence of stable joint molecules called **single-end invasion intermediates** (Hunter and Kleckner, 2001). The free 3´-end in this intermediate is used as a substrate by the DNA polymerase to extend the 3´-ended ssDNA, increasing the size of the displaced DNA strand called **displacement-loop** (D-loop). The D-loop can be either dissociated or stabilized. In the former case, the **synthesis-dependent strand annealing** (SDSA) pathway drives to the formation of **non-COs** (NCOs), non-reciprocal exchanges that can lead to gene conversion events (Allers and Lichten, 2001). In contrast, D-loop stabilization implies the formation of the **double Holliday junction** (dHj) (Osman *et al.*, 2011). At this point, dHjs can be resolved or dissolved. In the case of resolution, dHjs lead to the formation of COs or NCOs depending on how these recombination intermediates are resolved to release chromatids (Snowden *et al.*, 2004). If the dHj is dissolved, single Holliday junctions (Hjs) migrate one towards the other to finally be decatenated, giving rise to an NCO product (Bizard and Hickson, 2014) (Figure 6).

Focusing on the CO formation mechanisms, two types of COs have been described in *A. thaliana* (as in most species), called **class I and class II** (Copenhaver *et al*., 2002) (Figure 6). In this species, the mean chiasma frequency in pollen mother cells (PMCs) is from 8 to 12 (Sanchez-Moran *et al.*, 2002). **Class I** COs are sensitive to positive interference, non-randomly distributed, and responsible for approximately **85 % of total COs** (Higgins *et al*., 2004). These class I COs are also necessary for **CO assurance**, a mechanism by which at

least one CO must occur between each homologue pair, referred to as the obligate CO (Jones and Franklin, 2006). A wide number of genes encode proteins that underlie the control and formation of this CO pathway. Among these proteins are **MSH4 and MSH5,** related to the prokaryotic MutS proteins (Higgins *et al.*, 2004, 2008a). These proteins could play an important role in the stabilization of the dHjs. In *A. thaliana,* they are localized on the chromosome axis from leptonema to pachynema and their presence depends on the formation of DSBs. During the leptotene stage, numerous AtMSH5 foci are observed and this number is gradually reduced during zygonema until pachynema (Higgins *et al.*, 2008a). AtMSH4 and AtMSH5 proteins belong to a heterogeneous group of proteins called **ZMM** (Figure 6). Besides these two proteins, other members of this group are: **ZIP2/SHOC1, ZIP4, MER3/RCK, AtPTD** (reviewed in Osman *et al.*, 2011) and the last described **HEI10/ZIP3** (Chelysheva *et al.*, 2012). These are proteins involved in the class I CO pathway, probably having a regulatory or auxiliary role. All the corresponding mutants present a similar reduction of chiasma frequency and, unlike the situation in budding yeast, they apparently display normal synapsis. In addition, two other proteins have been reported to have a major function resolving the dHj to form interference-sensitive COs. These proteins are **MLH1 and MLH3**, which are homologues of the prokaryotic MutL (Franklin *et al.*, 2006) (Figure 6). In wild-type individuals, it is possible to observe around nine foci of these two proteins co-localizing at the pachytene stage (Jackson *et al.*, 2006). In the mutant *Atmlh3,* the presence of AtMLH1 is compromised, although pairing, synapsis, and dHj formation are apparently normal. The absence of AtMLH3 implies 60 % reduction in the number of chiasmata per cell (Franklin *et al.*, 2006; Jackson *et al.*, 2006).

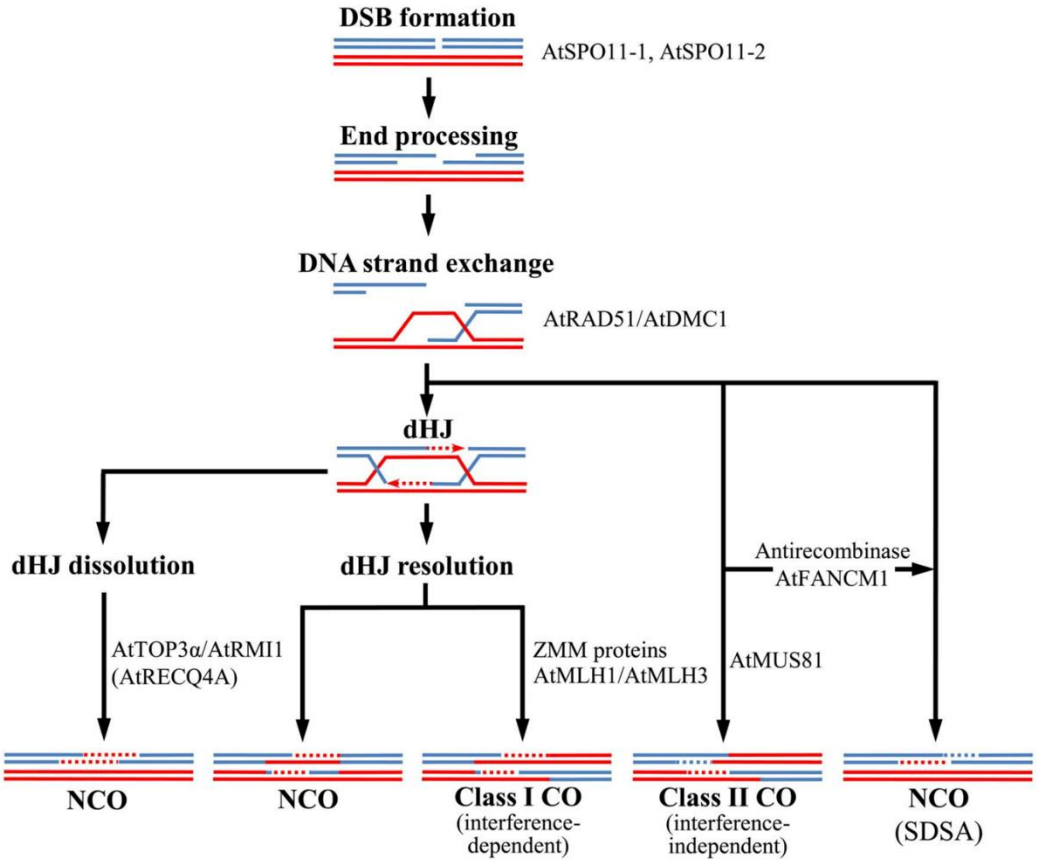


Figure 6. Model for CO and NCO formation pathways in *Arabidopsis thaliana*. After the formation of DSBs, 5´ends are resected to generate 3´-ended ssDNAs. The ssDNA invades a homologous DNA duplex, forming a D-loop intermediate. If the other ssDNA is captured and both DNA strands are ligated, a dHjs intermediate is formed. The dHjs can be resolved forming either class I COs or NCOs but can also be dissolved forming NCOs. Alternatively, D-loop intermediates can be processed forming class II COs or NCOs. The main proteins involved in these pathways are shown. Modified from Pradillo *et al.* (2014).

In *A. thaliana*, the analysis of different mutants of the ZMM genes revealed that the COs they show (around **15 % of total COs**) fit a Poisson distribution and hence have no interference as it has been reported in other organisms such as budding yeast (Copenhaver *et al.*, 2002; Mercier *et al.*, 2005). These COs belong to the so-called **class II** and are dependent on the heterodimer formed by MUS81 and EME1A/B proteins. The single mutant *Atmus81* presents a moderate decrease in COs in genetic intervals on the chromosomes 1 and 3, although this reduction does not affect fertility (Berchowitz *et al.*, 2007). However, the double mutant *mus81/msh4* was reported to have a significant reduction in COs (0.8) compared to the *msh4* single mutant (1.25). The presence of some residual COs in double mutants defective for the formation of both CO classes suggests the existence of at least

another pathway of CO formation. Recent research suggest that the protein **FANCD2** could be involved in a third pathway (Higgins *et al*., 2004; Kurzbauer *et al.*, 2018).

In addition to the pro-CO proteins, there are others that limit the number of COs. During the last years several **anti-recombinases** have been reported, such as **FANCM** (Crismani *et al.*, 2012; Girard *et al.*, 2014), **RMI1** (Chelysheva *et al.*, 2008; Hartung *et al.*, 2008), **TOP3α** (Séguéla-Arnaud *et al.*, 2015, 2017), and **FIGL1** (Girard *et al.*, 2015) (Figure 6). The gene *AtFANCM* is the *A. thaliana* homologue of the human *FANCM* (*Fanconi anaemia complementation group M*). This gene was discovered in a screening to find suppressors of the phenotype generated by the *Atzip4* mutation (Chelysheva *et al.*, 2007). In the single *Atfancm* mutant, it was observed a threefold increase in genetic distance in eight genetic intervals distributed in four different chromosomes compared to the control, although this result was not obvious by a chiasma counting assessment. Crismani *et al.* (2012) also reported an absence of interference among the COs formed in *Atfancm.* In addition, the "extra" COs that appear in this mutant cannot be detected by immunolocalization of AtMLH1. These results suggested a specific function for AtFANCM limiting the formation of class II COs. Moreover, it seems that the CO increase produced by mutations in *AtFANCM* are not observed in hybrids obtained from two different *A. thaliana* accessions (Girard *et al.*, 2015; Ziolkowski *et al.*, 2015).

Another protein that might play an essential role regulating the formation of COs is **CAND1**. In mammals, this protein regulates the ubiquitination process by getting bound to the unneddylated CULLIN1 (CUL1) (Liu *et al.*, 2002; Zheng *et al.*, 2002; Hwang *et al.*, 2003; Oshikawa *et al.*, 2003). In *A. thaliana*, the ***hve-1*** mutant, defective for CAND1, was discovered by Alonso-Peral *et al.* (2006) in a screening for natural variations in the leaf venation pattern. The mutant presents defects in fertility, inflorescences with reduced size and simple venation patterns. Studies conducted in our lab, which have not yet been published, have revealed that this mutant is also defective in CO formation, displaying univalents at metaphase I.

Once DSBs have been repaired, meiocytes enter the **diplotene stage**. During this stage, the SC disappears and the homologous chromosomes progressively separate from each other, except from the sites where chiasmata are located. Chromosomes are progressively condensed during **diakinesis** (Figure 5C) and acquire the maximum condensation at **metaphase I** (Figure 5D). At this stage, the five bivalents are co-oriented on the spindle and the homologous centromeres are directed towards opposite poles. Each bivalent contains from one to three chiasmata. During **anaphase I** (Figure 5E), the cohesion between sister chromatids is lost (except in centromeric regions) and the homologous chromosomes migrate towards opposite poles of the cell. The two groups of five chromosomes are partially decondensed during **telophase I**. In *Arabidopsis*, the organelles form a barrier between the two newly formed nuclei that is visualized during the whole second meiotic division. The transition between the first and the second meiotic division is rapid and characterized by the absence of DNA replication and cytokinesis. During **prophase II** (Figure 5F), the five chromosomes in each nucleus progressively get condensed again until **metaphase II** (Figure 5G). Then, sister chromatids are separated to opposite poles at **anaphase II** (Figure 5H). At **telophase II,** four nuclei within a common cytoplasm are visualized. Finally, the nuclear envelope is regenerated again, cytokinesis occurs, chromosomes are decondensed again, and a **tetrad** with the four haploid products can be visualized that eventually will give rise to gametes (Figure 5I) (Armstrong and Jones, 2003).

1.9. *Arabidopsis* as a model for the study of polyploid meiosis

Arabidopsis is a good model not only for the study of meiosis but also for understanding mechanisms associated with diploidization after genome duplication. Both *Arabidopsis* autopolyploids and allopolyploids occur in nature and can be easily generated in the laboratory using colchicine, thus providing an attractive system for comparing meiotic chromosome behavior among neopolyploids and established polyploids. However, until the date, no gene has been described to be involved in the control of the diploidization process in this species.

In the naturally evolved autotetraploid *A. arenosa*, the meiotic axis gene *ASY1* appears to have undergone strong ploidy specific differentiation. A specific allele found in tetraploid individuals (consisting on a modification that generates an amino acid substitution in a highly conserved HORMA domain of the corresponding protein) acts to shift chiasmata to a more distal position along the chromosomes in comparison with the situation in diploid plants (Hollister *et al*., 2012). In this context, studies have also indicated that *A. arenosa* autotetraploids experience a reduction in CO frequency per bivalent compared to diploid plants. It has been suggested that this phenomenon helps to stabilize meiosis by inhibiting multivalent formation and preventing multiple COs occurring among more than two homologous chromosomes (Yant *et al*., 2013).

In *A. thaliana*, Santos *et al.* (2003) compared the meiotic behavior of recently colchicine-induced autopolyploid lines with several established autopolyploid lines that were maintained at least 13 generations after the WGD induction in a Columbia background. They found that established autotetraploid lines had lower multivalent frequencies than the new autotetraploid lines, suggesting a rapid adaptation to genome doubling by cytological diploidization. Moreover, the reduction in multivalents was higher in the smallest chromosomes (2 and 4). Santos and colleagues suggested that some factors such as chromosome size and/or chromosome structure, as well as the epigenetic features, could affect the cytological diploidization levels at least at the very early stages of the process.

In this thesis, several approaches have been conducted to shed some light into the meiotic HR process in polyploids of *Arabidopsis thaliana* under different circumstances. Firstly, the study has been focused on the cytological and molecular comparison of polyploid

individuals at different stages of the diploidization process. For this purpose, we have used synthetic autotetraploid lines, established autotetraploid lines, and natural autotetraploid accessions. Specifically, we have identified possible changes during the diploidization process in the formation of COs but also in the expression levels of some meiotic recombination genes. Secondly, meiotic recombination has been cytologically characterized in intraspecific polyploid hybrids to analyze CO formation in a situation in which identical and homologous chromosomes are present. Finally, the consequences of doubling the sets of chromosomes under a situation in which the meiotic HR is compromised have been analyzed. A wide number of synthetic polyploid mutant lines with the chiasma frequency altered has been studied. These polyploid mutant lines were characterized by cytology, and in some of them, the expression levels of the principal meiotic recombination genes were assessed.

2. Thesis objectives

This thesis has been conducted as part of a Marie Curie Initial Training Network (ITN), "Control of meiotic recombination" (COMREC), funded by the European Union under the FP7 program. This research consortium aimed at determining the factors that regulate the frequency and distribution of crossovers during plant meiosis and translating this knowledge to develop novel strategies for improving breeding methods in key crop species. Specifically, our project was focused on understanding the control of meiotic recombination during the diploidization of autopolyploids in the model species *Arabidopsis thaliana*. This general objective has been addressed through a set of specific objectives:

- To gain a clearer understanding of changes in meiotic recombination that take place during different stages of the cytological diploidization process by analyzing synthetic, established, and natural autotetraploid lines.
- To determine the impact of intraspecific differences in the cytological diploidization process of autopolyploids.
- To understand the processes underlying the genetic control of diploidization in autopolyploids by inducing polyploidy in different key meiotic recombination mutants.

To reach these aims, we have developed an improved cytological protocol, in comparison with those previously used, to obtain proper spreads of metaphase I chromosomes in autotetraploids. The protocol is detailed in the first section of the results and it allows an accurate analysis of the different meiotic configurations and the assessment of the number of chiasmata formed in each tetrasome.

3. Materials and methods

3.1. Materials

In order to perform the experiments described in this thesis, the model organism *Arabidopsis thaliana* was used. Several features such as the small size, prolific seed production, short life cycle, completely sequenced genome (The Arabidopsis Genome Initiative, 2000), easy cross-pollination, vast number of mutant lines available in stock centers, easy obtention of polyploid individuals, and numerous database resources make this species the most used model organism for the study of angiosperms in a very diverse range of fields.

3.1.1. Natural accessions

Table 1 includes the natural accessions used in this thesis. Bla-5, M3385S and Wa-1 were selected since they are natural autopolyploids. The accessions Bla-1 and St-0 were used as diploid controls due to their geographical distribution, with proximity to those of Bla-5 and M3385S, respectively. In addition, the most commonly used accessions Col-0 and L*er*-1 were also analyzed.

Table 1. Polyploid and diploid accessions of *A. thaliana* used in this thesis

Accession	Geographical distribution	Ploidy level	NASC code
Bla-1	Blanes, Spain	2x	N970
Col-0	Columbia, USA	2x	N1092
L*er*-1	Landsberg, Germany	2x	N1642
St-0	Stockholm, Sweden	2x	N1534
Bla-5	Blanes, Spain	4x	N978
M3385S	Stockholm, Sweden	4x	N6183
Wa-1	Warsaw, Poland	4x	N1586

NASC: Nottingham Arabidopsis Stock Centre.

3.1.2. Established autopolyploid lines

The established autopolyploid lines 3151 (Col-1 background) and 3432 (Col-0 background) were obtained by colchicine treatment and maintained several generations after the WGD induction (Santos et al. 2003). At the beginning of this study, at least 13 generations had elapsed after the WGD. Analyses conducted in this thesis were performed using plants corresponding to at least the 20th generation after the WGD.

3.1.3. Mutant lines

Different diploid T-DNA insertion lines were used to produce mutant autopolyploid lines (Table 2). All of them belong to the same accession, Col-0. Seeds of the diploid mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC). These lines were selected because they present univalents at metaphase I and show no evidences of chromosome fragmentation. In addition, the lines *fancm*-1, defective for an anti-CO protein, which displays an increased mean cell chiasma frequency, and *mus81-2*, defective for the formation of class II CO, were also analyzed.

Table 2. T-DNA insertion lines used to obtain autopolyploid mutants

Gene	AGI code	Mutant line	Reference	T-DNA line
ASY1	At1g67370	*asy1-2*	Sanchez-Moran *et al.* (2007)	SALK_144182
ZIP4	At5g48390	*zip4-2*	Chelysheva *et al.* (2007)	SALK_068052
MSH5	At3g20475	*msh5-1*	Higgins *et al.* (2008)	SALK_110240
MER3	At3g27730	*mer3*-1/rck-*2*	Chen *et al.* (2005)	SALK_045941
HEI10	At1g53490	*hei10-2*	Chelysheva *et al.* (2012)	SALK_014624
MLH3	At4g35520	*mlh3-1*	Jackson *et al.* (2006)	SALK_015849
MUS81	At4g30870	*mus81-1*	Berchowitz *et al.* (2007)	SALK_107515
FANCM	At1g35530	*fancm-9*	Crismani *et al.* (2012)	SALK_069784
CAND1	At2g02560	*hve-2*	Alonso-Peral *et al.* (2006)	SALK_099479

AGI: Arabidopsis Genome Initiative.

3.2. Methods

3.2.1. Plant growth

Seeds were sowed in a sterilized mix of soil (75 %) and vermiculite (25 %) and grown under controlled environmental conditions: 16h light/ 8h night photoperiod, 19°C temperature, and 60 % of relative humidity.

For some experiments, seeds were sown in Petri dishes with MS medium (Murashige and Skoog, 1962) complemented with sucrose (10 g/L) and agar (10 g/L), pH 5.7. In these experiments, seeds were previously sterilized in a 2.5 % solution of bleach diluted in water for 5 minutes followed by 3 washes in sterilized deionized water (SDW).

3.2.2. Production of synthetic autopolyploid lines

Colchicine is an alkaloid substance known to stop microtubule polymerization, which takes place during the cell division (Cook and Loudon, 1952). Taking advantage of its effect, a treatment with this drug allows the production of polyploid individuals. Several methods can be used for this purpose (Yu *et al.*, 2009). In this thesis, the treatment with this drug consisted on applying a 10 µL drop of colchicine (0.25 % w/v) at the center of the plant rosette, prior to the first flowering. The concentration used was prepared by diluting the stock concentration (Sigma) in SDW. Aliquots can be stored at 4°C up to one month.

3.2.3. Genotyping of mutant lines

3.2.3.1. DNA extraction

DNA was extracted by squeezing a young leaf in 40 µL of the extraction buffer (100 mM Tris-HCl, pH 9.5; 250 mM KCl; 10 mM EDTA). Subsequently, samples were incubated at 95°C for 10 minutes. Then, samples were incubated on ice for at least 5 minutes, and then 40 µL of the dilution buffer (BSA 3 %) were added to each tube. Finally, the extracts were

centrifuged at maximum speed for 1 minute. Samples were stored up to 2 weeks at 4°C until needed.

3.2.3.2. PCR and electrophoresis

In order to check the presence of T-DNA insertions in the lines included in Table 2, PCRs were conducted. For this purpose, extracted DNA was amplified using the BioMix™ Red (Bioline). The presence of the T-DNA insertion was determined with a forward (LP), a reverse (RP), and a T-DNA primer (BP) (Table 3). Both, the LP and RP primers, were designed using the "T-DNA primer design tool" (http://signal.salk.edu/tdnaprimers.2.html) from the Salk institute. For all the lines analyzed, the BP primer used was LBb1.3 (5´ ATTTTGCCGATTTCGGAAC 3´). The use of the three primers allows the production of DNA amplicons with different sizes for the wild-type allele and the T-DNA insertion allele (mutant allele), thus, being able to determine whether an individual is wild-type (WT), homozygous (HM) or heterozygous (HZ) for the T-DNA insertion (Figure 7).

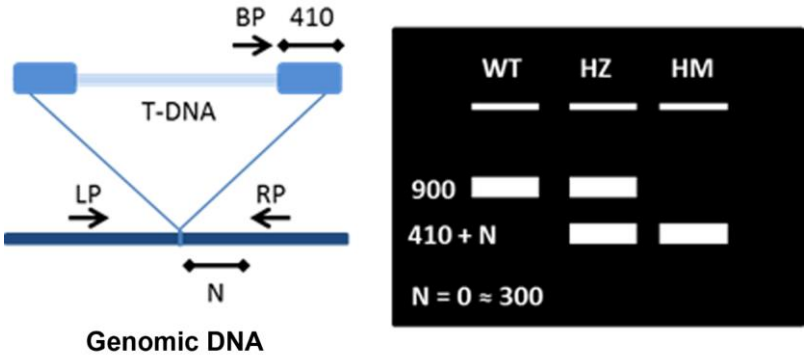


Figure 7. Genotyping of T-DNA insertion lines. The use of three primers (LP: "left primer"; RP: "right primer"; BP: "border primer") in the PCR allows the formation of a 900-1,100 bp product in wild-type individuals (WT) (due to the amplification with LP and RP) and a 410+N bp product in individuals homozygous (HM) for the T-DNA insertion (due to the amplification with RP and BP). The heterozygous individuals (HZ) present both products. N represents the distance between the T-DNA insertion point and the RP primer which is approximately 300 bp.

For the DNA amplification, the following program was used:

5 minutes at 94°C
30 seconds at 94°C
1 minute at 55°C
2 minutes at 72°C
} 35 cycles
10 minutes at 72°C

Agarose gels (1.5 % w/v) were prepared with TBE buffer 0.5x (for a 5x TBE stock: 54 g Tris-HCl; 27.5 g boric acid; 20 ml EDTA 0.5 M, pH 8; and make up to 1 L with SDW) and used for electrophoresis to evaluate the amplicons obtained in the PCR. To observe DNA molecules, the SYBR-green (Invitrogen) reagent was used. As a reference for the size of the DNA bands, a 100 bp ladder (Bioline) was also loaded in every gel.

Table 3. Primers to genotype the T-DNA insertion lines

Mutant line	5´-3´ sequence
asy1-2	LP: CAATAACACAGACCCTTCATC RP: CTCTGTTCAAGTTCTCTCAG
zip4-2	LP: TGTGTTCTTAGGTCTGCTGGG RP: AAGCTTAGAAGCCCTGCTCTG
msh5-1	LP: AACCGATCGTCATTTGTTCTG RP: CACTAAGGCCTGCTGAATTTG
mer3-1	LP: AATCAACCTGCCTGCACATAC RP: TCTGGTTTCAAAACAAAACCG
hei10-2	LP: CAGTGGAAGAACAAGCAAAGG RP: CCAAGAACCCGACTTTTTCTC
mlh3-1	LP: GTAGCCCCAAGAAAGTTTTGG RP: GCCTAGGAATGTCAAAGGGAC
mus81-2	LP: TGGTGAAATCTAGCAACCCAG RP: AATTTTCCACAAACCCTTTGG
fancm-1	LP: CCTCAATCTGCTGCATCAC RP: GGATCTAGGGTTCCAATAG
hve-2	LP: ATTTCGGATCCCATCAAAATC RP: AAGCTATACTCGCGAAGCTCC

3.2.4. RT-qPCR analysis

3.2.4.1. RNA extraction

In order to compare the expression levels of several meiotic genes among the different genetic backgrounds used in this thesis, total RNA extractions were obtained from both, 10-days seedlings (~50 seedlings per sample) and flower buds (from at least 10 different plants per sample). These extractions were performed using the commercial kit "InviTrap® Spin Plant RNA Mini Kit" (Invitrogen).

3.2.4.2. RT-qPCR

Gene expression analyses were conducted by the real time quantitative PCR (RT-qPCR) technique. For this purpose, a "Taqman" probe and a pair of primers is needed for each gene analyzed. All the probes used belong to the "Universal Probe Library" (UPL, Roche). The genes assessed as well as the primers and UPLs used are listed in Table 4. The RT-qPCR technique was conducted in the facilities of "Parque Científico de Madrid" at the Universidad Autónoma de Madrid using the LightCycler®480 system (Hsieh *et al.*, 2016). At least three technical replicates were done. In order to normalize the results, two reference genes were assessed, *ACTIN2* and *YLS8*. As the study involved the assessment and comparison of diploid and tetraploid plants, the reference genes were selected due to their suitability for this kind of experiments according to the data published by Wang *et al.* (2014).

Table 4. Genes analyzed by RT-qPCR, UPL probes, and primers

Gene	AGI code	UPL probe	Primers (5´-3´ sequence)
SPO11-1	At3g13170	143	LP: TTCCCAAACAGTGTCTTTTGC RP: TTCAAGTTCCAACCTCCATTG
MRE11	At5g54260	8	LP: GTTTCCGCCAGTCTCAAAGA RP: TTCTCCAATGGTGGAAGCA
RAD51	At5g20850	91	LP: CATGCCACCACAACAAGG RP: ACATGGCGAGCTTATCACTTTAC
DMC1	At3g22880	31	LP: TCAACGTTGCTGTCTACATGACT RP: GACCACCTGCTGGCTTTTT
SMC1	At3g54670	91	LP: CGTGACATGGAACAGCTCTC RP: TCAAGTATGAAAAAGGGTGAAGG
HEI10	At1g53490	3	LP: GACACCAAGAACCCGACTTT RP: GTCCTGGTGTTGCAGGAGAG
MLH3	At4g35520	47	LP: GACTGAAGCAGACCTCACTTTG RP: GCCTTCAAATCGACAAGAGG
MUS81	At4g30870	29	LP: GATATGTACCCAACGCTTTTGTC RP: CTTCTTGCGCCGAGACAT
FANCM	At1g35530	143	LP: TCGCGGTAAAAAGCACATTA RP: GACATCTCTGCTCCCGAAGA
RECQ4A	At1g10930	25	LP: GCAAAGTACTCTTCCACTTACAACAG RP: ACATGTTGGCAGAGAGATTCAC
CAND1	At2g02560	156	LP: ACCTGAAGATGCTTTGTCATCTTAT RP: TGGTTCCACAAGAGAATCCAG
ZYP1a	At1g22260	4	LP: TGTCAAAAATATTGAAGAAAGCTCA RP: CTTCATACTCACGATGTGTCACC
ZYP1b	At1g22275	119	LP: TCGTCGGTCAAAGTGAAAGA RP: CACAGATGTTACTTTTGCCTTTACA
ASY1	At1g67370	22	LP: CATTAGCAACAAGGCACAGC RP: GTTGACTGTCTCGGCTTCCT
ASY3	At2g46980	134	LP: TCAGAGCTGAAAGGAAGCATAA RP: CATCGTCCAGTTTTGTCTCG
SYN1	At5g05490	1	LP: AGCTTGTTAAGAGATTCCACTTGAG RP: AATCAGACGAGATGATAACAGATAGC
PDS5B	At1g77600	33	LP: TTAATTCTAAAAGCAACTCTCACAAGA RP: CATGGATTCATGGCTCAGG
MRD1	At1g53480	119	LP: TTTTCGTGTCATTGGGTGAA RP: CTACCCAACCGAAATTCACTG
ACTIN2	At3g18780	30	LP: CCGCTCTTTCTTTCCAAGC RP: CCGGTACCATTGTCACACAC
YLS8	At5g08290	22	LP: TTGGTGATTGCTCCAAAAGA RP: AGTGTTGGGAAGCTCGATTAGT

3.2.4.3. RT-qPCR data analysis

The data obtained from the RT-qPCR were analyzed in order to find differences in gene expression among the different genetic backgrounds used in this study. For this purpose, the ΔCt method was performed according to Schmittgen and Livak (2008). Thus, a mean Ct value was obtained from the Ct values of the three technical replicates conducted for each gene in every genetic background. Then, the mean Ct value was normalized by the mean of the Ct values obtained for the reference genes *ACT2* and *YLS8* ($\Delta Ct = \text{mean } Ct_{\text{gene of interest}} - \text{mean } Ct_{\text{reference genes}}$) (Riedel and Bock, 2014). Then, in order to do pairwise comparisons of the expression levels of each gene among the different genetic backgrounds assessed, the difference of the ΔCt values of a determined gene in two genetic backgrounds was calculated; e.g., the difference in the expression levels of the gene "a" between the genetic background "x" and "z" is calculated as $\Delta Ct_{a \text{ in } x} - \Delta Ct_{a \text{ in } z} = \Delta\Delta Ct$. In order to determine the statistical significance at the level of 99 % of the pairwise comparison, the confidence intervals of the ΔCt values were compared. As the interpretation of the ΔΔCt value is difficult from a biological point of view, the RQ value ($2^{-\Delta\Delta Ct}$) was calculated and represented in plots (Schmittgen and Livak, 2008). This value refers to the fold change in the expression of a gene between two different genetic backgrounds.

3.2.5. Cytological analysis of male meiocytes

3.2.5.1. Chromosome preparations by spreading

For the obtention of chromosome preparations, the spreading technique was conducted using flower buds in order to assess PMCs at the metaphase I stage. This was carried out following the procedure previously developed by Fransz *et al*. (1998). However, due to the use of polyploid material, several modifications had to be made to obtain a proper chromosome spreading (Parra-Nunez *et al*., 2020).

Flower buds were fixed in Carnoy fixative solution (6 parts of absolute ethanol, 3 of chloroform and 1 of glacial acetic acid) during around one week and no more than two, at room temperature (RT). Time of fixation is important because the less time the material is

fixed, the better is the spreading. After the fixation of the flower buds, inflorescences were washed twice during 2 minutes in 3:1 fixative solution (3 parts of absolute ethanol and 1 of glacial acetic acid). Then, flower buds were individualized and those with a proper size were selected (0.3 - 0.4 cm). After that, they were washed in citrate buffer 1x (obtained from the dilution of a 10x citrate buffer 10 mM, pH 4.6 with SDW) twice for 2 minutes. Next, flower buds were placed in a moist chamber and digested with 500 µL of the enzyme mix (0.3 % w/v celulase, 0.3 % w/v pectolyase, and 0.3 % citohelicase diluted with 10x citrate buffer) for 2 hours and 30 minutes, at 37°C. After the digestion, the reaction was stopped by adding 1 mL of ice-cold SDW. For each slide, a single flower bud was placed with a small volume of buffer and squeezed with a needle. Then, a 15 µL drop of 60 % ice-cold acetic acid (glacial acetic acid diluted with SDW) was applied and the slide was placed on a hot plate at 41°C for 1 minute. During this time, the drop was spread making circles by the mean of a needle. After that time, another 15 µL drop of 60 % ice-cold acetic acid was applied. Then, 200 µL of ice-cold 3:1 fixative solution were applied making a circle around the material. Additionally, 200 µL of ice-cold 3:1 fixative were applied at the top of the material and 200 µL more to wash the slide. Finally, the slide was dried using a hair dryer and stored at RT. To observe the chromosome spreads under an epi-fluorescence microscope, each slide was stained with 10 µL of DAPI (4', 6-diamidino-2-phenylindole) at the concentration of 1 µg/mL diluted in the antifade mounting medium Vectashield® and mounted placing a coverslip. Once the slides were mounted, they were stored at 5°C.

3.2.5.2. Fluorescence *in situ* hybridization

This technique allowed the identification of each chromosome using the rDNA probes 5S (cloned in the pCT4.2 plasmid) from *A. thaliana* (Campell *et al.*, 1992) and 45S (cloned in the pTa71 plasmid) from *Triticum aestivum* (Gerlach and Bedbrook, 1979). In the accessions Bla-1, Bla-5, Wa-1, M3385S, and St-0 the use of these two probes allowed the identification of the chromosomes 2, 4, and 5 (Chr 2, 4, and 5) whereas the differentiation between the chromosomes 1 and 3 (Chr 1 and 3) was not possible due to the lack of the 5S rDNA locus in the chromosome 3 (Figure 8). This technique was performed following the protocol described by Sanchez Moran et al. (2001) with minor changes.

- **Probe labelling:** To be able to detect probes on the chromosomes after the FISH protocol, they had to be labelled by a nick translation reaction. Biotin-dUTP and digoxigenin-dUTP molecules were used for this purpose. The first one was used to label the 5S probe and the second one for the 45S probe. Nick translation reactions were performed using the commercial kit of Enzo Life Sciences®.

- **Slide preparation for hybridization:** slides previously stained with DAPI, were washed in a coupling jar with 4T buffer (4x SSC from 20x SSC diluted with SDW and 0.05 % v/v of Tween 20, pH 7) at RT until the coverslip fell. If the coverslip had immersion oil, it was removed washing slides in a coplin jar with absolute ethanol at RT. After that, slides were washed in 4T buffer a minimum of 1 hour (usually overnight, ON). To prepare the slides for the hybridization step, they were washed in 2x SSC buffer (obtained diluting 20x SSC buffer, 0.3 M NaCl, 0.03 M sodium citrate at pH 7) for 10 minutes at RT. Then, they were incubated in a pepsin solution (0.01 g of pepsin, 99 mL of SDW and 1 mL of HCl 1 M) for 90 seconds at 37°C. After that, they were washed again in 2x SSC buffer for 10 minutes at RT. To fix the material, slides were incubated in 4 % paraformaldehyde (4 % w/v, pH 8) for 10 minutes at RT. Then, slides were rinsed in SDW twice for 5 minutes at RT and dehydrated in an ethanol series of 70 %, 90 % (diluted with SDW) and 100 % for 2 minutes each concentration. After the dehydration, slides were placed in darkness for a minimum of 30 minutes at RT until the ethanol was completely evaporated.

- **Hybridization:** To prepare the probe mixture, per each slide, 3 µL of each probe were added and the final volume was brought up to 20 µL with the hybridization mixture (5 mL of deionized formamide, 1 mL of 20x SSC buffer, and 1 g of dextran sulfate at pH 7). The probe mixture was denatured for 10 minutes at 80°C. Later, the mixture was placed on ice for 5 minutes. Then, 20 µL of the mixture were applied on a coverslip and each slide was mounted with it. The denaturation of the slides with the probe mixture was performed on a hot plate for 4 minutes at 72°C. Finally, slides were incubated ON at 37°C.

- **Slide preparation for the detection of probes:** Coverslips from slides were removed and then slides were washed twice in 50 % formamide-2x SSC buffer (for a 200 mL final

volume: 100 mL of deionized formamide, 20 mL of 20x SSC, and 80 mL of SDW) for 7 minutes each one. Then, slides were washed in 2x SSC buffer for 5 minutes and in 4T buffer for another 5 minutes, both at 45°C. Finally, they were washed again in 4T buffer for 5 minutes at RT.

- **Detection of probes:** the detection mixture was prepared by diluting FITC-anti-digoxigenin (final concentration 200 ng/µL) and Cy3-streptavidin (final concentration 1 ng/µL) in TNB (100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.5 % w/v of Boehringer blocking reagent). 50 µL of the detection mixture were used per each slide. This volume was applied on a piece of Parafilm® and this was put on the slide. Then, slides were incubated for 1 hour at 37°C. After that, the Parafilm® piece was removed, and slides were washed three times in 4T buffer for 5 minutes each one, at RT. Finally, slides were stained and mounted applying 10 µL of the DAPI-antifade solution.

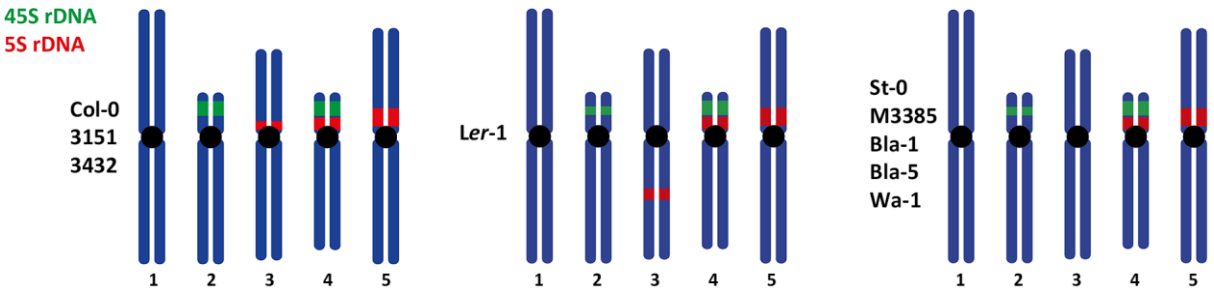


Figure 8. Ideograms of the different genetic backgrounds used. Schematic representation of the 45S rDNA regions (represented in green) and the 5S rDNA regions (represented in red) detected by FISH. The size of the green and red labels approximately represents the differences observed in the 5S and 45S regions among the different genetic backgrounds.

3.2.5.3. Chiasma frequency analysis

The chiasma frequency analysis per cell and per chromosome in the diploid lines was performed at the metaphase I stage of PMCs according to the criteria described by Sanchez Moran *et al*. (2001). For the polyploid lines, the criteria used to analyse the chiasma frequency per cell, per chromosome and per chromosome association (bivalents, trivalents and quadrivalents) at the metaphase I stage is detailed in the section 4.1.

3.2.5.4. Immunolocalization of proteins by spreading

This technique allowed a qualitative and semi-quantitative study of proteins related to the homologous recombination process. It was performed following the protocol described by Armstrong *et al*. (2009), with minor modifications.

Around 10 fresh flower buds were transferred onto a polylysine slide and dissected with 10 µL of the enzyme mixture (prepared with 0.1 g cytohelicase; 0.375 g sucrose; 0.25 g polyvinylpyrrolidone and 25 mL of Milli-Q water) to isolate the anthers. Another 10 µL drop of the enzyme mixture was applied to digest the anthers for 5 minutes at 37°C in a moist chamber. After that, anthers were squeezed using a needle and another 10 µL of the enzyme mixture were added to digest the material for 7 minutes more at 37°C in a moist chamber. Then, the material was squeezed again to ensure a proper maceration and 20 µL of lipsol (1 % v/v, diluted with SDW) were added. For 2 minutes, the material was shacked making circles to enhance the effect of the detergent and get a proper spread of the chromosomes. Later, the material with lipsol was incubated for 4 minutes at RT. Finally, 35 µL of paraformaldehyde (4 % v/v, pH 8) were added and the material was spread on the slide, subsequently letting it dry for 2 hours at RT (until the paraformaldehyde is dry but not precipitated).

To prepare the slides for the incubation with antibodies, they were washed 3 times in PBS-T (PBS 1x buffer with a 0.1 % v/v of Triton-X-100 detergent) for 5 minutes each one, at RT, and then incubated in a blocking medium of BSA 3 % (diluted with PBS-T 0.1%) for 45 minutes at 37°C.

A volume of 75 µL of the primary antibodies mixture in BSA 3 % was applied on each slide and then, the preparations were covered with Parafilm® and incubated ON at 5°C in a moist chamber. All the primary antibodies used in this thesis were kindly provided by Prof. Chris Franklin (University of Birmingham, UK). The primary antibodies were used at the following concentrations: α-MLH1 (rabbit, 1:500) (Jackson *et al.*, 2006) and α-ZYP1 (rat, 1:1000) (Higgins *et al.*, 2005). Next, slides were washed with shaking in PBS-T 5 times for 5 minutes each one at RT. Then, a secondary antibodies mixture (in PBS-T BSA 1 %) was applied to the slides and again a piece of Parafilm® was placed covering them. The secondary antibodies used were: α-rabbit-Cy3 (1:100, Agrisera) and α-rat-FITC

(1:50, Agrisera). Then, the slides were incubated for 1 hour and 30 minutes at RT in a moist chamber. Finally, slides were washed with shaking in PBS-T 5 times for 5 minutes each one at RT. Then, they were stained with 25 µL of DAPI + antifade solution and mounted using a glass coverslip. Slides were stored at 5°C for up to 1 month.

3.2.5.5. Image capture and analysis

For both the FISH and immunolocalization slides, a motorized epifluorescence microscope (Olympus BX61) equipped with DAPI, FITC and CY3 filters as well as a digital camera CCD (Olympus DP71) was used. This allowed the obtention of images from the different fluorochromes applied to the sample and different stacks of the PMCs.

To adjust the brightness and contrast of images as well as to merge the different channel images, Adobe Photoshop© and Image J were used. The maximum intensity projection of the immunolocalization images with more than one stack was performed using the "Z project" tool provided by the Image J software. Image deconvolution was implemented using the iterative algorithm "Richardson-Lucy" (DeconvolutionLab2, ImageJ plugin). This tool improves the discrimination of the signals. In order to reduce bias, immunolocalization images from both the wild-type and mutants were pooled prior to analysis (blind analysis).

3.2.6. Bioinformatic tools

Several bioinformatics tools have been used such as the database TAIR ("The Arabidopsis Information Resource"; http://www.arabidopsis.org) and the NCBI ("National Center for Biotechnology Information"; http://www.ncbi.nlm.nih.gov), which contain wide information about genes, genetic maps, DNA sequences and accessions. In addition, the NASC database, the ABRC ("Arabidopsis Biological Resource Center") (http://arabidopsis.info), and the tool SIGnAL ("The Salk Institute Genome Analysis Laboratory"; http://signal.salk.edu/cgiSbin/tdnaexpress) were used in order to get the information about the seeds available for each mutant line used in this thesis. To design the primers needed for genotyping, the tool "T-DNA Primer Design" (http://signal.salk.edu/tdnaprimers.2.html) was used. The UPL

probes as well as the LP and RP primers for the RT-qPCR experiments were designed for each gene with the tool “Universal Probe Library Assay Design Center” provided by ROCHE (https://lifescience.roche.com/en_gb/brands/universal-probe-library.html). All the statistical analyses were conducted using the GraphPad Prism© software.

3.2.7. Statistical analysis

The statistical analysis was chosen according to the characteristics of the data sets. When the average of two data sets were compared, the T-student test was conducted. When differences among averages of more than two data sets were compared, the ANOVA test was conducted. In order to perform a pair-wise comparison among all the data sets, the Holm-Sidak's multiple comparisons post-hoc test was conducted. When the pair-wise comparison was made between each data set and a reference data set, the Dunnett´s post-hoc test was performed. The cases in which the variances of the data sets were significantly different among each other (assessed by the Brown-Forsythe test), the Welch's ANOVA test was conducted. In that case, the post-hoc test used for the pair-wise comparisons was the Dunnett's T3 test, regardless the comparison was among all data sets or just between each data set and a reference data set. When differences were tested between percentages, the Fisher´s exact test was performed. Finally, in order to test the goodness of fit of a data set to an expected ratio, the Chi-square test was conducted. When the goodness of fit of a data set to a calculated Poisson distribution was tested, the Kolmogorov-Smirnov test was performed. In all these cases, the significance level was set to 0.05.

4. Results

4.1. Chiasma scoring in *Arabidopsis thaliana* (diploids and polyploids)

In this thesis, a detailed cytological study was conducted to obtain information on the average number of chiasmata per cell in both diploid and tetraploid lines. In **diploids**, chiasma frequency was assessed following the criteria described by (Sanchez-Moran *et al.*, 2001). Rod bivalents are formed by a minimum of one chiasma bounding either the short or the long arms of the chromosomes. On the other hand, ring bivalents are formed by a minimum of two chiasmata having at least one per each pair of chromosome arms (Figure 9). Nevertheless, depending on the morphology of the bivalents, it is also possible to distinguish rod bivalents with two chiasmata and ring bivalents with three or even more chiasmata (examples in Figure 9).

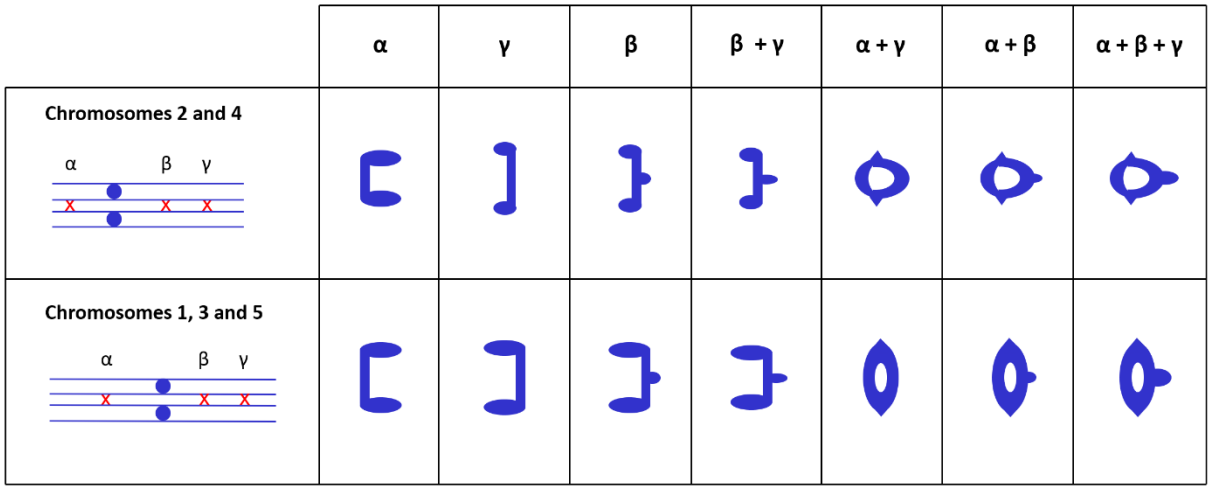


Figure 9. Schematic representation of bivalent configurations. Bivalent configurations observed at metaphase I in *Arabidopsis thaliana*. The different configurations can be explained by variations in the position and number of chiasmata as well as the chromosome morphology. Modified from Sanchez-Moran *et al*. (2001).

In **polyploids**, the chiasma frequency assessment was more complex since not only bivalent associations were observed but multivalents as well. Chiasma scoring in multivalents is a challenging analysis since a wide number of different configurations can be observed at metaphase I. The analysis was performed following the examples given by Sybenga (1975) and Santos *et al.* (2003). Each multivalent was associated to a minimum number of chiasmata necessary to explain the configuration observed. In the case of trivalents (III), most of them presented two or three chiasmata (Figure 10). For the quadrivalents (IV), we observed a wide number of different configurations, ranging from three (minimum number of chiasmata required to form a IV) (Figure 11) to six chiasmata (Figure 12). In addition, the chiasma frequency analysis was performed after applying FISH with the 5S and 45S rDNA probes to distinguish individual chromosomes and chromosome arms (except chromosomes 1 and 3 in some accessions; see section 3.2.5.2, Figure 8). The use of these probes also made easier the interpretation of different chromosome configurations in bivalents (II), III, and IV. In order to perform a proper interpretation, it is necessary to bear in mind that although the metaphase I images analyzed are in 2D, the actual structure of the chromosome configurations is in 3D. There are several aspects that should be considered for this interpretation: the morphology of the chromosomes involved in the configuration (acrocentric/submetacentric), the number and location of chiasmata (distal, subdistal, interstitial), the position of the FISH signals, the degree of variation in DAPI signal intensity, etc. Even though, we might be underestimating the number of chiasmata with this cytological approach, it should be noted that according to the results from previous publications, the cytological data agree with the recombination rates obtained by genetic estimations (Sanchez-Moran *et al.*, 2001).

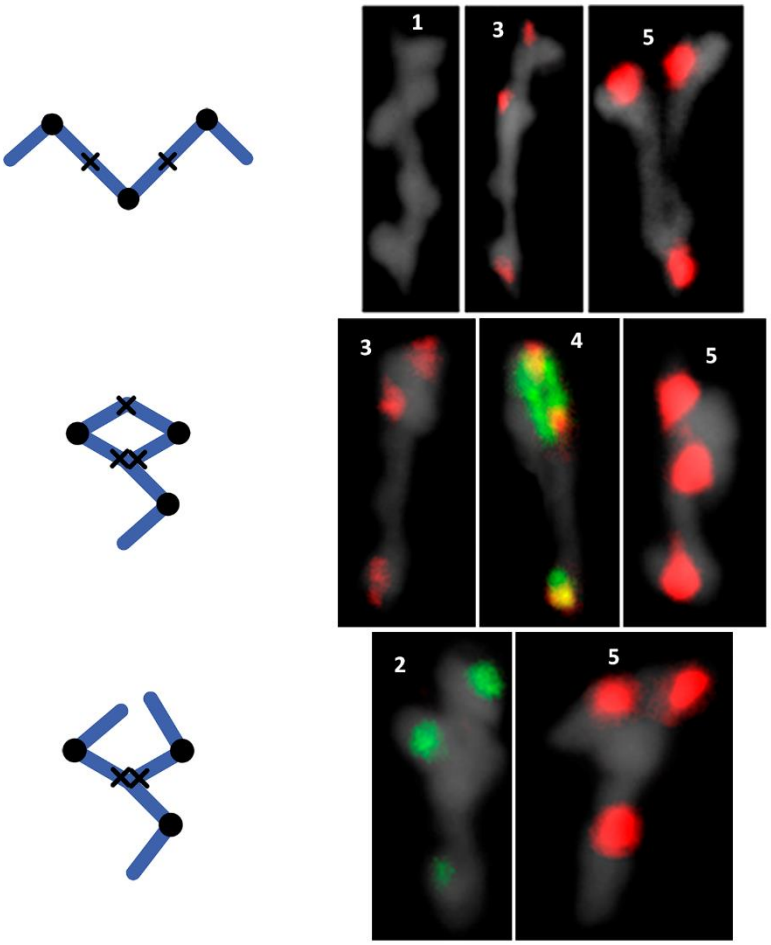


Figure 10. Examples of trivalents. Pictures in the same row show the same trivalent configuration formed by different chromosomes. On the left, there are schemes indicating the minimum number of chiasmata necessary to explain each trivalent configuration (black crosses). Centromeres are represented with black circles. Chromosomes have been identified by FISH with 5S (red) and 45S rDNA (green) probes. Numbers identify the chromosomes of the complement.

All the chiasma frequency analyses of this thesis were conducted in PMCs at metaphase I, and a minimum of three plants were analyzed for each line assessed. Since no significant differences in the mean chiasma frequencies per cell were observed among plants, individual plant data were grouped.

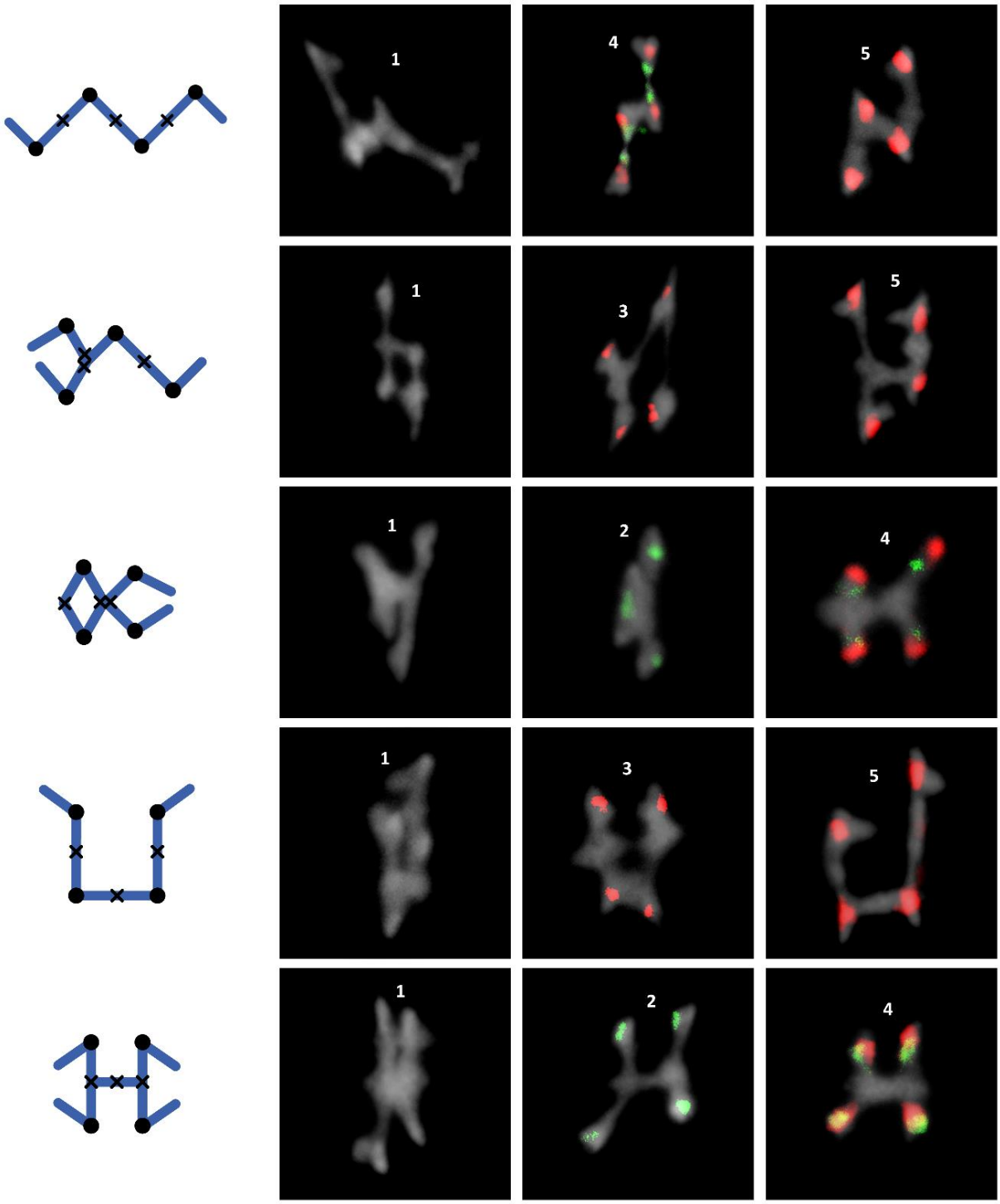


Figure 11. Examples of quadrivalents with three chiasmata. Pictures in the same row show the same quadrivalent configuration formed by different chromosomes. On the left, there are schemes indicating the minimum number of chiasmata necessary to explain each quadrivalent configuration (black crosses). Centromeres are represented with black circles. Chromosomes have been identified by FISH with 5S (red) and 45S rDNA (green) probes. Numbers identify the chromosomes of the complement.

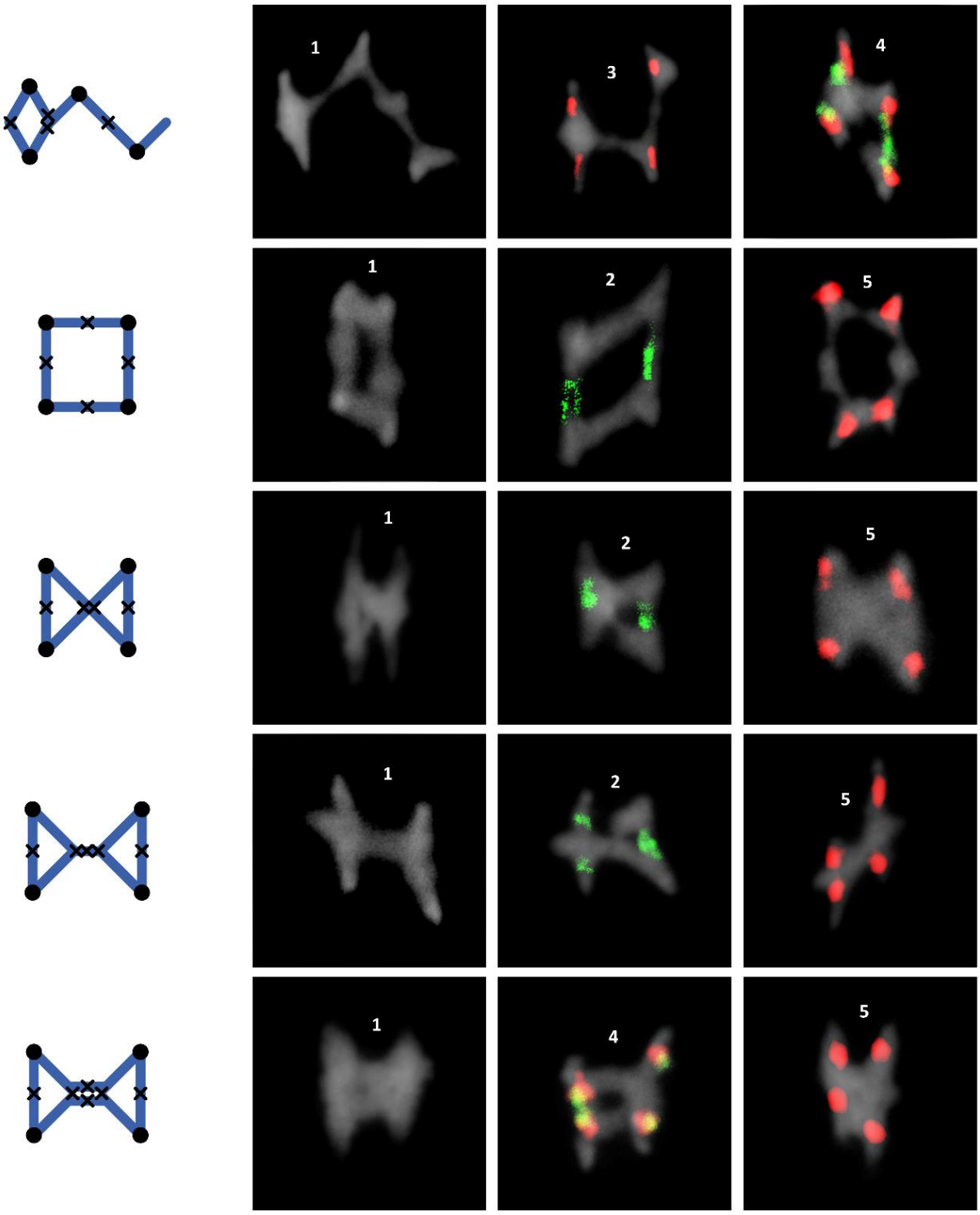


Figure 12. Examples of quadrivalents with more than three chiasmata. Pictures in the same row show the same quadrivalent configuration formed by different chromosomes. On the left, there are schemes indicating the minimum number of chiasmata necessary to explain each quadrivalent configuration (black crosses). Centromeres are represented with black circles. Chromosomes have been identified by FISH with 5S (red) and 45S rDNA (green) probes. Numbers identify the chromosomes of the complement.

4.2. Analysis of meiotic recombination in different autotetraploid lines

4.2.1. Chiasma frequency analysis of different diploid accessions

One objective of this thesis was to analyze by cytology different autotetraploid lines to compare their behavior in terms of **chiasma frequency**. In order to perform an adequate study of these lines, we initially studied the diploid lines from which they were obtained (in the case of synthetic autotetraploids) or geographically close diploids (in the case of natural autotetraploids) (Table 1). This analysis in the diploid lines was necessary to properly characterize how WGD affects lines with different mean chiasma frequencies. Figure 13 includes some examples of metaphase I PMCs from the **diploid lines** analyzed: Col-0 2x, L*er*-1 2x, St-0 2x, and Bla-1 2x.

Chiasma frequencies were scored in metaphase I PMCs according to the criteria previously detailed in section 4.1. These ranged from 8.35 (St-0 2x) to 10.20 (Col-0 2x) (Figure 14). The ANOVA test revealed significant differences among the four diploid accessions analyzed [$F(3, 393) = 31.55$; $p < 0.001$]. Subsequently, the Holm-Sidak´s test was conducted in order to do a pairwise comparison among these four diploid accessions. The analysis revealed that the chiasma frequency per cell in Col-0 2x (10.20 ± 0.14, n = 70) was significantly higher than those of the other three accessions ($p < 0.001$ for the three comparisons). It was also noticed that L*er*-1 2x (9.13 ± 0.10, n = 158) and Bla-1 2x (9.19 ± 0.15, n = 57) had similar chiasma frequencies, whereas St-0 2x (8.35 ± 0.13, n = 112) showed the lowest levels among these four diploid accessions ($p < 0.001$ for the three comparisons) (Figure 14, Table 5).

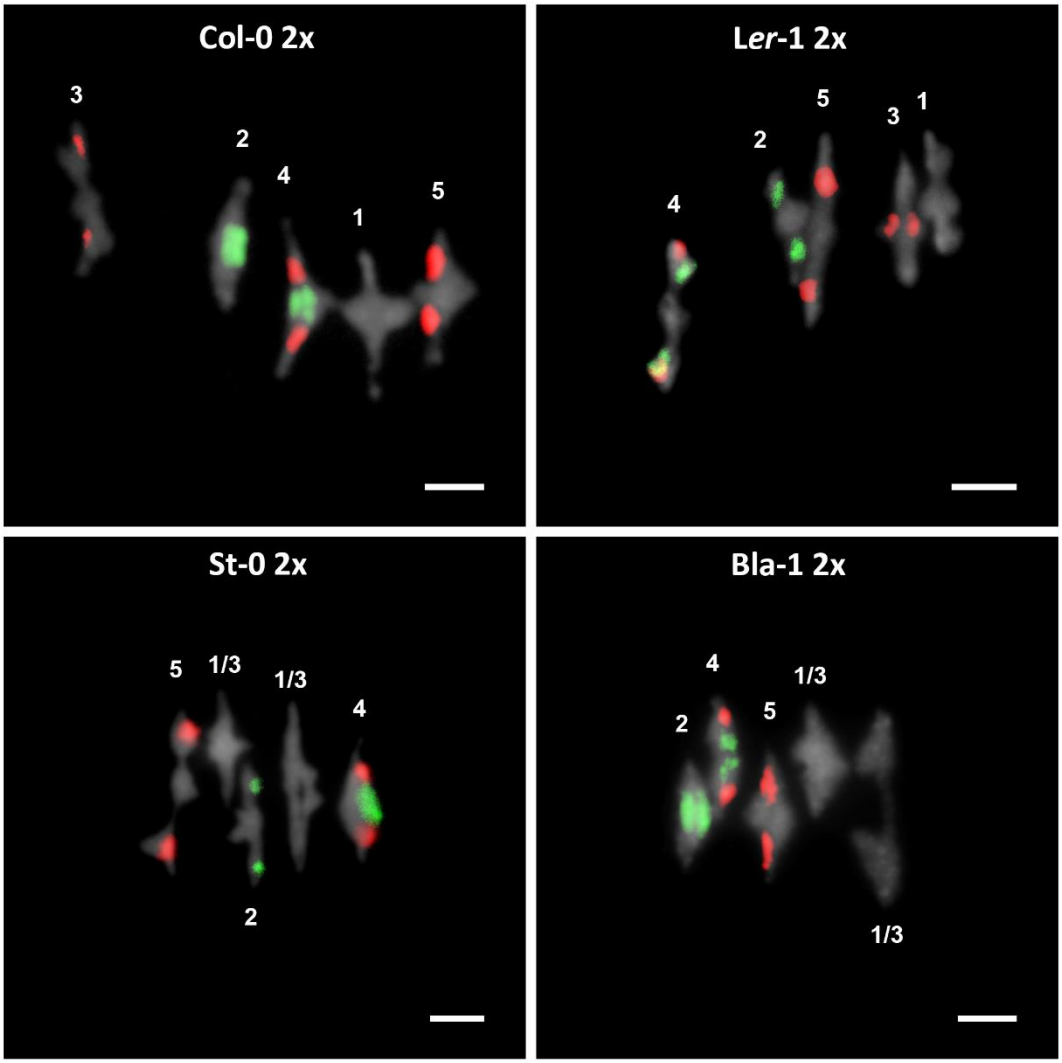


Figure 13. Examples of PMCs at metaphase I in diploid accessions. Representative examples of metaphases I from Col-0 2x, L*er*-1 2x, St-0 2x, and Bla-1 2x. Chromosomes were identified by FISH using 5S (red) and 45S rDNA (green) probes. The identification of Chr 1 and 3 was possible only in Col-0 2x and L*er*-1 2x. Numbers indicate the chromosomes of the complement. Bars represent 5 μm.

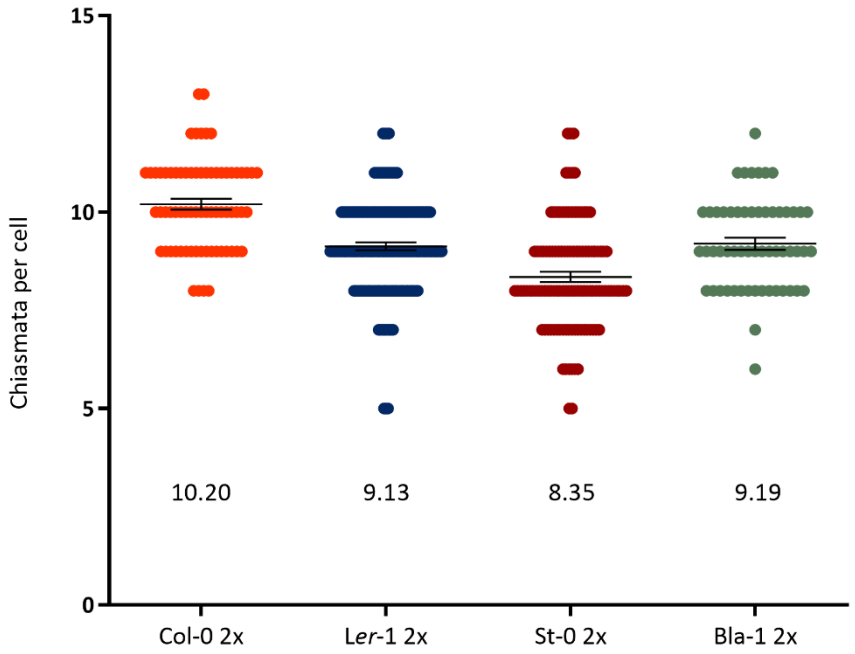


Figure 14. Analysis of chiasma frequency per cell in diploid accessions. Scatter plot representing the total mean chiasma frequency per cell of the diploid accessions Col-0 2x, L*er*-1 2x, St-0 2x, and Bla-1 2x. Numbers show the calculated value of the mean. Error bars represent the standard error of the mean.

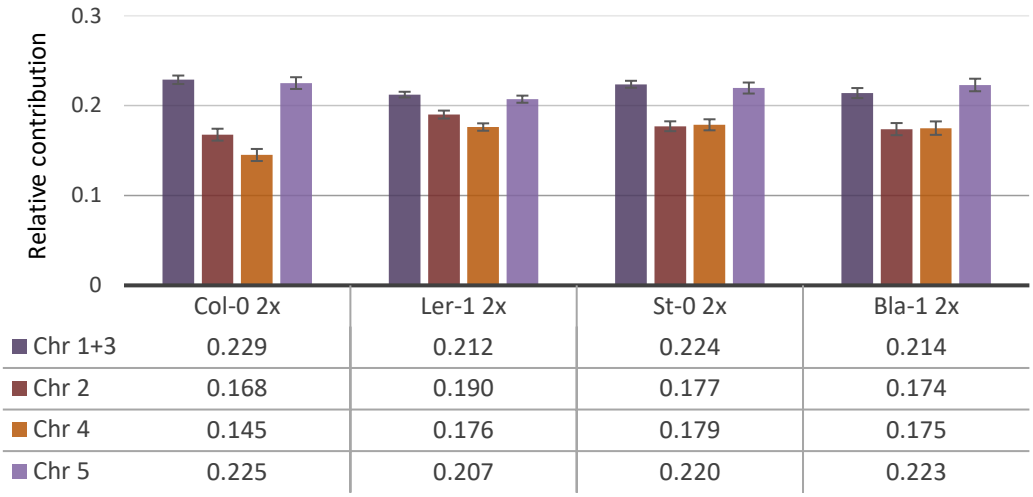
Table 5. Statistical differences obtained from the Holm-Sidak´s test comparing the mean chiasma frequencies among the different diploid accessions analyzed

	Col-0 2x		
L*er*-1 2x	***	**L*er*-1 2x**	
St-0 2x	***	***	**St-0 2x**
Bla-1 2x	***	ns	***

*Stars indicate *** p < 0.001. ns = not significant.*

In addition, by means of FISH, it was possible to analyze the chiasma frequency of each chromosome of the complement (see section 3.2.5.2, Figure 8), and then, assess their **relative contribution to the total chiasma frequency**. As it was not possible to distinguish between Chr 1 and 3 in the accessions St-0 and Bla-1 (Figure 13), the data obtained for these two chromosomes were merged. Thus, the relative contribution of each chromosome to the total chiasma frequency was compared among the four diploid accessions (Figure 15). The ANOVA statistical test showed significant differences for the merge of Chr 1 and 3 (Chr 1+3) [$F(3, 393) = 4.20$, $p < 0.01$], Chr 2 [$F(3, 393) = 3.21$, $p < 0.05$], and Chr 4 [$F(3, 393) = 6.00$, $p < 0.001$]. On the contrary, no significant differences were found for the contribution of the

Chr 5 [F (3, 393) = 2.39, p = 0.068]. Subsequently, the Holm-Sidak´s test showed that the contribution of the Chr 1+3 in Col-0 2x (0.230 ± 0.004) was significantly higher than in L*er*-1 2x (0.213 ± 0.003, p < 0.01) and St-0 (0.212 ± 0.004, p < 0.01). Regarding the Chr 2, only L*er*-1 2x (0.190 ± 0.004) and Col-0 2x (0.168 ± 0.007, p < 0.05) displayed significantly different contributions. As for the Chr 4, the contribution of this chromosome in Col-0 2x (0.145 ± 0.007) was significantly lower than in the rest of the accessions (L*er*-1 2x: 0.176 ± 0.004, p = 0.001; St-0 2x: 0.179 ± 0.006, p < 0.001; Bla-1 2x: 0.175 ± 0.007, p < 0.05), which did not present significant differences when they were compared with each other (Table 6). Additionally, although Col-0 2x presented the highest total mean chiasma frequency, it also showed the acrocentric chromosomes with the lowest contributions (Figure 15, Table 6). In general, in all the diploid accession assessed in this study, it was possible to observe an unequal contribution of the different chromosomes to the total chiasma frequency and this was corroborated by conducting the ANOVA test [Col-0 2x: F (3, 346) = 47.90, p < 0.001; L*er*-1: F (3, 786) = 18.36, p < 0.001; St-0: F (3, 556) = 24.20, p < 0.001; Bla-1: F (3, 281) = 13.48, p < 0.001]. Subsequently, by means of the Holm-Sidak´s test, it was observed that in these four genetic backgrounds the Chr 2 and Chr 4, which are the shortest and acrocentric chromosomes, presented a tendency to have lower chiasma contributions than the Chr 1+3 and Chr 5, which are the longest and metacentric/submetacentric chromosomes. This was also observed in previous studies in a wide number of *A. thaliana* accessions (Sanchez-Moran *et al.*, 2002; López *et al.*, 2012). None of the accessions analyzed in the present study displayed differences between the Chr 1+3 and 5 in terms of their contribution to the total chiasma frequency, however, the Chr 2 and 4 showed slight but significant differences with each other in Col-0 2x (p < 0.05) and L*er*-1 2x (p < 0.05) (Figure 15, Table 7). These results are pointing out that the different diploid genetic backgrounds analyzed not only showed different chiasma frequencies (Figure 14) but also differences in how chromosomes contribute to them.



	Col-0 2x	Ler-1 2x	St-0 2x	Bla-1 2x
Chr 1+3	0.229	0.212	0.224	0.214
Chr 2	0.168	0.190	0.177	0.174
Chr 4	0.145	0.176	0.179	0.175
Chr 5	0.225	0.207	0.220	0.223

Figure 15. Relative chromosome contribution to the total chiasma frequency per cell in diploid accessions. Bar chart represents the relative contribution of chromosomes (Chr) 1+3 (merge of 1 and 3), 2, 4, and 5 to the total mean chiasma frequency per cell in the diploid accessions Col-0 2x, L*er*-1 2x, St-0 2x, and Bla-1 2x. Error bars represent the standard error of the mean.

Table 6. Statistical differences obtained from the Holm-Sidak´s test comparing the relative contribution of each chromosome to the total chiasma frequency among different diploid accessions

Chr 1+3	**Col-0 2x**		
L*er*-1 2x	**	**L*er*-1 2x**	
St-0 2x	**	ns	**St-0 2x**
Bla-1 2x	ns	ns	ns
Chr 2	**Col-0 2x**		
L*er*-1 2x	*	**L*er*-1 2x**	
St-0 2x	ns	ns	**St-0 2x**
Bla-1 2x	ns	ns	ns
Chr 4	**Col-0 2x**		
L*er*-1 2x	***	**L*er*-1 2x**	
St-0 2x	***	ns	**St-0 2x**
Bla-1 2x	*	ns	ns
Chr 5	**Col-0 2x**		
L*er*-1 2x	ns	**L*er*-1 2x**	
St-0 2x	ns	ns	**St-0 2x**
Bla-1 2x	ns	ns	ns

Chromosomes (Chr)
*Stars indicate *** p < 0.001, ** p < 0.01, and * p < 0.05. ns = not significant.*

Table 7. Statistical differences obtained from the Holm-Sidak´s test comparing the relative contribution of each chromosome to the total chiasma frequency within each diploid accession

Col-0 2x	**Chr 1+3**		
Chr 2	***	**Chr 2**	
Chr 4	***	*	**Chr 4**
Chr 5	ns	***	***
L*er*-1 2x	**Chr 1+3**		
Chr 2	***	**Chr 2**	
Chr 4	***	*	**Chr 4**
Chr 5	ns	*	***
St-0 2x	**Chr 1+3**		
Chr 2	***	**Chr 2**	
Chr 4	***	ns	**Chr 4**
Chr 5	ns	***	***
Bla-1 2x	**Chr 1+3**		
Chr 2	***	**Chr 2**	
Chr 4	***	ns	**Chr 4**
Chr 5	ns	***	***

Chromosomes (Chr)
*Stars indicate *** p < 0.001 and * p < 0.05. ns = not significant.*

4.2.2. Chiasma frequency analysis of synthetic, established, and natural autotetraploids

As the cytological diploidization is a long-term process where polyploid organisms end up with a diploid-like meiosis, different stages with intermediate degrees of diploidization can be expected. In several species, different degrees of diploidization have been described (see more in section 1.6). In *A. thaliana,* Santos *et al.* (2003) reported different levels of cytological diploidization among different synthetic autotetraploid lines with at least thirteen generations after WGD. Following this study, in order to shed more light on the diploidization process at the cytological level, the following lines were analyzed: i) colchicine-induced autotetraploids lines from different accessions right after the WGD (G1 synthetic lines: Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x); ii) colchicine-induced autotetraploids lines with more than 20 generations after the polyploidy induction

(G20+ established lines: 3151 4x and 3432 4x); iii) and three natural autotetraploid accessions (Bla-5 4x, Wa-1 4x, and M3385S 4x; see section 3.1.1, Table 1).

Figure 16 includes some examples of metaphase I PMCs from the **synthetic tetraploid lines** analyzed: Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x. As in diploid lines, the analysis of the total mean **chiasma frequency per cell** and the relative contribution of each chromosome to the total chiasma frequency at metaphase I were conducted by means of FISH. These synthetic autotetraploids presented chiasma frequencies ranging from 17.80 (St-0 4x) to 19.99 (Col-0 4x) (Figure 17). It is worth to comment that all the autotetraploid lines used in this study presented not only II but also multivalents and univalents (I) (see more in section 4.2.3). Moreover, in all the cells analyzed, the formation of multivalents always involved homologous chromosomes. The chiasma numbers obtained for these synthetic lines were compared conducting the Welch´s ANOVA statistical test (used when there are significantly different variances among data sets, see more in section 3.2.7). The results obtained indicated that these four *de novo* autotetraploid lines presented differences in terms of chiasma formation [$W(3.0, 95.06) = 39.31$, $p < 0.001$]. The Dunnett´s T3 multiple comparison test showed that Col-0 4x (19.99 ± 0.11, n = 186) presented the highest mean chiasma frequency compared to the other three synthetic autotetraploid lines analyzed (L*er*-1 4x: 18.50 ± 0.27, n = 50, $p < 0.001$; St-0 4x: 17.80 ± 0.23, n = 71, $p < 0.001$; Bla-1 4x: 17.88 ± 0.27, n = 33, $p < 0.001$). However, the other three autotetraploid lines presented no statistical differences when they were compared with each other (Table 8). The genotype Col-0, which was the one with the highest mean cell chiasma frequency among the diploid lines, also presented the greatest value for this parameter among the autotetraploids. However, the differences between St-0 (the accession with the lowest chiasma frequency) and the other lines disappeared after WGD, since no differences were observed when St-0 4x was compared with L*er*-1 4x and Bla-1 4x.

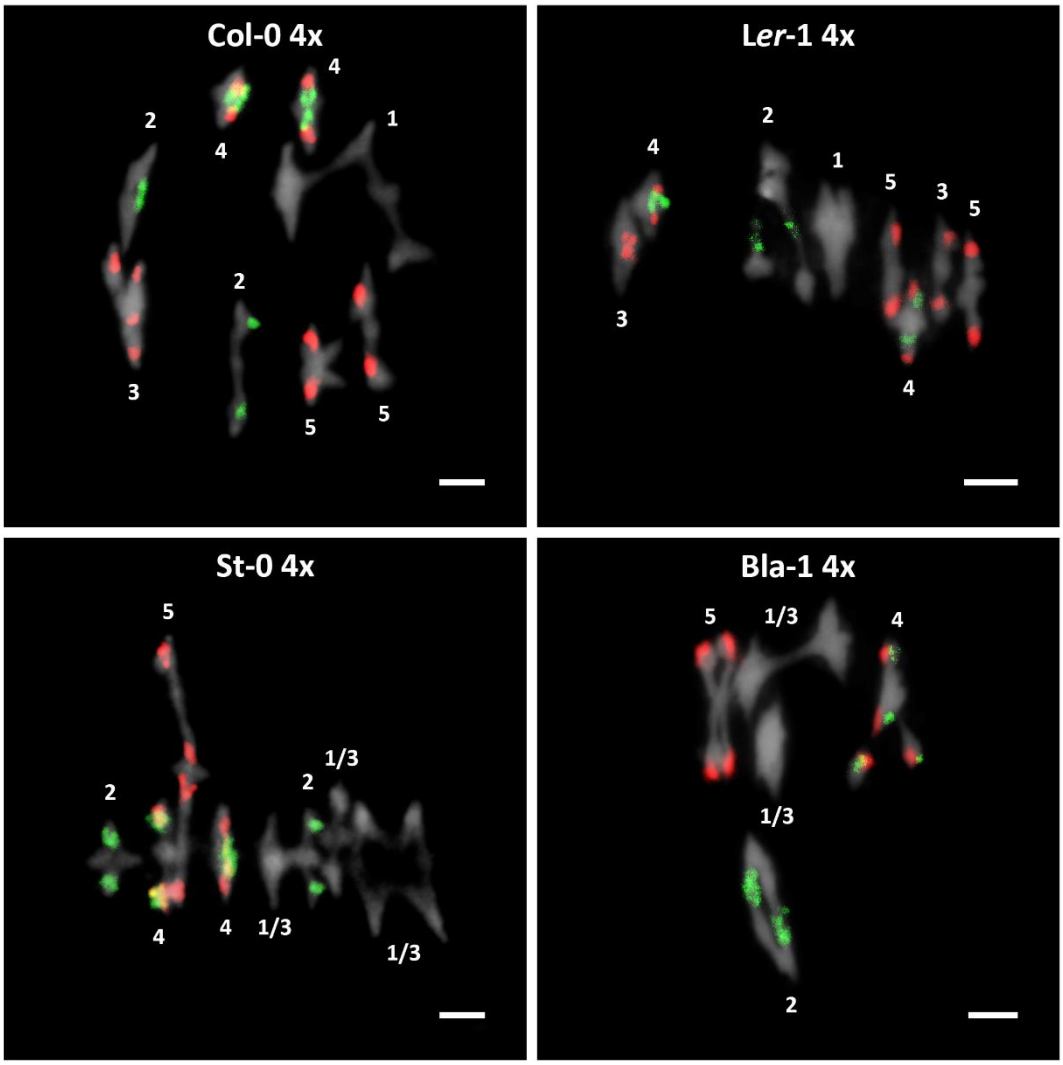


Figure 16. Examples of PMCs at metaphase I in synthetic autotetraploid lines. Representative examples of metaphases I from Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x. Chromosomes were identified by FISH using 5S (red) and 45S rDNA (green) probes. The identification of Chr 1 and 3 was possible only in Col-0 4x and L*er*-1 4x. Numbers indicate the chromosomes of the complement (one number per each chromosome association). In these examples, Chr 1 and 3 in Col-0 4x, Chr 1 and 2 in L*er*-1 4x, and Chr 1/3 and 5 in St-0 are forming multivalents. In Bla-1 4x, all the chromosomes are forming multivalents. Bars represent 5 μm.

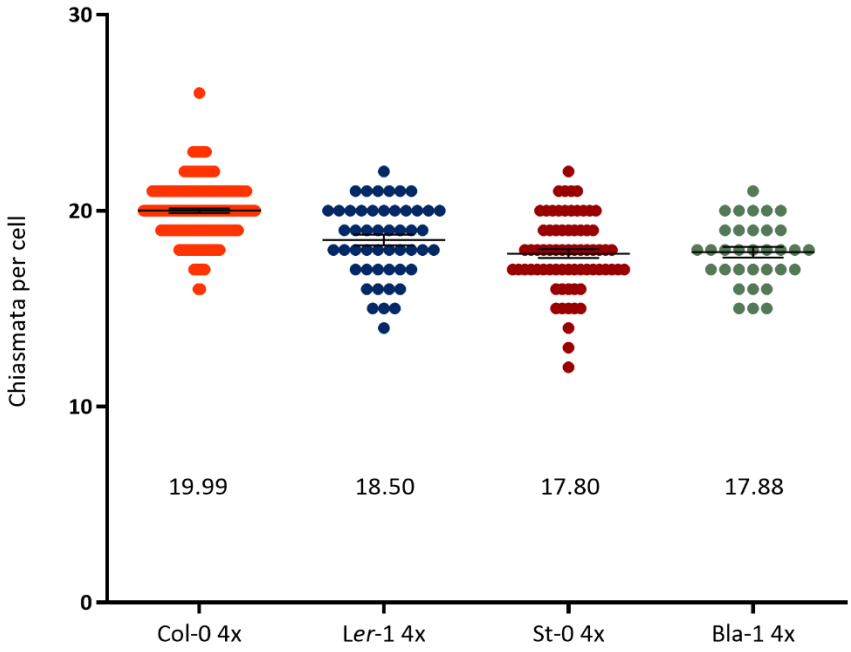


Figure 17. Analysis of chiasma frequency per cell in synthetic autotetraploids. Scatter plot representing the total mean chiasma frequency per cell of the synthetic autotetraploid lines Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x. Numbers show the calculated value of the mean. Error bars represent the standard error of the mean.

Table 8. Statistical differences obtained from the Dunnett´s T3 test comparing the mean chiasma frequencies among the different synthetic autotetraploid lines analyzed

	Col-0 4x		
L*er*-1 4x	***	**L*er*-1 4x**	
St-0 4x	***	ns	**St-0 4x**
Bla-1 4x	***	ns	ns

*Stars indicate *** p < 0.001. ns = not significant.*

In order to compare not only the chiasma frequency at the tetraploid level but also how this feature changes as a response to the WGD induction, the **fold-change of chiasma frequency** was calculated for these four genetic backgrounds. This value was obtained by dividing the frequency of each tetraploid cell by the total mean chiasma frequency of the diploid counterpart line. In general, the total chiasma frequency was duplicated as a response to the WGD, however, when a Welch´s ANOVA test was performed, significant differences were observed among these four genetic backgrounds [W (3.0, 91.98) = 12.61, p < 0.001] (Figure 18). When the pairwise comparison was performed by the Dunnett´s T3

test, it was observed that St-0 presented significantly the highest fold-change of chiasma frequency ($p < 0.001$ vs Col-1; $p < 0.05$ vs L*er*-1; $p < 0.001$ vs Bla-1) (Table 9), whereas the remaining genetic backgrounds showed very similar responses to the polyploidy induction.

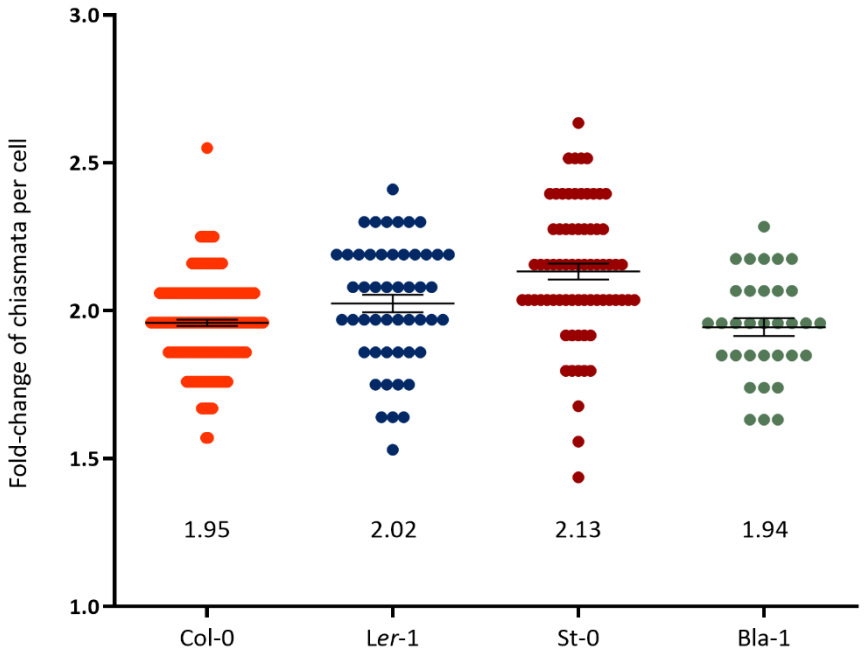


Figure 18. Fold-change of chiasma frequency per cell after WGD in different accessions. Scatter plot representing the fold-change of the total chiasma frequency per cell in the accessions Col-0, L*er*-1, St-0, and Bla-1 after chromosome duplication. Numbers represent the calculated value of the mean fold-change between the diploid and the autotetraploid lines. Error bars represent the standard error of the mean.

Table 9. Statistical differences obtained from the Dunnett´s T3 test comparing the mean fold-change of the chiasma frequencies among different accessions

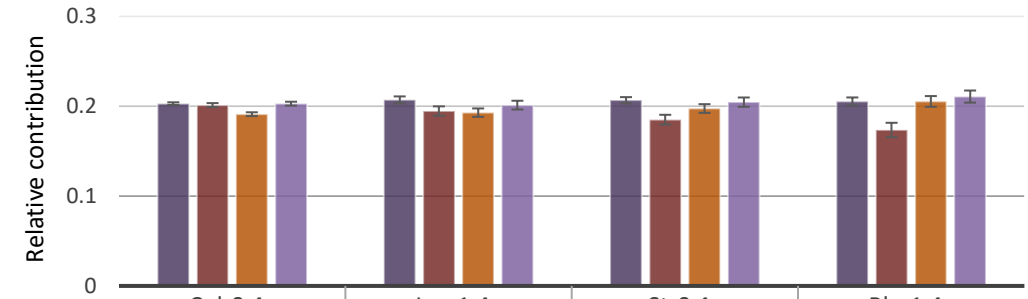
	Col-0		
L*er*-1	ns	**L*er*-1**	
St-0	***	*	**St-0**
Bla-1	ns	ns	***

*Stars indicate *** $p < 0.001$ and * $p < 0.05$. ns = not significant.*

Thus, the results obtained from the chiasma frequency analysis performed in diploid and synthetic autotetraploid individuals of the accessions Col-0, L*er*-1, St-0, and Bla-1 showed significant differences among them in both ploidy levels. Besides, St-0 displayed a significantly higher fold-change of chiasma frequency after WGD compared to the other three accessions analyzed.

Finally, the **relative chromosome contributions** of the Chr 1+3, 2, 4, and 5 were analyzed in these synthetic autotetraploid lines (Figure 19). The Welch´s ANOVA test revealed that only the Chr 2 [$W(3.0, 90{,}8) = 5.64$, $p < 0.01$] presented differences among these four synthetic autotetraploid lines, whereas the rest of the chromosomes presented similar contributions when they were compared among these four synthetic lines [Chr 1+3: $W(3.0, 92.6) = 0.97$, $p = 0.409$; Chr 4: $W(3.0, 94.2) = 1.94$, $p = 0.129$; Chr 5: $W(3.0, 92.7) = 0.49$, $p = 0.690$]. The Dunnett´s T3 pairwise comparison showed that the relative contribution of the Chr 2 in Col-0 4x (0.201 ± 0.002) was significantly higher than in St-0 4x (0.185 ± 0.005, $p < 0.05$) and Bla-1 4x (0.173 ± 0.008, $p < 0.05$) (Table 10). However, these results differ from the results obtained at the diploid level (section 4.2.1, Figure 15, Table 6). Diploid lines showed some differences for the relative contribution of the Chr 1+3, 2, and 4; whereas their synthetic tetraploid counterparts only displayed differences for the Chr 2. In this chromosome, at the diploid level, differences were found between Col-0 2x and L*er*-1 2x. However, these two genotypes did not present differences in polyploids, instead, differences between Col-0 4x and the lines St-0 4x and Bla-1 4x were found at this level. Additionally, as it was performed for the diploid lines, the contribution of the different chromosomes was compared with each other within each synthetic tetraploid line. The ANOVA test showed that all the lines [Col-0 4x: $F(3, 926) = 7.429$, $p < 0.001$; St-0 4x: $F(3, 351) = 4.363$, $p < 0.01$; Bla-1 4x: $F(3, 161) = 6.631$, $p < 0.001$] except L*er*-1 4x [$F(3, 246) = 2.239$, $p = 0.084$] presented differences (Figure 19). When the Holm-Sidak´s post hoc test was performed, it was found that in Col-0 4x, only the Chr 4 (vs Chr 1+3, $p < 0.001$; vs Chr 2, $p < 0.01$; vs Chr 5, $p < 0.001$) had a differential contribution compared with the other chromosomes, whereas the Chr 2 presented no differences compared with the longest chromosomes. In St-0 4x and Bla-1 4x, the Chr 2 displayed differences respect to the Chr 1+3 and 5, whereas the Chr 4 showed similar contribution compared to those. In Bla-1 4x, as in

Col-0 4x, differences between the shortest chromosomes were also observed ($p < 0.01$) (Table 11).



	Col-0 4x	Ler-1 4x	St-0 4x	Bla-1 4x
Chr 1+3	0.203	0.207	0.207	0.205
Chr 2	0.201	0.195	0.185	0.174
Chr 4	0.191	0.193	0.197	0.205
Chr 5	0.203	0.201	0.205	0.211

Figure 19. Relative chromosome contribution to the total chiasma frequency per cell in the synthetic autotetraploids. Bar chart representing the relative contribution of chromosomes (Chr) 1+3 (merge of 1 and 3), 2, 4, and 5 to the total mean chiasma frequency per cell in the synthetic autotetraploid lines Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x. Error bars represent the standard error of the mean.

Table 10. Statistical differences obtained from the Dunnett´s T3 test comparing the relative contribution of each chromosome to the total chiasma frequency among different synthetic autotetraploid lines

Chr 1+3	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	ns	ns	**St-0 4x**
Bla-1 4x	ns	ns	ns
Chr 2	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	*	ns	**St-0 4x**
Bla-1 4x	*	ns	ns
Chr 4	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	ns	ns	**St-0 4x**
Bla-1 4x	ns	ns	ns
Chr 5	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	ns	ns	**St-0 4x**
Bla-1 4x	ns	ns	ns

Chromosomes (Chr)
*Stars indicate * $p < 0.05$. ns = not significant.*

Table 11. Statistical differences obtained from the Holm-Sidak´s test comparing the relative contribution of all the chromosomes within each synthetic autotetraploid line

Col-0 4x	**Chr 1+3**		
Chr 2	ns	**Chr 2**	
Chr 4	***	**	**Chr 4**
Chr 5	ns	ns	***
L*er*-1 4x	**Chr 1+3**		
Chr 2	ns	**Chr 2**	
Chr 4	ns	ns	**Chr 4**
Chr 5	ns	ns	ns
St-0 4x	**Chr 1+3**		
Chr 2	**	**Chr 2**	
Chr 4	ns	ns	**Chr 4**
Chr 5	ns	*	ns
Bla-1 4x	**Chr 1+3**		
Chr 2	***	**Chr 2**	
Chr 4	ns	**	**Chr 4**
Chr 5	ns	***	ns

Chromosomes (Chr)
*Stars indicate *** p < 0.001, ** p < 0.01, and * p < 0.05. ns = not significant.*

At the diploid level, it was observed that in all the accessions analyzed, the shortest and acrocentric chromosomes have the tendency to contribute less to the total chiasma frequency than the longest and metacentric/submetacentric ones. However, analyzing the synthetic tetraploid lines, it was found that, at least in the genetic backgrounds analyzed in this study, there is a tendency in all the chromosomes to have a more uniform contribution to the total chiasma frequency. Thus, these results suggest that one of the responses after WGD could be the change of how each chromosome of the complement contributes to the total chiasma frequency.

As for the **established autotetraploid lines** 3432 4x (with the same genetic background as Col-0 4x) and 3151 4x (background Col-1, same origin as Col-0) (Figure 20), the purpose was to compare the total mean **chiasma frequency** of these two established lines (at least 20 generations after WGD) and the synthetic autotetraploid Col-0 4x (G1 plants after WGD) (Figure 21). Although a similar comparison was already conducted by Santos *et al.* (2003), in this thesis, individuals corresponding to seven generations later than those previously analyzed were examined. According to the data obtained in that study, the process of diploidization in synthetic autopolyploids can occur quickly and can be accompanied by a reduction in the total chiasma frequency. In that paper, it was reported that the established lines E_1 and E_4 (here referred as 3151 4x and 3432 4x, respectively), with at least 13 generations after the WGD, showed slightly lower mean chiasma frequencies than the synthetic Col-0 4x, although no statistical analysis was performed. Then, one could expect greater differences in the present study between Col-0 4x and these two established lines as another seven generations have passed (due to time limitations, further generations could not be analyzed). The assessment of the PMCs at metaphase I and the subsequent ANOVA statistical analysis [$F(2, 314) = 14.58$, $p < 0.001$], together with the Holm-Sidak´s test, revealed that 3151 4x (19.20 ± 0.17, n = 66) and 3432 4x (18.94 ± 0.22, n = 65) showed similar chiasma levels, significantly lower than those of the synthetic autotetraploid line Col-0 4x (19.99 ± 0.11, $p < 0.001$ in both cases) (Table 12).

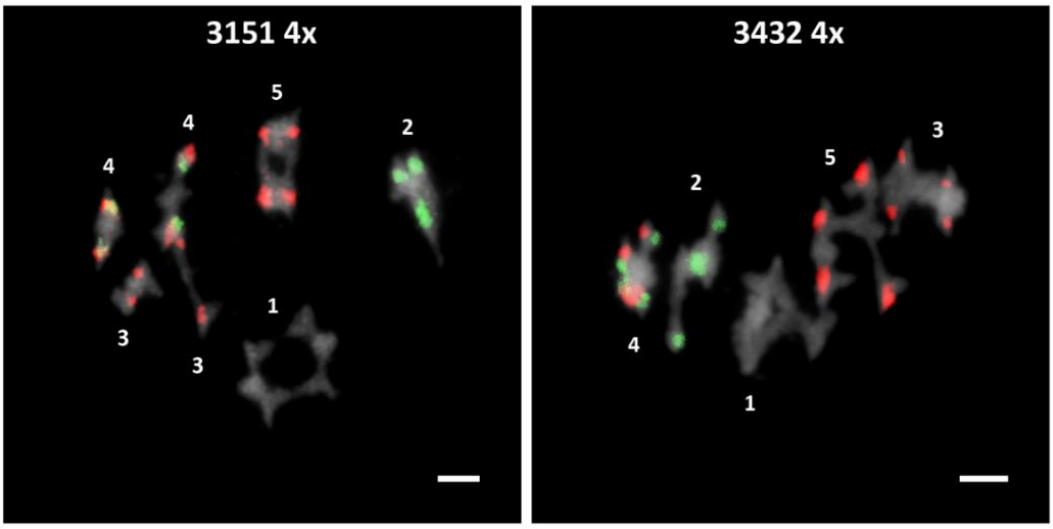


Figure 20. Examples of PMCs at metaphase I in established autotetraploid lines. Representative examples of metaphases I from 3151 4x and 3432 4x. Chromosomes were identified by FISH using 5S (red) and 45S rDNA (green) probes. Numbers indicate the chromosomes of the complement (one number per each chromosome association). In 3151 4x Chr 1, 2, and 5 are forming multivalents, whereas in 3432 all the chromosomes appear as multivalents. Bars represent 5 μm.

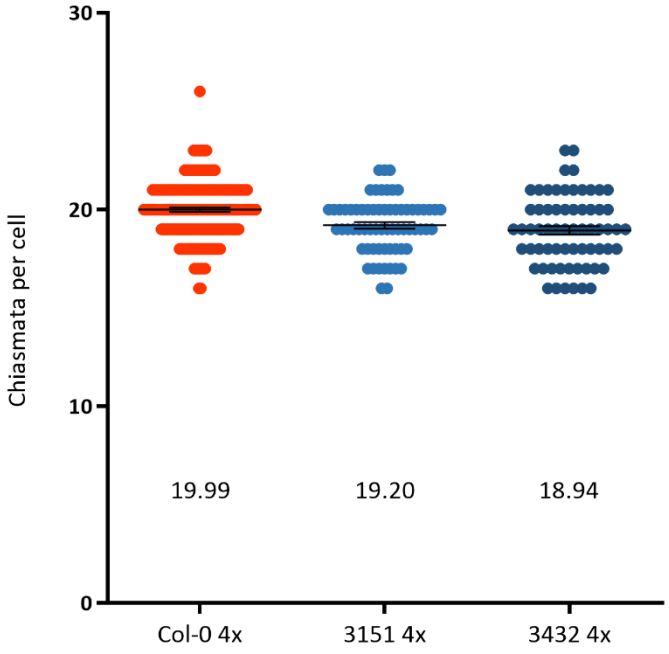


Figure 21. Analysis of chiasma frequency per cell in established autotetraploids. Scatter plot representing the total mean chiasma frequency per cell of the synthetic autotetraploid line Col-0 4x and the established autotetraploid lines 3151 4x and 3432 4x. Numbers show the calculated value of the mean. Error bars represent the standard error of the mean.

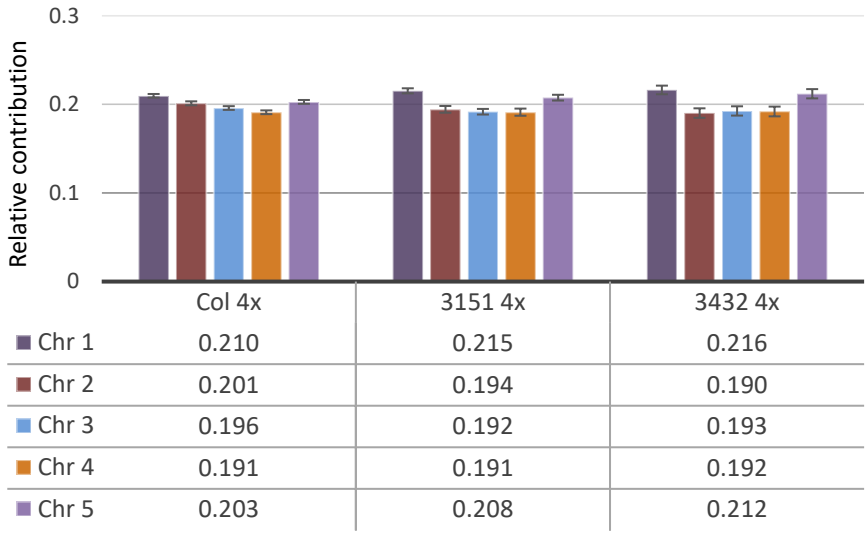
Table 12. Statistical differences obtained from the Holm-Sidak´s test comparing the mean chiasma frequencies among the two established autotetraploid lines and Col-0 4x

	Col-0 4x	
3151 4x	***	**3151 4x**
3432 4x	***	ns

*Stars indicate *** p < 0.001. ns = not significant.*

The results obtained in this study are similar to those reported by Santos *et al.* (2003), being the mean cell chiasma frequency of the synthetic line (Col-0 4x) significantly greater than that of the established lines (at least 20 generations after WGD). The mean cell chiasma frequency of 3151 4x was slightly higher than that of 3432 4x, yet no statistical significance was found.

As for the **contribution of each chromosome** to the total chiasma frequency, again 3151 4x and 3432 4x were compared with each other and the synthetic counterpart line Col-0 4x. In this case, it was possible to differentiate all the chromosomes of the complement using the 5S and 45S FISH probes. The Welch´s ANOVA test showed that the established lines and Col-0 4x had a similar behavior in terms of chromosome contribution to total chiasma frequency [Chr 1: W (2.0, 127.9) = 1.68, p = 0.191; Chr 2: W (2.0, 120.9) = 2.51, p = 0.086; Chr 3: W (2.0, 124.0) = 0.65, p = 0.525; Chr 4: W (2.0, 115.9) = 0.01, p = 0.988; Chr 5: W (2.0, 125.0) = 1.70, p = 0.186] (Figure 22).



	Col 4x	3151 4x	3432 4x
Chr 1	0.210	0.215	0.216
Chr 2	0.201	0.194	0.190
Chr 3	0.196	0.192	0.193
Chr 4	0.191	0.191	0.192
Chr 5	0.203	0.208	0.212

Figure 22. Relative chromosome contribution to the total chiasma frequency per cell in established autotetraploids. Bar chart representing the relative contribution of chromosomes (Chr) 1, 2, 3, 4, and 5 to the total mean chiasma frequency per cell in the established autotetraploid lines 3151 4x and 3432 4x, and the synthetic line Col-0 4x. Error bars represent the standard error of the mean.

Finally, the chiasma frequency was also assessed in the **natural autotetraploid lines** (Figure 23). These lines are accessions of *A. thaliana* that were described as polyploids by means of flow cytometry (reviewed in Novikova *et al*., 2018). In our study, these accessions were confirmed as autotetraploids by cytology, being the first time that meiosis has been assessed in natural polyploids of *A. thaliana*. These three accessions come from different regions of Europe (see section 3.1.1) and in the case of Bla-5 and M3385S, the diploid accessions geographically close to these ones, Bla-1 and St-0, respectively, were also assessed in this study (see section 4.2.1). The mean **chiasma frequency** analysis revealed that Bla-5 4x (18.45 ± 0.12, n = 192) presented slightly lower frequency than Wa-1 4x (18.58 ± 0.19, n = 78) and M3385S 4x (18.87 ± 0.18, n = 90) (Figure 24). However, the ANOVA test showed that these differences were not significant [F (2, 357) = 1.89, p = 0.153].

The mean chiasma frequencies showed by the natural autotetraploids analyzed in this study (Bla-5 4x, Wa-1 4x, and M3385S 4x) were similar. The values were not very different from those found in the synthetic lines, showing lower numbers than Col-0 4x and L*er*-1 4x and higher than St-0 4x and Bla-1 4x.

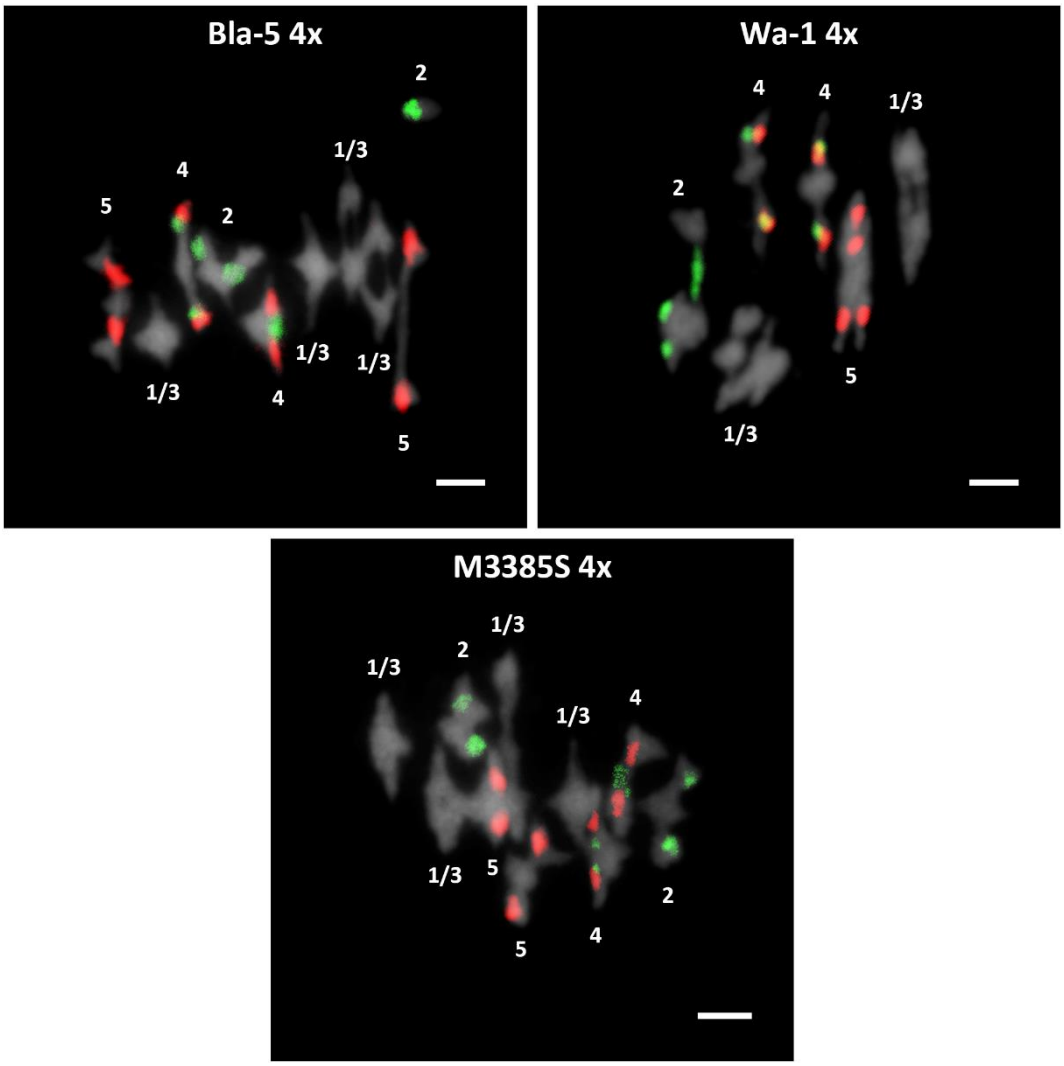


Figure 23. Examples of PMCs at metaphase I in natural autotetraploid accessions. Representative examples of metaphases I from Bla-5 4x, Wa-1 4x, and M3385S 4x. Chr 2, 4, and 5 are identified by FISH with 5S (red) and 45S rDNA (green) probes. Numbers indicate the chromosomes of the complement (one number per each chromosome association or univalent). In Bla-5 4x Chr 2 is forming a trivalent, in Wa-1 4x Chr 1/3 and 5 appear as multivalents, whereas in M3385S 4x all chromosomes are forming bivalents. Bars represent 5 μm.

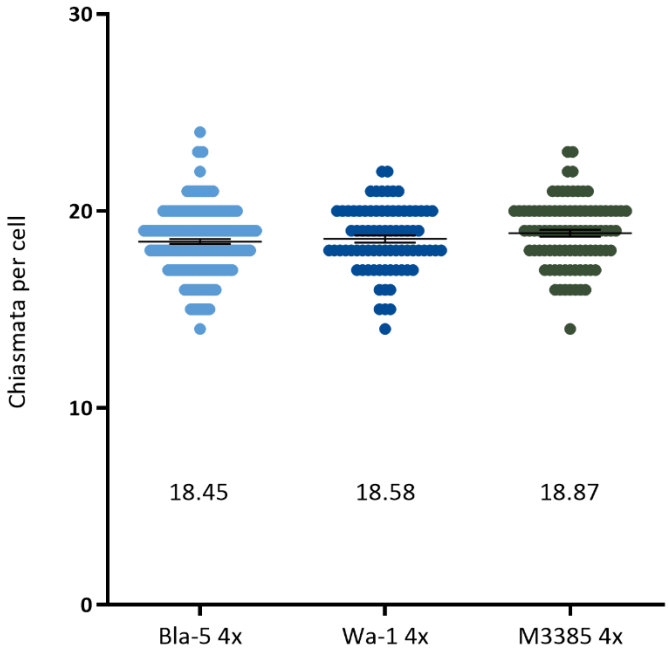


Figure 24. Analysis of chiasma frequency per cell in natural autotetraploids. Scatter plot representing the total mean chiasma frequency per cell of the natural autotetraploid accessions Bla-5 4x, Wa-1 4x, and M3385S 4x. Numbers show the calculated value of the mean. Error bars represent the standard error of the mean.

Besides, the relative **contribution of each chromosome** to the total chiasma frequency was assessed for the natural autotetraploid lines (Figure 25). The Welch´s ANOVA test showed no differences among Bla-5 4x, Wa-1 4x, and M3385S 4x for any of the chromosomes [Chr 1+3: W (2.0, 190.0) = 2.56, p = 0.080; Chr 2: W (2.0, 192.0) = 2.96, p = 0.054; Chr 4: W (2.0, 187.0) = 1.16, p = 0.315; Chr 5: W (2.0, 188.0) = 0.60, p = 0.552].

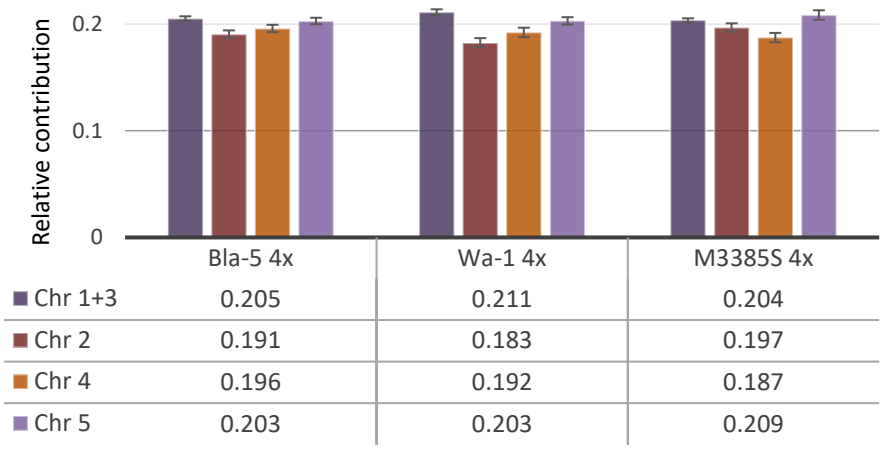


Figure 25. Relative chromosome contribution to the total chiasma frequency per cell in natural autotetraploid accessions. Bar chart representing the relative contribution of chromosomes (Chr) 1+3 (merge of 1 and 3), 2, 4, and 5 to the total mean chiasma frequency per cell in the natural autotetraploid accessions Bla-5 4x, Wa-1 4x, and M3385S 4x. Error bars represent the standard error of the mean.

4.2.3. Analysis of chromosome configurations at metaphase I in synthetic, established, and natural autotetraploids

Another feature assessed in the autotetraploid lines was the frequency of II, IV, III, and I in metaphase I PMCs. In autotetraploid lines, the presence of four homologous chromosomes for each chromosome of the complement gives rise to a situation where there is more than one partner to recombine with. Then, the different chromosome associations and configurations depend on the final pattern of CO formation among homologous chromosomes. Thus, in this analysis, we were able to observe II, IV, III, and I in all the lines analyzed but also different configurations with different shapes for each chromosome association (see section 4.1). As the diploidization process at the cytological level is related to a progressive increase of II to the detriment of multivalents (see section 1.6), the II frequency analysis gave us the opportunity to find out the diploidization levels of each autotetraploid line used in this study. As we assessed three different kind of autotetraploid lines (synthetic, established and natural) that *a priori* were at different stages of the diploidization process, one could expect different levels of diploidization for each polyploid group, having the natural lines higher levels of diploidization than the established lines, and these ones more than the synthetic lines. In addition, the frequencies of III and I were also

assessed as both are related with chromosome mis-segregations at anaphase I and aneuploidies at the second meiotic division.

As for the **synthetic autotetraploids**, results showed that Bla-1 4x (4.33 ± 0.37) presented the highest mean frequency of II per cell, followed by St-0 4x (3.11 ± 0.26), L*er*-1 4x (2.74 ± 0.27), and Col-0 4x (2.41 ± 0.15) (Figure 26). The ANOVA test revealed significant differences [$F(3, 336) = 8.93$, $p < 0.001$]. The pairwise comparison conducted by the Holm-Sidak´s test showed that the II numbers of Bla-1 4x were significantly higher than those of the other three synthetic lines (vs Col-0 4x, $p < 0.001$; vs L*er*-1 4x, $p < 0.01$; vs St-0 4x, $p < 0.05$). Additionally, St-0 4x displayed significantly higher II levels than Col-0 4x ($p < 0.05$) (Table 13).

Regarding the IV levels, the synthetic lines behaved as it was expected according to their frequency of II (the higher the II frequency, the lower the IV frequency). Thus, Col-0 4x (which was the line with the lowest II frequency) presented the highest levels of IV per cell (3.70 ± 0.08). Bla-1 4x exhibited the lowest numbers (2.61 ± 0.18), whereas L*er*-1 4x (3.42 ± 0.14) and St-0 4x (3.23 ± 0.13) showed intermediate values (Figure 26). The ANOVA test revealed significant differences among these lines [$F(3, 336) = 11.6$, $p < 0.001$] and the Holm-Sidak´s test showed that those differences were significant between Col-0 4x and St-0 4x ($p < 0.01$), Col-0 4x and Bla-1 4x ($p < 0.001$), L*er*-1 4x and Bla-1 4x ($p < 0.01$), and between St-0 4x and Bla-1 4x ($p < 0.05$) (Table 13).

As for the mean III and I frequencies, the ANOVA test showed that the synthetic autotetraploid lines analyzed did not present considerable differences in III frequencies [$F(3, 336) = 1.57$, $p = 0.197$], whereas they did display significant differences in I frequencies [$F(3, 336) = 6.86$, $p < 0.001$] (Figure 26). The Holm-Sidak´s test showed that the I frequency of Col-0 4x (0.11 ± 0.03) was significantly lower than that of L*er*-1 4x (0.37 ± 0.10, $p < 0.05$), St-0 4x (0.37 ± 0.09, $p < 0.01$), and Bla-1 4x (0.45 ± 0.15, $p < 0.01$). However, no differences were found among L*er*-1 4x, St-0 4x, and Bla-1 4x when they were compared with each other (Table 13). It is important to point out that in general the frequencies of III and I observed in this study in all the synthetic, established, and natural lines were relatively low, showing all of them an average of less than one I or III per cell.

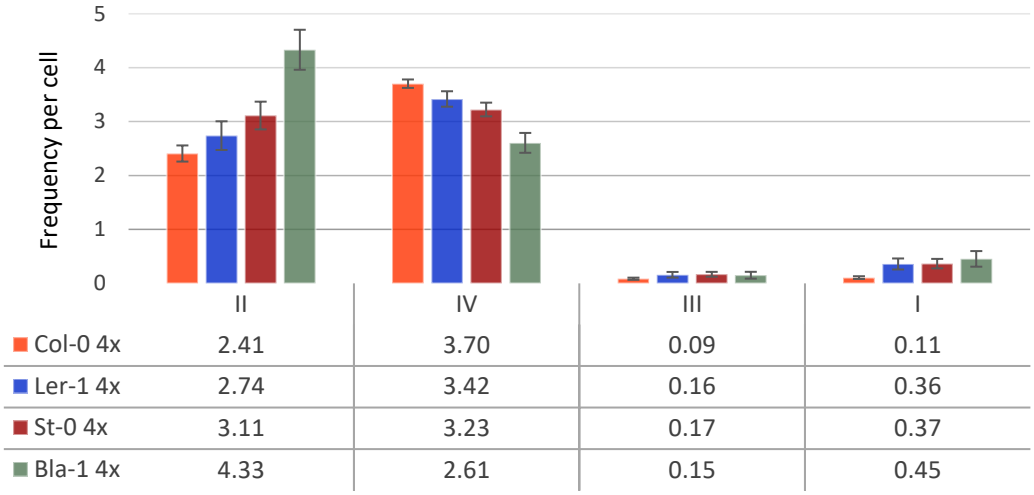


Figure 26. Mean frequency per cell of the different chromosome associations in the synthetic autotetraploids. Bar chart representing the mean frequency of bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I) per cell in the synthetic autotetraploid lines Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x. Error bars represent the standard error of the mean.

Table 13. Statistical differences obtained from the Holm-Sidak´s test comparing the mean frequency of bivalents, quadrivalents, trivalents, and univalents among the different synthetic autotetraploid lines analyzed

II	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	*	ns	**St-0 4x**
Bla-1 4x	***	**	*
IV	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	**	ns	**St-0 4x**
Bla-1 4x	***	**	*
III	**Col-0 4x**		
L*er*-1 4x	ns	**L*er*-1 4x**	
St-0 4x	ns	ns	**St-0 4x**
Bla-1 4x	ns	ns	ns
I	**Col-0 4x**		
L*er*-1 4x	*	**L*er*-1 4x**	
St-0 4x	**	ns	**St-0 4x**
Bla-1 4x	**	ns	ns

Bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I).
*Stars indicate *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. ns = not significant.*

From the chiasma frequency analysis and the chromosome association analysis for these four synthetic autotetraploid lines, it can be concluded that Col-0 4x, the line with the highest chiasma frequency, displayed the lowest levels of II and I, and the highest of IV. However, other lines with similar chiasma frequencies showed significant differences in terms of II levels, which was the case of St-0 4x and Bla-1 4x. In the same direction, L*er*-1 4x and St-0 4x displayed significant differences in terms of chiasma frequency but very similar I levels. Therefore, a clear correlation cannot be made between the chiasma frequency and the different chromosome associations.

The scoring of different chromosome associations was also performed for the **established autotetraploid lines** 3151 4x and 3432 4x. The data of these lines were compared with each other and with the synthetic line Col-0 4x (Figure 27). Performing the ANOVA test and the Holm-Sidak´s post hoc test [II: $F(2, 314) = 4.59$, $p < 0.05$; IV: $F(2, 314) = 5.04$, $p < 0.01$], it was observed that 3432 4x presented significantly higher numbers of II (3.29 ± 0.29) and lower of IV (3.22 ± 0.15) than Col-0 4x (2.41 ± 0.15 II, $p < 0.05$; 3.70 ± 0.08 IV, $p < 0.01$) and similar to 3151 4x (2.88 ± 0.25 II, $p > 0.999$; 3.50 ± 0.13 IV, $p = 0.553$). However, no significant differences were found between 3151 4x and Col-0 4x (II, $p = 0.225$; IV, $p = 0.249$) (Table 14). In addition, the statistical analyses found no significant differences neither in the number of III [$F(2, 314) = 0.26$, $p = 0.768$] nor in the number of I [$F(2, 314) = 1.85$, $p = 0.159$] comparing 3151 4x (0.06 ± 0.03 III and 0.09 ± 0.04 I), 3432 4x (0.09 ± 0.04 III and 0.22 ± 0.08 I), and Col-0 4x (0.09 ± 0.02 III and 0.11 ± 0.03 I) (Table 14).

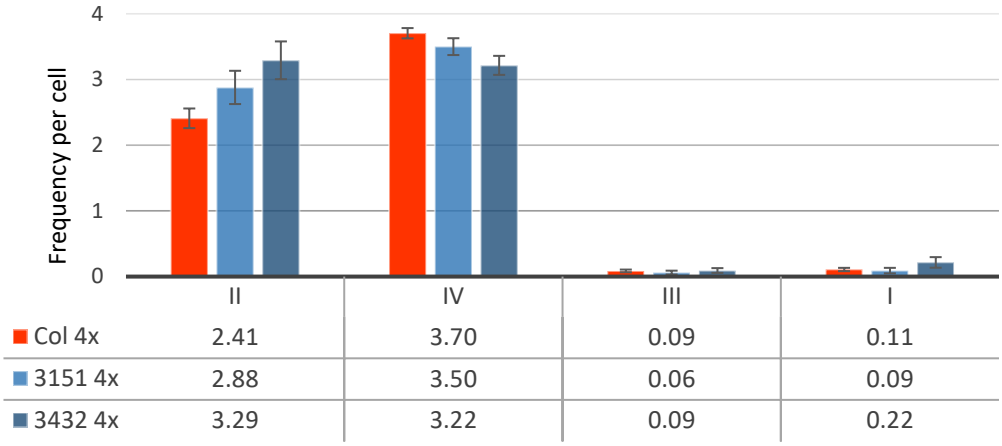


Figure 27. Mean frequency per cell of the different chromosome associations in the established autotetraploids. Bar chart representing the mean frequency of bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I) per cell in the established autotetraploid lines 3151 4x and 3432 4x, and the synthetic line Col-0 4x. Error bars represent the standard error of the mean.

Table 14. Statistical differences obtained from the Holm-Sidak´s test comparing the mean frequency of bivalents, quadrivalents, trivalents, and univalents among the two established autotetraploid lines analyzed and Col-0 4x

II	**Col-0 4x**	
3151 4x	ns	**3151 4x**
3432 4x	*	ns
IV	**Col-0 4x**	
3151 4x	ns	**3151 4x**
3432 4x	**	ns
III	**Col-0 4x**	
3151 4x	ns	**3151 4x**
3432 4x	ns	ns
I	**Col-0 4x**	
3151 4x	ns	**3151 4x**
3432 4x	ns	ns

Bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I).
*Stars indicate ** $p < 0.01$ and * $p < 0.05$. ns = not significant.*

In line with the results obtained by Santos *et al.* (2003), 3432 4x presented higher cytological diploidization levels (II frequency) than 3151 4x and the synthetic counterpart Col-0 4x.

Regarding the **natural autotetraploid accessions**, mean frequencies of II, IV, III, and I were also evaluated for Bla-5 4x, Wa-1 4x, and M3385S 4x. In terms of II and IV, these three autotetraploid accessions showed a similar behavior since the ANOVA statistical test showed no significant differences among them [in II: $F(2, 357) = 2.55$, $p = 0.080$; in IV: $F(2, 357) = 0.80$, $p = 0.451$]. Although differences were not significant, the mean frequency of II showed by M3385S 4x (3.26 ± 0.23) was higher than that of Wa-1 4x (2.81 ± 0.23) and Bla-5 4x (2.64 ± 0.16).). In addition, due to the geographical proximity of the accessions Bla-1 and Bla-5, the synthetic autotetraploid Bla-1 4x and the natural autotetraploid Bla-5 4x were compared in terms of II formation ($t = 4.17$, $p < 0.001$). The two-tailed t-test revealed that Bla-1 4x presented significantly higher II numbers than those of Bla-5 4x. As for the frequency of IV, Wa-1 4x (3.31 ± 0.12) presented the highest value followed by Bla-5 4x (3.16 ± 0.09) and M3385S 4x (3.09 ± 0.12) (Figure 28). On the contrary, the ANOVA test revealed significant differences for the frequencies of III and I among these natural autotetraploids [in III: $F(2, 357) = 5.91$, $p < 0.01$; in I: $F(2, 357) = 4.64$, $p < 0.05$]. As for III, the Holm-Sidak´s test revealed that the levels observed in Bla-5 4x (0.43 ± 0.04) were significantly higher than in Wa-1 4x (0.24 ± 0.06, $p < 0.05$) and M3385S 4x (0.22 ± 0.05, $p < 0.01$), but no differences were found when Wa-1 4x and M3385S 4x were compared ($p = 0.966$) (Table 15). Regarding the I frequency assessment, the post hoc test revealed that again Bla-5 4x (0.77 ± 0.07) presented significantly higher numbers of I than those of Wa-1 4x (0.47 ± 0.11, $p < 0.05$) and M3385S 4x (0.47 ± 0.09, $p < 0.05$). Finally, as it was observed for the III frequency, Wa-1 4x and M3385S 4x did not show significant differences in terms of I levels ($p = 0.988$).

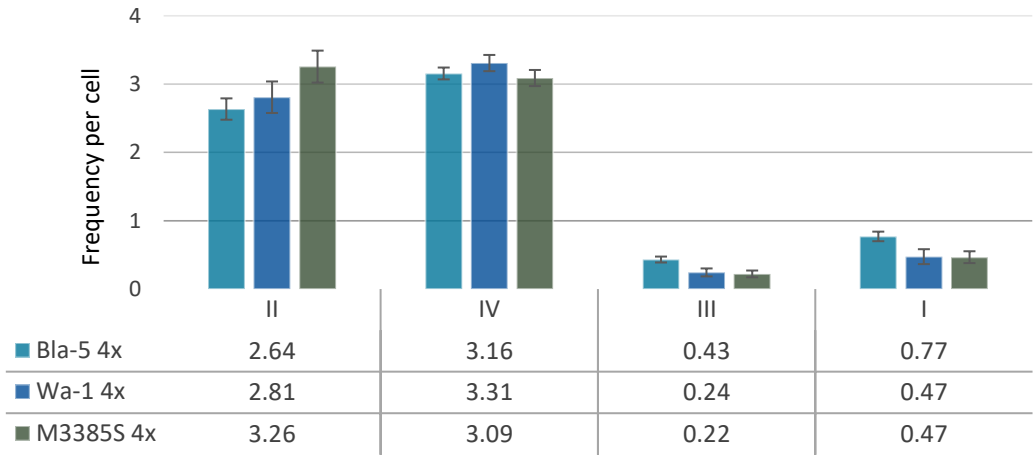


Figure 28. Mean frequency per cell of the different chromosome associations in natural autotetraploid accessions. Bar chart representing the mean frequency of bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I) per cell in the natural autotetraploid accessions Bla-5 4x, Wa-1 4x, and M3385S 4x. Error bars represent the standard error of the mean.

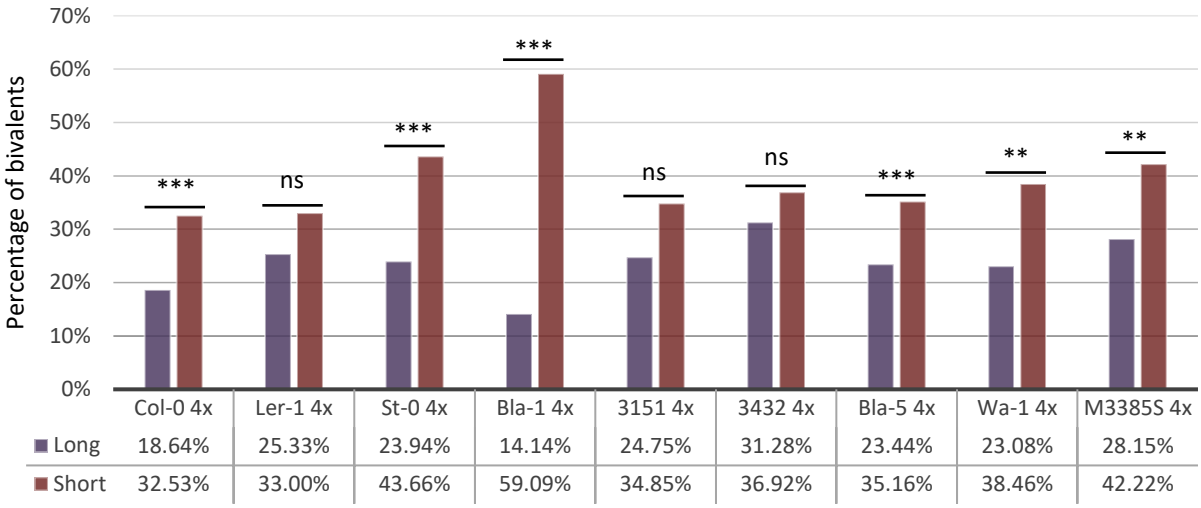
Table 15. Statistical differences obtained from the Holm-Sidak´s test comparing the mean frequency of bivalents, quadrivalents, trivalents, and univalents among different natural autotetraploid accessions

II	**Bla-5 4x**	
Wa-1 4x	ns	**Wa-1 4x**
M3385S 4x	ns	ns
IV	**Bla-5 4x**	
Wa-1 4x	ns	**Wa-1 4x**
M3385S 4x	ns	ns
III	**Bla-5 4x**	
Wa-1 4x	*	**Wa-1 4x**
M3385S 4x	**	ns
I	**Bla-5 4x**	
Wa-1 4x	*	**Wa-1 4x**
M3385S 4x	*	ns

Bivalents (II), quadrivalents (IV), trivalents (III), and univalents (I).
*Stars indicate ** $p < 0.01$ and * $p < 0.05$. ns = not significant.*

Comparing the results obtained from the chiasma frequency analysis and from the levels of the different chromosome associations, as it was observed in the synthetic lines, the level of I cannot be clearly associated to the chiasma frequency as the three natural autotetraploids assessed presented similar chiasma frequencies, whereas the I levels were significantly higher in Bla-5 4x than in the other two natural autotetraploid lines. Interestingly, the II frequency showed by the natural autotetraploid Bla-5 4x was lower than that of the synthetic line Bla-1 4x.

Finally, the contribution of the different chromosomes to the diploidization levels was assessed in all the autotetraploid lines analyzed in this study. In order to make simpler the comparison, the data obtained were grouped according to the features of the different chromosomes. Thus, the data from the Chr 1+3 and 5 were merged as these are metacentric/submetacentric and the longest chromosomes and the data of the Chr 2 and 4 were grouped as both are acrocentric and the shortest chromosomes in this species. In this way, the **percentage of II** in the **long** (Chr 1, 3, and 5) and **short** (Chr 2 and 4) chromosomes was analyzed. The levels of II in these two groups of chromosomes was compared with each other in each autotetraploid line (Figure 29). The statistical analysis was made by means of the Fisher´s exact test. In general, a tendency of higher II percentages in the short chromosomes compared to the percentages obtained from the long ones was observed. In detail, among the synthetic lines, apart from L*er*-1 4x ($p = 0.200$), the remaining lines presented significantly higher II levels in the short than in the long chromosomes (all of them $p < 0.001$). The established lines 3151 4x and 3432 4x also displayed higher percentages of II in the short than in the long chromosomes, although they did not show statistical significance (3151 4x, $p = 0.062$; 34332, $p = 0.338$). Finally, all the natural autotetraploid accessions used in this study, Bla-5 4x, Wa-1 4x, and M3385S 4x presented significant differences between these two groups of chromosomes in terms of II formation (Bla-5 4x, $p < 0.001$; Wa-1, $p < 0.01$; M3385S, $p < 0.01$) (Figure 29).



	Col-0 4x	Ler-1 4x	St-0 4x	Bla-1 4x	3151 4x	3432 4x	Bla-5 4x	Wa-1 4x	M3385S 4x
Long	18.64%	25.33%	23.94%	14.14%	24.75%	31.28%	23.44%	23.08%	28.15%
Short	32.53%	33.00%	43.66%	59.09%	34.85%	36.92%	35.16%	38.46%	42.22%

Figure 29. Percentage of bivalents in long (1, 3, and 5) and short (2 and 4) chromosomes of different autotetraploid lines. Bar chart representing the percentage of long (blue) and short (red) chromosomes forming bivalents in the synthetic autotetraploid lines Col-0 4x, L*er*-1 4x, St-0 4x, and Bla-1 4x; the established lines 3151 4x and 3432 4x; and the natural lines Bla-5 4x, Wa-1 4x, and M3385S 4x. Statistical comparison made between the long and short chromosomes for each autotetraploid line. Stars indicate *** $p < 0.001$ and ** $p < 0.01$. ns = not significant.

Although autotetraploid lines from different groups (synthetic, established, and natural) were not statistically compared as they differ not only in the number of generations after the WGD event but also in their genetic backgrounds, a general comparison of the results might be worth to be done in order find out possible differences in chromosome behavior. Firstly, the chiasma frequency could not be directly related with the autotetraploid type as most the frequencies observed from the synthetic lines (except Col-0 4x) were similar to those obtained in the established and natural lines. Furthermore, the synthetic lines St-0 4x and Bla-1 4x displayed the lowest chiasma frequencies. Secondly, regarding the chromosome associations at metaphase I, the results obtained from these three different groups were again unexpected due to the presence of similar levels of II, IV, and I among the synthetic, established, and natural lines. As for the I levels, some of the lines also presented comparable frequencies. Thirdly, the line that showed the highest levels of diploidization was the synthetic Bla-1 4x, being its frequency of II from 30 % to 60 % higher than that of the established and natural autotetraploids analyzed in this study. Finally, there seems to be a general trend to have higher diploidization levels in the shortest and acrocentric chromosomes (2 and 4) than in the longest and metacentric/submetacentric chromosomes (1, 3, and 5).

4.2.4. Expression analysis of genes involved in homologous recombination in diploid and autotetraploid lines

Following WGD, adaptation to polyploidy might involve changes in gene expression levels. In order to shed light on the gene expression changes that may occur because of WGD events and those derived from the processes of adaptation to polyploidy, the mRNA levels of genes that are important for the meiotic HR were assessed in different diploid and autotetraploid lines of *A. thaliana*. Thus, **transcription levels** of genes essential for the formation and resection of DSBs (*SPO11-1* and *MRE11*); genes necessary for the invasion of DNA strands to homologous sequences (*RAD51* and *DMC1*); genes important for both class I (*MLH3* and *HEI10*) and class II (*MUS81*, *FANCM*, and *RECQ4A*) CO formation pathways; genes involved in the organization of the axial/lateral (*ASY1* and *ASY3*) and central (*ZYP1a* and *ZYP1b*) elements of the SC; and genes related to sister chromatid cohesion (*SYN1*, *SMC1*, and *PDS5B*) (see section 1.8) were assessed by means of the RT-qPCR technique. Additionally, the transcription levels of *CAND1* were assessed as it was observed in this study that this gene is important for normal CO levels in both diploid and tetraploid plants (see sections 4.4.1 and 4.4.2). Finally, expression levels of *MRD1* were also analyzed. We included this gene in the analysis because Yu *et al.* (2010) observed significant variations in the mRNA levels when several diploid accessions of *A*. *thaliana* were compared with each other and with their synthetic autotetraploid counterparts. Although little is known about the role of *MRD1*, it has been described that this gene is transcriptionally suppressed in the *mto1-1* mutant background, which is characterized by an over-accumulation of soluble methionine in young rosettes (Goto and Naito, 2002). In addition, we found interesting to assess the expression level of this gene as its sequence overlaps with that of *HEI10* (a gene important for class I CO formation) at the Chr 1, being both genes in opposite directions.

The autotetraploid lines analyzed in this study were the synthetic line Col-0 4x, the established line 3151 4x, and the natural accession Bla-5 4x. The diploid accession Col-0 2x was used in this analysis because it has the same genetic background as the synthetic line Col-0 4x and similar to the 3151 4x background (Col-1). The other diploid line used for this analysis, Bla-1 2x, was selected due to its geographical proximity to the natural autotetraploid accession Bla-5 4x. Thus, in order to evaluate changes in the transcription levels of these genes during meiosis, mRNA was isolated from flower buds with a proper

size. Additionally, genes that showed significant differences in transcription levels between two genotypes using flower bud samples and with RQ values higher than 2 or lower than 0.5 (double or half of the mRNA levels, respectively; see section 3.2.4.3), were also analyzed in seedling samples in order to find out whether the differential levels observed were meiosis-specific.

Firstly, a comparison between the diploid accessions **Col-0 2x** and **Bla-1 2x** was made (Figure 30). Results showed that ***SPO11-1*** (RQ = 0.09), ***RAD51*** (RQ = 0.47), **DMC1** (RQ = 0.47), and ***SYN1*** (RQ = 0.30) presented significantly lower transcription levels in flower buds of Bla-1 2x than those of Col-0 2x. The difference showed by *SPO11-1* was especially remarkable as its transcription levels in Bla-1 2x were more than ten times lower than in Col-0 2x. As for *RECQ4A* (RQ = 0.55) and *ASY1* (RQ = 0.52), they also showed significantly lower levels in Bla-1 2x than in Col-0 2x, although in these cases the RQ values were not lower than 0.5. On the contrary, *ZYP1a* displayed a 2.67-fold higher transcription levels in Bla-1 2x compared to those in Col-0 2x (Figure 30A). As for the rest of the genes analyzed, no significant differences were found.

The mRNA levels of the genes that showed significant differences in the flower bud samples were also analyzed in seedling samples (Figure 30B). *RAD51* (RQ = 0.54) and *DMC1* (RQ = 0.10) presented significantly lower levels in Bla-1 2x than in Col-0 2x, however, only *DMC1* displayed an RQ value lower than 0.5.

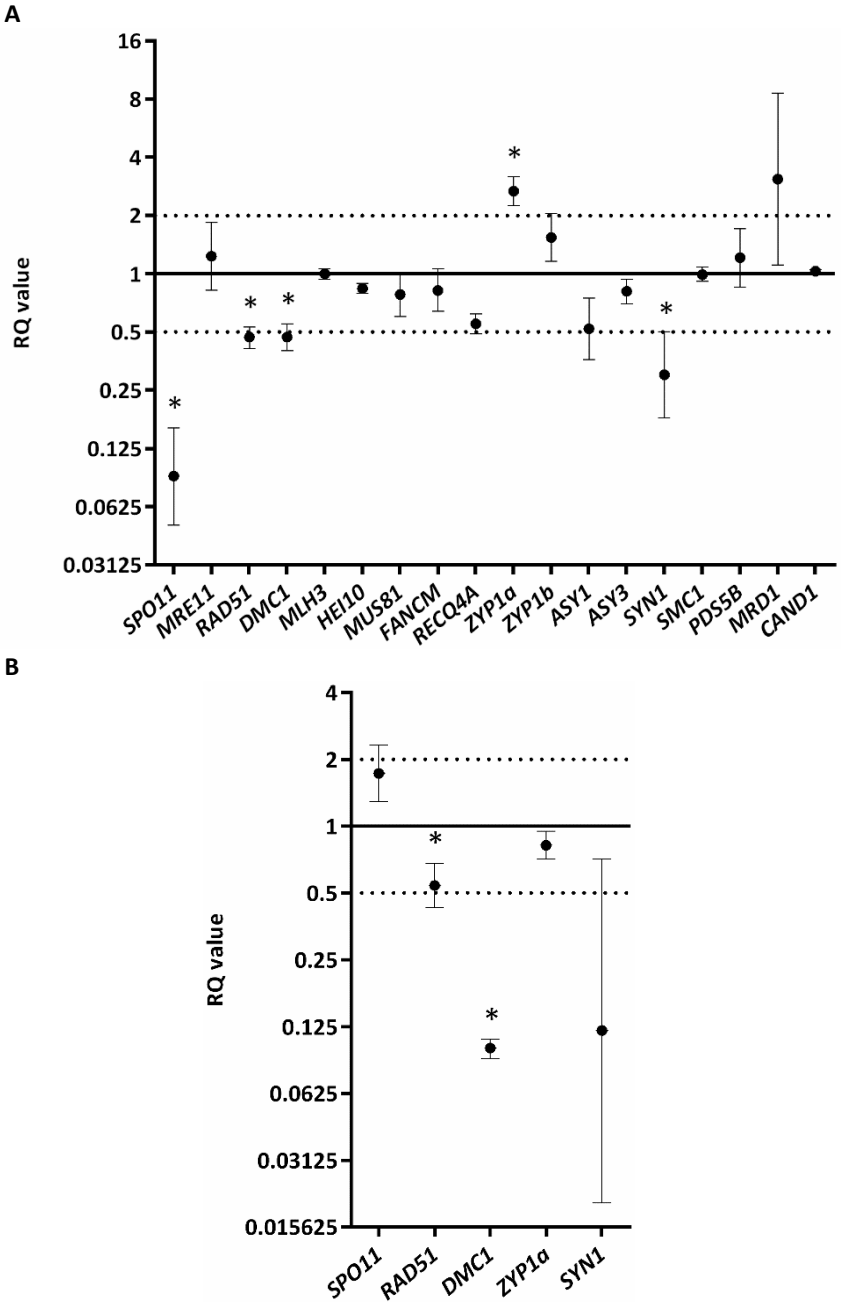


Figure 30. Relative transcription levels of several genes involved in meiotic HR comparing diploid accessions: Bla-1 2x vs Col-0 2x. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of Bla-1 2x respect to the levels of Col-0 2x. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels, respectively). Stars indicate significant differences, 99 % CI.

Also, a comparison between the synthetic autotetraploid line **Col-0 4x** and the natural autotetraploid accession **Bla-5 4x** was made (Figure 31). In this case, the experiment showed that the mRNA levels of ***SYN1*** in flower buds (RQ = 0.31) and especially those of ***SPO11-1*** (almost 10 times, RQ = 0.14) were significantly higher in Col-0 4x than in Bla-5 4x. Regarding ***CAND1***, this gene also showed significantly lower levels in the natural tetraploid than in the synthetic one, however the RQ value was not lower than 0.5 (RQ = 0.67). The other genes that presented different levels between these two autotetraploid backgrounds were ***HEI10***, with more than four times higher levels in Bla-5 4x than in Col-0 4x (RQ = 4.16); and ***PDS5B***, with a 3.31-fold difference (Figure 31A).

SPO11-1 (RQ = 0.08) and *SYN1* (RQ = 0.48) also had considerably lower expression levels in Bla-5 4x than in Col-0 4x seedlings (Figure 31B). *PDS5B* showed significantly greater levels in Bla-5 4x compared to those of Col-0 4x. Although this result was similar in flower bud samples, in this case, the difference was smaller and the RQ value was below 2 (RQ = 1.12). On the contrary, *HEI10* presented the opposite behavior in seedlings than in flower bud samples, with a slight but still significant lower expression in Bla-5 4x than in Col-0 4x (RQ = 0.60). Therefore, apart from *SPO11-1*, differences observed for *HEI10*, *SYN1*, and *PDS5B* in flower bud samples seem to be meiosis-specific.

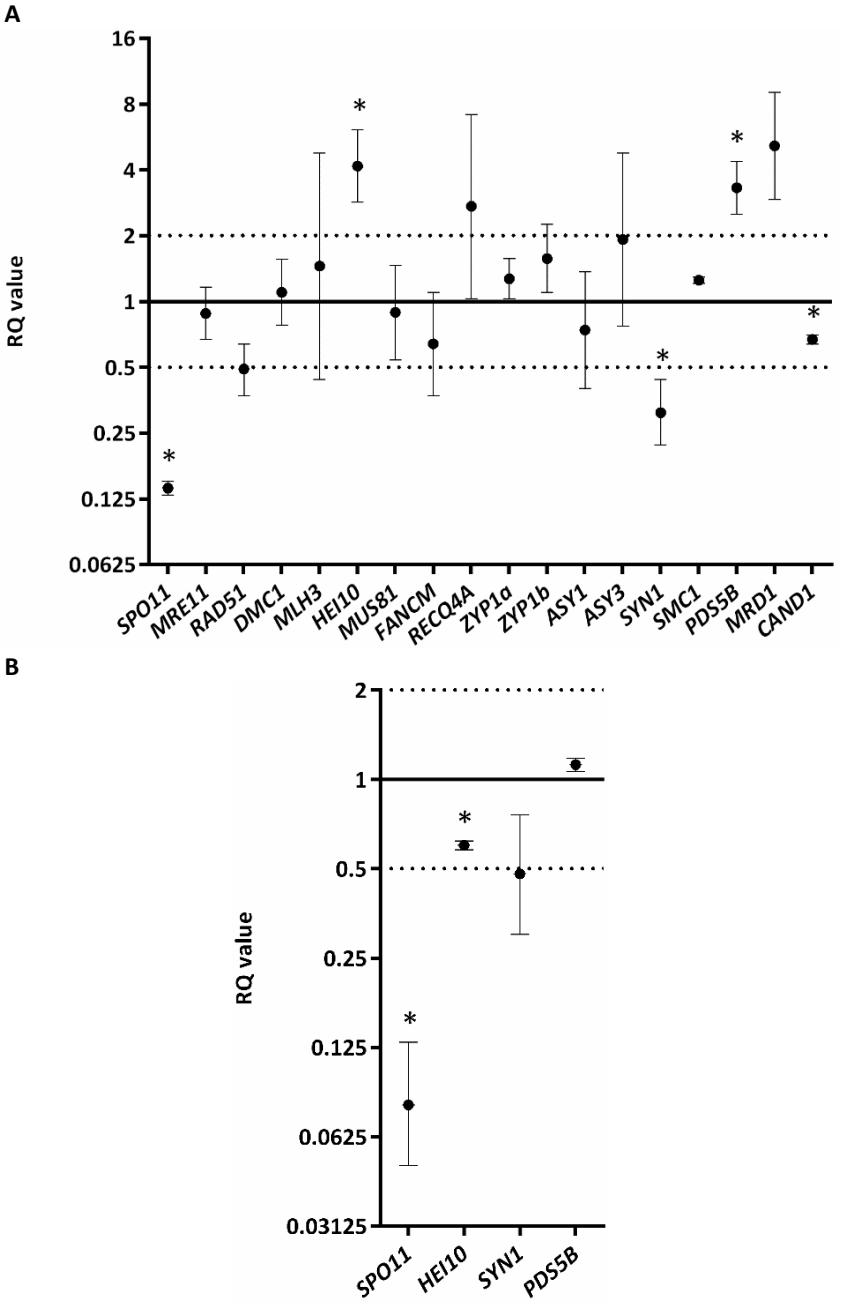


Figure 31. Relative transcription levels of several meiotic recombination genes comparing synthetic and natural autotetraploids: Bla-5 4x vs Col-0 4x. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of Bla-5 4x respect to the levels of Col-0 4x. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

Next, the comparison was made between the synthetic autotetraploid line **Col-0 4x** and the established autotetraploid **3151 4x** (Figure 32). In this case, the greatest differences in transcription levels were found for the genes ***HEI110*** (RQ = 0.23), ***MUS81*** (RQ = 0.18), ***RECQ4A*** (RQ = 0.31), ***ZYP1b*** (RQ = 0.34), and ***MRD1*** (RQ = 0.01). All of them presented higher levels in Col-0 4x than in 3151 4x, but probably the most striking result was the difference found between the *MRD1* levels being 100 times lower in 3151 4x than in Col-0 4x. In addition, slight differences were also observed in the expression levels of *DMC1* (RQ = 1.51), *FANCM* (RQ = 0.61), *ZYP1a* (RQ = 0.66), and *CAND1* (RQ = 1.36) between this two autotetraploid lines (Figure 32A).

Again, the mRNA levels of the genes that showed the greatest differences in flower bud samples were also compared in seedling samples (Figure 32B). Results displayed that *HEI10* (RQ = 0.24) and *MRD1* (RQ = 0.02) also presented significantly lower levels in 3151 4x than in Col-0 4x in somatic tissues. On the contrary, *MUS81* (RQ = 0.83), *RECQ4A* (RQ = 0.76), and *ZYP1b* (RQ = 0.68) displayed no significant differences in seedlings between this two genetic backgrounds, then the differences of their mRNA expression levels in flower bud samples might be meiosis-specific.

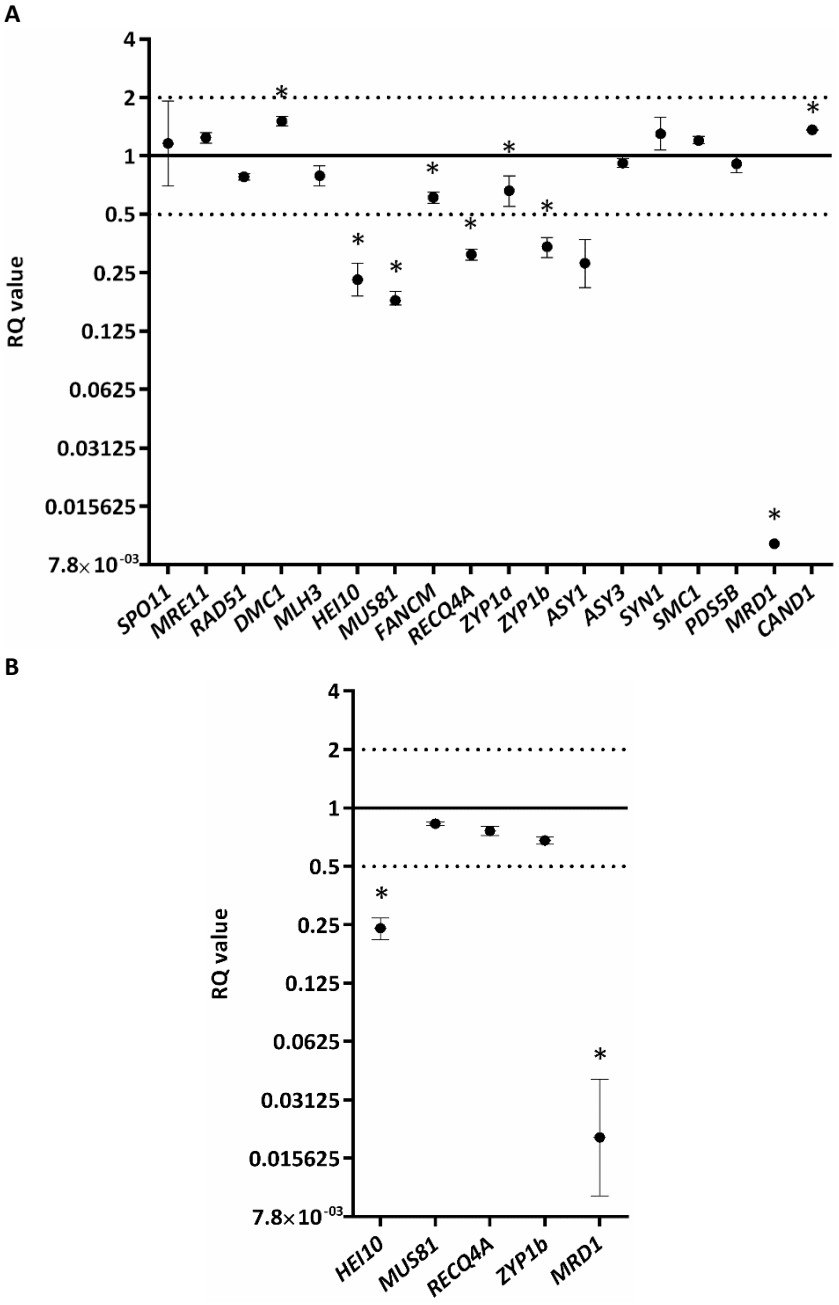


Figure 32. Relative transcription levels of several meiotic recombination genes comparing synthetic with established autotetraploids: 3151 4x vs Col-0 4x. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of 3151 4x respect to the levels of Col-0 4x. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

In order to assess differences in transcription levels that can occur in the same genetic background immediately after a WGD event, the diploid line **Col-0 2x** and its synthetic autotetraploid counterpart **Col-0 4x** were compared (Figure 33). The analysis in flower bud samples presented significant differences in ***ZYP1a***, being the Col-0 4x levels almost 4 times higher than those of Col-0 2x (RQ = 3.57). ***SYN1*** also showed a significantly greater expression in the synthetic polyploid line compared to its diploid counterpart (RQ = 2.62). In addition, *CAND1* presented significantly greater levels in the tetraploid than in the diploid (RQ = 1.69), however, in this case the RQ value was below 2 (Figure 33A). On the other hand, although the RQ value of *MRD1* was close to 4 (RQ = 3.61), the statistical reveled no significant differences between Col-0 4x and Col-0 2x. This result differs from the result obtained by Yu *et al.* (2010), since they found up to 110-fold increase in the synthetic tetraploid compared to the diploid, however, their comparison was conducted in leaves and seedlings.

When the mRNA levels of *ZYP1a* (RQ = 1.42) and *SYN1* (RQ = 0.83) were compared in seedling samples, no differences were observed (Figure 33B). Then, it might be that the changes observed after the WGD induction for these two genes in Col-0 are meiosis-specific.

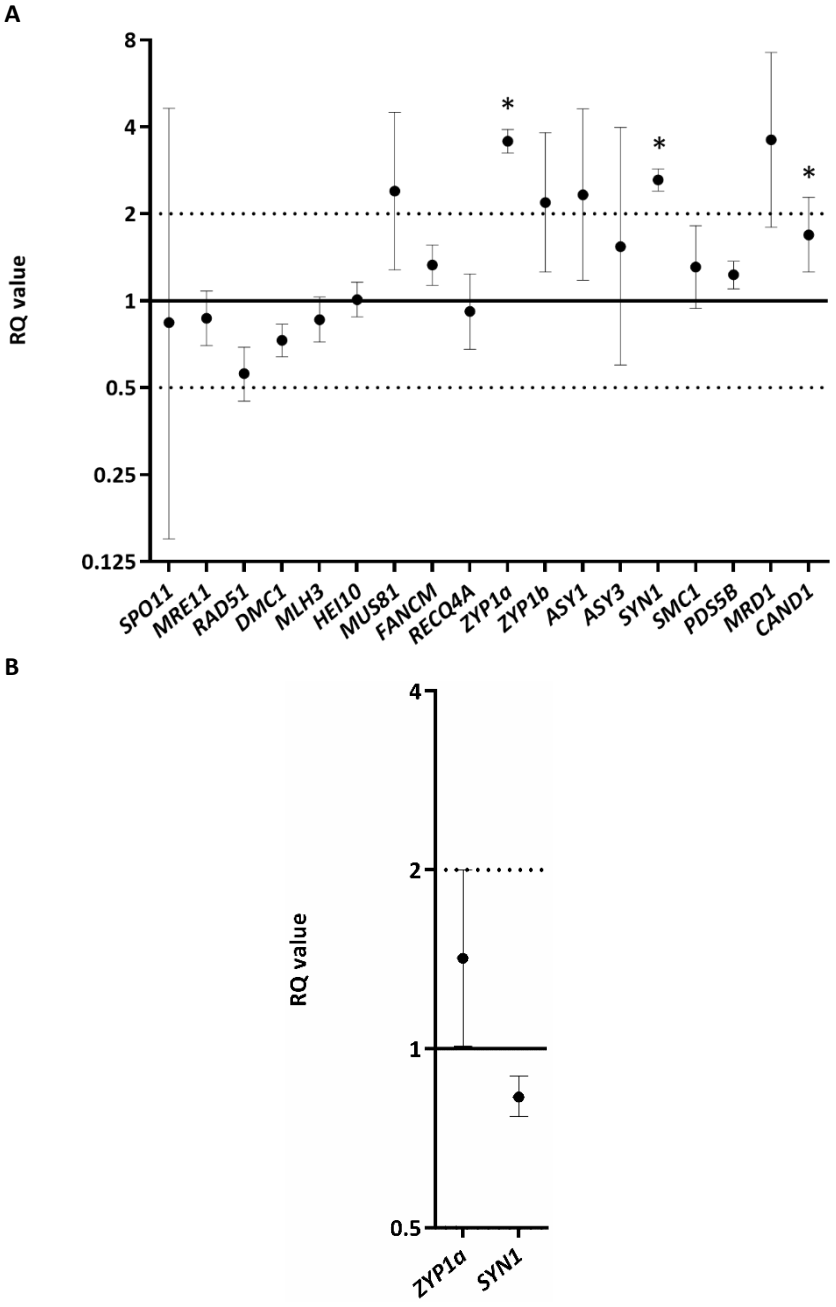


Figure 33. Relative transcription levels of several meiotic recombination genes comparing a diploid accession with its synthetic tetraploid counterpart: Col-0 4x vs Col-0 2x. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of Col-0 4x respect to the levels of Col-0 2x. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

Finally, a comparison between the diploid accession **Bla-1 2x** and the natural autotetraploid **Bla-5 4x** was performed (Figure 34). In this case, the analysis conducted in flower bud samples displayed that the transcription levels of both ***HEI10*** (RQ = 5.65) and ***RECQ4A*** (RQ = 5.25) were almost 6 times higher in the natural autotetraploid than in this diploid accession that come from the same geographical region. Besides, significant differences were noticed when the expression levels of ***PDS5B*** were compared. The mRNA levels of this gene were more than 3 times higher in Bla-5 4x than in Bla-1 2x (RQ = 3.35). Moreover, narrow but significant differences were found for *SMC1* (RQ = 1.66) and *CAND1* (RQ = 0.90) (Figure 34A).

The transcription levels of *HEI10* (RQ = 3.77), *RECQ4A* (RQ = 4.20), and *PDS5B* (RQ = 40.32) were also assessed in Bla-5 4x and Bla-1 2x seedlings (Figure 34B). This analysis revealed significantly higher levels of these three genes (especially *PDS5B*) in the autotetraploid accession than in the diploid accession, although the difference exhibited by *PDS5B* in seedling samples was considerably greater than in flower buds.

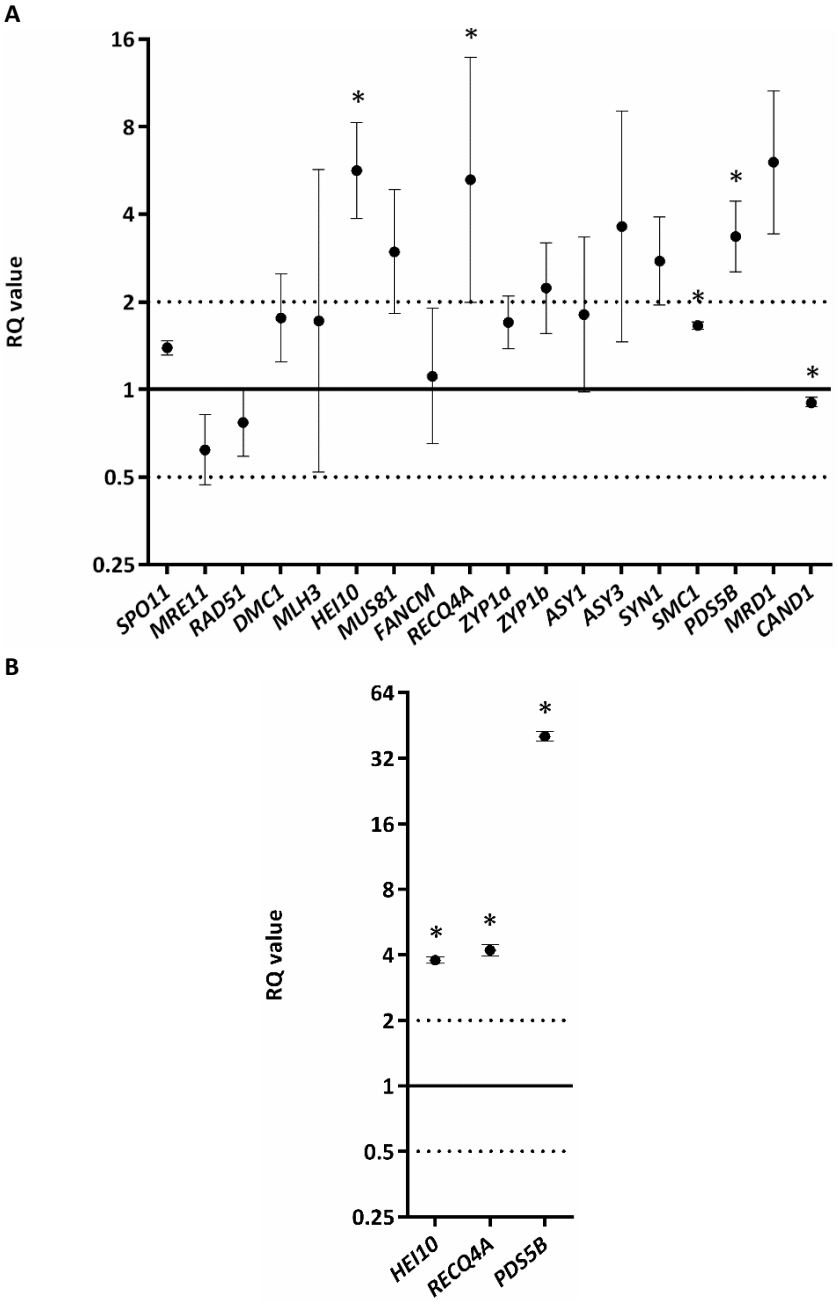


Figure 34. Relative transcription levels of several meiotic recombination genes comparing a diploid accession with a natural autotetraploid accession: Bla-5 4x vs Bla-1 4x. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of Bla-5 4x respect to the levels of Bla-1 2x. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

The statistical significance of the results obtained from the gene transcription analyses performed in different diploid and autotetraploid lines are summarized in Tables 16 and 17.

Table 16. Variations in transcription levels of several meiotic genes among different diploid and autotetraploid genotypes using flower bud samples

	Bla-1 2x vs Col-0 2x	Bla-5 4x vs Col-0 4x	3151 4x vs Col-0 4x	Col-0 4x vs Col-0 2x	Bla-5 4x vs Bla-1 2x
SPO11-1	* ↓	* ↓	ns	ns	ns
MRE11	ns	ns	ns	ns	ns
RAD51	* ↓	ns	ns	ns	ns
DMC1	* ↓	ns	* ↑	ns	ns
MLH3	ns	ns	ns	ns	ns
HEI10	ns	* ↑	* ↓	ns	* ↑
MUS81	ns	ns	* ↓	ns	ns
FANCM	ns	ns	* ↓	ns	ns
RECQ4A	ns	ns	* ↓	ns	* ↑
ZYP1a	* ↑	ns	* ↓	* ↑	ns
ZYP1b	ns	ns	* ↓	ns	ns
ASY1	ns	ns	ns	ns	ns
ASY3	ns	ns	ns	ns	ns
SYN1	* ↓	* ↓	ns	* ↑	ns
SMC1	ns	ns	ns	ns	* ↑
PDS5B	ns	* ↑	ns	ns	* ↑
MRD1	ns	ns	* ↓	ns	ns
CAND1	ns	* ↓	* ↑	* ↑	* ↓

** Significant differences with 99 % confidence interval. ns = not significant.*
Arrows indicate higher (↑) or lower (↓) relative transcription levels of the first genotype respect to the second one.
Dark cells refer to comparisons that show both, significant differences and RQ values greater than 2 or lower than 0.5.

Table 17. Variations in transcription levels of several meiotic genes among different diploid and autotetraploid genotypes using seedling samples

	Bla-1 2x vs Col-0 2x	Bla-5 4x vs Col-0 4x	3151 4x vs Col-0 4x	Col-0 4x vs Col-0 2x	Bla-5 4x vs Bla-1 2x
SPO11-1	ns	* ↓	—	—	—
RAD51	* ↓	—	—	—	—
DMC1	* ↓	—	—	—	—
HEI10	—	* ↓	* ↓	—	* ↑
MUS81	—	—	ns	—	—
RECQ4A	—	—	ns	—	* ↑
ZYP1a	ns	—	—	ns	—
ZYP1b	—	—	ns	—	—
SYN1	ns	ns	—	ns	—
PDS5B	—	ns	—	—	* ↑
MRD1	—	—	* ↓	—	—

** Significant differences with 99 % confidence interval. ns = not significant.*
Arrows indicate higher (↑) or lower (↓) relative transcription levels of the first genotype respect to the second one.
Dark cells refer to comparisons that show both, significant differences and RQ values greater than 2 or lower than 0.5.
Lines refer to comparison not conducted.

4.3. Competition for chiasma formation between identical and homologous chromosomes in autotetraploids

4.3.1. Mean cell chiasma frequencies of the diploid accessions Col-0, L*er*-1, and the hybrid Col/L*er*

By means of the FISH technique, the **chiasma frequencies per cell and per chromosome** of the complement were assessed for the accessions **Col-0 2x, L*er*-1 2x, and the hybrid** obtained by crossing these two (Col/L*er* 2x) (Figure 36, Table 18). Col-0 2x (10.20 ± 0.14, n = 70) showed a significantly higher mean chiasma frequency per cell than L*er*-1 2x (9.13 ± 0.10, n = 158; t = 6.20, p < 0.001). However, the hybrid Col/L*er* 2x presented an intermediate value (9.48 ± 0.11, n = 120) which was significantly different respect to the frequency showed by Col-0 2x (t = 4.14, p < 0.001) and L*er*-1 (t = 2.39, p < 0.05).

Table 18. Chiasma frequencies observed for the different chromosomes in metaphase I PMCs of Col, L*er,* and Col/L*er* diploid plants

	Chromosomes						
	1	**2**	**3**	**4**	**5**	$\bar{X}$	**N**
Col-0 2x	2.55 (25.0)	1.73 (17.0)	2.16 (21.2)	1.50 (14.7)	2.28 (22.4)	10.20	70
L*er*-1 2x	2.09 (22.9)	1.74 (19.1)	1.79 (19.6)	1.61 (17.6)	1.90 (20.8)	9.13	158
Col/L*er* 2x	2.14 (22.6)	1.76 (18.6)	1.85 (19.5)	1.70 (17.9)	2.04 (21.5)	9.48	120

Values showed in parentheses represent the contribution in percentage of each chromosome to the total mean chiasma frequency ($\bar{X}$).
Number of cells analyzed (N).

In L*er*-1 2x, the 5S rDNA region of the **Chr 3** divides the **long arm** of this chromosome in two regions: a proximal-interstitial region between the centromere and the 5S rRNA genes, and an interstitial-distal region from these genes to the telomere (Figure 35). This feature allowed a more accurate **analysis of the chiasma distribution on this arm**, not only in this accession but also in the Col/L*er* 2x hybrid (Figure 36 D-G). In these two backgrounds, results showed that about 50 % of chiasmata took place in each region (L*er*-1 2x: $X^2_{(1)} = 0.70$, p = 0.40; Col/L*er* 2x: $X^2_{(1)} = 1.20$, p = 0.27).

Thus, the results showed that the accession Col-0 2x presented a greater total chiasma frequency than that of L*er*-1 2x. Interestingly, the diploid hybrid obtained from the cross of the two lines displayed an intermediate chiasma frequency. Moreover, when the chiasma localization was assessed at the Chr 3 in L*er*-1 2x and the hybrid, chiasmata were evenly distributed at both sides of the 5S rDNA region which divides the long arm of this chromosome.

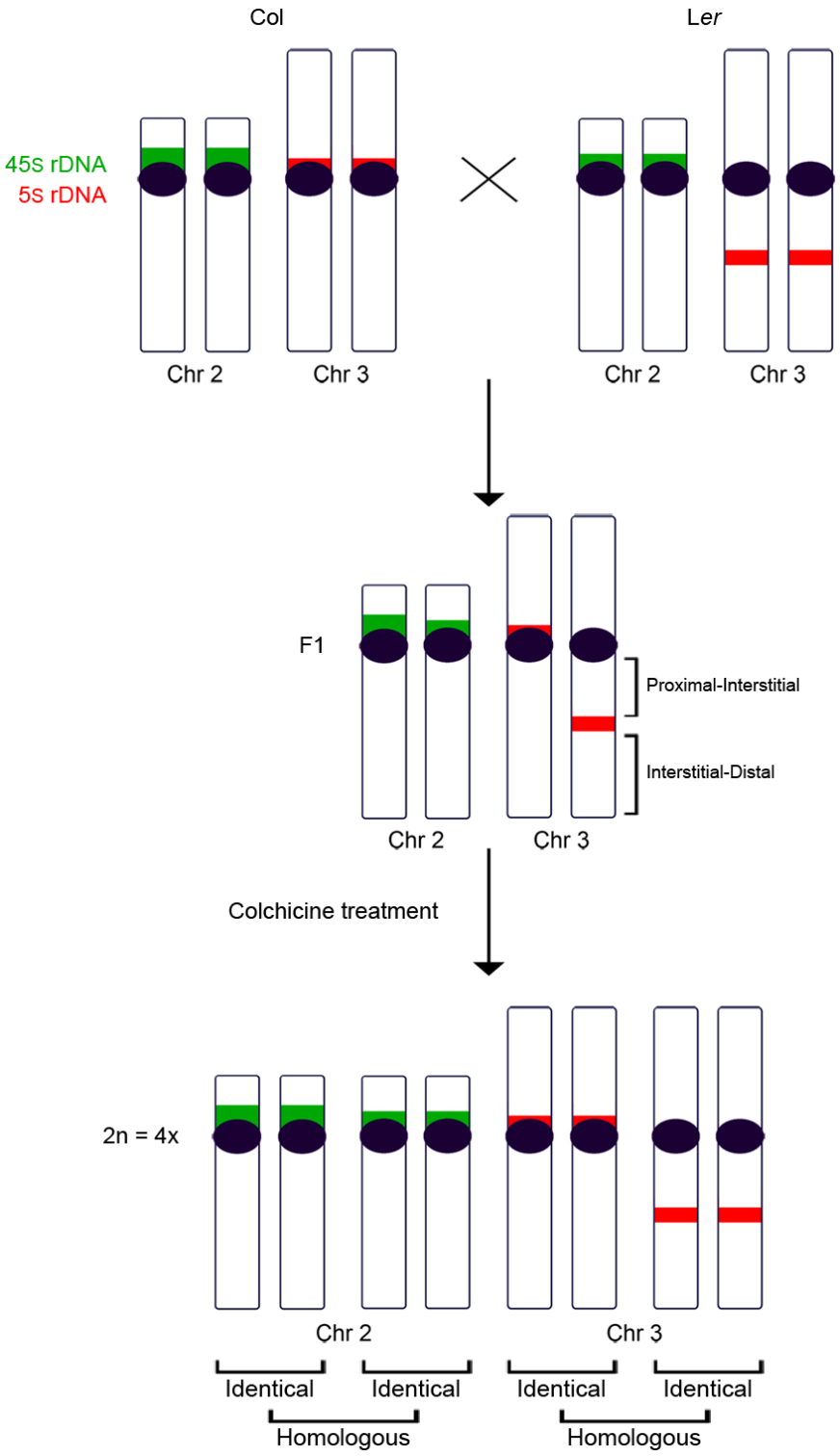


Figure 35. Ideogram of chromosomes 2 and 3 in Col-0, L*er*-1, and in the hybrid progeny obtained from the cross between both accessions. In the hybrid, the ideogram is represented before and after the chromosome duplication. The Chr 2 and 3 of Col-0 and L*er*-1 can be distinguished to each other after applying FISH with the 5S (red) and 45S (green) rDNA probes due to the size and position of these loci. These differences allow the identification of the parental origin of each chromosome in the hybrid, before and after the colchicine treatment. In the tetraploid hybrid, each chromosome is accompanied by one identical chromosome and two homologous chromosomes.

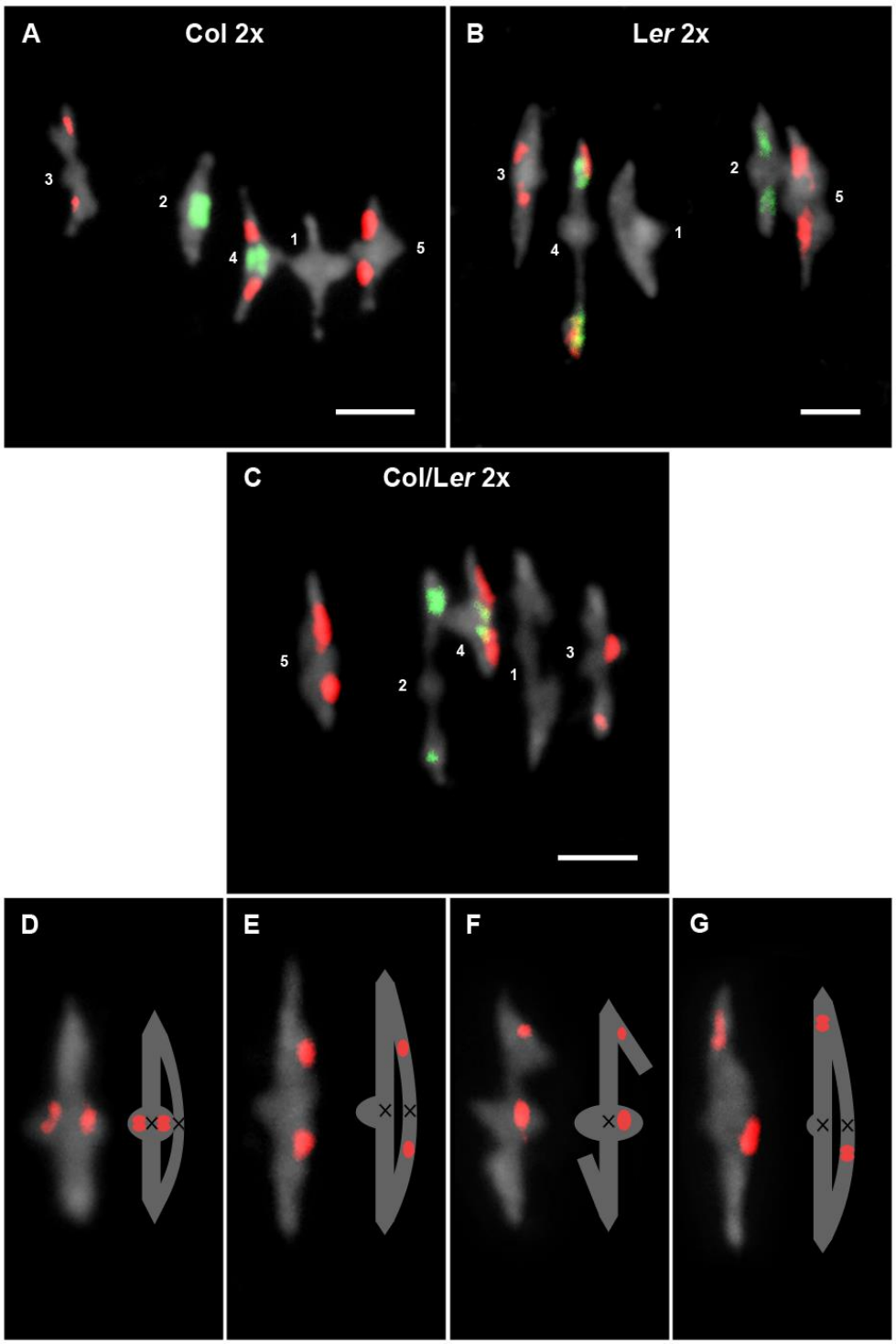


Figure 36. Cytological analysis of Col-0 2x, L*er*-1 2x, and Col/L*er* 2x PMCs at metaphase I. Representative examples of metaphases I from Col-0 2x (**A**), L*er*-1 2x (**B**), and Col/L*er* 2x (**C**). Chromosomes were identified by FISH using the 5S (red) and 45S rDNA (green) probes. Numbers indicate which chromosomes of the complement are forming each bivalent. Images and representations of bivalents formed by Chr 3 in which the chiasma (black cross) has been formed in the central-interstitial region (**D**, **F**) or in the interstitial-terminal region (**E**, **G**) in L*er* (**D**, **E**) and in Col/L*er* (**F**, **G**) backgrounds. Bars represent 5 μm.

4.3.2. Mean cell chiasma frequencies and chromosome configurations of autotetraploid plants of Col-0, L*er*-1, and the hybrid Col/L*er*

Chiasma frequencies were also assessed for the tetraploids **Col-0 4x, L*er*-1 4x, and the hybrid** Col/L*er* 4x. Results showed that Col-0 4x had the highest mean cell chiasma frequency (19.99 ± 0.11, n = 186) followed by Col/L*er* 4x (19.03 ± 0.15, n = 139), whereas L*er*-1 4x presented the lowest frequency (18.5 ± 0.27, n = 50) (Table 19). These values were about two-fold the corresponding mean cell chiasma frequencies of the diploid counterparts (Table 18). The data showed significant differences between the means of Col-0 4x and L*er*-1 4x ($t = 5.19$, $p < 0.001$), and between Col-0 4x and the tetraploid hybrid ($t = 3.24$, $p < 0.001$), however, no significant differences between L*er*-1 4x and the hybrid were found ($t = 1.19$, $p = 0.08$). The relative contribution of each chromosome to the total mean cell chiasma frequency was similar in these three backgrounds, except for a slightly lower contribution of the Chr 3 in the hybrid (Table 19). Besides, the **chiasma localization** was also analyzed in more detail on the **long arm of the Chr 3** in L*er*-1 4x and the tetraploid hybrid, but only in cells in which the Chr 3 were associated as II. As it was observed in diploid cells, about 50 % of chiasmata were located at the proximal-interstitial region (centromere – 5S rDNA) and the remaining 50 % at the interstitial-distal region (5S rDNA – telomere) (L*er* 4x: $X^2_{(1)} = 1.58$, $p = 0.30$; Col/L*er* 4x: $X^2_{(1)} = 1.09$, $p = 0.21$).

Table 19. Multivalents, bivalent pairs, and chiasma frequency observed for the different chromosomes in metaphase I PMCs of Col-0 4x, L*er*-1 4x, and Col/L*er* 4x plants

		Chromosomes							
		1	**2**	**3**	**4**	**5**	**Total**	$\bar{X}$	**N**
Col-0 4x	M	153 (82.3)	129 (69.4)	150 (80.6)	122 (65.9)	151 (81.2)	705 (75.9)	3.79	186
	IIs	33 (17.7)	57 (30.6)	36 (19.4)	63 (34.1)	35 (18.8)	224 (24.1)	1.20	
	Xta	4.19 (21.0)	4.02 (20.1)	3.91 (19.6)	3.82 (19.1)	4.05 (20.2)		19.99	
L*er*-1 4x	M	40 (80)	31 (64.6)	36 (73.5)	33 (70.2)	39 (73.6)	179 (72.5)	3.58	53
	IIs	10 (20)	17 (35.4)	13 (26.5)	14 (29.8)	14 (26.4)	68 (27.5)	1.36	
	Xta	4.00 (21.6)	3.60 (19.5)	3.64 (19.7)	3.54 (19.1)	3.72 (20.1)		18.50	
Col/L*er* 4x	M	97 (69.8)	70 (51.5)	70 (50.7)	89 (64)	99 (71.2)	425 (61.5)	3.06	139
	IIs	42 (30.2)	66 (48.5)	68 (49.3)	50 (36)	40 (28.8)	266 (38.5)	1.91	
	Xta	4.07 (21.4)	3.78 (19.9)	3.60 (18.9)	3.59 (18.9)	3.99 (20.9)		19.03	

Values showed in parentheses represent the percentage of multivalents (M) and pairs of bivalents (IIs) to the total cells (N) and the contribution in percentage of each chromosome to the total mean chiasma frequency ($\bar{X}$).

Additionally, frequencies corresponding to the different **chromosome associations** observed at metaphase I were scored for each chromosome in each genotype (Table 19). Chromosomes were associated as IV, III, and II (Figure 37 A-C). The frequency of III was very low in the three genetic backgrounds analyzed (16/186 = 9 % in Col-0 4x; 8/50 = 16 % in L*er*-1 4x; 11/139 = 8 % in Col/L*er* 4x), therefore no distinction was made between IV and III, scoring both as multivalents (Table 19).

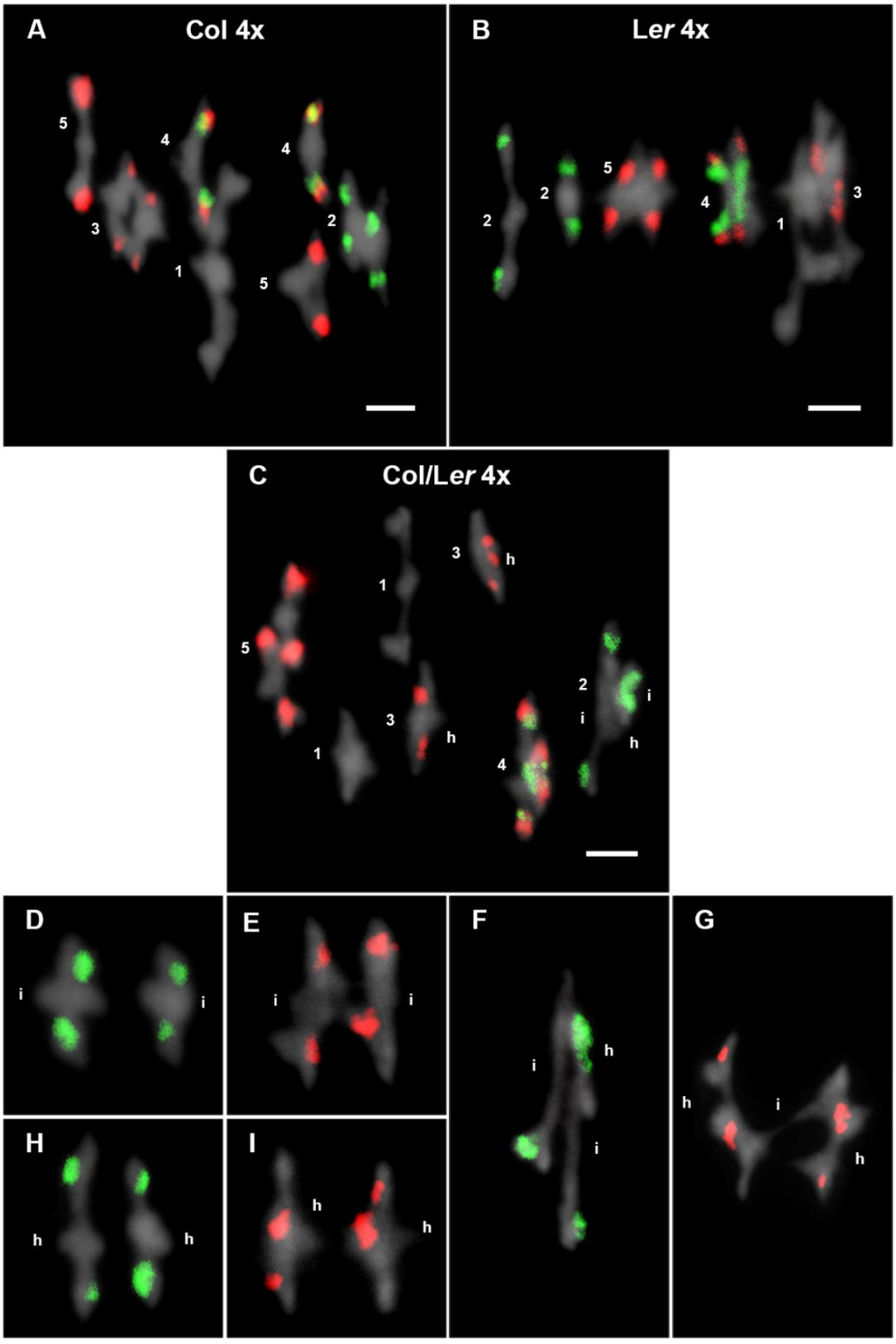


Figure 37. Cytological analysis of Col-0 4x, L*er*-1 4x, and Col/L*er* 4x PMCs at metaphase I. Representative examples of metaphases I from Col-0 4x (**A**), L*er*-1 4x (**B**), and Col/L*er* 4x (**C**). Chromosomes were identified by FISH using the 5S (red) and 45S rDNA (green) probes. Numbers indicate which chromosomes of the complement are forming each quadrivalent or bivalent. The figure also includes examples of associations between identical chromosomes in bivalents formed by Chr 2 (**D**) and Chr 3 (**E**). Examples of associations between homologous chromosomes in bivalents formed by Chr 2 (**H**) and Chr 3 (**I**). Examples of quadrivalents formed by chromosomes 2 (**F**) and 3 (**G**). Letters "i" and "h" indicate associations between identical and homologous chromosomes, respectively. Bars represent 5 μm.

Synaptic associations in autotetraploids with metacentric chromosomes have usually been estimated under the following premises (reviewed in Sybenga, 1975): (i) the presence of two independent synapsis initiation points per chromosome, one at each end; (ii) the absence of synaptic preferences; (iii) the existence of the same probabilities for chiasma formation in all meiotic configurations; and (iv) the possibility of free partner switches between the two synapsis initiation points at each chromosome. In this context, there are nine possible configurations to be formed among each group of homologous chromosomes (tetrasome), of which six are IV (2/3) and the remaining three are bivalent pairs (1/3). Thus, the **expected ratio of multivalents to bivalent pairs** at prophase I would be **2:1**. The ratios observed for multivalents to bivalent pairs were tested for the agreement with the theoretical 2:1 ratio for each chromosome in the three autotetraploid genotypes analyzed (Table 20). The rate of multivalent formation over the five chromosomes (705 multivalents, 76 % *vs.* 224 bivalent pairs, 24 %) displayed by Col-0 4x significantly exceeded the 2:1 ratio expected (66.66 % multivalents) ($X^2_{(1)}$ = 35.55; $p < 0.001$). In this accession, at the chromosomal level, only the three largest chromosomes of the complement (1, 3 and 5) showed multivalent frequencies consistently in excess of the 66.66 %. In the case of L*er*-1 4x, it also presented an excess of multivalents (72.5 %) but with a value that is at the limit of the significance level ($X^2_{(1)}$ = 3.74; $p = 0.053$). In this case, only the Chr 1 showed a significant excess of multivalents, while the other four fitted to the random theoretical expectations. Conversely, there was a significant excess of bivalent pairs in Col/L*er* 4x (38.5 %) ($X^2_{(1)}$ = 8.28; $p < 0.01$), mainly due to the behavior of Chr 2 and 3 (Table 20).

Table 20. Chi-square test values testing goodness of fit to 2:1 ratio of multivalents : bivalent pairs for the different chromosomes in PMCs from Col-0 4x, L*er*-1 4x and Col/L*er* 4x plants

	Chromosomes					
	1	**2**	**3**	**4**	**5**	**T**
Col-0 4x	> 20.35***	= 0.60^{ns}	> 16.35***	= 0.04^{ns}	> 17.64***	> 35.55***
L*er*-1 4x	> 4.00*	= 0.09^{ns}	= 1.02^{ns}	= 0.64^{ns}	= 0.64^{ns}	= 3.74^{ns}
Col/L*er* 4x	= 0.61^{ns}	< 14.13***	< 15.78***	= 0.44^{ns}	= 1.30^{ns}	< 8.28**

Less than and greater than symbols indicate direction of deviation: < (<2:1), > (>2:1).
*Stars indicate *** p < 0.001, ** p < 0.01, and * p < 0.05. ns = not significant.*

As in the results obtained at the diploid level, the tetraploid Col-0 4x showed higher chiasma numbers than that of L*er*-1 4x and the hybrid Col/L*er* 4x which displayed again an intermediate frequency. When the chiasma localization at the Chr3 was assessed in the tetraploids L*er*-1 4x and the hybrid, their behavior was again similar to their diploid counterparts showing an even distribution of the chiasmata at both sides of the 5S rDNA region. Finally, when the ratio of multivalents : bivalent pairs was analyzed, Col-0 4x showed an excess of multivalents according to the 2:1 ration predicted, whereas L*er*-1 4x presented a no significant excess and the hybrid displayed significantly higher percentage of bivalent pairs. Locking at the behavior of each chromosome individually, different proportions of multivalents were observed comparing chromosomes within the same background but also comparing the same chromosome among different backgrounds.

4.3.3. Competition for chiasma formation between identical and homologous chromosomes in the autotetraploid hybrid Col/L*er*

By means of the FISH technique, accessions **Col-0 and L*er*-1 can be distinguished by the position of 5S rDNA genes in the Chr3** (short arm in Col-0 and long arm in L*er*-1). Moreover, during this study, another difference between these two accessions was detected. The NOR region located on the Chr 2 is bigger in Col-0 than in L*er*-1 (Figures 35, 36 A-C, and 37 A-C). Thus, this study allowed to analyze whether chromosome intraspecific differences in autotetraploids are enough to determine preferences in terms of chiasma formation. In Col/L*er* 4x hybrids, there is one pair of identical chromosomes from Col-0 and another identical pair from L*er*-1. These two pairs of identical chromosomes are almost homologous but non-identical to each other (Figure 35). Then, two types of two-by-two metaphase I associations are possible for any chromosome arm: between identical chromosomes or between homologous chromosomes (Figure 37 D-G). If the chiasma formation takes place randomly among the four members of the tetrasome, homologous associations would be twice as common as identical ones.

Following the criteria established by Benavente and Orellana (1991), in this analysis, cells with at least one chiasma between identical or homologous chromosomes were taken into account (regardless of the chromosome configuration adopted by the corresponding tetrasome) to test randomness (Figure 37 D-G, Table 21). When data from multivalents and bivalent pairs were grouped, we detected a different behavior of Chr 2 and 3. We observed random chiasma formation between identical and homologous arms of the Chr 2, and homologous preferences in both arms of the Chr 3. This tendency was also maintained when only the data from bivalent pairs were considered, although in this case the excess of chiasmata between homologous short arms of the Chr 3 was at the limit of the significance level.

Table 21. Number of Col/L*er* 4x chromosome configurations with at least one chiasma between identical or homologous chromosomes in bivalents and multivalents + bivalents and the goodness of fit to the expected ratio 1 identical : 2 homologous

	Chr 2						Chr 3					
	Short arm			Long arm			Short arm			Long arm		
	i	h	X^2_1	i	h	X^2_1	i	h	X^2_1	i	h	X^2_1
M+IIs	36	63	0.41	34	67	0.01	21	78 ↑	6.58*	20	85 ↑	9.64*
IIs	24	42	0.27	22	44	0.00	15	51	3.34	14	54 ↑	4.97*

Chromosomes 2 (Chr 2) and 3 (Chr 3).
Identical (i) and homologous (h) chromosomes.
Chi-square test values obtained (X^2_1).
Bivalent pairs (IIs) and multivalents (M).
Values that exceed the 1:2 (i:h) ratio expected at random (↑).
*Chi-square test values statistically significant, * $p < 0.05$.*

Thus, the assessment of preferences in chiasma formation between homologous or identical chromosomes revealed that different chromosomes of the complement (like in this case Chr 2 and 3) can show different preference patterns.

4.4. Analysis of chromosome behavior after a WGD event in mutants defective in meiotic recombination

Another objective of this thesis was to analyze the behavior of several meiotic recombination mutants as a response to WGD. We wondered whether in a tetraploid situation, where four copies of each chromosome are present, the behavior of several meiotic recombination mutants could change respect to the phenotype that they show at the diploid level.

4.4.1. Cytological analysis of PMCs at metaphase I in diploid mutants defective in meiotic recombination

In this study, up to nine different mutant lines that show defects or alterations in CO formation (see section 1.8), most of them previously characterized at the diploid level, were treated with colchicine to obtain the corresponding autotetraploid mutant lines. In order to assess the chiasma frequency both in diploid (Figures 38 and 39) and tetraploid lines (Figures 40 and 41), as well as the different chromosome association in the last ones (Figure 40, 43, and 44), a cytological analysis was conducted by means of FISH.

Thus, mutant lines for the genes that encode the axis-related protein ASY1; the ZMM proteins ZIP4, MSH5, MER3, and HEI10; the mismatch repair protein MLH3 important for class I COs; the endonuclease MUS81 important for class II COs; the anti-recombinase FANCM; and the neddylation-related protein CAND1 (mutant called *hve*) (Table 2) were analyzed (Figure 38). In the case of CAND1, the role of this protein in meiosis has not been reported yet and currently is being characterized in our laboratory. None of these mutant shows defects in pairing or synapsis except *asy1*, and none of them shows chromosome fragmentation. In this study, the accession Col-0 was used as a control as it is the genetic background of all these mutant lines.

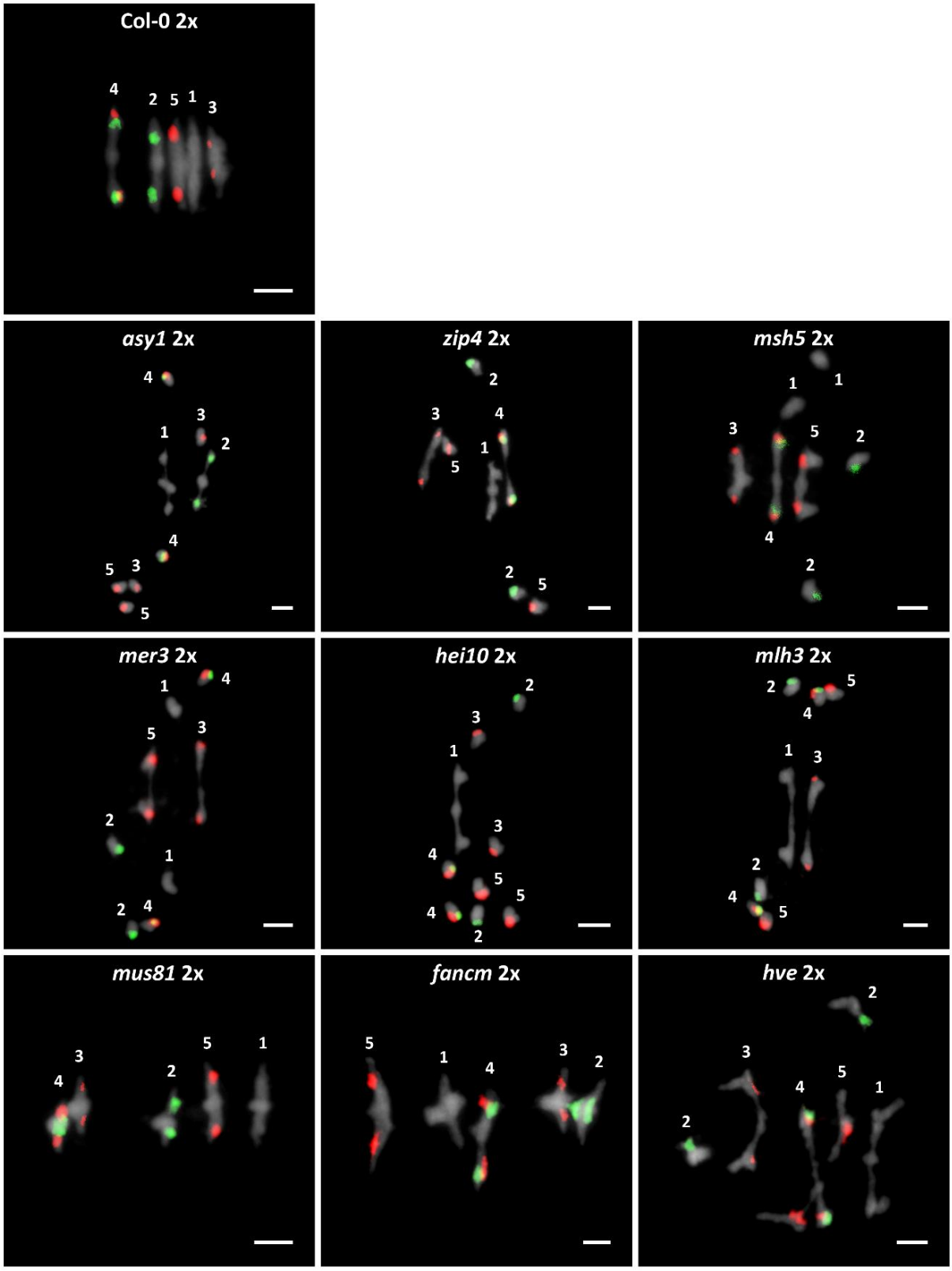


Figure 38. Cytological analysis of PMCs at metaphase I in different mutant lines defective for HR. Representative examples of metaphases I from the diploid mutant lines *asy1* 2x, *zip4* 2x, *msh5* 2x, *mer3* 2x, *hei10* 2x, *mlh3* 2x, *mus81* 2x, *fancm* 2x, and *hve* 2x, as well as the control Col-0 2x. Chromosomes were identified by FISH using the 5S (red) and 45S rDNA (green) probes. Numbers indicate which chromosomes of the complement are forming each bivalent or univalent. Bars represent 5 μm.

In order to compare the **total chiasma frequencies** obtained from the **diploid mutant lines** and the WT, the Welch´s ANOVA statistical test was performed. As it was expected, significant differences were noticed [W (9.0, 232.2) = 807.2, p < 0.001]. Subsequently, a pairwise comparison of each diploid mutant line with the control (Figure 39) was performed using the Dunnett´s T3 post hoc test. Results showed that the diploid mutants ***asy1* 2x** (2.10 ± 0.13, n = 101, p < 0.001)*,* ***zip4* 2x** (1.65 ± 0.08, n = 255, p < 0.001), ***msh5* 2x** (1.77 ± 0.14, n = 57, p < 0.001), ***mer3* 2x** (3.36 ± 0.22, n = 36, p < 0.001), ***hei10* 2x** (2.07 ± 0.24, n = 29, p < 0.001), and ***mlh3* 2x** (4.18 ± 0.19, n = 91, p < 0.001) present a significant decrease of the total mean chiasma frequency per cell respect to the control (10.20 ± 0.14, n = 70).***hve* 2x** (3.36 ± 0.27, n = 78, p < 0.001), which its mean chiasma frequency has been reported in this study for the first time, also showed a lower chiasma rate compared with that of Col-0 2x. All these diploid mutants presented a mean chiasma frequency under 5 (less than one chiasma per pair of homologous chromosomes) and high frequencies of I. This means that the obligatory chiasma that ensures the correct segregation of homologous chromosomes at anaphase I is lost in these mutant lines. However, the homology recognition is not affected in any of these mutants, as the chiasmata are invariably formed between homologous chromosomes. Regarding the mutants that affect the class II COs, ***mus81* 2x** (10.31 ± 0.12, n = 101, p > 0.999) showed no differences compared with the control. The single mutant *mus81* is firstly described by cytology in this thesis. Previous publications characterized the function of MUS81 in CO formation by genetic maps, observing 15 % reduction respect to the WT in the corresponding mutant (Berchowitz *et al.*, 2007). Regarding ***fancm* 2x** (11.25 ± 0.19, n = 52, p > 0.001), it displayed a significant increase in chiasma levels. As it was previously reported by Crismani *et al.* (2012), this mutant presents a dramatic increase of CO formation in several genetic intervals, however, in this study, only a slight (but significant) increase of the mean chiasma frequency was detected. In addition, the total chiasma frequencies of the *zmm* diploid mutants (*zip4* 2x, *msh5* 2x, *mer3* 2x, and *hei10* 2x) and *mlh3* 2x were compared to each other by means of the Welch´s ANOVA test. Significant differences among these mutant backgrounds were observed [W (4.0, 103.4) = 46.04, p < 0.001]. When the pairwise comparison was conducted by the Dunnett´s T3 test, it was found that *mer3* 2x and *mlh3* 2x present similar chiasma frequencies to each other whereas their numbers are significantly higher than those of *zip4* 2x (p < 0.001 vs both *mer3* 2x and *mlh3* 2x), *msh5* 2x (p < 0.001 vs both *mer3* 2x and

mlh3 2x), and *hei10* 2x ($p < 0.01$ vs *mer3* 2x; $p < 0.001$ vs *mlh3* 2x), which are similar to each other (Table 22).

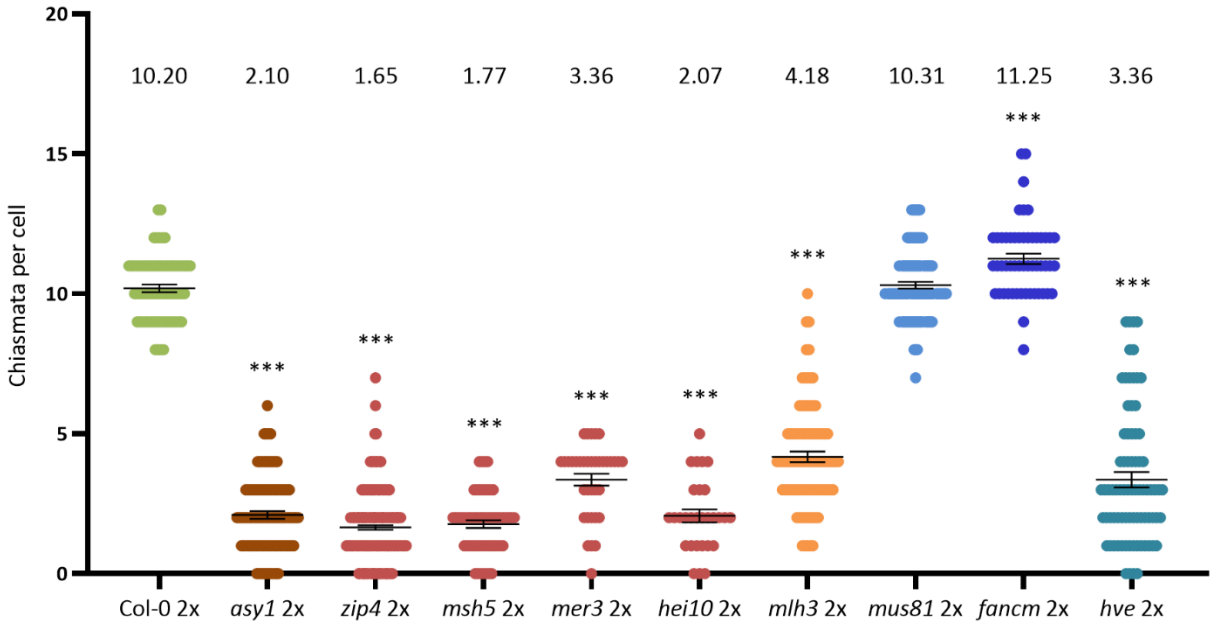


Figure 39. Analysis of total mean chiasma frequency per cell in different mutant lines defective for HR. Scatter plot representing the chiasma frequency per cell of the diploid mutant lines *asy1* 2x, *zip4* 2x, *msh5* 2x, *mer3* 2x, *hei10* 2x, *mlh3* 2x, *mus81* 2x, *fancm* 2x, and *hve* 2x, as well as the control Col-0 2x. Numbers represent the calculated value of the mean. Error bars represent the standard error of the mean. Statistical comparisons were made between each mutant line and Col-0 2x. Stars indicate *** $p < 0.001$.

Table 22. Statistical differences obtained from the Dunnett´s T3 test comparing the mean chiasma frequencies among the diploid *zmm* mutants analyzed as well as *mlh3* 2x

	***zip4* 2x**			
***msh5* 2x**	ns	***msh5* 2x**		
***mer3* 2x**	***	***	***mer3* 2x**	
***hei10* 2x**	ns	ns	**	***hei10* 2x**
***mlh3* 2x**	***	***	ns	***

*Stars indicate *** $p < 0.001$ and ** $p < 0.01$. ns = not significant.*

4.4.2. Cytological analysis of PMCs at metaphase I in autotetraploid mutants defective in meiotic recombination

Once the diploid mutants and the control were assessed, the **chiasma frequency analysis** was also conducted for the autotetraploid mutants ***asy1* 4x, *zip4* 4x, *msh5* 4x, *mer3* 4x, *hei10* 4x, *mlh3* 4x, *mus81* 4x, *fancm* 4x, and *hve* 4x**, as well as for Col-0 4x (Figure 40). Again, the Welch´s ANOVA test was conducted in order to find out whether these autotetraploid mutant lines present differences in the comparison with the autotetraploid control Col-0 4x. Thus, as it was displayed by the diploid mutants, significant differences were found [W (9.0, 183.1) = 1132, $p < 0.001$]. Then, the Dunnett´s T3 multiple comparison test was conducted (Figure 41). As in the diploid lines, a dramatic decrease in chiasma frequencies of the lines *asy1* 4x (6.02 ±0.32, n = 54, $p < 0.001$), *zip4* 4x (3.85 ± 0.43, n = 26, $p < 0.001$), *msh5* 4x (2.58 ± 0.17, n = 113, $p < 0.001$), *mer3* 4x (8.31 ± 0.56, n = 42, $p < 0.001$), *hei10* 4x (6.92 ± 0.37, n = 61, $p < 0.001$), *mlh3* 4x (9.57 ± 0.32, n = 61, $p < 0.001$), and *hve* 4x (9.29 ± 0.66, n = 24, $p < 0.001$) were noticed when they were compared with the control (19.99 ± 0.11, n = 186). Surprisingly, the *fancm* 4x mutant (18.54 ± 0.21, n = 96, $p < 0.001$) also showed significantly lower chiasma levels than the WT, which is the opposite situation to what was observed in the diploid lines. Finally, in the same direction as the result observed at the diploid level, the *mus81* 4x mutant displayed no significant differences respect to the autotetraploid control (19.52 ± 0.22, n = 66, $p = 0.376$). In addition, the total chiasma frequencies of the *zmm* tetraploid mutants (*zip4* 4x, *msh5* 4x, *mer3* 4x, and *hei10* 4x) and *mlh3* 4x were also compared to each other by means of the Welch´s ANOVA test. As in diploids, significant differences among these mutant backgrounds were found [W (4.0, 98.5) = 115.10, $p < 0.001$]. When Dunnett´s T3 multiple comparison test was conducted, it was observed that again *mer3* 4x and *mlh3* 4x present similar mean chiasma frequencies. Contrary to the diploid mutants, at the tetraploid level, *hei10* 4x displayed a chiasma frequency non-significantly different compared with *mer3* 4x but still significantly lower than that of *mlh3* 4x ($p < 0.001$). Interestingly, opposite to the results obtained at the diploid level, the frequency of *hei10* 4x was significantly higher than that of *zip4* 4x and *msh5* 4x ($p < 0.001$ in both cases), which again presented similar frequencies to each other (Table 23).

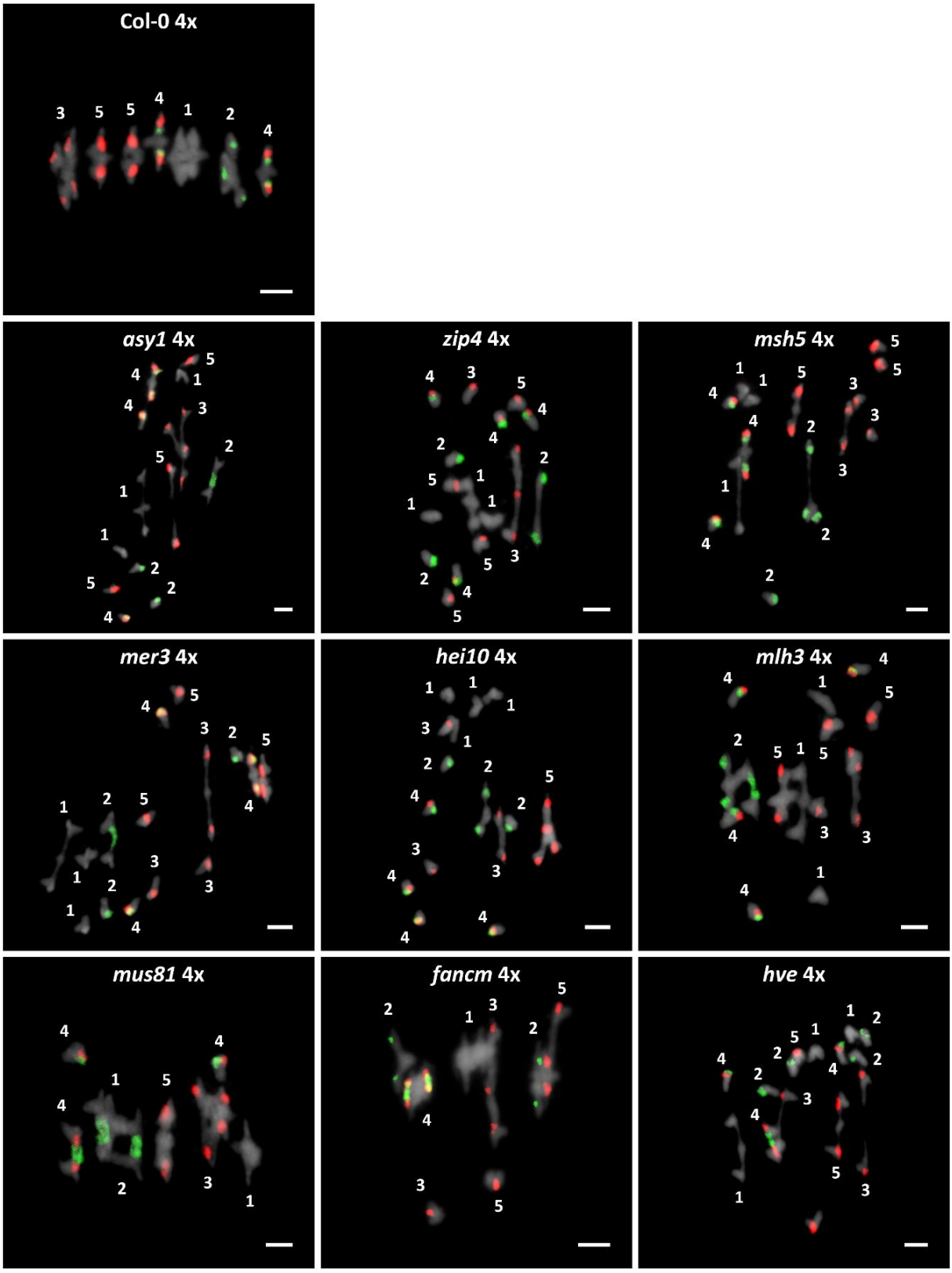


Figure 40. Cytological analysis of PMCs at metaphase I in different autotetraploid mutant lines defective in HR. Representative examples of metaphases I from the autotetraploid mutant lines *asy1* 4x, *zip4* 4x, *msh5* 4x, *mer3* 4x, *hei10* 4x, *mlh3* 4x, *mus81* 4x, *fancm* 4x, and *hve* 4x, as well as the control Col-0 4x. Chromosomes were identified by FISH using the 5S (red) and 45S rDNA (green) probes. Numbers indicate the chromosomes of the complement (one number per each chromosome association or univalent). Bars represent 5 μm.

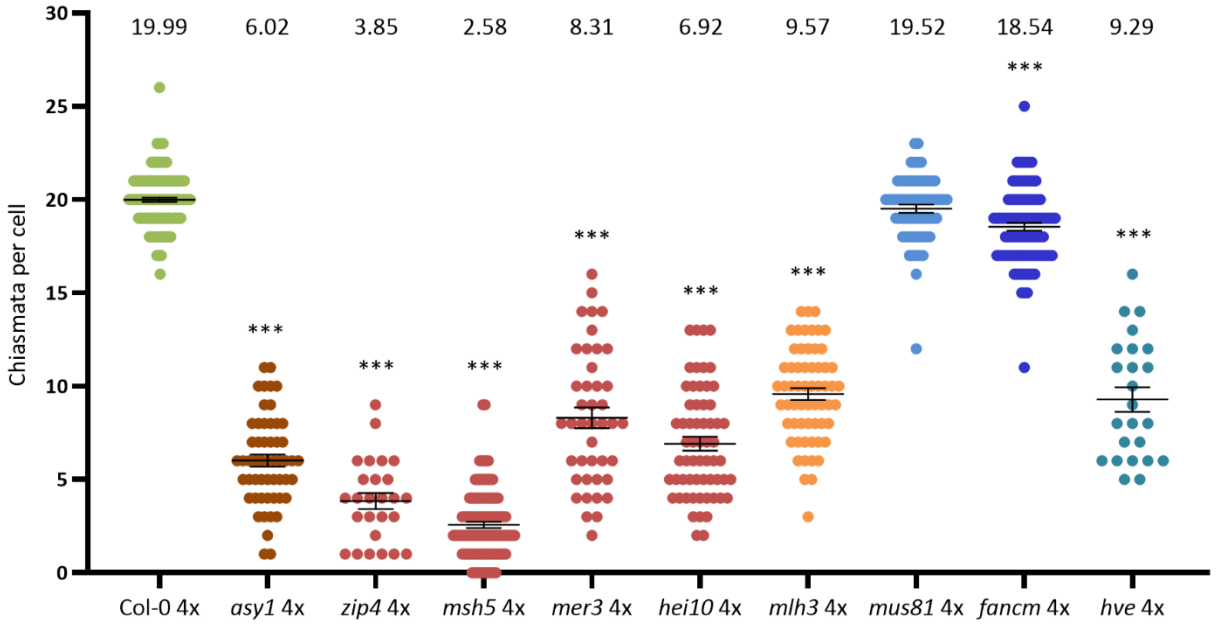


Figure 41. Analysis of total mean chiasma frequency per cell in different autotetraploid meiotic recombination mutant lines and the control. Scatter plot representing the chiasma frequency per cell of the polyploid mutant lines *asy1* 4x, *zip4* 4x, *msh5* 4x, *mer3* 4x, *hei10* 4x, *mlh3* 4x, *mus81* 4x, *fancm* 4x, and *hve* 4x, as well as the control Col-0 4x. Numbers represent the calculated value of the mean. Error bars represent the standard error of the mean. Statistical comparisons were made between each mutant line and Col-0 4x. Stars indicate *** $p < 0.001$ and * $p < 0.05$.

Table 23. Statistical differences obtained from the Dunnett´s T3 test comparing the mean chiasma frequencies among the tetraploid *zmm* mutants analyzed as well as *mlh3* 4x

	***zip4* 4x**			
***msh5* 4x**	ns	***msh5* 4x**		
***mer3* 4x**	***	***	***mer3* 4x**	
***hei10* 4x**	***	***	ns	***hei10* 4x**
***mlh3* 4x**	***	***	ns	***

*Stars indicate *** $p < 0.001$ and * $p < 0.05$. ns = not significant.*

Thus, the chiasma frequency comparisons among the tetraploid mutants and the control showed similar qualitative results compared with the study of the diploid lines (except for *fancm*). However, from a quantitative point of view, the **increase in the mean chiasma frequency per cell due to chromosome duplication** was different (Figure 42). The control showed a 1.96 ± 0.01-fold increase of total chiasma frequency per cell. When this value was compared with the values calculated for all the mutant backgrounds used in this

study, significant differences were observed by the Welch´s ANOVA test [W (9.0, 177.1) = 42.92, p < 0.001]. Trying to find out which lines behaved differentially compared with the control, the Dunnett´s T3 test was performed. This statistical analysis showed that *asy1* (2.87 ± 0.15, p < 0.001), *mer3* (2.47 ± 0.17, p < 0.05), *hei10* (3.34 ± 0.18, p < 0.001), *mlh3* (2.29 ± 0.08, p < 0.001), and *hve* (2.77 ± 0.20, p < 0.01) had a significantly higher fold-change of total chiasma frequency than the control. On the other hand, the mutant backgrounds *msh5* (1.45 ± 0.10, p < 0.001) and *fancm* (1.65 ± 0.02, p < 0.001) exhibited the opposite behavior with a significantly lower fold-change variation than the control. Finally, the mutant backgrounds *zip4* (2.33 ± 0.26, p = 0.777) and *mus81* (1.89 ± 0.02, p = 0.055) displayed no significant differences in the fold-change of chiasma frequency after the chromosome duplication.

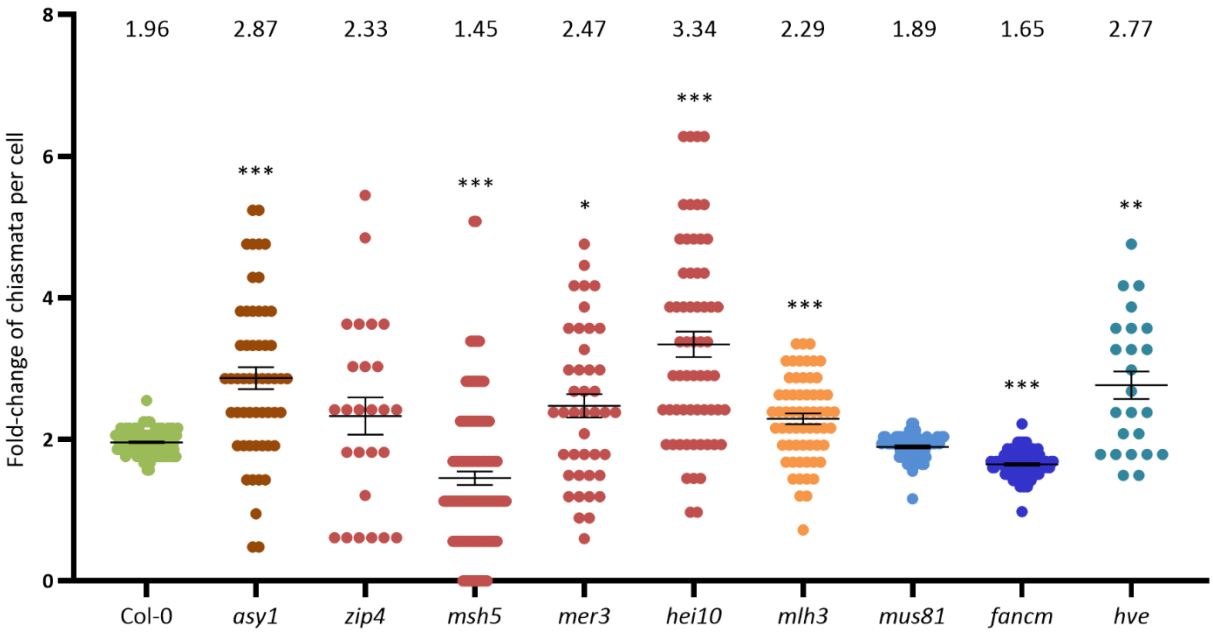


Figure 42. Fold-change of chiasma frequency per cell after WGD in different mutant lines defective for HR. Scatter plot representing the fold-change of the total chiasma frequency per cell in the mutant backgrounds *asy1*, *zip4*, *msh5*, *mer3*, *hei10*, *mlh3*, *mus81*, *fancm*, and *hve,* as well as the control Col-0 after chromosome duplication. Numbers represent the calculated value of the mean fold-change between the diploid and the autotetraploid lines. Error bars represent the standard error of the mean. Statistical comparisons were made between each mutant background and Col-0. Stars indicate ***p < 0.001.

Taking into account the chiasma frequency data obtained from the autotetraploid mutant lines analyzed in this study and compared to the data obtained from their diploid counterparts, it can be concluded that meiotic recombination mutant backgrounds with similar mean cell chiasma frequencies at the diploid level can show differences in this parameter when they are analyzed at the polyploid level. Mutants such as *zip4*, *msh5*, and *hei10*, which all belong to the so-called ZMM group, differ only in a maximum of around 0.5 chiasmata per cell when diploid individuals were compared. Conversely, they displayed much larger differences when the comparison was made among autotetraploid individuals with a maximum difference of around 4.5 chiasmata per cell. This fact could be observed more clearly when the fold-change of chiasma frequency as a response to WGD was calculated. For instance, the *msh5* mutant background showed a mean fold-change of 1.45 whereas *hei10* displayed a fold-change of 3.34. This implies that in the WT situation, the chiasma frequency is almost doubled as a response to WGD (1.96) whereas in mutants deficient for ZMM proteins (with very similar chiasma frequency phenotypes at the diploid level) the response to the WGD is completely different. Additionally, the study revealed that all the meiotic recombination mutants analyzed, except *zip4* and *mus81*, exhibited a differential response to polyploidy compared with the control.

It is also worth to mention that the autotetraploid *fancm* displayed an unexpected behavior. Whereas at the diploid level this mutant background showed a mean cell chiasma frequency significantly higher than the control, at the polyploid level, this mutant presented a significant decrease in the mean number of chiasmata per cell compared to the control.

4.4.3. Study of the different chromosome associations at metaphase I in different autotetraploid meiotic recombination mutants

Besides the total chiasma frequency analysis (section 4.4.2), FISH performed in PMCs at metaphase I also helped us to assess the different **chromosome associations**. The frequencies of II, IV, III, and I per cell were determined for the different autotetraploid mutant lines and the control (Figure 43 and 44). This analysis was performed to assess how these mutations that affect CO formation have an effect on the formation of the different chromosome associations.

Firstly, the ANOVA test was conducted to compare the mean **frequency of II and IV** per cell of the mutant lines *mus81* 4x and *fancm* 4x to the control (Figure 43). This comparison was made only in these two autotetraploid mutant backgrounds as they presented the highest chiasma frequencies (the chiasma frequencies in the remaining mutants were very low, hence, very low frequencies of II and IV were expected in these mutants). The results indicated that the II frequency per cell was not significantly different neither in *mus81* 4x (2.46 ± 0.21) nor in *fancm* 4x (2.22 ± 0.20) compared with Col-0 4x (2.41 ± 0.15) [$F(2, 345) = 0.39$, $p = 0.680$]. However, when the IV frequency was compared by the ANOVA test between these mutant backgrounds and the control, significant differences were found [$F(2, 345) = 8.47$, $p < 0.001$]. The post hoc test of Dunnett revealed that the IV levels of *mus81* 4x (3.62 ± 0.11, $p = 0.825$) were not significantly different compared to the control, whereas the levels of *fancm* 4x (3.16 ± 0.12, $p < 0.001$) were significantly lower (Figure 43).

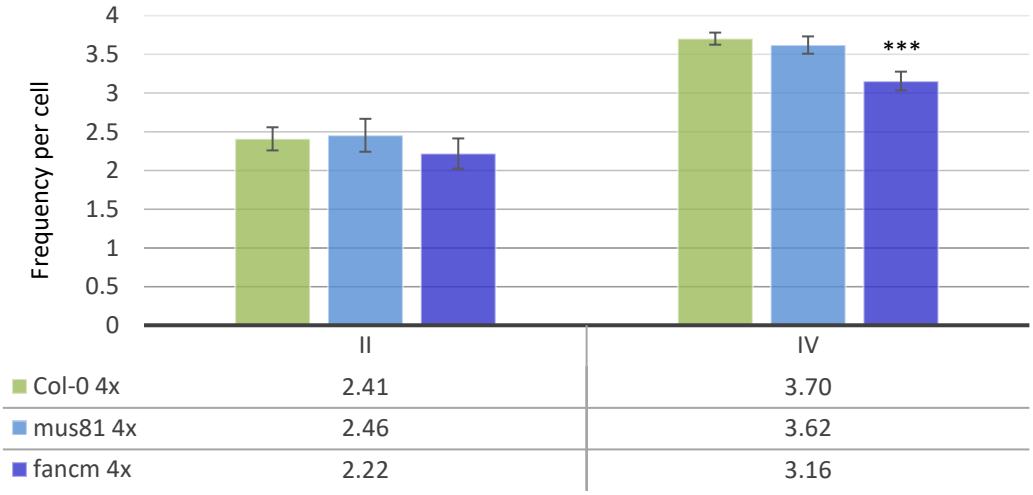


Figure 43. Frequencies of bivalents and quadrivalents per cell in the autotetraploid meiotic mutants *mus81* 4x and *fancm* 4x and the control. Bar chart representing the mean frequency of bivalents (II) and quadrivalents (IV) per cell in the mutant lines *mus81* 4x and *fancm* 4x, as well as the control Col-0 4x. Error bars represent the standard errors of the mean. Statistical comparisons have been made respect to Col-0 4x. Stars indicate *** $p < 0.001$.

In terms of **III frequency**, when the Welch´s ANOVA test was performed, it revealed that, in general, the autotetraploid mutant backgrounds assessed showed significant differences compared with the control [W (9.0, 279.2) = 11.94, p < 0.001]. The mutant lines *asy1* 4x (0.54 ± 0.11, p < 0.01), *mer3* 4x (0.69 ± 0.13, p < 0.001), *hei10* 4x (0.56 ± 0.10, p < 0.001), *mlh3* 4x (0.70 ± 0.10, p < 0.001), *fancm* 4x (0.63 ± 0.08, p < 0.001), and *hve* 4x (0.96 ± 0.19, p < 0.01) exhibited considerably higher frequencies than Col-0 4x (0.09 ± 0.02) (Figure 44A). However, mutants such as *zip4* 4x (0.31 ± 0.11, p = 0.364) and *msh5* 4x (0.21 ± 0.04, p = 0.072) did not present an increase in III levels probably due to their very low chiasma frequencies (see section 4.4.2). For a correct interpretation of the results, it is important to consider that all the mutant genetic backgrounds and the control showed a relatively low frequency of III.

Finally, the Welch´s ANOVA test revealed that there were differences in the **frequency of I** among the autotetraploid mutant lines analyzed in this study and the control [W (9.0, 172.6) = 590.7, p < 0.001]. The mutant lines *zip4* 4x (14.15 ± 0.56, p < 0.001) and *msh5* 4x (15.01 ± 0.26, p < 0.001) displayed the highest frequencies of I. As for the rest of the mutant lines, except for *mus81* 4x (0.29 ± 0.09, p = 0.381), which presented a frequency of I similar to that of the control (0.11 ± 0.03), a significant increase was observed: *asy1* 4x (10.63 ± 0.41, p < 0.001), *mer3* 4x (8.91 ± 0.61, p < 0.001), *hei10* 4x (9.53 ± 0.50, p < 0.001), *mlh3* 4x (7.10 ± 0.36, p < 0.001), and *hve* 4x (7.29 ± 0.70, p < 0.001). Interestingly, the mutant line *fancm 4x* (1.02 ± 0.11, p < 0.01) also showed a significantly higher I frequency than Col-0 4x (Figure 43B).

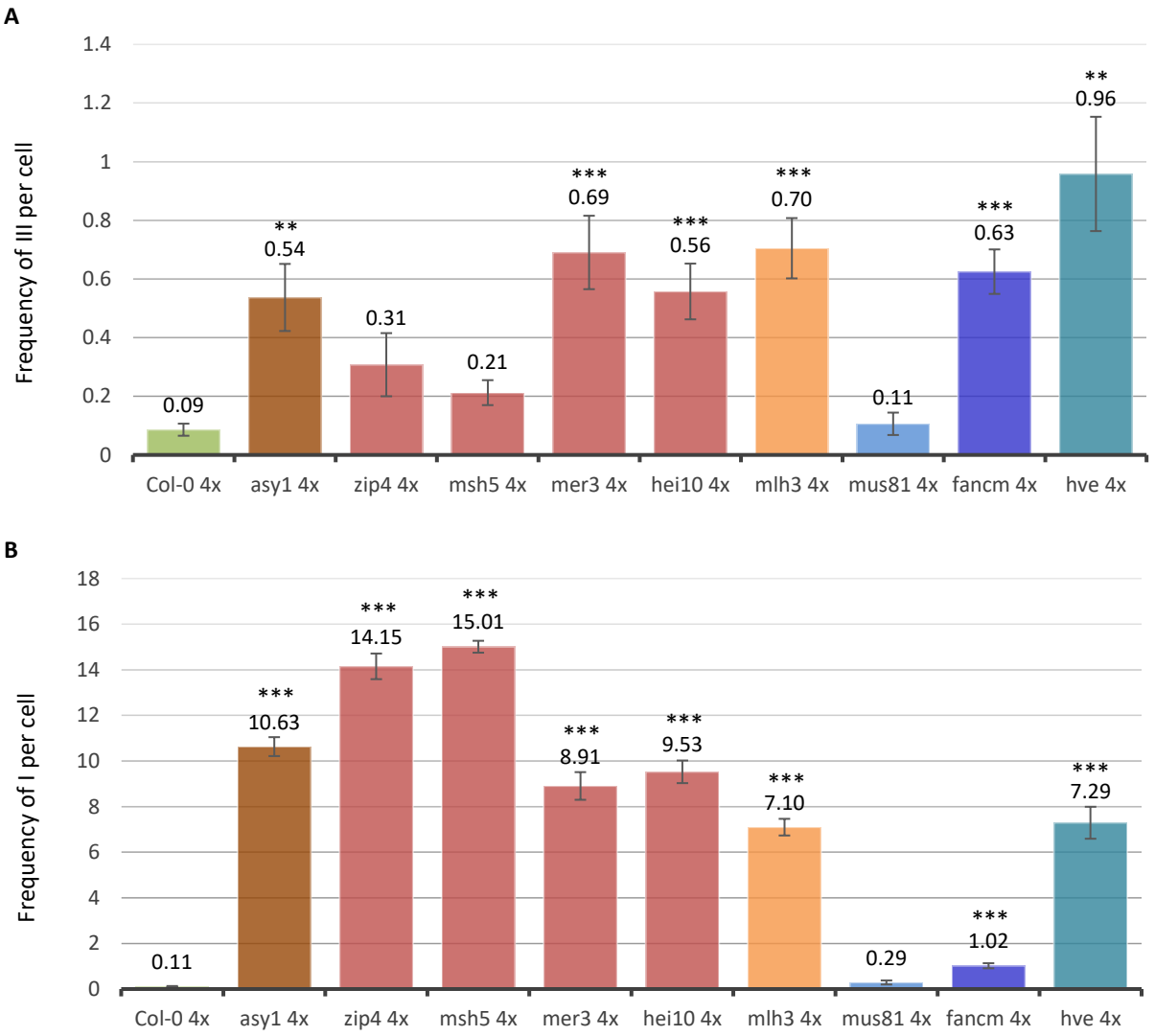


Figure 44. Frequencies of trivalents and univalents per cell in different autotetraploid meiotic recombination mutant lines and the control. Bar chart representing the mean frequency of trivalents (**A**) and univalents (**B**) per cell in the mutant lines *asy1* 4x, *zip4* 4x, *msh5* 4x, *mer3* 4x, *hei10* 4x, *mlh3* 4x, *mus81* 4x, *fancm* 4x, and *hve* 4x, as well as the control Col-0 4x. Error bars represent the standard errors of the mean. Statistical comparisons have been made respect to Col-0 4x. Stars indicate *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

The most interesting result obtained from the analysis of the different chromosome associations at metaphase I is probably again the one obtained from the *fancm* mutant. As it is reported above, the chiasma frequency of this mutant background at the tetraploid level displayed an unexpected reduction compared to that of the WT. Assessing the level of II and IV, it was also observed that this mutant displayed slightly lower levels of II and significantly lower levels of IV than the autotetraploid control. The reduction in the frequency of II and IV is explained by a significant increase in the frequency of III and I compared to Col-0 4x.

Additionally, another interesting result was obtained from *mus81*. It was observed that this mutant background did not show any differences neither in terms of chiasma frequency at the diploid and the tetraploid level nor in terms of II, IV, III, and I compared to the control.

Finally, the rest of the mutant backgrounds analyzed displayed an expected phenotype with significantly higher frequencies of III and I than the WT, due to their severe defects in CO formation. It is worth to note that mutants such as *hve* 4x or *mlh3* 4x, which showed a chiasma frequency close to the minimum needed to ensure the obligatory CO per pair of homologous chromosomes, displayed high frequencies of I, implying high randomness in CO distribution. This situation also applies for the mutant lines *asy1* 4x, *zip4* 4x, *msh5* 4x, *mer3* 4x, and *hei10* 4x, as they displayed a higher amount of I than expected from their chiasma frequencies, in case the COs were evenly distributed. These results are the consequence of severe defects in CO formation. In the case of *hve*, no chiasma frequency analysis was reported before. From the results obtained at the diploid and tetraploid, it is clear that this mutant background presents severe defects in CO formation.

4.4.4. Analysis of class I COs in diploid and autotetraploid lines of *fancm* and Col-0

An **immunolocalization** approach to detect **MLH1** (a class I CO marker) was conducted to get further insights into the unexpected decrease of the total mean chiasma frequency per cell observed in *fancm 4x* compared with the autotetraploid control. Besides, it could explain the significantly higher mean frequency of III and I in this tetraploid mutant compared with the WT (see sections 4.4.2 and 4.4.3). Thus, the MLH1 foci were scored in PMCs from Col-0 2x, Col-0 4x, *fancm* 2x, and *fancm* 4x at the pachytene stage, using the central element protein ZYP1 as a cytological marker (Figure 45).

Results obtained from this study (Figure 46) showed an average of 8.33 ± 0.31 MLH1 foci in **Col-0 2x** (n = 27). This value is in line with the studies previously conducted by Varas *et al.* (2015). As for the diploid mutant ***fancm* 2x**, the mean frequency scored was 7.46 ± 0.38 (n = 24). This result was expected as it was published that the CO increase in the *fancm* mutant is due to a boost in class II COs (Crismani *et al.*, 2012). Regarding the autotetraploid lines, **Col-0 4x** displayed an average of 11.50 ± 0.76 (n = 24) and ***fancm* 4x** an average of 10.44 ± 0.66 (n = 18). The ANOVA statistical test revealed significant differences among these four lines [$F(3, 89) = 12.10$, $p < 0.001$]. The Holm-Sidak´s post-hoc test revealed no differences neither between *fancm* 2x and Col-0 2x ($p = 0.356$) nor between *fancm* 4x and Col-0 4x ($p = 0.356$). However, a significant increase in the number of MLH1 foci in the tetraploid lines respect to the diploid lines was observed (Col-0 2x vs Col-0 4x, $p < 0.001$; *fancm* 2x vs *fancm* 4x, $p < 0.01$) (Figure 46). However, in both cases, the increase in class I COs (MLH1 foci) was not proportional to the increase in chiasma frequency (section 4.4.2) as a response to the WGD.

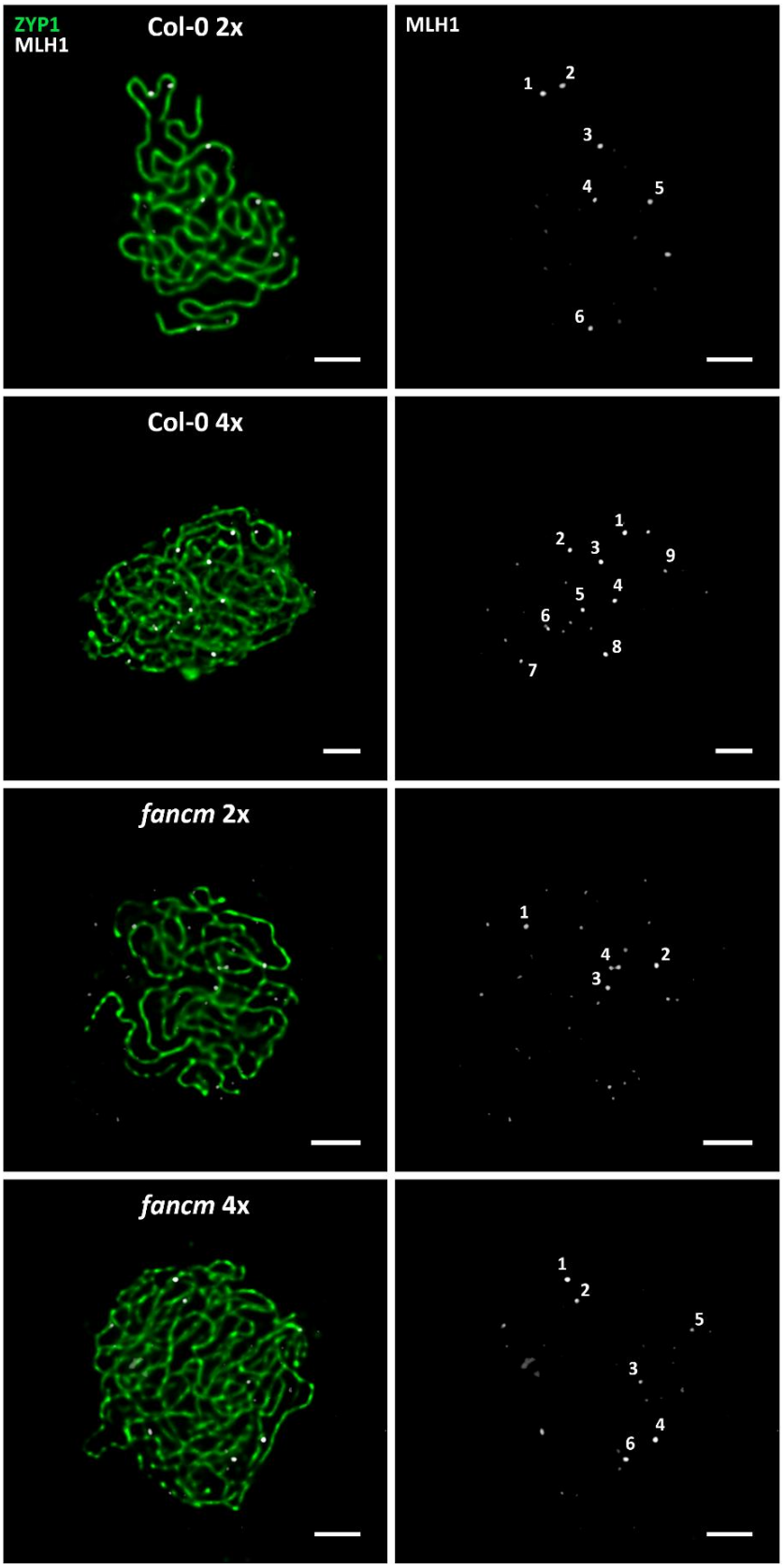


Figure 45. Immunolocalization of MLH1 and ZYP1 in diploid and autotetraploid lines of Col-0 and *fancm*. Representative examples of immunolocalizations of MLH1 (grey) and ZYP1 (green) in PMCs at the pachytene stage of *fancm* 2x, *fancm* 4x and their controls Col-0 2x and Col-0 4x, respectively. Numbers counting the MLH1 foci. Bars represent 5 μm.

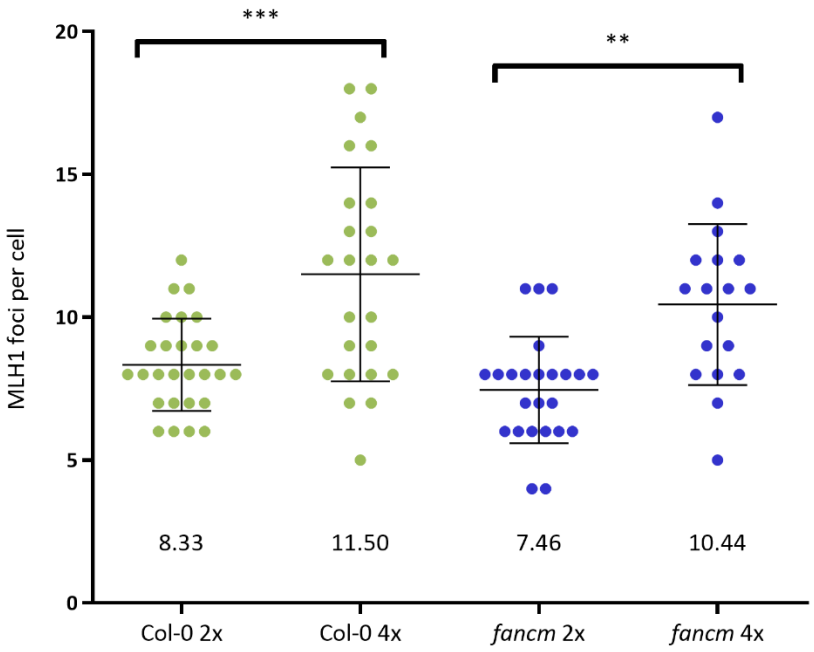


Figure 46. MLH1 foci in diploid and autotetraploid PMCs from Col-0 and *fancm*. Representation of the number of MLH1 foci scored in each PMC of Col-0 2x, Col-0 4x, *fancm* 2x, and *fancm* 4x. Bars in the middle represent the mean value and error bars represent the standard error of the mean. Stars indicate *** $p < 0.001$ and ** $p < 0.01$.

The analysis of the interference-sensitive CO levels by the immunolocalization of the protein MLH1 revealed that both backgrounds *fancm* and the WT present similar frequencies of MLH1 foci regardless their ploidy level. Moreover, although the amount of foci in the tetraploid lines was significantly higher than those of the diploid counterparts, the number of MLH1 foci was not doubled as a response to WGD neither in *fancm* nor in the control.

4.4.5. Expression analyses of several meiotic genes in diploid and autotetraploid lines of *hei10* and *fancm*

In addition to the cytological techniques conducted to analyze chiasma levels and the rate of the different chromosome associations, several expression analyses by **qRT-PCR** were also performed using diploid and autotetraploid individuals of the mutant backgrounds *fancm* and *hei10*, as well as the control. These mutant backgrounds were selected for this study due to the interesting results obtained in the cytological analysis of the autotetraploid mutants (see sections 4.4.2 and 4.4.3). As it has been mentioned above (section 4.4.1), the gene *FANCM* has been described to limit the class II COs and *HEI10* is essential for normal levels of class I COs in *A. thaliana*.

The mutant line ***fancm* 4x** showed a lower fold-change than the control in terms of chiasma frequency as a response to the WGD and a lower total mean chiasma frequency per cell. Moreover, this autotetraploid mutant line showed significantly higher frequencies of III and I than those of Col-0 4x. In the case of ***hei10* 4x**, this mutant background displayed the highest fold increase in the total chiasma frequency as a response to polyploidy among all the mutant lines assessed in this study. For these reasons, this analysis was conducted to determine whether some changes in the transcription levels of some of the most important meiotic genes could be underlying the differential response to polyploidy observed in these two mutant backgrounds.

Several qRT-PCR analyses were conducted in flower buds from diploid and autotetraploid individuals of *hei10, fancm*, and Col-0. We evaluated mRNA levels of several genes related to the formation and processing of DSBs such as *SPO11, RAD51, DMC1*; class I CO formation as *MLH3* and *HEI10*; class II CO formation, such as *MUS81;* and NCO formation, such as *FANCM* and *RECQ4A*. The gene related to the AEs, *ASY1*, and the gene affected by the *hve* mutation, *CAND1*, were also analyzed in this study. Besides, in order to find out whether changes were meiosis-specific, the transcription analyses were also performed in 10-days old seedlings for those genes that presented significant differences and a greater increase or decrease in expression (RQ > 2 or RQ < 0.5) respect to the genetic background used as a reference.

Firstly, we compared the mRNA levels in the mutant background *hei10*, both at the diploid (Figure 47) and at the tetraploid level (Figure 48), respect to the WT. Regarding the diploid mutant ***hei10* 2x** (Figure 47), the expression of *CAND1* was almost two-fold compared to what was observed in the control (RQ = 1.95). On the other hand, this mutant presented a slight reduction in the expression level of *RECQ4A* compared with that in Col-0 2x (RQ = 0.71).

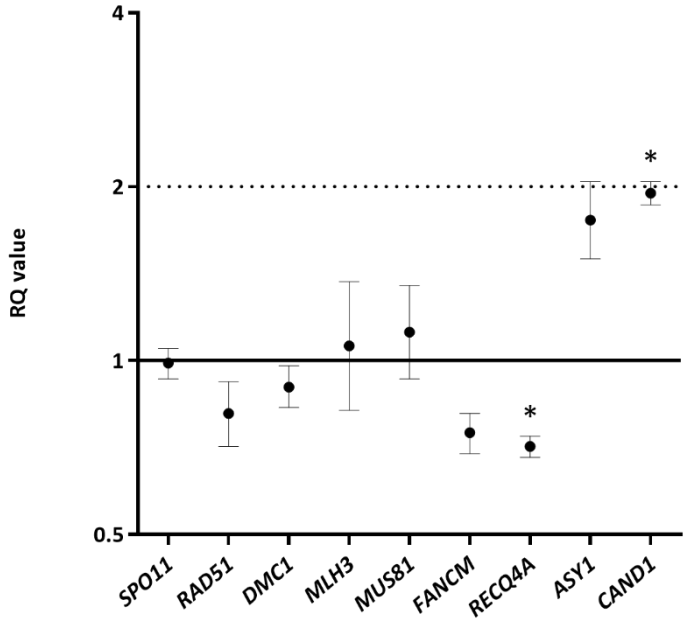


Figure 47. Relative transcription levels of meiotic recombination genes in flower bud samples from the diploid mutant line *hei10* 2x respect to its control. Graph showing the relative transcription levels of some meiotic recombination genes in flower bud samples of *hei10* 2x respect to the levels of Col-0 2x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

Opposite to the situation found at the diploid level, the transcription levels of *CAND1* were slightly but significantly lower in ***hei10* 4x** than in the control (RQ = 0.85). On the other hand, the expression of ***SPO11*** (RQ = 3.69), ***RAD51*** (RQ = 2.28), *DMC1* (RQ = 1.66), and *MLH3* (RQ = 1.74) presented a significant increase in this tetraploid mutant compared with that of the control. When the seedling samples were analyzed, we did not find significant differences neither in *SPO11* (RQ = 1.11) nor in *RAD51* (RQ = 1.17), suggesting that the increase in the expression of these genes in *hei10* 4x is meiosis-specific.

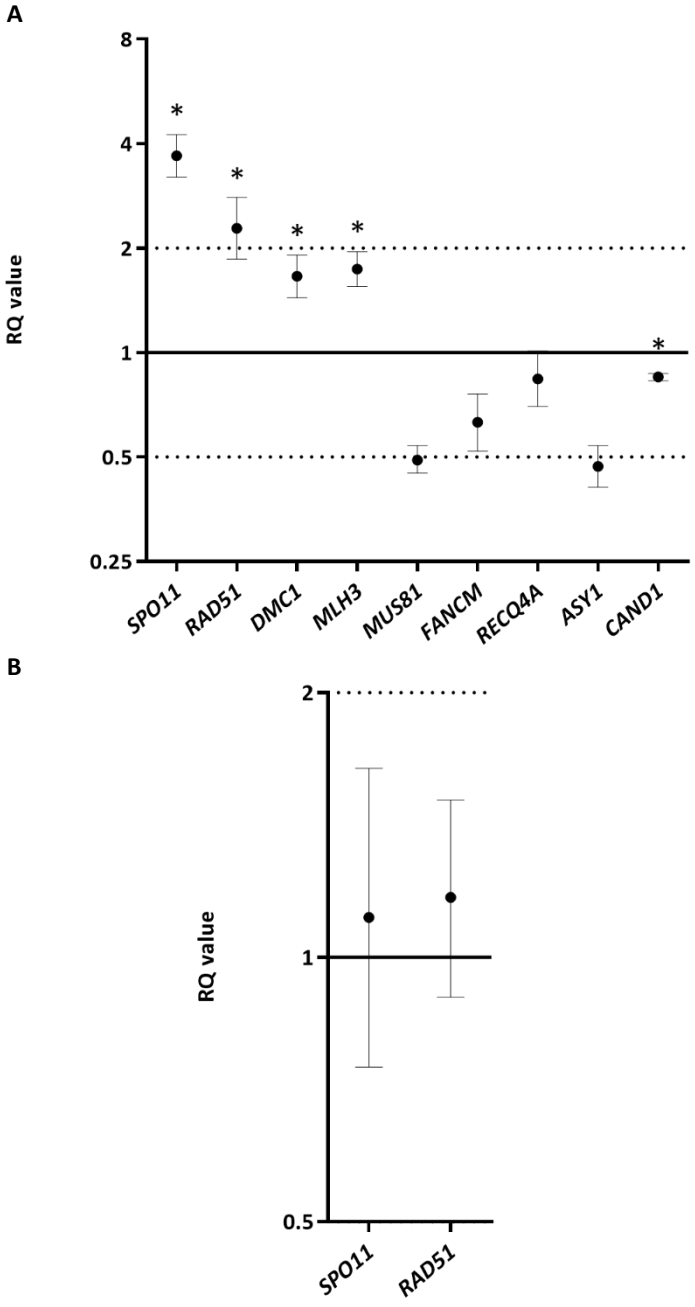


Figure 48. Relative transcription levels of several meiotic recombination genes comparing the autotetraploid mutant line *hei10* 4x respect to its control. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of *hei10* 4x respect to the levels of Col-0 4x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

As for the diploid mutant ***fancm* 2x**, results showed significantly lower mRNA levels of ***HEI10*** compared to those in the control (RQ = 0.31) (Figure 49). Additionally, the transcription levels of *MUS81* (RQ = 0.73) and *RECQ4A* (RQ = 0.75) were slightly but significantly lower in this diploid mutant than in the WT. On the other hand, *DMC1* (RQ = 1.28) and ***CAND1*** (RQ = 2.08) presented higher transcription levels in *fancm* 2x than in the control. The downregulation of *HEI10* (RQ = 0.43) and the upregulation of *CAND1* (RQ = 1.66) were also confirmed in the seedling samples.

Regarding the comparison between the autotetraploid mutant ***fancm* 4x** and the control (Figure 50), it also revealed differences in ***HEI10*** (RQ = 0.43). Moreover, *MUS81* (RQ = 0.41) and *ASY1* (RQ = 0.33) showed a similar reduction in *fancm 4x* compared with Col-0 4x, however, in both cases, the statistical analysis revealed that those differences were not significant. The reduction in the expression of HEI10 was also observed in somatic tissues (RQ = 0.17).

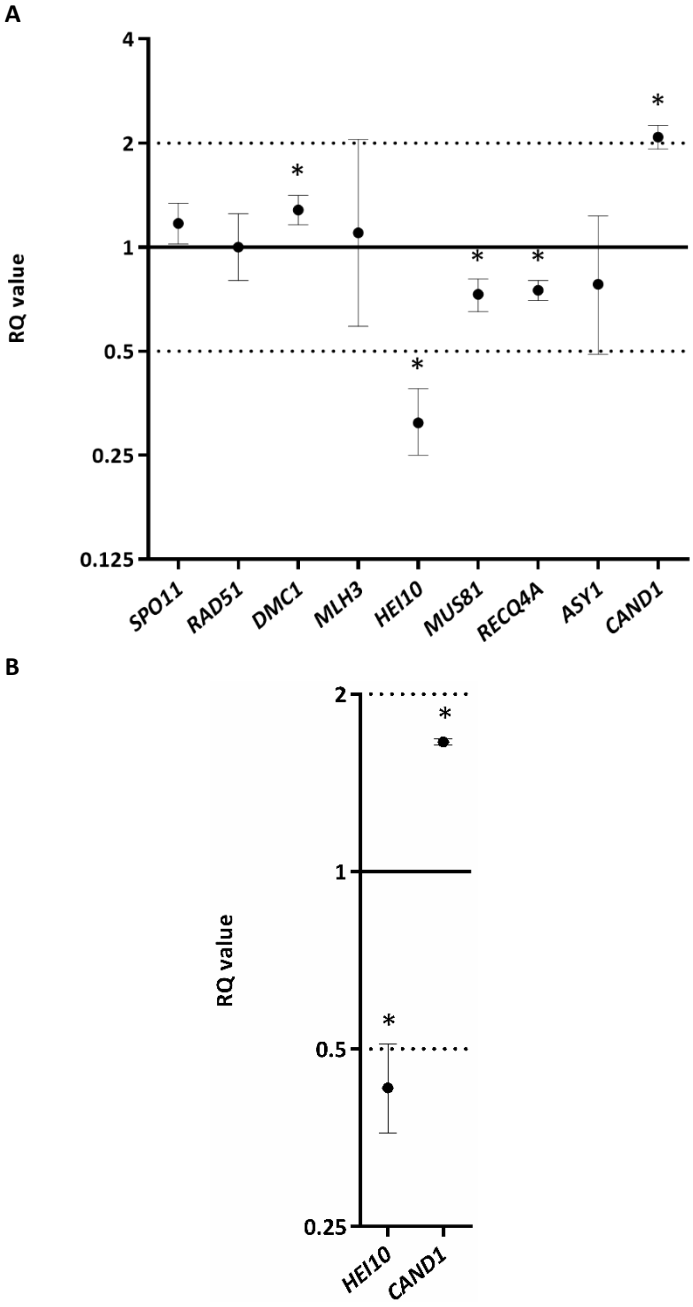


Figure 49. Relative transcription levels of several meiotic recombination genes comparing the autotetraploid mutant line *fancm* 2x respect to its control. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of *fancm* 2x respect to the levels of Col-0 2x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

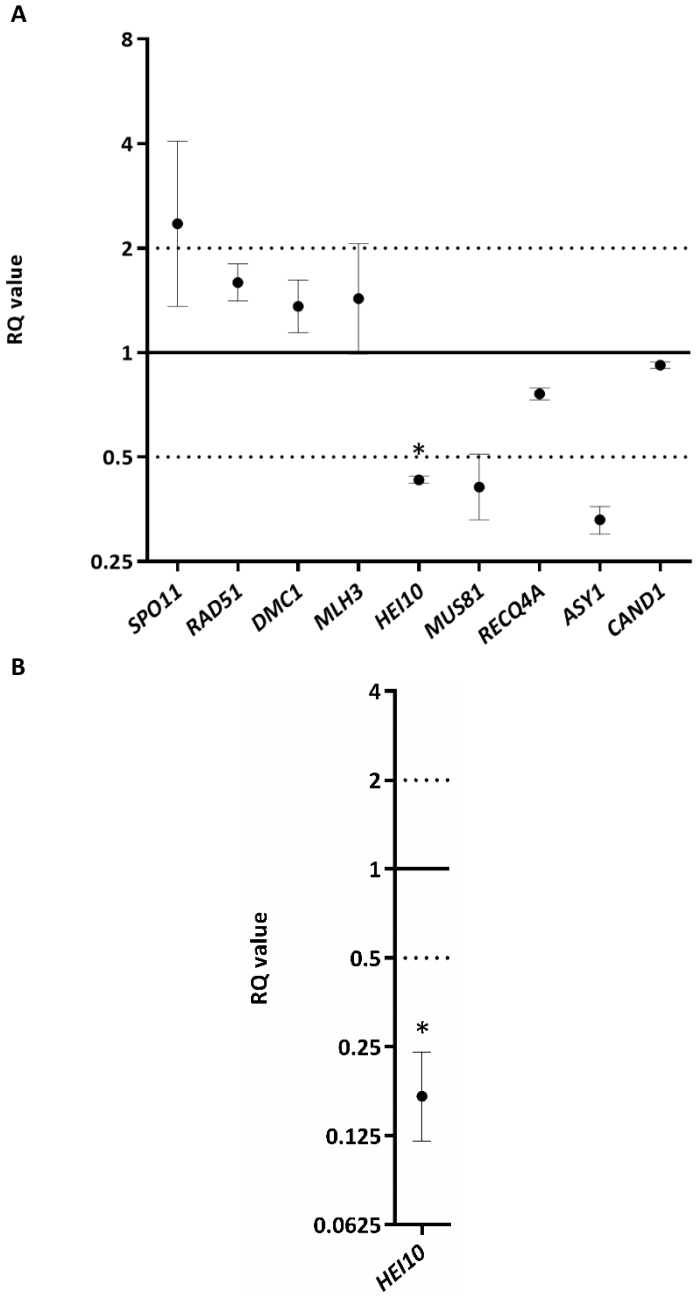


Figure 50. Relative transcription levels of several meiotic recombination genes comparing the autotetraploid mutant line *fancm* 4x respect to its control. Graph showing the relative transcription levels (RQ values) of some meiotic recombination genes in flower buds (**A**) and seedlings (**B**) of *fancm* 4x respect to the levels of Col-0 4x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

Finally, in order to establish which of the gene expression differences detected could take place in these genetic backgrounds due to the WGD induction, we analyzed the comparison between the diploids and tetraploids of the same genetic background. Results displayed only significant differences in *CAND1* (RQ = 1.69) between the diploid and the tetraploid controls **Col-0 2x** and **Col-0 4x**, although the RQ value was lower than 2 (Figure 51). As it was presented above (section 4.2.4), other meiotic genes such as *ZYP1α* and *SYN1* also presented significantly higher levels in the tetraploid than in the diploid flower bud samples (Figure 33).

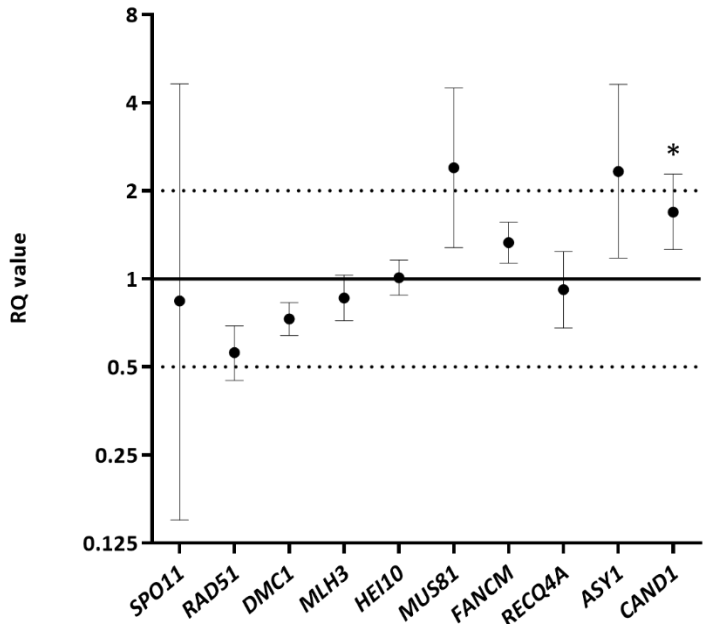


Figure 51. Relative transcription levels of meiotic recombination genes in flower bud samples from Col-0 4x respect to its diploid counterpart. Graph showing the relative transcription levels of some meiotic recombination genes in flower bud samples of Col-0 4x respect to the levels of Col-0 2x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

In the comparison between ***hei10* 2x** and ***hei10* 4x**, only *SPO11* displayed significant differences in mRNA levels being higher in *hei10* 4x than in its diploid counterpart, however, the RQ value obtained was under 2 (RQ = 1.55) (Figure 52).

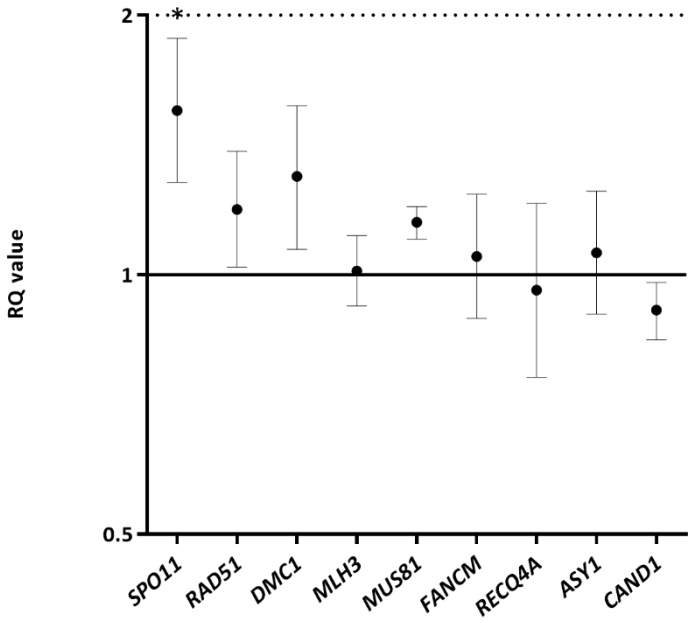


Figure 52. Relative transcription levels of meiotic recombination genes in flower bud samples from the autotetraploid mutant line *hei10* 4x respect to its diploid counterpart. Graph showing the relative transcription levels of some meiotic recombination genes in flower bud samples of *hei10* 4x respect to the levels of *hei10* 2x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

Regarding the comparison between ***fancm* 2x** and ***fancm* 4x**, the results obtained revealed a slight but significant reduction of the *RECQ4A* transcription levels in *fancm* 4x (RQ = 0.81) compared with its diploid counterpart (Figure 53).

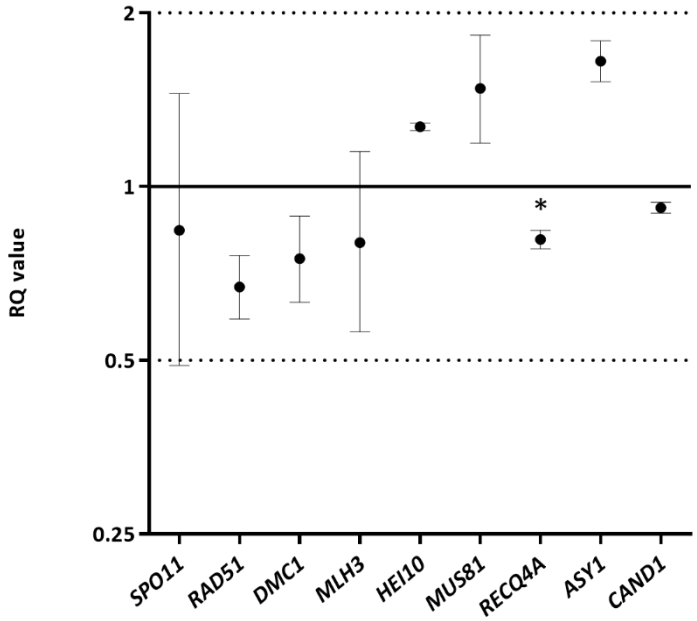


Figure 53. Relative transcription levels of meiotic recombination genes in flower bud samples from the autotetraploid mutant line *fancm* 4x respect to its diploid counterpart. Graph showing the relative transcription levels of some meiotic recombination genes in flower bud samples of *fancm* 4x respect to the levels of *fancm* 2x. Error bars represent the standard deviation of the RQ value. Solid line represents the RQ reference value (no differences between both genetic backgrounds). Dotted lines represent RQ values of 2 and 0.5 (double and half transcription levels respectively). Stars indicate significant differences, 99 % CI.

The statistical significance of the results obtained from the gene transcription analyses performed in different diploid and autotetraploid mutant lines is summarized in Tables 24 and 25.

Table 24. Variations in transcription levels of several meiotic genes among different diploid and autotetraploid genotypes using flower bud samples

	hei10 2x vs Col-0 2x	*hei10* 4x vs Col-0 4x	*fancm* 2x vs Col-0 2x	*fancm* 4x vs Col-0 4x	Col-0 4x vs Col-0 2x	*hei10* 2x vs *hei10* 4x	*fancm* 2x vs *fancm* 4x
SPO11-1	ns	*↑	ns	ns	ns	*↑	ns
RAD51	ns	*↑	ns	ns	ns	ns	ns
DMC1	ns	*↑	*↑	ns	ns	ns	ns
MLH3	ns	*↑	ns	ns	ns	ns	ns
HEI10	—	—	*↓	*↓	ns	—	ns
MUS81	ns	ns	*↓	ns	ns	ns	ns
FANCM	ns	ns	—	—	ns	ns	—
RECQ4A	*↓	ns	*↓	ns	ns	ns	*↓
ASY1	ns	ns	ns	ns	ns	ns	ns
CAND1	*↑	*↓	*↑	ns	*↑	ns	ns

** Significant differences with 99 % confidence interval; ns = not significant*
Arrows indicate higher (↑) or lower (↓) relative transcription levels of the first genotype respect to the second one
Dark cells refer to comparisons that show both, significant differences and RQ values greater than 2 or lower than 0.5
Lines refer to comparison not conducted

Table 25. Variations in transcription levels of several meiotic genes among different diploid and autotetraploid genotypes using seedling samples

	hei10 4x vs Col-0 4x	*fancm* 2x vs Col-0 2x	*fancm* 4x vs Col-0 4x
SPO11-1	ns	—	—
RAD51	ns	—	—
HEI10	—	*↓	*↓
CAND1	—	*↑	—

** Significant differences with 99 % confidence interval; ns = not significant*
Arrows indicate higher (↑) or lower (↓) relative transcription levels of the first genotype respect to the second one
Dark cells refer to comparisons that show both, significant differences and RQ values lower than 0.5
Lines refer to comparison not conducted

5. Discussion

One of the aims of this thesis was to analyze possible changes in meiotic recombination as a response to WGD events and those that could take place during the subsequent adaptation process. For this purpose, several *Arabidopsis thaliana* autotetraploid lines were assessed in this study. The duplication of the entire genome entails a wide number of challenges to the newly formed polyploids. The evolutionary adaptation to overcome those changes is the so-called diploidization process which takes place both at genetic and cytological levels (Ma and Gustafson, 2005). Meiosis is one of the processes affected by WGD since each chromosome has more than one partner to interact with. This situation can lead to the formation of multiple associations during the meiotic prophase I called multivalents. Some of these multivalents persist until metaphase I, which in some cases drive to uneven chromosome segregations at anaphase I (Sybenga, 1975). As a consequence of the diploidization process, the interactions of the homologous chromosomes are regulated favoring the formation of bivalents as in a diploid meiosis (Cifuentes *et al.*, 2010). How meiotic recombination responds immediately after a WGD event and gets adapted in the long term has been subject of study in several plants species (reviewed in Grandont *et al*., 2013). Although most of the research has been focused on allopolyploid species such as *Triticum aestivum* or *Brassica napus*, the meiotic behavior of several autopolyploids has been also analyzed. The most characterized species is the allohexaploid wheat, which presents several loci that prevent the formation of COs between homeologous chromosomes being the most important *Ph1* and *Ph2* (reviewed in Sears, 1976; Cifuentes *et al.*, 2010). Similarly, the *PrBn* locus has been identified in the allotetraploid *Brassica napus* as the major determinant locus among several quantitative trait loci that influences the homologous recombination levels between homeologous chromosomes (Jenczewski *et al.*, 2003). On the contrary, no locus has been described yet as a main factor of diploidization in autopolyploids. However, recent studies in *Arabidopsis arenosa* have proposed the presence of allelic variants of several meiotic genes as possible factors underlying the high rates of cytological diploidization observed in natural autotetraploid populations of this species (Hollister *et al.*, 2012; Wright *et al.*, 2015; Morgan *et al.*, 2020). In *Arabidopsis thaliana*, only a few studies have been conducted addressing the meiotic behavior of autopolyploid individuals (Santos *et al.*, 2003; Weiss and Maluszynska, 2000; Parra-Nunez *et al.*, 2019). The

study conducted by Santos *et al.* (2003) compared the chiasma frequency and chromosome association levels of colchicine-induced autotetraploid lines and several established autotetraploid lines of *A. thaliana*. They found a partial diploidization just few generations after the WGD induction. In this thesis, in addition to the newly formed Col-0 4x line, synthetic autotetraploids from another three *A. thaliana* accessions were obtained and assessed for the first time (L*er*-1 4x, St-0 4x, and Bla-1 4x). Moreover, the established autotetraploid lines 3151 4x and 3432 4x, previously assessed by Santos *et al.* (2003), were maintained another 7 generations and analyzed in this study. Besides, this thesis presents the first cytological and molecular analysis of meiotic recombination in natural autotetraploids of *A. thaliana* (Bla-5 4x, Wa-1 4x, and M3385S 4x). In addition, how intraspecific differences affect the meiotic behavior of autopolyploids in terms of chiasma formation was another objective of this thesis. For this purpose, the synthetic autotetraploid hybrid Col/L*er* was analyzed. Finally, the present study aimed to characterize how mutants deficient for the meiotic HR process respond to WGD in order to find out more details about the steps of this process in which they are affected. For this purpose, up to nine different meiotic mutant backgrounds (*asy1*, *zip4*, *msh5*, *mer3*, *hei10*, *mlh3*, *mus81*, *fancm*, *hve*) were analyzed at the tetraploid level and compared to their diploid counterparts.

5.1. Cytological and molecular study of synthetic, established, and natural autotetraploid lines

5.1.1. The genetic background, rather than the time elapsed since the WGD event, determines the mean cell chiasma frequency in *Arabidopsis* autotetraploids

During diploidization, polyploids tend to get a diploid-like state at metaphase I where homologous chromosomes are only associated as bivalents. This process is substantially different between allopolyploids and autopolyploids. In allopolyploids, differences in sequence and structure between homeologous chromosomes seem to play an important role to prevent the formation of COs between them, establishing preferences between homologous chromosomes, and therefore, forming mainly bivalents (Le Comber *et al.*, 2010; Glover *et al*., 2016). On the contrary, such differences among homologous chromosomes are not present in autopolyploids, making more difficult to establish chiasma formation preferences (Figure 54). Thus, the formation of multivalents, especially during the first stages of the diploidization process, is more likely to happen in autopolyploids than in allopolyploids. In addition, it is known that in established allopolyploids such as *T. aestivum* or *B. napus*, there are several loci that act as a genetic control ensuring the strict formation of bivalents (reviewed in Jenczewski and Alix, 2004). However, in autopolyploids no major locus have been described yet to be responsible for the cytological diploidization. Thus, it is possible that a high rate of bivalents in autopolyploids is obtained due to an accumulation of a wide number of quantitative traits favored by natural selection. In *A. arenosa*, it was reported when diploid and autotetraploid populations were compared that a number of genes important for the meiotic process such as *ASY1*, *ASY3*, *SYN1*, *SMC1*, *PDS5*, *ZYP1a*, and *ZYP1b* have experienced selection (Hollister *et al.*, 2012; Wright *et al.*, 2015). Despite *ASY1*, and in a lesser extent *ASY3*, seem to have an impact on cytological diploidization levels in this species (Morgan *et al.*, 2020), they are not as determinant as *PrBn* or *Ph1* in the allopolyploids *B. napus* and *T. aestivum*, respectively.

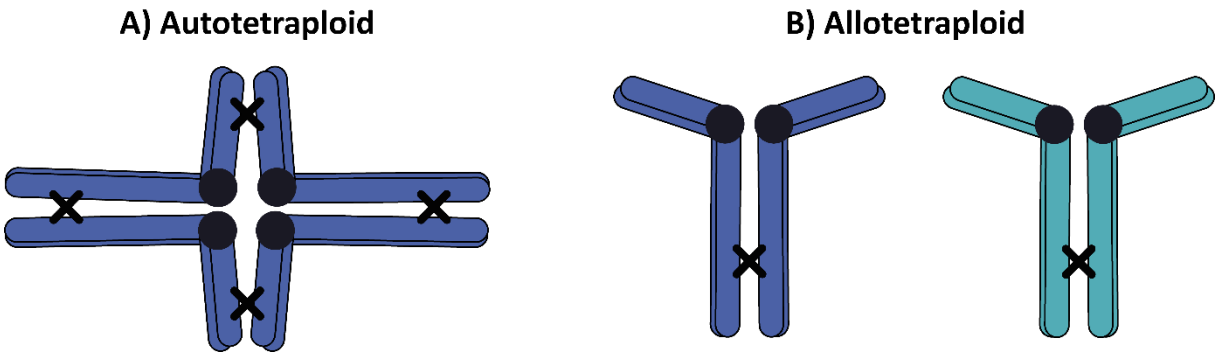


Figure 54. Typical chromosome associations during meiosis in autotetraploids and allopolyploids. Differences between homologous/homeologous chromosomes are important to determine the pattern of CO formation during meiosis. In autopolyploids (**A**), since there are only homologous chromosomes the formation of multivalents is frequent, whereas in allotetraploids (**B**), the differences between homeologous chromosomes make less likely this situation favouring the formation of bivalents instead. Figure modified from Glover *et al*. (2016).

One of the mechanisms proposed as a key factor in the diploidization process of autopolyploids is the reduction of the recombination frequency between homologous chromosomes (Bomblies *et al.*, 2016). It has been reported that adapted autotetraploids tend to have lower total chiasma frequencies than the newly formed autotetraploids (Morrison and Rajhathy, 1960; Mulligan, 1967; Reddi, 1970; Yant *et al.*, 2013; Wu *et al.*, 2014) and also proportionally lower than the diploids from which they were originated (Mulligan, 1967; Yant *et al.*, 2013). Several studies reported that in experiments where synthetic autotetraploids were under selective pressure and individuals with higher diploidization levels were favored, these individuals also showed a reduction in the number of chiasmata (Bremer and Bremer-Reinders, 1954; Povilaitis and Boyes, 1956; Hilpert, 1957; Morrison and Rajhathy, 1960; Lavania *et al.*, 1991; Santos *et al.*, 2003). Furthermore, in some species, the chiasma frequency is reduced to one per pair of bivalents, which is the minimum amount (named the obligatory CO) to ensure a correct segregation of the homologous chromosomes. This way, the presence of multivalent associations at metaphase I is prevented. Some examples are the autotetraploids of *Physaria vitulifera*, *Lotus corniculatus*, and *Arabidopsis arenosa* which present a mean cell frequency of chiasmata about 1.1 per pair of chromosomes (Mulligan, 1967; Davies *et al*., 1990; Yant *et al.*, 2013). However, exceptions have also been reported. For instance, adapted autotetraploids of the species

Dactylis glomerata show more quadrivalents and higher chiasma frequencies than the newly formed autotetraploids (McCollum, 1958).

In this study, it was observed that the general tendency of *A. thaliana* is to duplicate the number of chiasmata as a response to WGD, however, as the number of chromosomes is also doubled, the rate of chiasmata per pair of chromosomes is maintained. This is in line with the results obtained in this species by Santos *et al.* (2003) and contrasts with the data published by Pecinka *et al.* (2011). They reported a net increase in the recombination frequency of the chromosome 3. It is important to keep in mind that the approach conducted by Pecinka and colleagues detected all the recombination events that have happened between non-sister chromatids regardless these are COs or NCOs. However, the chiasma frequency analysis only detects COs as these are the only kind of recombination event that can produce such chromosome associations. Thus, it would be possible that the NCO events could be underlying the increased recombination detected by Pecinka and colleagues without an increase of COs. Besides, these differences may be explained by the fact that in the present study and in the study conducted by Santos and colleagues, the data are obtained from the whole chromosome complement, whereas Pecinka and colleagues assessed only one region at the chromosome 3. Thus, the increase observed could have only happened in certain regions and compensated by a reduction in other regions that were not analyzed. In the present study, the four synthetic autotetraploid lines with different genetic backgrounds presented significant differences. Col-0 4x displayed the highest chiasma levels, whereas L*er*-1 4x, Bla-1 4x, and St-0 4x did not differ significantly (Figure 17, Table 8). Interestingly, when these accessions were also analyzed at the diploid level, differences were found. Although Col-0 2x showed a significantly higher chiasma frequency than L*er*-1 2x, Bla-1 2x, and St-0 2x; in this case, St-0 2x displayed significantly lower levels than the other three accessions (Figure 14, Table 5). Moreover, when the **fold-change of the chiasma frequency** due to the WGD induction was analyzed, **different behaviors were observed in these genetic backgrounds**. St-0 displayed a significantly higher fold-change than the one exhibited by Col-0, L*er*-1, and Bla-1 (Figure 18, Table 9).

As it was already shown by Sanchez-Moran *et al.* (2002) and López *et al.* (2012) but also observed in this study (Figure 14, Table 5), different genetic backgrounds of *A. thaliana* can display different levels of chiasmata. It was reported by Ziolkowski *et al.* (2017) that up

to 56.9 % of the CO variation between the accessions Col-0 2x and L*er*-1 2x can be explained by two major recombination quantitative trait loci (rQTL), mapping one of them to semidominant polymorphisms in *HEI10*, which is a gene essential for the formation of class I COs in *A thaliana* and other species (Chelysheva *et al.*, 2012) (see section 1.8). Hence, it seems that chiasma frequency is modulated by different allelic variants that are present in the different genetic backgrounds. However, the different responses to WGD found in the accessions assessed in this study brought out that the CO levels may be also determined by other factors. This is supported by the fact that the genetic background St-0 presented differences with the accessions L*er*-1 and Bla-5 at the tetraploid (Figure 17, Table 8) but not at the diploid level (Figure 14, Table 5). It would be possible that other factors such as changes in the gene expression regulation (see more below) or protein levels after the duplication of the entire genome could be playing an important role. It has been documented that differences at the protein level take place after the induction of polyploidy. The study conducted by Ng *et al.* (2012) revealed that from a thousand proteins analyzed only around a 6.8 % of them presented different levels in the comparison between an autotetraploid line of *A. thaliana* and its diploid counterpart. Interestingly, although the percentage of proteins with differential levels between ploidies roughly matches the percentage of genes found in other studies with altered transcription due to the WGD, a very low amount of these proteins match the differentially expressed genes of the previous analysis (Del Pozo and Ramirez-Parra, 2014). One possible explanation to this fact is that the post-translational modifications could play an important role modifying protein levels in autopolyploids.

When the **natural autotetraploid** accessions Bla-5 4x, Wa-1 4x, and M3385S 4x were analyzed, no significant differences were observed among them in terms of chiasma frequency (Figure 24). This situation might reflect a tendency to standardize the chiasma frequency as an adaptation to polyploidy. As it is mentioned above, most of the established autopolyploids analyzed in several studies present a tendency to reduce their chiasma levels per chromosome compared to the newly formed autotetraploid and diploid counterparts (reviewed in Bomblies *et al.*, 2016). In the same line are the results obtained from the established lines 3151 4x and 3432 4x. As it was previously observed by Santos *et al.* (2003) and in this study, these **established lines**, which share the genetic background with the

accession Col, presented a significant **reduction in chiasma frequency** compared to that of the synthetic line Col-0 4x (Figure 21, Table 12). As a result of the study conducted in established autotetraploids of *A. arenosa*, Bomblies *et al.* (2016) proposed an increase in the effective distance of the CO interference, either by a chromosome axis shortening (already observed in this species by Morgan *et al.*, 2020) or by an increase of the interference *per se*, as a mechanism to reduce the number of chiasmata, favoring in polyploids the formation of bivalents. In that species, the natural autotetraploids show substantially lower levels of chiasmata per pair of bivalents than the synthetic autotetraploid lines (Yant *et al.*, 2013). Besides, it cannot be ruled out that other factors such as changes in the morphology of the chromosomes could also be playing an important role in the reduction of the chiasma levels. It has been observed that in recent autopolyploids of *A. thaliana* (although in a lesser extent than in allopolyploids of the *Arabidopsis* species), chromosome modifications such as sequence duplications take place (Bento *et al.*, 2015). Weiss and Maluszynska (2000) reported that an established autotetraploid line (at least 30 generations after the WGD) of the *A*. *thaliana* accession Wilna presented a translocation of the 45S rDNA locus from the chromosome 4 to the chromosome 3. Duplications of highly repeated sequences such as rDNA, where CO formation is prevented (Sims *et al*., 2019), might be one of the factors that underlies the tendency of autopolyploids to reduce their chiasma frequency during their process of adaptation.

5.1.2. The duplication of the entire genome equalizes the contribution of the chromosomes to the total chiasma frequency

Several studies have demonstrated that autopolyploids suffer changes in meiotic recombination during their adaptation to polyploidy such as variations in chiasma frequency and/or distribution. It has been observed that there is a general tendency to reduce the number of COs to one per pair of homologous chromosomes but also their formation is favored at more distal regions (Bomblies *et al.*, 2016). However, how polyploidy affects the CO levels for each individual chromosome of the complement has only been analyzed in a few studies (Santos *et al.*, 2003). In contrast to other studies where the meiotic recombination events are assessed only in certain chromosome regions or evaluated for the whole complement without distinguishing the different chromosomes, in the present study,

the FISH technique allowed the assessment of the chiasma frequency for each chromosome of the complement. This is a very valuable information since it is known that morphologically different chromosomes, present differences in meiotic recombination that could finally affect the response and adaptation of each one of them to the WGD. Recently, Murakami *et al.* (2020) reported in *S. cerevisiae* that the association with the chromatin of certain proteins involved in the formation of DSBs is differentially regulated between short and long chromosomes to ensure the allocation of DSBs in the shortest chromosomes. In *A. thaliana*, differences in chiasma levels have been reported between short and long chromosomes in a wide number of diploid accessions (Sanchez-Moran *et al.*, 2002; López *et al.*, 2012).

In this thesis, the **contribution of each chromosome** to the total chiasma frequency was analyzed in autotetraploid and diploid lines. The chromosome contribution pattern showed by the diploid accessions assessed in this study is in line with the results described before by Sanchez-Moran *et al.* (2002) and López *et al.* (2012). As in most of the accessions analyzed in previous studies, Col-0 2x, L*er*-1 2x, Sto-0 2x, and Bla-1 2x showed a pattern where the shortest and acrocentric chromosomes (2 and 4) presented lower contributions to the total chiasma frequency than the longest and metacentric/submetacentric chromosomes (1+3 and 5) (Figure 15, Table 7). These data might be showing a positive correlation between the size of the chromosomes and their chiasma levels, however, other factors seem to be affecting since the contributions of the chromosomes 2 and 4, which have similar sizes, displayed significant differences when they were compared with each other in Col-0 2x and in L*er*-1 2x (Figure 15, Table 7). These differences between chromosomes 2 and 4 were also noticed in other ecotypes. In the studies conducted by Sanchez-Moran *et al*. (2002) and López *et al*. (2012), several accessions showed differences in the chiasma levels of these two chromosomes being especially remarkable the case of the accession Cvi which showed a markedly higher contribution of the chromosome 4 than of the chromosome 2. These chromosomes have different contents of rDNA sequences. Chromosome 2 has a 45S rDNA sequence on the short arm, whereas the chromosome 4, in addition to a 45S rDNA sequence, also has a 5S rDNA sequence on the short arm (Figure 8). Probably, this feature, in addition to other sequence and morphological differences between these two chromosomes, could play an important role modifying their chiasma levels.

As it was observed with the total chiasma frequency, the contribution of each chromosome seems to be modulated at a certain level by the genetic background. In this study, the contributions of the chromosomes 1+3, 2, and 4 presented significant differences among the diploid accessions analyzed (Figure 15, Table 6). In all the cases, Col-0 2x showed a differential pattern compared to the other three accessions. As it was noticed during the cytological analysis carried out in this study (Parra-Nunez *et al.*, 2019), but also in previous studies (Fransz *et al.*, 1998; Sanchez-Moran *et al.*, 2002), Col-0 has differences in the rDNA content of the chromosomes 2 and 3 compared to L*er*-1. Also, in this study, the accessions Bla-1 and St-0 presented differences at the chromosome 3 compared to Col-0 and L*er*-1 (Figure 8). Besides, analyses in different *A. thaliana* accessions have revealed that they harbor wide sequence variability in subtelomeric regions (Kuo *et al*., 2006; Wang *et al.*, 2010), rDNA, and retrotransposons (Fransz *et al.*, 2000; Davison *et al*., 2007). For instance, it has been published that Col-0 and L*er*-1 differ in almost 50 inversions and over 500 transpositions (Ziolkowski *et al.*, 2009; Zapata *et al.*, 2016). Thus, it may be possible that differences in the sequence of certain chromosome regions could determine their structure and ultimately their capacity to form COs. Probably, these, among other features, underlie the differences observed among accessions in the contribution of chromosomes to the total chiasma frequency.

Interestingly, the synthetic autopolyploid lines showed **changes in the pattern of chiasma distribution** compared to their diploid counterparts, in general reducing the differences found among diploid accessions between the long (1+3 and 5) and the short (2 and 4) chromosomes (Figures 15 and 19, Tables 7 and 11). Furthermore, when the chromosome contributions of the established (Figure 22) and natural autotetraploid lines (Figure 25) were analyzed, the same pattern was found. Thus, it seems clear that the presence of more than two copies of each chromosome (in this case four), remarkably alters the capacity of the different chromosomes to form COs. Hence, **WGD modifies** the pattern of **chromosome contribution** to the total chiasma frequency that is usually observed in diploids of *A. thaliana*, **homogenizing the contributions of short and long chromosomes**, and probably this tendency is maintained during the following generations after the WGD event. Similar to these results, in *S. tuberosum*, differences in the chiasma levels of the

chromosomes 1 and 2 were found between three natural autotetraploids and a diploid line (Choudhary *et al.*, 2020).

5.1.3. The genetic background has a great impact on bivalent and quadrivalent frequencies

The feature that defines the cytological diploidization levels is the proportion of chromosomes forming bivalents at metaphase I in meiosis. The more chromosomes are associated as bivalents in a polyploid, the greater is this diploidization. The formation of quadrivalents and especially trivalents + univalents, which are related with chromosome mis-segregation and reduced fertility, has been proposed to be one of the major challenges that a newly formed polyploid has to face (reviewed in Ramsey and Schemske, 2002; Comai, 2005; Grandont *et al*., 2013). Thus, an increase in the number of bivalents, and therefore a reduction of multivalents, would prevent the formation of unbalanced gametes. This seems to be an evolutionary tendency since paleopolyploid organisms always show a diploid-like meiosis where only bivalents can be observed. Due to the presence of species with mesopolyploid populations such as *Arabidopsis arenosa* (Yant *et al.*, 2013), *Actinidia chinensis* (Wu *et al.*, 2014), and *Agropyron elongatum* (Charpentier *et al*., 1986), which present high levels of diploidization but still the presence of sporadic multivalents and univalents at metaphase I, one can assume that the complete cytological diploidization is achieved throughout a long process. Thus, one of the objective of this thesis was to assess the bivalent and multivalent levels of synthetic, established, and natural autotetraploid lines of *A. thaliana*.

When the frequency of the different **chromosome associations** was assessed and compared among the **synthetic lines**, remarkable results were obtained. The line that presented the highest chiasma levels, Col-4x (Figure 17, Table 8), also presented lower numbers of bivalents than St-0 4x and Bla-1 4x, but similar to L*er*-1 4x (Figure 26, Table 13). Additionally, although the synthetic lines Bla-1 4x, L*er*-1 4x, and St-0 4x displayed similar mean chiasma frequencies, they showed significant differences in terms of bivalent and quadrivalent formation. Bla-1 4x showed significantly higher levels of bivalents and lower of quadrivalents than those displayed by L*er*-1 4x and St-0 4x. These results suggest that, at

least in ***A. thaliana*** **neotetraploids**, the **chiasma frequency is not the only factor that determines the bivalent levels** at metaphase I. Other aspects such as CO distribution could be playing an important role. In *Secale cereale*, the study conducted by Hazarika and Rees (1967) also reported differences in bivalent and quadrivalent rates among several synthetic autotetraploids with different genetic backgrounds, but in this case, the levels of bivalents and the chiasma frequency presented a clear indirect correlation. The fact that different genetic backgrounds show different bivalent rates immediately after the WGD demonstrates that the cytological diploidization levels might be affected (at least partially) by different allelic combinations and/or differences in the morphology of chromosomes (see more above). In fact, the studies published by Hollister *et al.* (2012) and Wright *et al.* (2015) suggest that the selection of certain allelic variants of meiotic genes such as *ASY1*, *ASY3*, *PDS5*, *SMC3*, *ZYP1a*, and *ZYP1b* in autotetraploid populations of *A. arenosa* could be underlying their high cytological diploidization levels. Moreover, in a recent study conducted by Morgan *et al.* (2020), it was confirmed by cytology that autotetraploid individuals that harbor "tetraploid" allelic variants of *ASY1* and *ASY3* present lower levels of multivalents than those that carry "diploid" variants. These genes are involved in the formation and remodeling of the chromosome axis during meiosis and are essential for the formation of COs (Armstrong *et al.*, 2002; Sanchez-Moran *et al.*, 2008; Ferdous *et al.*, 2012). Then, it seems that in autopolyploids, a polygenic solution could adapt the meiotic recombination avoiding the presence of multivalents at metaphase I. Thus, one possible explanation to understand the results obtained in our study could be that allelic variants of some meiotic genes are determining the different bivalent levels observed in the different genetic backgrounds analyzed. To prove that, it would be interesting to perform whole genome sequencing followed by a QTL analysis with accessions such as Col-0 (low number of bivalents) and Bla-1 (high number of bivalents) which presented remarkable differences. Thus, it would determine which genes are potentially responsible of such variation in the bivalent levels immediately after the WGD.

It has been observed in several species that the diploidization levels of synthetic autotetraploids get increased after several generations of self-pollination (Reviewed in Pelé *et al*., 2018). In fact, a study in *A. thaliana* described a partial diploidization after just a few generations (Santos *et al.*, 2003). They found that several lines with at least 13

generations after the induction of polyploidy presented higher proportion of bivalents than their synthetic autotetraploid counterpart. Among these lines, E_1 showed the lowest bivalent levels, whereas E_4 presented the highest numbers. It is possible to find several examples of autotetraploids that underwent rapid decreases of quadrivalents. For instance, species like *Amaranthus caudatus*, *Amaranthus edulis* (Pal and Pandey, 1982), *Brassica rapa* (Swaminathan and Sulbha, 1959), *Hyoscyamus niger* (Lavania *et al*., 1991)*, Tithonia speciosa* (Pandey, 1993) or *Pennisetum typhoides* (Jauhar, 1970) showed up to 50 % reduction of quadrivalents after a few generations of self-fertilization. In this thesis, the aim was to assess whether the cytological diploidization levels showed by the autotetraploid **established lines** of *A. thaliana* E_1 and E_4 (here referred as 3151 4x and 3432 4x, respectively) were increased during the following generations. For this purpose, these two lines were self-fertilized during another seven generations. The line 3432 4x presented a significantly higher frequency of bivalents and lower of quadrivalents than its synthetic counterpart Col-0 4x, whereas 3151 4x displayed non-significant differences neither with 3432 4x nor with Col-0 4x (Figure 27, Table 14). Comparing the results obtained by Santos and colleagues (plants with at least 13 generations after the WGD) and the results obtained in this study (plants with at least 20 generations after the WGD) an increase in the number of multivalents (and a decrease of the bivalent levels) was observed both in 3432 4x and 3151 4x. Therefore, **the lines have not advanced in the cytological diploidization process during these seven generations**, since the results even indicate a setback in this process when compared to those obtained by Santos and colleagues (2003). In the same line, in species such as *Lolium perenne* (Crowley and Rees, 1968) and *Phlox drummondii* (Raghuvanshi and Pathak, 1975; Dar *et al*., 2017b) there are increases in the frequency of quadrivalents in established polyploid lines after the self-fertilization of synthetic autotetraploid lines. In *P. drummondii*, the increase of quadrivalents was accompanied by changes in repetitive and non-repetitive sequences

(Dar *et al*., 2017b). Therefore, it cannot be ruled out that a constant rearrangement of chromosomes after the induction of polyploidy and during the following generations can promote low rates of multivalents but also they could lead to the opposite phenotype. Nevertheless, it is important to take into account that our observation window is very limited (only seven generations). In addition, the apparently contradictory results compared with those obtained by Santos *et al.* (2003) might be also explained by slight differences in

growth conditions between both studies. As it was reported by Lloyd *et al.* (2018), SC length and COs can be affected by environmental factors such as temperature. This may also explain the fact that all, the established lines 3151 4x and 3432 4x but also the synthetic line Col-0 4x presented in this study higher chiasma frequency than in the study conducted by Santos *et al.* (2003).

The apparently **rapid cytological diploidization observed in the established autopolyploid lines contrasts with the data obtained for the natural autotetraploids**. These autotetraploid accessions have most likely passed many more generations after the WGD event than the established lines although this cannot be confirmed as we do not have information about the number of generations that the natural lines have spent as polyploids. Surprisingly, the bivalent and quadrivalent levels of the natural lines (Figure 28) were comparable to those showed by the synthetic (Figure 26) and established lines (Figures 27). Strikingly, the autotetraploid line which showed the highest levels of bivalents in the present study was the synthetic line Bla-1 4x. In other species such as *Dactylis glomerata* (McCollum, 1958) and *Lathyrus pratensis* (Khawaja *et al*., 1997), natural and synthetic autotetraploids also show similar quadrivalent and bivalent frequencies. Although it seems clear that during the first generations after the WGD rapid genetic and epigenetic changes let autopolyploid plants achieve certain levels of diploidization, it would be possible that the diploidization process could get stranded. After the initial response to the shock of duplicate the entire genome, autotetraploids might get adapted presenting acceptable frequencies of multivalents and univalents that allow them to have enough fertility to survive in their environments without the necessity of increasing their levels of bivalents. However, the presence of species such as *A. arenosa* (Yant *et al.*, 2013), *A. chinensis* (Wu *et al.*, 2014), *A. elongatum* (Charpentier *et al*., 1986), or *S. cereale* (Santos *et al.*, 1995) that present markedly higher levels of bivalents in their natural autotetraploids compared to synthetic lines suggests that after a period of time, in which the process might be paused, the accumulation of genetic and/or epigenetic changes can contribute to keep increasing the cytological diploidization levels. For instance, as it is mentioned above, autotetraploids of *A. arenosa* present allelic variants of several meiotic genes that are not present in diploid populations (Hollister *et al.*, 2012; Yant *et al.*, 2013; Morgan *et al.*, 2020). These could explain greater diploidization levels than the ones observed during the very first generations

after the WGD. Also other factors such as differences between homologous chromosomes could play an important role (especially at the very early stages after the WGD) since in this study, the autotetraploid hybrid Col/L*er* 4x presented a substantially greater bivalent pair frequency than that of the autotetraploid individuals of its parental accessions Col-0 4x and L*er*-1 4x (Tables 19 and 20).

In this thesis, the **frequency of bivalents** was also analyzed for **each chromosome** of the complement. Grouping chromosomes according to their length, it was found that the **percentage of short chromosomes** (2 and 4) **forming bivalents** is constantly **higher** than that of **long chromosomes** (1+3 and 5) (Figure 29). Only the synthetic line L*er*-1 and the established lines 3151 4x and 3432 4x showed non-significant differences, but still in these cases, they showed higher percentage of short than long chromosomes forming bivalents. As it has been mentioned above, morphological differences between chromosomes such as rDNA sequences, their length, or the position of the centromere may affect the pattern of pairing and synapsis that can ultimately affect the distribution of COs and determine the chromosome associations. The fact that different chromosomes show significant variations in bivalent levels would imply that the process of cytological diploidization occurs at different speeds within the genome.

Although a low number of cells presented **univalents and trivalents**, interesting results were also obtained when their frequencies were assessed. The synthetic autotetraploid lines displayed significantly different levels of univalents. Col-0 4x, the genotype that showed the highest chiasma rates, also presented significantly lower univalent numbers than L*er*-1 4x, St-0 4x, and Bla-1 4x (Figure 26, Table 13). However, when the natural autotetraploids were analyzed, Bla-5 4x revealed considerable higher values of trivalents and univalents than Wa-1 and M3385S (Figure 28 and Table 15) but similar chiasma frequencies (Figure 24). These results confirmed again that the genetic background has an important influence in the cytological diploidization process and that there is not a clear correlation between chiasma frequency and chromosome associations.

5.1.4. Gene transcription analysis of meiotic genes in diploid and autotetraploid lines

It is known that the duplication of the entire genome entails variations in gene expression. Although in a lesser extent than in allopolyploids, autopolyploids show changes in the transcriptome compared to their parental diploids (Osborn *et al.*, 2003; Comai, 2005). In species such as *Citrus limonia* (Allario *et al.*, 2011), *Solanum tuberosum* (Stupar *et al.*, 2007), *Zea mays* (Riddle *et al.*, 2010), *Chrysanthemum lavandulifolium* (Gao *et al.*, 2016), and *Paulownia fortunei* (Zhang *et al*., 2014), changes in gene expression have been reported as a response to WGD. However, in these species, only a low percentage of the genes analyzed showed alterations of their mRNA levels when polyploidy was induced. Similarly, in studies with *A. thaliana*, only 2 % of the genes presented different expression levels in synthetic autotetraploids when they were compared with their corresponding diploids (Del Pozo and Ramirez-Parra, 2014). However, in the related species *B. rapa*, the study conducted by Braynen *et al.* (2017) revealed a wide number of genes related with the replication, repair, and recombination of the DNA, as well as chromatin structure and dynamics, that were differentially expressed in flower buds between a synthetic autotetraploid and its diploid counterpart. Some of these genes such as *RPA*, *DMC1*, *RAD54*, *TAM*, *SYN1*, *MER3*, *ZYP1a*, and *ZYP1b* are known to play an important role in meiosis. Then, it seems that the expression of some genes that are essential in meiotic recombination could be altered when polyploidy is induced. On these grounds, in this thesis, gene transcription analyses were conducted in synthetic, established, and natural autotetraploids in order to shed some light on the gene expression changes that could take place in several meiotic genes after the WGD induction and during the process of adaptation to polyploidy. Comparisons between diploid and autotetraploid lines with identical genetic backgrounds (or with proximal localizations) were conducted to identify whether the transcription levels of meiotic recombination genes change as a response to the WGD in the short (Col-0 4x vs Col-0 2x) and/or in the long term (Bla-5 4x vs Bla-1 2x). For the purpose of this study, it was assumed that Bla-5 4x and Bla-1 2x are relatives due to their geographical proximity, however, no information to confirm it was available. Also, an analysis of how the transcription levels of these meiotic genes could have been modulated during the first generations of adaptation to polyploidy was assessed by the comparison of the synthetic line Col-0 4x and the established line 3151 4x. Finally, a comparison between the synthetic line Col-0 4x and the

autotetraploid accession Bla-5 4x was performed in order to discover long-term transcriptional variations that could occur during the adaptation of autotetraploids.

The comparison between the synthetic autotetraploid Col-0 4x and its diploid parental Col-0 2x revealed that only two genes were differentially transcribed in flower bud samples (Figure 33A). ***ZYP1a*** **and** ***SYN1*** showed significantly **higher** mRNA levels in the **synthetic line** than in the **diploid parental**. Moreover, these differences are meiosis-specific since the expression levels of these two genes were similar between Col-0 4x and Col-0 2x in the analysis conducted in seedling samples (Figure 33B). These results are in line with the study conducted in flower buds of *B. rapa* where a synthetic autotetraploid also presented differential levels of these two genes compared to its diploid counterpart (Braynen *et al.*, 2017). Furthermore, natural selection seems to be acting on these two genes in autotetraploid populations of *A. arenosa* since different allelic variants have been found compared to diploid populations (Hollister *et al.*, 2012; Wright *et al.*, 2015). Since the functions of the proteins ZYP1 and SYN1 (REC8) are essential for the correct synapsis of the homologous chromosomes (Cai *et al.*, 2003; Higgins *et al.*, 2005), it may be possible that their modification (either the protein sequence or protein levels) is necessary to make changes in pairing and synapsis that are essential for the adaptation of autopolyploids both in the short and in long term. On the contrary, the expression levels of ***SYN1*** were found to be significantly **lower** in flower bud samples of the natural autotetraploid **Bla-5 4x** than in those of the synthetic line **Col-0 4x** (Figure 31A). However, the **same result** was also observed comparing the diploid accessions **Col-0 2x** and **Bla-1 2x** (geographically close to Bla-5 4x) (Figure 30A). Moreover, Bla-5 4x did not showed any difference in the mRNA levels of this gene compared to that of the diploid accession Bla-1 2x. Thus, the transcription levels of this gene could be firstly increased as a response to the WGD and then reduced during the following generations. Nonetheless, it cannot be ruled out that the differences shown by Col-0 4x and Bla-5 4x might be due to genotype variations. Besides, ***PDS5B***, which is one of the four genes that encodes one of the cohesin cofactors in *A. thaliana*, presented significantly **higher** transcription levels in **Bla-5 4x** than in **Col-0 4x** (Figure 31A). It has been reported by Pradillo *et al.* (2015) that the mutant of this gene as well as the quadruple mutant *pds5a/pds5b/pds5c/pds5e* show a decrease in fertility, but no serious defects were found in meiosis apart from the appearance of some chromosome bridges at anaphase I.

Moreover, according to the Bio-Analytic Resource for Plant Biology (BAR), PDS5 is a possible interactor of SYN1 and SCC2 which is required for a correct meiotic sister chromatid cohesion (Sebastian *et al.*, 2009). Then, it is possible that the increase in the mRNA levels of *PDS5B* in Bla-5 4x compared to Col-0 4x is related to the differences also observed in ***SYN1*** between these two autopolyploids (Figure 31A). However, the mRNA levels of other genes essential for the sister chromatid cohesion like **SMC1** were similar in both the synthetic and the natural autotetraploids (Figure 31A). Thus, immunolocalizations of SYN1 and other cohesins would provide information about whether polyploidy alters the dynamics of the sister chromatid cohesion.

Interestingly, in the comparison made between Col-0 4x and 3151 4x, the transcription of ***HEI10*** and ***MUS81*** showed marked **lower** levels in the **established** than in the **synthetic line** (Figure 32A). These results can explain the significantly lower chiasma levels showed by 3151 4x compared to Col-0 4x (Figure 21, Table 12) since it has been described that these two genes have an important role in CO formation (Higgins *et al.*, 2008b; Chelysheva *et al.*, 2012). In addition, ***RECQ4A*** also showed **lower** mRNA levels in **3151 4x** than in **Col-0 4x** (Figure 32A). The protein RECQ4A plays an important role in the dissolution of dHjs promoting the formation of NCOs (Chelysheva *et al.*, 2008; Hartung *et al.*, 2008; Knoll *et al*., 2014). In *A. thaliana*, its absence has been related to a strong increase of class II CO levels (Séguéla-Arnaud *et al.*, 2015). Thus, it would be expected that the decrease in the transcription levels of this gene could promote higher chiasma levels, however, the cytological analysis revealed significantly higher chiasma levels in Col-0 4x than in 3151 4x (Figure 21, Table 12). It is possible that the marked decrease of *HEI10* expression, and therefore, a dramatic reduction of the class I CO levels could be compensated by increasing the class II CO levels due to the decrease in the expression of *RECQ4A*. However, with this situation, higher frequencies of univalents in 3151 4x than in Col-0 4x (not observed) would have been expected since the class II COs are randomly distributed. Another option that could explain this situation is that the decrease in *HEI10* mRNA levels is underlying the lower chiasma frequency observed in 3151 4x respect to Col-0 4x and the decrease in *RECQ4A* levels is compensated by the decrease in *MUS81* maintaining the class II CO pathway with similar rates in both autotetraploid lines.

In the comparison between synthetic and established plants, ***MRD1*** also showed a marked **lower** transcription levels in **3151 4x** compared to **Col-0 4x**, both in flower bud samples and in seedling samples (Figure 32). This gene partially overlaps with the sequence of *HEI10* and has significantly higher mRNA levels in somatic tissue samples of Col-0 4x than in Col-0 2x (Yu *et al.*, 2010). Although in our study no statistical differences were found between flower bud samples of Col-0 2x and Col-0 4x, the autotetraploid showed noticeable higher levels (more than 4 times) (Figure 33A). These differences between the present study and the one conducted by Yu and colleagues (2010) could be due to either the low sensitivity of this technique or differences in the region of the gene targeted in each study. Then, the transcription levels of this gene may be substantially increased in somatic tissues (and probably in meiocytes) as a response to the WGD, but when the autotetraploids have several generations of adaptation to polyploidy, its transcription dramatically decreases displaying levels similar to the diploid line. In line with this, the comparison between the diploid accession Bla-1 2x and the autotetraploid accession Bla-5 4x revealed no differences in the expression of this gene (Figure 34A). Furthermore, in the study conducted by Yu *et al.* (2010), they showed that the differential levels showed by *MRD1* between the tetraploid and the diploid of Col-0 were not observed in other genetic backgrounds such as L*er*-0 due to a strong methylation of the gene.

When the comparison between the natural autotetraploid accession Bla-5 4x and the synthetic autotetraploid line Col-0 4x was made, it was also observed that the transcription levels of *SPO11* and *HEI10* had differences between these two autotetraploids. ***SPO11*** displayed significantly **lower** mRNA levels in **Bla-5 4x** than in **Col-0 4x** (Figure 31A). These results are in accordance with the chiasma frequency levels of these two lines, since Col-0 4x showed significantly higher chiasma numbers than those of Bla-5 4x ($t = 9.41$, $p < 0.001$). This situation was also found when the diploid accessions Bla-1 2x and Col-0 2x were compared (Figure 30A), which could be pointing out to genetic background differences. SPO11 is a highly conserved protein that has been reported to catalyze the formation of the programmed DSBs in meiosis (Hartung and Puchta, 2000; Grelon *et al.*, 2001). Thus, the low levels showed by both Bla-1 2x and Bla-5 4x could indicate that in these two genetic backgrounds the number of DSBs is reduced compared to those of Col-0 2x and Col-0 4x, respectively. Besides, the transcription levels of *SPO11* showed by Bla-1 2x and Bla-5 4x in

flower buds were similar (Figure 34A). At this point it is important to bear in mind that a reduction or increase in mRNA levels of a gene are not always accompanied by a change in protein levels (reviewed in Del Pozo and Ramirez-Parra, 2015). For this reason, this result ought to be corroborated by other approaches such as immunolocalization or western blot.

Regarding ***HEI10***, the transcription levels were significantly **higher in the natural autotetraploid** than in the **synthetic one** (Figure 31A). As it has been mentioned above, HEI10 is a ZMM protein essential for the formation of the class I COs (Chelysheva *et al.*, 2012) and its increase in *A. thaliana* has been reported to have a boosting effect in that CO pathway (Ziolkowski *et al.*, 2017; Serra *et al.*, 2018). Therefore, this result conflicts with the result obtained from the cytological analysis since Bla-5 4x (18.45 ± 0.12) showed a substantially lower chiasma levels than Col-0 4x (19.99 ± 0.11) (Figures 24 and 17, respectively). Moreover, differences were also found when a comparison between the natural autotetraploid accession Bla-5 4x and the proximally located diploid accession Bla-1 2x was performed (Figure 34A). **Bla-5 4x** presented significantly **higher** mRNA levels of ***HEI10*** than **Bla-1 2x**. Again, further analyses are necessary to find out whether these differences in transcription reflect actual differences in the amount of protein.

5.2. Chiasma formation competition between identical and homologous chromosomes in *Arabidopsis thaliana* hybrids

When the chromosome complement of a diploid individual is duplicated, each chromosome is accompanied by **one identical and two homologous chromosomes** within the same nucleus. In this situation, a competition in pairing/synapsis/chiasma formation between identical and homologous chromosomes can occur. Several studies have been conducted in different species to address whether there are preferences in CO formation between identical rather than between homologous chromosomes. Attempts to address this issue have been performed mainly in plants since it is very easy to obtain autotetraploids by a colchicine treatment, with only a few examples in animals. Possible preferences between chromosomes of a tetrasome were inferred from analyses to determine the segregation of genetic and/or chromosomal markers (Giraldez and Santos, 1981; Santos *et al*., 1983; Benavente and Orellana, 1991; Curole and Hedgecock, 2005). The most exhaustive cytological studies were conducted on rye, taking advantage of the existence of C-bands polymorphisms, especially in the nucleolar organizing region (NOR)-bearing chromosome 1R (Orellana and Santos, 1985; Benavente and Orellana, 1989, 1991; Benavente and Sybenga, 2004). In general, this species showed preferences for identical over homologous associations at metaphase I. This tendency is greater in hybrids with higher chromosomal divergence between the parental diploid plants. This fact indicates that chromosome differentiation could play a relevant role in the establishment of such preferences (Benavente and Orellana, 1991; Jenkins and Chatterjee, 1994). Thus, these preferences could contribute to the diploidization process of autopolyploids. In the present study, the analysis by FISH of chromosome configurations at metaphase I in synthetic autotetraploid hybrids Col/L*er* (these two accessions harbor differences in rDNA sequences in chromosomes 2 and 3, Figure 35) allowed us to evaluate not only the chiasma frequency of each chromosome of the complement but also the chiasma formation preferences between identical and homologous chromosomes, and thus, determine if those chromosome intraspecific differences in this autotetraploid hybrid are enough to determine preferences in terms of chiasma formation.

5.2.1. Heterozygosity has a great impact on bivalent levels

The multivalent frequency observed in **Col-0 4x significantly exceeds the 2:1 ratio** (66.66 % multivalents) expected by the random-end pairing model (Table 20), which means that, despite their small size, *A. thaliana* chromosomes have more than two autonomous synaptic initiation sites (López et al., 2008), and more than one synaptic partner switch per tetrasome. However, the autotetraploid **Col/L*er* hybrid showed a significant excess of bivalents** (Tables 19 and 20) which might be produced, at least partially, as a consequence of the heterozygosity.

Around 16,000 single feature polymorphisms between Col-0 and L*er*-1 accessions were detected in ~8,000 of the ~26,000 genes represented in a 44,000-feature exon-specific oligonucleotide array (Singer et al., 2006). Furthermore, both genomes differ in 564 transpositions and 47 inversions that comprise around 3.6 Mb (Ziolkowski *et al.*, 2009; Zapata *et al.*, 2016). Increases in bivalent frequency are strongly chromosome dependent and are generally ascribed to overall decreases in chiasma frequency and/or changes in chiasma distribution, with a more rapid response of the shortest chromosomes to these alterations. This differential response of the shortest chromosomes to polyploidy was observed in this study as a general trend in most of the autopolyploid lines analyzed (Figure 29). In this context, the behavior of the chromosome 3 in L*er*-1 can shed some light on this issue since it carries a 170-kb inversion on the short arm (Zapata *et al.*, 2016). Hence, Col/L*er* hybrid is heterozygous for such inversion, and it is well known that heterozygosity for inversions suppresses meiotic recombination.

The mean cell chiasma frequencies of the chromosome 3 in L*er*-1 4x and Col/L*er* 4x were similar (3.64 vs. 3.60; $t = 0.31$, $p = 0.76$), but significant differences between the bivalent pairs levels were observed (0.26 vs. 0.49; $t = 3.03$, $p \leq 0.001$) (Table 19), which is a similar situation to what was observed in this study when synthetic autotetraploid lines from different accessions were analyzed (Tables 8 and 13). Thus, other factors, in addition to **chromosome rearrangements**, such as **genotypic, epigenetic or cryptic structural differences** along chromosomes, may be **involved in the increase of the bivalent frequency** observed not only in this hybrid but also, for instance, in established autotetraploid lines of *A. thaliana* (Santos *et al.*, 2003). On these grounds, Zhang *et al.* (2015) reported that, after 49 self-pollinated generations, autotetraploid rice showed a significant increase in the

methylation of class II transposons in relation to its diploid donor that may affect gene expression. Also, Dar *et al*. (2017b) observed differences in the frequency of both quadrivalents and bivalents between synthetic autotetraploid lines of *Phlox drummondii* right after the WGD and lines with two generations after the polyploidy induction. These differences were associated with changes in both repetitive and non-repetitive regions.

Polyploidy is a major process in plant speciation. The potential evolutionary success of polyploids has been linked, among other hypotheses, to the buffering of mutations and sub- and neo-functionalization of duplicated genes (reviewed in Otto, 2007; Soltis and Soltis, 2009; Parisod *et al*., 2010; Zielinski and Mittelsten Scheid, 2012). It has been reported that polyploids of *Gossypium* and *Arabidopsis* enhance meiotic recombination compared with diploids (Desai *et al.*, 2006; Pecinka *et al.*, 2011). Increases in chiasma frequency could help to the establishment of new polyploid species by rapid creation of genetic diversity when population sizes are small. The data reported in this thesis unfit to this proposal since the autotetraploids showed chiasma frequencies about two-fold in comparison with their diploid counterparts (Figure 18 and Tables 18 and 19). However, the possibility to obtain an increase in recombination in certain chromosome regions cannot be ruled out. It would be interesting to test this hypothesis by examining chiasma frequencies not only in other *A. thaliana* accessions but also in other non-related species.

5.2.2. Preferences for chiasma formation among identical or homologous chromosomes are not present in all the chromosomes of the complement

Benavente and Orellana (1991) analyzed preferences for chiasma formation in synthetic autotetraploids of *Secale cereale* obtained from heterozygous hybrids for telomeric C-bands at the chromosome 1R. They found a clear tendency for preferences between identical partners in inter-subspecific hybrids. This tendency is increased in inter-specific hybrids with a higher chromosomal divergence between homologous chromosomes. These results reflect the potential effect of chromosomal differentiation on chiasma preferences in polyploids (see also Jenkins and Chatterjee, 1994). However, the hybrids resulting from crosses between inbred lines showed a wide range of preferential associations. Therefore, chiasmata between identical partners are not always favored.

In the present study, we have observed in the **hybrid Col/L*er* 4x** that although **chromosomes 2 and 3** exhibited similar frequencies of bivalent pairs (0.47 and 0.49, respectively) and chiasmata (3.78 and 3.60, respectively) (Table 19), they presented **different preferences in chiasma formation**. Chiasmata were randomly formed between identical and homologous chromosomes 2, but preferentially established between homologous chromosomes 3 (Table 21). These results indicate that although chromosome differentiation between related genomes may be the main cause of the excess of bivalents in the hybrid, the formation of bivalents between identical chromosomes is not necessarily favored (Sybenga, 1992; Bourke *et al.*, 2015). In this regard, random chiasma formation among identical and homologous chromosomes 2 could be related to their close spatial nuclear location as a consequence of bearing the NOR region on the short arm, since differences in the number of 45S tandem repeats (Rabanal *et al.*, 2017) do not seem to have an influence. On the other hand, preferences for chiasma formation between homologous chromosomes 3 could be more related to specific features of specific chromosome regions. Actually, in L*er*, the 5S rDNA region on the long arm of this chromosome is close (~6 Mb) to the 170 kb inversion mentioned before (Simon *et al.*, 2018). This means that the genomes of the two accessions are different in a large region, which would have important consequences for meiotic recombination. However, recent meiotic recombination analyses have suggested that high levels of sequence divergence are not necessarily inhibitors of meiotic recombination (Barth *et al.*, 2001; Singer *et al.*, 2006; Salomé *et al.*, 2012). This idea is in agreement with a positive correlation of ancestral recombination frequencies and regions with high sequence divergence (Kim *et al.*, 2007). In addition, heterozygous regions increase chiasma formation when are juxtaposed with homozygous regions, which reciprocally decrease (Ziolkowski *et al.*, 2015; Blackwell *et al*., 2020). In relation to the chromosome 3, Barth *et al*. (2001) found a strong negative correlation between genetic similarities of ecotypes and recombination frequencies for two adjacent markers located on the long arm of this chromosome, but not for other genomic regions. In general, there are difficulties in mapping and sequencing this chromosome, consequently this fact suggests the existence of unusual chromatin-related features respect to the other chromosomes of the complement (Schmidt, 2018).

When the chromosome complement of a diploid individual is duplicated, the degree of relationship between two chromosomes within each tetrasome may be greater than mere homology. In this situation, there is a competition for chiasma formation between identical and homologous chromosomes. In this study, it was observed that the presence of intraspecific differences among homologous chromosomes in autopolyploids could favor greater levels of diploidization. However, that situation did not imply a greater preference for chiasma formation between identical chromosomes. In fact, it was observed that this competition between identical and homologous can be resolved through different ways depending on the chromosome. Accordingly, identical and homologous chromosome regions will persist in each tetrasome in a differential pattern throughout generations. This chromosomal genetic variation has not been considered in current models about tetrasomic and disomic inheritance and it could produce a relevant impact on haplotypes.

5.3. The study of polyploid mutants as a tool to better describe the function of meiotic recombination genes

One of the objectives of this thesis was to analyze the behavior of several meiotic recombination mutants as a response to the duplication of the entire genome. Although most of the mutant lines used in this study were already described, it was done in diploid individuals and very little is known about the effect of these mutations in a polyploid situation. Interestingly, the results obtained in this thesis showed that polyploidy provides features in meiosis, and specifically in homologous recombination, that can be useful to better characterize the function of genes involved in this process. The presence of more than two homologous chromosomes that can pair, synapse, and recombine with each other, together with the fact that the gene dose is increased and the expression of some genes altered, make a scenario where polyploid mutants can offer new insights. Although the characterization of polyploid meiotic mutants is scarce in the literature, there are some examples. In *A. thaliana*, Tian *et al.* (2011) analyzed the meiotic behavior of the colchicine-induced tetraploid mutants *asy1 and dmc1*. They found different levels of fertility comparing these two tetraploid mutants with their parental diploids.

5.3.1. Polyploidy reveals chiasma frequency differences among *zmm* mutants

ZMM refers to a heterogeneous group of structurally diverse proteins (Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, and Mer3) essential for the formation of the SC and class I COs in *S. cerevisiae* (Börner *et al*., 2004). In this species, these proteins are thought to play an important role coordinating some recombination events and during the assembly of the SC, providing a link between these two processes (Börner *et al*., 2004; Lynn *et al*., 2007; Shinohara *et al.*, 2008). In plants and animals, putative orthologues of the genes encoding ZMM proteins have been found. As in budding yeast, *zmm* mutants in *A. thaliana* are defective in the formation of interference-sensitive COs. However, in this species, except ZYP1, which is the main component of the central element of the SC (ortholog of Zip1), the remaining ZMM proteins are not essential in the SC formation, unlike the situation in *S. cerevisiae* (reviewed in Osman *et al.*, 2011). Studies performed in budding yeast showed that all the *zmm* mutants present a reduction of approximately 85 % in their CO levels respect to the WT. Besides, analysis of the

phenotype of double mutants revealed that *ZMM* genes belong to the same epistatic group (Börner *et al*., 2004). Similarly, studies in *A. thaliana* showed that *ZIP4*, *MSH4/5*, *MER3*, and *HEI10* are also epistatic due to their effect on CO levels (Chelysheva *et al.*, 2007, 2012). The cytological analyses of the diploid mutants *zip4*, *msh5*, *mer3*, and *hei10* showed that they present low rates of chiasma formation (Mercier *et al.*, 2005; Chelysheva *et al.*, 2007, 2012; Higgins *et al.*, 2008a). It has been proposed that the COs lost in these *zmm* mutants belong to the class I, whereas the remaining COs observed seem to be randomly distributed, and therefore, belong to the class II (reviewed in Osman *et al.*, 2011). In order to have a global information about the phenotype of *zmm* mutants, we have analyzed them at the diploid level, and we have found slight differences in terms of chiasma formation. The results obtained in this study showed that the diploid lines *zip4* 2x, *msh5* 2x, and *hei10* 2x had no differences among their chiasma frequencies, whereas *mer3* 2x presented significantly higher frequency than that of the other three *zmm* mutants (Figure 39, Table 22). It is important to point out that this is the first study that makes a comparison of four *zmm* mutants at the same time in *A. thaliana*. Surprisingly, when the chiasma frequencies of the **tetraploid *zmm* mutants** were assessed, **significant differences** were observed not only between *mer3* 4x and the rest of *zmm* but also **between *hei10* 4x and *zip4* 4x and *msh*5 4x** (Figure 41, Table 23). Moreover, when the **fold-change in chiasma frequency** was calculated for each mutant background, there was also a **different response among the *zmm* mutants** analyzed. While the WT presented a 1.96-fold increase in its chiasma frequency as a response to the WGD, *mer3* and *hei10* displayed a significantly higher fold-change. On the contrary, *msh5* showed lower fold-change and *zip4* presented no differences than the control (Figure 42). Additionally, in line with the chiasma frequency results, although all the *zmm* mutants analyzed presented high levels of **univalents**, the numbers displayed by ***zip4* 4x and *msh5* 4x** were significantly **higher** than those of ***hei10* 4x** (Figure 44). Therefore, the duplication of the entire genome reveals some differences in terms of chiasma formation among *zmm* mutants that are not observed at the diploid level.

Interestingly, when the **immunolocalization of MLH1** (widely used as class I CO marker, De Boer *et al.*, 2006; Lhuissier *et al.*, 2007; Chelysheva *et al.*, 2010) was performed, Col-0 2x revealed an average of 8.33 foci per cell, whereas Col-0 4x displayed an average of 11.50 foci per cell (Figure 46). These results, together with the chiasma frequencies

obtained, suggest that the CO events are duplicated as a response to polyploidy, however, the class I COs are not. In other words, whereas at the **diploid level** the **class I COs** roughly represent the **82 %** of the total number of COs (consistent with previous studies reviewed in Osman *et al.*, 2011), at the **polyploid level** the MLH1-dependent COs represent only the **58 %**. Thus, the induction of the WGD in *A. thaliana* seems to modulate the proportion of class I/class II COs, being the number and proportion of class II CO much higher than in a diploid situation. This could shed some light into the differences observed in this study among the tetraploid *zmm* mutants. As the tetraploid WT shows 19.99 chiasmata per cell and 11.50 MLH1 foci per cell, it means that around 8 chiasmata could be formed due to the class II COs and 12 chiasmata by the class I CO pathway. Thus, the chiasma frequency reduction of *hei10* 4x would fit with the number of COs that are dependent on MLH1, and therefore, as it has been proposed for the diploid mutant (Chelysheva *et al.*, 2012), the remaining chiasmata in *hei10* 4x could be only produced by class II COs. On the contrary, the chiasma reduction showed by *zip4* 4x and *msh5* 4x does not match with those numbers, being the loss of chiasmata showed by these mutants higher than the average number of MLH1 foci detected in the tetraploid WT. Thus, a possible explanation for these results could be that, in a polyploid scenario the ZMM proteins might have a role in other CO formation pathways than the canonical one. However, it cannot be ruled out that depending on the *zmm* mutant background, the response from other CO formation pathways could be different, then, explaining the different chiasma numbers observed among the *zmm* mutants at the tetraploid level (see more below).

5.3.2. The mutants *mlh3* and *mer3* might not have the class I COs completely depleted

In *mer3*, the chiasma frequency observed in the diploid mutant exceeds the predicted number of class II COs, and this situation was also seen in the tetraploid mutant. The mean number of chiasmata displayed by ***mer3* 2x is significantly higher than that of other *zmm*** mutants such as *zip4* 2x, *msh5* 2x, and *hei10* 2x (Figure 39, Table 22). This situation was **also observed** when the **tetraploid mutants** were analyzed (Figure 41, Table 23). This suggests that the *mer3* background could be partially (but not totally) limiting the number of class I COs, whereas the class II COs might be normally formed. This fits with the results obtained by Chelysheva *et al.* (2012), since they reported that the double mutant *hei10*/*mer3* and the

single mutant *hei10* had similar chiasma frequencies and both displayed significantly lower frequency than *mer3*. Furthermore, Macaisne *et al*. (2011) also showed that the double mutants *shoc1*/*mer3* and *mer3*/*msh4* have significantly lower chiasma levels than *mer3*. Chen *et al.* (2005) suggested that the greater chiasma formation of the *mer3* mutants (*rck* in that study) compared to other *zmm* mutants can be explained by the fact that MER3 may be partially redundant with other proteins. For example, the predicted protein encoded by At5g61140 has 30 % sequence identity to MER3. However, recombination analysis of adjacent intervals revealed that probably the remaining COs in *mer3* are not interference-sensitive (Mercier *et al.*, 2005). This is supported by the fact that the remaining chiasmata in this mutant are distributed not significantly different to a Poisson distribution (Chen *et al.*, 2005). On the other hand, it has been reported in *Oryza sativa* that the contributions of MER3 and ZIP4 in the formation of COs are not equivalent since the double mutant *mer3*/*zip4* presented lower chiasma levels than the respective single mutants (Shen *et al.*, 2012). Also, they observed that the MER3 loading during prophase I depends on ZIP4 but not vice versa although no direct interactions between these two proteins was found by a yeast two-hybrid analysis. In budding yeast, Mer3, which is 51 % similar to the *Arabidopsis* MER3 (Mercier *et al.*, 2005), has been described to be a DExH-box-type DNA helicase that unwinds duplex DNA in the 3´ to 5´ direction (Nakagawa *et al*., 2001) and stimulates the extension of the DNA heteroduplex in vitro (Mazina *et al.*, 2004). Besides, the meiotic recombination-related DNA synthesis is compromised in *mer3* (Terasawa *et al.*, 2007). These evidences suggest a role of Mer3 in the stabilization and extension of the DNA heteroduplex molecules formed by the action of Dmc1 and Rad51. Therefore, according to the phenotype showed by *mer3* in this study, but also in previous studies, the putative role of MER3 could be necessary, but not essential, for the formation of the class I COs, and this protein would accomplish its function downstream of ZIP4 in the HR pathway. However, further research should be conducted to check whether the remaining COs in the absence of MER3, both in diploid and tetraploid meiosis, belong exclusively to the class II COs or, on the contrary, a few interference-sensitive CO are also being formed. Immunolocalizations of class I CO markers (MLH1, MLH3, or HEI10) in the diploid and tetraploid *mer3* would give more information.

Similar to the results obtained in *mer3*, the ***mlh3*** mutant background also showed **higher chiasma frequencies** compared to the ***zmm* mutants** analyzed both at **diploid** (Figure 39, Table 22) **and tetraploid** levels (Figure 41, Table 23). This situation could be expected as it is known that this protein acts downstream the ZMMs in the class I CO formation pathway. *MLH3* is homologue of the prokaryotic *MutL* mismatch repair gene. In a wide number of species, it has been reported that MLH3 is essential for normal levels of CO formation. In fact, it has been demonstrated that it acts together with another MutL homologue called MLH1 resolving dHjs into class I COs (reviewed in Osman *et al.*, 2011). In *A. thaliana*, the chiasma frequency reduction in absence of MLH3 (60 %) is lower than in *zmm* mutants like *msh4* (85 %) (Jackson *et al.*, 2006). In line with this, *mlh3* 2x exhibited in this study significantly higher chiasma levels than *zip4* 2x, *msh5* 2x, *mer3* 2x, and *hei10* 2x (Figure 39, Table 22). Besides, this situation did not change when these mutant backgrounds were analyzed at the tetraploid level. *mlh3* 4x still displayed significantly higher chiasma numbers than all the tetraploid *zmm* mutants analyzed (Figure 41, Table 23). Moreover, when the fold-change in chiasma frequency as a response to WGD was analyzed, it was observed that this mutant background also presented higher increase chiasma numbers than the WT (Figure 42). Therefore, these results suggest that the absence of MLH3 could partially affect the formation of class I COs, having still some COs formed through this pathway. However, Jackson *et al.* (2006) observed that MLH1 cannot be detected by immunolocalization in the *mlh3* mutant and its remaining chiasmata are randomly distributed following a pattern similar to a Poisson distribution. Then, it could be possible that the class II COs are boosted in absence of MLH3. Unfortunately, a lack of a class II CO immunolocalization marker make difficult to prove it. Furthermore, no double mutants of *mlh3* with a *zmm* mutant has been analyzed so far. These could shed light into the possibility that the class I CO pathway is still working in absence of MLH3.

5.3.3. The study of the tetraploid mutant *hei10* 4x suggests an increase in class II COs as a response to the WGD

The *HEI10* gene of *A. thaliana* is an orthologue of both the mammalian *HEI10* and the yeast *Zip3* (Chelysheva *et al.*, 2012). The protein harbors a RING-domain that, in budding yeast, has been associated with a SUMO E3 ligase activity (Cheng *et al.*, 2006), while in humans, this domain have E3 ubiquitin ligase activity in vitro (Toby *et al.*, 2003). In budding yeast, Zip3, as the rest of the ZMM proteins, is essential for the formation of both the SC and class I COs (Börner *et al*., 2004). However, in mammals and plants, although HEI10 also has role in meiotic recombination, the process of synapsis seems to be unaltered when this protein is absent (Ward *et al.*, 2007; Chelysheva *et al.*, 2012). In Arabidopsis, HEI10 is loaded in a wide number of recombination sites during leptotene. As the prophase I progresses, the number of foci observed by immunolocalization is reduced until the pachytene stage, where the number of foci is around 9 and they co-localize with the MLH1 foci (Chelysheva *et al.*, 2012). Thus, it seems that HEI10 loads into the early recombination intermediates, however, as these intermediates mature, HEI10 only remains in those that are designated to be processed as class I COs. Due to the putative function of this protein in ubiquitination/SUMOylation and the phenotype showed by *hei10*, it seems that during the maturation process of the recombination intermediates, HEI10 could play an important role degrading or modifying other proteins, and that is essential for the class I CO pathway. However, it is still unknown if this protein is acting at each step from the early intermediates until the formation of the class I COs or if it only acts during the last stages (Chelysheva *et al.*, 2012).

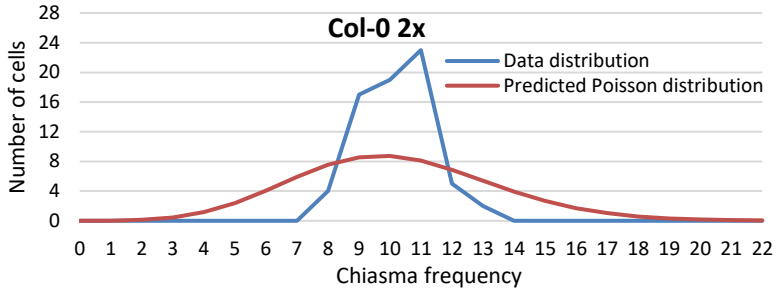
As it was observed in previous studies but also in this thesis, the diploid mutants ***zip4* 2x and *msh5* 2x** showed around a 15 % of the WT **chiasma levels** (Figure 39) (Chelysheva *et al.*, 2007; Higgins *et al.*, 2008a), which is **not significantly different** compared to the chiasma frequency showed by ***hei10* 2x** (Table 22) (this study and Chelysheva *et al.*, 2012). However, when the chiasma frequency was assessed at the **tetraploid level** (Figure 41), important **differences were observed** among the *zmm* mutants analyzed (Table 23). While *zip4* 4x and *msh5* 4x still displayed similar frequencies, ***hei10* 4x** presented significantly **higher chiasma levels** than the former ones. One possibility to explain this situation could be that the *hei10* mutant allele used for this study (*hei10-2*) still produces

some amount of functional protein. In fact, in previous analysis, *hei10-2* showed some transcription levels of *HEI10* in flower buds (Chelysheva *et al.*, 2012). From the position of the T-DNA insertion in this allele, it was predicted that a truncated protein of 279 amino acids (instead of 304 in the WT) could be still produced. Thus, it might be possible that in the diploid mutant, the amount of truncated protein is not enough to produce any class I CO, showing *hei10* 2x only a 20 % of the WT chiasma levels. However, *hei10* 4x could have higher levels of the truncated protein that might be enough to produce, apart from the class II COs, some class I COs, thus, explaining the increased chiasma frequency that this tetraploid mutant displayed respect to *zip4* 4x and *msh5* 4x. In fact, it has been demonstrated that HEI10 acts in a dose-dependent manner increasing the amount of COs in *A. thaliana* (Ziolkowski *et al.*, 2017). Nevertheless, immunolocalizations of MLH1 and HEI10 conducted by Chelysheva *et al.* (2012) in this diploid mutant line showed no detection of these two proteins at prophase I. Moreover, the MLH1 immunolocalization assessment conducted in the diploid and tetraploid WTs (Figure 46) suggests that the class I/class II CO proportion is modified as a response to the WGD, having Col-0 4x higher percentage of class II and lower of class I than Col-0 2x (see more above). As the reduction in chiasma frequency showed by *hei10* 4x roughly fits the mean number of MLH1 foci (class I COs), it might indicate that *hei10* 4x presents only class II COs (Chelysheva *et al.*, 2012). Moreover, when the **distribution of the chiasma frequency** per cell was analyzed for ***hei10* 2x** and ***hei10* 4x** by the Kolmogorov-Smirnov test, it was observed that the distribution in both mutants is not significantly different than a **Poisson distribution** ($p = 0.878$ for hei10 2x; $p = 0.287$ for hei10 4x), whereas the diploid and tetraploid controls showed significant differences ($p < 0.001$ in both cases) (Figure 55). These results are a strong evidence that the residual chiasmata in both *hei10* 2x and *hei10* 4x are randomly distributed, which is a feature that has been associated to an absence of CO interference (class II COs) (Higgins *et al.*, 2004, 2008a, 2008b).

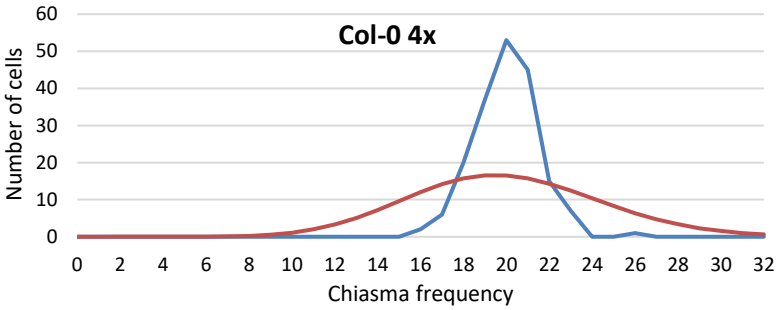
When the **transcription levels** of several meiotic genes were assessed in *hei10* 2x and ***hei10* 4x**, it was observed that in the tetraploid (Figure 48), but not in the diploid mutant (Figure 47), ***SPO11-1***, ***RAD51***, and in a lesser extent ***DMC1*** presented **slightly higher** mRNA levels ($RQ < 2$) than in the **WT**, being these differences meiosis-specific. This response cannot be only attributed to WGD as the comparison between the diploid and tetraploid WTs did

not show any differences. SPO11-1 is necessary for the formation of the programed DSBs in meiosis, whereas RAD51 and DMC1 are essential to repair those DSBs through the HR repair pathway (reviewed in Osman *et al.*, 2011). Thus, these results could suggest that in a situation in which the class I COs are reduced but also the number of chromosomes is duplicated, more DSBs could be generated and repaired in order to compensate defects in CO formation. Similarly, in *S. cerevisiae*, it was reported that *zmm* mutants have greater DSB numbers than the WT (Thacker *et al.*, 2014). However, that situation has been related to the synapsis problems that *zmm* mutants have in yeast, which is not the case in *A. thaliana*. In any case, the results obtained in this study should be complemented with other approaches such as immunolocalizations to confirm that the increased transcription levels of *SPO11*, *RAD51*, and *DMC1* are actually translated into increased levels of DSBs.

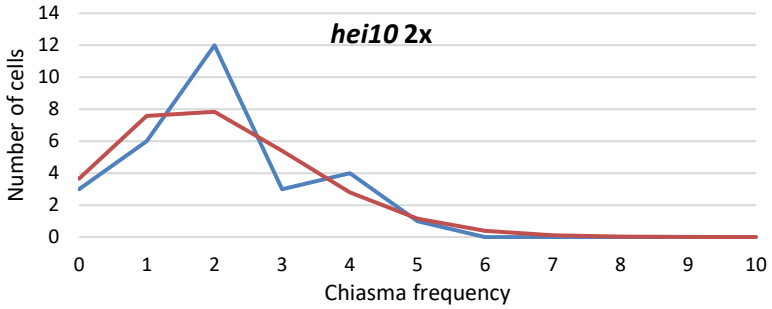
A



B



C



D

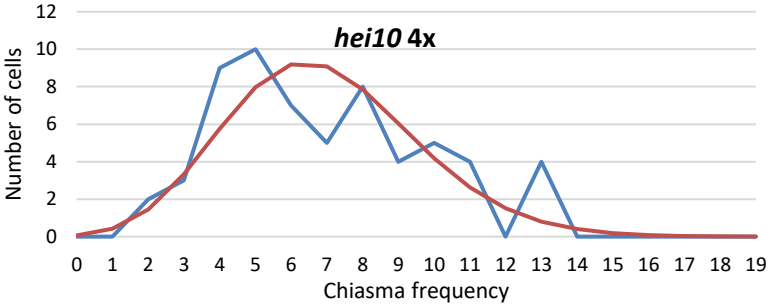


Figure 55. Observed and Poisson-predicted distributions of chiasma frequency per cell in the diploid and tetraploid of *hei10* and the control. Line chart representing the distribution of the chiasma frequency per cell (blue) and the Poisson-predicted distribution (red) of Col-0 2x (**A**), Col-0 4x (**B**), *hei10* 2x (**C**), and *hei10* 4x (**D**).

5.3.4. *zip4* and *msh5* present significantly lower chiasma numbers than *hei10* at the tetraploid level but not at the diploid level

In budding yeast, Zip4, along with Zip2 (SHOC1 in Arabidopsis) and Zip3 (HEI10 in *Arabidopsis*), could have a role in ubiquitination and/or SUMOylation (Perry *et al*., 2005). Although it is still unclear, Perry and colleagues (2005) suggested that in yeast, Zip4 could act together with Zip2 as shuttle components bringing substrate to the Zip3 (E3 ligase) or helping this protein to get localized at the recombination complexes. However, this putative function for ZIP4 in *Arabidopsis* is still unclear.

As for MSH5, several studies have suggested that this protein could form a heterodimer together with MSH4. Snowden *et al.* (2004) found that in vitro the heterodimer formed by the human MSH4 and MSH5 bounds to the cores of Hjs and structures like D-loops. They suggested that the affinity for these structures is consistent to the hypothesis that these proteins stabilize early recombination intermediates by a sliding clamp mechanism that embraces duplex DNA. This would enable first the conversion of the Hj into a dHj and later its resolution into either a CO or NCO. In *A. thaliana*, immunolocalizations showed that antibodies specific for MSH4 and MSH5 co-localized on chromosome axis during prophase I (Higgins *et al.*, 2008a). In the same study, it was also revealed that during leptotene MSH5 can be detected as lots of foci that are decreased in number as prophase I progresses.

The results obtained in this study showed that while at the diploid level ***zip4* 2x**, ***msh5* 2x**, and ***hei10* 2x** did **not** present **significant differences** in terms of **chiasma frequency** (Figure 39, Table 22), at the tetraploid level, ***zip4* 4x** and ***msh5* 4x** displayed a significantly **lower** chiasma frequency than ***hei10* 4x** (Figure 41, Table 23). In *hei10* 4x, the reduction of chiasma numbers fits with the number of MLH1 foci found in the WT (see more above), whereas in *zip4* and *msh5*, the reduction is markedly higher. Thus, one of the options to explain this situation is that the absence of ZIP4 or MSH5 could also affect the formation of MLH1-independent COs.

According to the results obtained by Shen *et al.* (2012) in rice, ZIP4 could be playing its function earlier than other ZMMs such as MER3, probably making a proper environment to recruit other proteins that are necessary to mature recombination intermediates. Then,

the role of ZIP4 would be necessary to process those intermediates that mainly will be resolved as interference-sensitive COs, however, some of those intermediates may also be needed for other kind of COs. Interestingly, Luo *et al.* (2013) found also in *O. sativa* that MSH5 is needed for the correct localization of ZIP4 and MER3 during prophase I. Also, the localization of MER3 depends on ZIP4. The hierarchy of these proteins in *O. sativa* fits with the results obtained in this thesis where *msh5* 4x showed lower chiasma levels than *zip4* 4x (although not statistically significant) and this one lower than *mer3* 4x (Figure 41, Table 23). Thus, the results obtained in previous studies, together with the results observed in this thesis, indicate that MSH5 could have a role stabilizing the early recombination intermediates that later can be used for the formation of COs through different pathways, although mainly interference-sensitive. In the class I CO pathway, the function of this heterodimer could be necessary from early to very late stages according with the dynamics observed during prophase I. The lack of significant differences between *hei10* 2x, *zip4* 2x, and *msh5* 2x compared to the tetraploid situation, where *hei10* 4x showed significantly higher chiasma frequency than *zip4* 4x and *msh5* 4x, could be explained either if most of the interference-insensitive COs formed in the tetraploid meiosis are produced by a pathway that is not active in diploids, or if the low number of class II COs formed in diploids (only one or two) does not allow to detect differences among the mutants in their formation. However, another option mentioned above is that the remaining transcription levels of the *hei10* allele used in this study are enough at the tetraploid level (but not at the diploid level) to still produce some class I COs, thus, explaining the differences in terms of chiasma formation that *hei10* 4x displayed compared to *zip4* 4x and *msh5* 4x, that present a null allele for *ZIP4* and *MSH5*, respectively (Chelysheva *et al.*, 2007; Higgins *et al.*, 2008a). Analysis of tetraploid double mutants such as *hei10*/*msh5* or *hei10*/*zip4* would be necessary to confirm the results obtained in the tetraploid single mutants, and therefore, to determine if the absence of either MSH5 or ZIP4 affects the formation of the remaining chiasmata in *hei10* 4x. Also, immunolocalization of class I CO markers (MLH1 or MLH3) could be useful to determine if some of the remaining chiasmata in *hei10* 4x are formed by the class I CO pathway.

5.3.5. *mus81* is indistinguishable from the wild-type, whereas *fancm* shows unexpected chiasma numbers at the tetraploid level

In budding yeast, around 15 % of the COs are interference-insensitive (De los Santos *et al.*, 2003; Börner *et al*., 2004) and dependent on the heterodimer Mus81-Mms4 (De los Santos *et al.*, 2003). However, in *Schizosaccharomyces pombe*, Mus81 forms a heterodimer with Eme1 and it is required for the formation of the majority of COs, which do not present interference either (Smith *et al.*, 2003). In *A. thaliana*, MUS81 was firstly analyzed for its role in the repair of somatic DNA (Hartung *et al.*, 2006). Later, in meiosis, an analysis of genetic intervals on chromosomes 1 and 3 revealed a moderate decrease of COs in *mus81* compared to the WT (Berchowitz *et al.*, 2007). Furthermore, it has been reported that the double mutant *msh4*/*mus81* (0.8 chiasmata per cell) has lower chiasma levels than the *msh4* mutant (1.25 chiasmata per cell) (Higgins *et al.*, 2008b). Therefore, these results suggest that MUS81 could also have a role in the formation of class II COs in *A. thaliana*. However, the remaining chiasmata found in the double *msh4*/*mus81* also indicate that there is at least another pathway that can produce class II COs. Kurzbauer *et al.* (2018) have proposed that in *A. thaliana*, FANCD2 could also be necessary for the formation of class II COs. Furthermore, when they compared the bivalent levels between the double mutant *msh4*/*mus81* and the triple *fancd2*/*msh4*/*mus81*, it was observed that FANCD2 seems to be responsible of an 80 % of the remaining COs formed in the absence of MSH4 and MUS81. However, few bivalents were still observed in the triple mutant, suggesting that there could be more pathways or proteins apart from FANCD2 and MUS81 that are involved in the formation of class II COs.

In this study, cytological analyses of the diploid and tetraploid *mus81* were performed. Both, ***mus81* 2x** and ***mus81* 4x**, presented **similar levels of chiasmata** compared to their respective **WTs** (Figures 39 and 41). Besides, when the chromosome associations were analyzed, *mus81* 4x and its control presented **similar numbers of bivalents, quadrivalents, trivalents, and univalents** (Figures 43 and 44). Due to the role that has been assigned to MUS81 in the formation of class II COs in *A. thaliana*, one could expect that both the diploid and especially the tetraploid (higher proportion of class II COs, discussed above) *mus81* mutants presented a substantial reduction of the chiasma frequency compared to their respective WTs. The absence of such phenotype in the diploid and tetraploid *mus81* could indicate that some of the class II COs formed are due to a CO pathway or pathways

that do not depend on MUS81. As it has been mentioned above, other pathways (such as the one in which FANCD2 is involved) might be also behind the formation of this kind of COs. Then, some of the class II COs formed in the tetraploid meiosis could depend on FANCD2. Thus, the analysis of a tetraploid *fancd2* mutant would provide information in this respect. In addition, Kurzbauer *et al.* (2018) proposed that some of the recombination intermediates that are processed by MUS81, in the absence of that protein, could be processed by FANCD2. Therefore, it is also possible that MUS81 is responsible for the formation of the class II COs, but when this protein is absent, FANCD2 may replace its function. Thus, the study of the double mutant *mus81*/*fancd2* at the tetraploid level would also be useful to define better the role of these proteins in the formation of the interference-insensitive COs and the possible differences derived from the WGD.

In order to better characterize the class II COs in tetraploids, the mutants *fancm* 2x and *fancm* 4x were also analyzed. Crismani *et al.* (2012) proposed that FANCM, with its putative helicase function, processes the meiotic DSB repair intermediates towards the formation of NCOs or sister chromatid repair. In the absence of FANCM, these intermediates would be repaired by MUS81, increasing the class II COs. Crismani and colleagues (2012) showed that the *fancm* mutant has increased meiotic recombination in several genetic intervals. They also reported that the absence of FANCM restores the bivalent formation in several *zmm* mutants due to an increase in class II COs. In line with the results obtained previously, we found that the diploid mutant ***fancm* 2x** presented a slight but significant **increase in chiasma frequency** compared to the **control** (Figure 39). Strikingly, when the tetraploid mutant ***fancm* 4x** was analyzed, it displayed a significant **decrease in chiasma numbers** respect to the **WT** (Figure 41). These results were also accompanied by a significant increase in univalents and trivalents respect to those of the WT (Figure 44). In addition, an immunolocalization of MLH1 was conducted to assess whether these defects are due to a reduction in class I COs. This assessment showed that both, *fancm* 2x and *fancm* 4x, presented similar number of MLH1 compared to their respective controls (Figure 46). Therefore, the chiasma reduction in the tetraploid mutant respect to its control is probably be due to a loss of class II COs. Interestingly, there are other situations in which the absence of FANCM does not promote an increase of COs. When the CO formation was assessed by

marker segregation analysis in the diploid hybrid Col/L*er* with the *fancm* mutation, no significant differences were found respect to the control (Girard *et al.*, 2015).

In order to find out the reason of this differential behavior of *fancm* 4x respect to its diploid counterpart and the WT, a **gene transcription analysis** was also performed. The results showed that the transcription levels of ***HEI10*** are **reduced** in the absence of FANCM compared to the WT, both in ***fancm* 2x** (Figure 49A) and ***fancm* 4x** (Figure 50A). These results are intriguing since HEI10 seems to be essential for the formation of class I COs, whereas *fancm* 2x and *fancm* 4x presented WT levels of MLH1 foci (class I CO marker). It would be possible that a reduction of HEI10 could favor the increase of the class II COs in the absence of FANCM in a diploid situation, but it would be necessary to check whether the *HEI10* transcription reduction is accompanied by a reduction of the protein levels, and also if the dynamics of HEI10 during the early prophase I is modified in the absence of FANCM. In *fancm* 4x other factors (e.g. interactions between pathways that are producing class II COs) could be preventing the formation of COs.

Interestingly, the transcription analysis also revealed that the mRNA levels of ***CAND1*** were **duplicated** in ***fancm* 2x** compared to the **WT**, whereas that was **not** the case when ***fancm* 4x** was compared to its control. Besides, the tetraploid WT also showed greater *CAND1* mRNA levels than Col-0 2x. This result could suggest that CAND1 could be regulating the formation of class II COs. However, it is important to take into account that the severe defects in chiasma formation observed in the mutants *hve* 2x (Figure 39) and *hve* 4x (Figure 41) (with a mutation in *CAND1*) suggest that this protein could also be playing a role in the formation of class I COs (see more below).

In *A. thaliana,* apart from FANCM, there are other proteins with anti-recombinase activity such as RECQ4A/B and FIGL1 that seem to act over different pathways of class II COs (Fernandes *et al.*, 2018). It would be interesting to check whether in the absence of these anti-recombinases, the behavior of the tetraploid is different to what was observed in *fancm* 4x. In addition, the double mutant *hei10*/*fancm* 4x would be useful to check if in the absence of class I COs and FANCM, the formation of class II COs is also compromised.

5.3.6. *hve* presents severe meiotic recombination defects both at diploid and tetraploid levels

Another gene analyzed in this study was *CAND1*. It has been demonstrated in mammals that the corresponding protein has a role in ubiquitination (Liu *et al.*, 2002; Zheng *et al.*, 2002; Hwang *et al.*, 2003; Oshikawa *et al.*, 2003). More precisely, it seems to regulate the formation and activity of the SCF complexes (Skp, Cullin, F-box containing complex), which are essential in that process. CAND1 binds to the unneddylated CUL1 preventing the union of this protein to the other two components of the SCF complex, SKP1 and the F-box. The neddylation (rubylation in plants) of CUL1 release CAND1 and makes possible the subsequent steps of ubiquitination. Although this seems to be the principal task of the rubylation process, other non-cullin substrates have been recently identified (reviewed in Schwechheimer, 2018). The data obtained from pull-down experiments by Alonso-Peral *et al.* (2006) confirmed the interaction between CAND1 and the SCF complexes in *A. thaliana*. These authors also showed that the absence of this protein (*hve* mutant) has a pleiotropic effect that includes altered roots, branched inflorescences, and a simple vascular pattern in leaves. However, experiments to describe which are the meiotic problems that underlay the fertility defects showed by *hve* mutants have not been conducted yet. In this thesis, both *hve* 2x and *hve* 4x presented a significant reduction of the chiasma numbers compared to their respective WTs (Figures 39 and 41). Due to this defect in chiasma formation, the levels of univalents and trivalents showed by *hve* 4x were significantly higher than those of the WT (Figure 44). Alonso-Peral *et al.* (2006) observed that the phenotype displayed by *hve* and *axr1* are similar. AXR1 is a E1 enzyme of the rubylation complex that activates ubiquitination through the rubylation of CUL1. It has been observed in *A. thaliana* that the absence of this protein leads to variations in the distribution of class I COs, reduced chiasma levels, and defects in synapsis (Jahns *et al.*, 2014; Martinez-Garcia *et al.*, 2020).

In this study, the **chiasma frequencies** showed by ***hve***, both at the **diploid** and **tetraploid** levels, were **similar to** those obtained from ***mer3*** and ***mlh3*** (Figures 39 and 41). Besides, as it was observed in most of the mutants analyzed, the fold-change in chiasma frequency due to the WGD found in *hve* was significantly greater than the control (Figure 42). Thus, it is clear that the absence of CAND1 affects the formation of COs both in diploids and tetraploids. Moreover, the qPCR analyses conducted in *fancm* 2x, *fancm* 4x, and

their respective WT suggested a potential role of CAND1 in the regulation of the class II CO formation (see more above). However, the data obtained from the cytological analysis cannot reveal the actual problems behind the defects in chiasma formation. Due to the similar phenotype at the diploid and tetraploid levels compared to *mer3* and *mlh3*, it would be possible that, as it has been proposed for these two mutant backgrounds, the absence of CAND1 affects the class I CO numbers but is not completely essential for their formation (see section 5.3.2). Further experiments such as immunolocalization of class I CO markers, ZYP1 (central element of the SC), or double mutants of *hve* with mutants defective for class I (*zmm*) and class II COs (*mus81* or *fancd2*) would provide more information about the CO pathway or pathways in which CAND1 is involved and if the defects showed in the absence of this protein are similar to those displayed by *axr1*.

5.3.7. Polyploidy increases the capacity of *asy1* to form chiasmata

It has been reported in different organisms, including plants, that the proteins associated to the chromosome axis and the SC are not only essential for a correct synapsis of the homologous chromosomes but also they play a key role in HR (reviewed in Zickler and Kleckner, 2016). Mutations that produce defects in the chromosome axis or the SC, usually present dramatic effects on CO levels. Interestingly, in most of the organisms and in all of the plants studied, the relationship between HR and SC formation seems to be reciprocal. In absence of proteins that are essential for the HR, the formation of the SC is compromised and *vice versa*. Although in wide number of species, included *A. thaliana*, the ZMM proteins are not necessary for normal levels of synapsis, other proteins involved in HR such as SPO11, RAD51, DMC1, ASY1, ASY3, or PCH2 are essential (Couteau *et al.*, 1999; Grelon *et al.*, 2001; Armstrong *et al.*, 2002; Li *et al.*, 2004; Ferdous *et al.*, 2012; Lambing *et al.*, 2015). Thus, it is well stablished that synapsis is needed for normal levels of COs and HR is essential for synapsis (with exceptions in some species like *C. elegans* and *D. melanogaster*).

The protein ASY1, known as Hop1 in budding yeast, is the most extensively studied meiotic chromosome axis-related protein in plants. In *A. thaliana*, although this protein is associated to the axial elements throughout prophase I, it is not necessary for the formation of the meiotic chromosome axis (Pradillo *et al.*, 2007). However, in absence of this protein,

there are severe defects in synapsis and chiasma formation (Ross *et al.*, 1997). In addition, *asy1* shows problems in the stabilization of DMC1 on the chromosomes, which can explain its severe deficiency in interhomolog recombination (Sanchez-Moran *et al.*, 2007). It has been proposed that ASY1 is part of an interface between the meiotic axis and the chromatin, which coordinates the axis/SC morphogenesis and recombination promoting CO interference (Sanchez-Moran *et al.*, 2007; Lambing et al., 2020).

In this thesis, in line with previous studies, the diploid mutant ***asy1* 2x** displayed a marked **decrease in chiasma formation** compared to the **WT** (Figure 39). When ***asy1* 4x** was analyzed, **also defects** in chiasma formation were observed (Figure 41). Yet, when the **fold-change** in chiasma frequency (as a response to the WGD induction) was calculated, it was noticed that this **mutant background** (2.87) presented significantly **higher** fold-change than the **control** (1.96) (Figure 42). As well as in *zmm* mutants, the higher fold-change showed by *asy1* compared to the WT could suggest that in absence of ASY1, the formation of class I COs, but not class II, is affected. However, the immunolocalizations performed by Sanchez-Moran *et al.*, (2007) showed that some PMCs of *asy1* had MLH3 signal (class I CO marker) on the chromosomes. Therefore, another option is that the duplication of the number of homologous chromosomes could influence in general the probability of CO formation in this mutant background. In absence of ASY1, pairing and synapsis are severely compromised (Armstrong *et al*., 2001), and the interhomolog recombination events can only happen in subtelomeric regions (which form a transient bouquet prior to their pairing) (Roberts *et al.*, 2018; Lambing et al., 2020). It could be the case that in the tetraploid mutant, each homologous chromosome has three potential partners to interact with and this fact increases substantially the chances that the chromosomes must stabilize recombination intermediates during the transient bouquet which is essential to form COs. Then, the chiasma frequency boost showed by *asy1* 4x as a response to the WGD compared to the WT could be due to a general increase in the capacity to form COs.

However, in order to explain better the behavior observed by *asy1* 4x, it would be necessary to further characterize the pathway or pathways that are forming COs in absence of ASY1. The analysis of the double mutants *asy1*/*hei10* and *asy1*/*fancd2* or *asy1*/*mus81* could shed some light into this. In addition, immunolocalizations of interference-sensitive CO

markers (MLH1, MLH3 or HEI10) would also prove if the chiasmata observed in *asy1* 4x are formed through that pathway.

6. Conclusions

From the results obtained in this thesis, the following conclusions can be drawn:

1. Regarding the study of the changes in meiotic recombination that take place at different stages of the cytological diploidization process:

- There is no uniformity in the response to polyploidy of different accessions, having the genetic background an important role in the cytological diploidization process.

- Autotetraploid lines with similar chiasma frequencies show significant differences in terms of bivalent rates. Therefore, a clear correlation between the chiasma frequency and the different chromosome associations cannot be made, at least in *de novo* autotetraploids.

- In synthetic tetraploid lines, at least in the genetic backgrounds analyzed in this study, all the chromosomes show a tendency to have a more uniform contribution to the total chiasma frequency than in their diploid counterparts.

- All the autotetraploid lines used in this study display higher diploidization levels in the shortest and acrocentric chromosomes (2 and 4) than in the longest and submetacentric chromosomes (1, 3, and 5). Therefore, the size and/or the morphology of the chromosomes seem to be important in the process of diploidization.

- Santos *et al*. (2003) reported that a rapid cytological diploidization takes place in established autotetraploid lines during the first generations after the polyploidy induction. In this study, we have confirmed that there is a stabilization in the frequency of bivalents after the first generations and the diploidization process slows down. However, it must be considered that few generations have been studied and this situation could change by extending the study to more generations.

- The synthetic autotetraploid Bla-1 4x presents substantially higher bivalent numbers than any other synthetic, established, or natural lines analyzed in this study, included the geographically close natural autotetraploid Bla-5 4x.

- The duplication of the entire genome does not have a great impact on the transcription levels of the meiotic genes analyzed, whereas either the adaptation to polyploidy or the genetic background seem to have more impact on gene expression.

2. Regarding the study of the impact of intraspecific differences in the cytological diploidization process of autopolyploids:

- The autotetraploid intraspecific hybrid Col/L*er* 4x shows a significant excess of bivalent pairs respect to the autotetraploids obtained from its parental lines. It might be produced, at least partially, as a consequence of the heterozygosity.

- Although chromosome differentiation between related genomes may be the main cause of the excess of bivalents in the autotetraploid intraspecific hybrid Col/L*er* 4x hybrid, bivalent formation between identical chromosomes is not necessarily favored.

3. Regarding the analysis of the processes underlying the genetic control of diploidization in autopolyploids by inducing polyploidy in different key meiotic recombination mutants:

- Meiotic recombination mutants with similar mean cell chiasma frequencies at the diploid level, such as *zip4*, *msh5*, and *hei10*, show differences in this parameter when they are analyzed at the polyploid level.

- In both *mer3* and *mlh3*, the chiasma frequency observed in the diploid mutant exceeds the predicted number of interference-insensitive (class II) crossovers, and this situation

also occurs in the tetraploid mutants. It is possible that class I crossovers are not completely depleted in these mutants.

- The fold-changes in chiasma frequency displayed by the mutants *asy1* and *hve* as a response to genome duplication are greater than that of the wild-type. In these genetic backgrounds, the duplication of the number of homologous chromosomes seems to partially recover the meiotic recombination defects.

- The absence of MUS81 does not have a great impact on chiasma frequency regardless of the ploidy level.

- The hyper-recombinant phenotype produced by the absence of FANCM is not observed in a tetraploid background. Indeed, *fancm* 4x displays a significant number of univalents, although the number of class I crossovers seems to be the same as in the autotetraploid control. This result could compromise the potential application of this mutation in plant breeding programs designed for polyploid crops.

- The number of chiasmata is duplicated as a response to the duplication of the entire genome, whereas this duplication does not happen for class I crossovers. Polyploidy seems to modulate the proportion of class I/class II crossovers, being the number and proportion of class II crossovers much higher in polyploids than in diploids.

We have detected changes in the expression of some meiotic genes as a response to polyploidy. However, it would be necessary to extend the study to more mutants to confirm whether they are changes associated with genomic duplication or derive from a general deregulation resulting from problems in meiosis.

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8. Supplementary material

S1. ΔCt values and their standard deviations obtained by RT-qPCR from several meiotic recombination genes in flower bud samples of diploid and autotetraploid lines

	Col-0 2x		Col-0 4x		Bla-1 2x		Bla-5 4x		3151 4x	
	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D
SPO11-1	9.46	0.16	9.63	0.33	12.89	0.85	12.42	0.08	9.41	0.72
MRE11	3.51	0.20	3.71	0.31	3.21	0.58	3.89	0.39	3.40	0.10
RAD51	5.70	0.20	6.13	0.34	6.79	0.19	7.17	0.40	6.48	0.06
DMC1	5.07	0.20	5.47	0.22	6.15	0.24	5.34	0.50	4.87	0.08
MLH3	7.72	0.98	7.46	0.29	7.71	0.08	6.93	1.73	7.80	0.17
HEI10	3.95	0.34	3.75	0.22	4.20	0.09	1.70	0.55	5.89	0.29
MUS81	4.65	0.39	3.27	1.01	5.01	0.37	3.44	0.71	5.77	0.12
FANCM	6.55	0.48	6.04	0.26	6.83	0.36	6.68	0.78	6.76	0.10
RECQ4A	2.94	0.59	2.86	0.48	3.81	0.18	1.42	1.40	4.53	0.08
ZYP1a	7.18	0.05	5.35	0.14	5.77	0.25	5.01	0.30	5.94	0.25
ZYP1b	6.49	0.14	5.36	0.80	5.86	0.41	4.71	0.52	6.93	0.18
ASY1	5.47	0.27	5.11	1.10	6.40	0.53	5.55	0.88	6.97	0.41
ASY3	3.59	0.41	2.97	1.37	3.89	0.22	2.03	1.31	3.09	0.08
SYN1	12.14	0.32	10.75	0.12	13.89	0.74	12.42	0.50	10.38	0.28
SMC1	1.72	0.16	1.33	0.48	1.74	0.12	1.01	0.04	1.07	0.07
PDS5B	3.54	0.25	3.24	0.16	3.26	0.50	1.52	0.40	3.38	0.15
MRD1	8.08	2.65	6.22	1.00	6.45	1.48	3.86	0.81	12.48	0.12
CAND1	2.73	0.15	2.27	0.15	2.69	0.03	2.85	0.06	1.83	0.02

Criteria used to calculate the ΔCt explained in section 3.2.4.3
Standard deviation (Std. D)

S2. ΔCt values and their standard deviations obtained by RT-qPCR from several meiotic recombination genes in seedling samples of different diploid and autotetraploid lines

	Col-0 2x		Col-0 4x		Bla-1 2x		Bla-5 4x		3151 4x	
	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D
SPO11-1	10.86	0.11	10.03	0.34	10.07	0.42	13.66	0.74	—	—
RAD51	7.25	0.09	—	—	8.15	0.34	—	—	—	—
DMC1	7.34	0.12	—	—	10.67	0.10	—	—	—	—
HEI10	—	—	5.32	0.03	7.97	0.35	6.06	0.05	7.35	0.17
MUS81	—	—	6.96	0.42	—	—	—	—	7.23	0.17
RECQ4A	—	—	4.58	0.05	5.97	0.00	3.90	0.08	4.98	0.07
ZYP1a	9.81	0.04	9.30	0.49	—	—	—	—	—	—
ZYP1b	—	—	10.35	0.44	—	—	—	—	10.92	0.06
SYN1	13.80	0.52	14.07	0.11	16.82	2.53	15.13	0.66	—	—
PDS5B	—	—	4.62	0.06	9.79	0.45	4.46	0.08	—	—
MRD1	—	—	5.99	0.14	—	—	—	—	11.38	0.76

Criteria used to calculate the ΔCt explained in section 3.2.4.3
Standard deviation (Std. D)
Lines refer to measure not conducted

S3. ΔCt values and their standard deviations obtained by RT-qPCR from several meiotic recombination genes in flower bud samples of different diploid and autotetraploid mutant lines as well as their controls

	Col-0 2x		Col-0 4x		*hei10* 2x		*hei10* 4x		*fancm* 2x		*fancm* 4x	
	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D
SPO11-1	6.72	0.06	7.99	0.13	6.74	0.09	6.11	0.20	6.49	0.19	6.75	0.85
RAD51	5.15	0.29	6.40	0.34	5.46	0.19	5.21	0.30	5.15	0.32	5.73	0.15
DMC1	5.24	0.07	5.74	0.22	5.39	0.13	5.01	0.20	4.88	0.14	5.30	0.20
MLH3	7.03	0.56	7.73	0.29	6.94	0.37	6.93	0.16	6.89	0.89	7.21	0.53
HEI10	3.86	0.12	4.03	0.22	—	—	—	—	5.57	0.33	5.23	0.05
MUS81	4.92	0.12	3.55	1.01	4.76	0.27	4.56	0.13	5.39	0.16	4.82	0.30
FANCM	6.63	0.25	6.31	0.26	7.05	0.12	6.98	0.27	—	—	—	—
RECQ4A	2.82	0.06	3.13	0.48	3.32	0.06	3.38	0.27	3.23	0.09	3.54	0.03
ASY1	7.37	0.45	5.39	1.10	6.56	0.22	6.49	0.19	7.73	0.66	7.01	0.16
CAND1	3.13	0.11	2.08	0.08	2.17	0.07	2.31	0.04	2.08	0.12	2.20	0.03

Criteria used to calculate the ΔCt explained in section 3.2.4.3
Standard deviation (Std. D)
Lines refer to measure not conducted

S4. ΔCt values and their standard deviations obtained by RT-qPCR from several meiotic recombination genes in seedling samples of different diploid and autotetraploid mutant lines as well as their controls

	Col-0 2x		**Col-0 4x**		***hei10* 4x**		***fancm* 2x**		***fancm* 4x**	
	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D	ΔCt	Std. D
SPO11-1	—	—	8.37	0.16	8.22	0.56	—	—	—	—
RAD51	—	—	7.04	0.23	6.82	0.37	—	—	—	—
HEI10	5.70	0.24	5.32	0.03	—	—	6.93	0.26	7.90	0.52
CAND1	4.54	0.17	—	—	—	—	3.81	0.02	—	—

Criteria used to calculate the ΔCt explained in section 3.2.4.3
Standard deviation (Std. D)
Lines refer to measure not conducted

9. Publications

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Competition for Chiasma Formation Between Identical and Homologous (But Not Identical) Chromosomes in Synthetic Autotetraploids of *Arabidopsis thaliana*

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Polyploid organisms provide additional opportunities to study meiosis in a more complex context since more than two potential homologous chromosomes are available. When the chromosome complement of a diploid individual is duplicated, each chromosome is accompanied by one identical and two homologous chromosomes within the same nucleus. In this situation, a competition in pairing/synapsis/chiasma formation between identical and homologous (but not necessarily identical) chromosomes can occur. Several studies have been conducted in different species to address whether there are preferences in crossover formation between identical rather than homologous chromosomes. In this study, multivalent and chiasma frequencies were cytologically analyzed in synthetic autotetraploids of *Arabidopsis thaliana* including the accessions Col, L*er,* and the Col/L*er* hybrid. Fluorescence *in situ* hybridization was conducted to identify each chromosome at metaphase I. The new Col and L*er* tetraploids showed high multivalent frequencies, exceeding the theoretical 66.66% expected on a simple random end-pairing model, thus indicating that there are more than two autonomous synaptic sites per chromosome despite their small size. However, a significant excess of bivalent pairs was found in the Col/L*er* hybrid, mainly due to the contribution of chromosomes 2 and 3. The mean chiasma frequencies of the three artificial autotetraploids were about twofold the corresponding mean cell chiasma frequencies of their diploid counterparts. The relative contribution of each chromosome to the total chiasma frequency was similar in the three genotypes, with the exception of a lower contribution of chromosome 3 in the hybrid. Preferences for chiasma formation between identical and homologous chromosomes were analyzed in Col/L*er* 4x, taking advantage of the cytological differences between the accessions: variations in the size of the 45S rDNA region on the short arm of chromosome 2 and changes in the size and localization of the 5S rDNA region in chromosome 3. We observed a different behavior of chromosomes 2 and 3, i.e., random chiasma formation between identical and homologous chromosomes 2, and preferences for chiasma formation between homologous chromosomes 3. Hence, our results reveal the existence of chromosome-specific mechanisms responsible for these preferences.

Keywords: ***Arabidopsis thaliana,*** **autotetraploids, chiasma, homologous chromosomes, meiosis**

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INTRODUCTION

Meiosis is a specialized eukaryotic cell division which reduces the number of chromosomes in a parent diploid cell by half to produce haploid gametes. During meiosis, the correct segregation of homologous chromosomes at anaphase I is ensured by the combined action of sister chromatid cohesion and chiasma formation. In many species, chiasmata (the physical attachments between homologous chromosomes) are formed after the recognition of homologous chromosomes (pairing), the close association of paired chromosomes by the synaptonemal complex (SC), and the reciprocal exchange of sequences by the homologous recombination (HR) process.

Polyploids provide additional opportunities to study meiosis in a more complex context since more than two potential partners for these exchanges are available. Depending on their origin, they can show different meiotic behaviors (Sybenga, 1996). Polyploids resulting from the merging of two chromosomal sets from different species (allopolyploids) are expected to show disomic inheritance, with pairs of related chromosomes from the same parental forming preferentially bivalents (Le Comber et al., 2010; Lloyd and Bomblies, 2016). On the other hand, polyploids resulting from within-species duplication events (autopolyploids) generally show tetrasomic inheritance (random synapsis, recombination and segregation of all homologous chromosomes) as a consequence of an extensive multivalent formation (Soltis and Rieseberg, 1986; Wolf et al., 1989; Muthoni et al., 2015; Lloyd and Bomblies, 2016). In this landscape, synapsis and recombination preferences among the members of a tetrasome (set of four homologous chromosomes) can be responsible for cases that present an intermediate behavior between a disomic and a tetrasomic inheritance, and even for the diploidization process (Jannoo et al., 2004; Stift et al., 2008; Meirmans and Van Tienderen, 2013).

The degree of relationship of two chromosomes may be greater than mere homology. For example, when the chromosome complement of a diploid individual is duplicated, each tetrasome is formed by two pairs of completely identical chromosomes; i.e., each chromosome is accompanied by one identical and two homologous chromosomes within the same nucleus. In this landscape, a competition in pairing, synapsis, and recombination between identical and homologous (but not necessarily identical) chromosomes can occur. Attempts to address this issue have been performed mainly in plants since it is very easy to obtain autotetraploids by a colchicine treatment, with only a few examples in animals. Possible preferences between chromosomes of a tetrasome were inferred from analyses to determine the segregation of genetic and/or chromosomal markers (Giraldez and Santos, 1981; Santos et al., 1983; Benavente and Orellana, 1991; Curole and Hedgecock, 2005). The most exhaustive cytological studies were conducted on rye, taking advantage of the existence of C-bands polymorphisms, especially in the nucleolar organizing region (NOR)-bearing chromosome 1R (Orellana and Santos, 1985; Benavente and Orellana, 1989, 1991; Benavente and Sybenga, 2004). In general, in this species there is a trend to identical over homologous preferential associations at metaphase I. This tendency is greater in hybrids with higher chromosomal divergence between the parental diploid plants. This fact indicates chromosome differentiation may play a relevant role in the establishment of such preferences (Benavente and Orellana, 1991; Jenkins and Chatterjee, 1994). These preferences could contribute to the diploidization process of autopolyploids.

In this study, we have analyzed chromosome configurations at metaphase I in autotetraploid meiocytes from the plant model species *Arabidopsis thaliana*. Tetraploid plants were obtained by applying a colchicine treatment to hybrid diploid plants from the cross between Col-0 and L*er*-1 accessions (Col and L*er* onward). We have used the 45S and 5S rDNA sequences as cytological markers. These sequences show quantitative and qualitative variations in chromosomes 2 and 3 of these accessions (Sanchez-Moran et al., 2002). In *Arabidopsis*, the initiation and progression of meiotic recombination is required to establish the SC-mediated pairwise association between homologous chromosomes (Grelon et al., 2001). Therefore, we consider more appropriate the use of the term "chiasma formation preferences" instead of "pairing preferences" throughout this paper. This clarification is necessary because in the mid-20th century and first decade of the current century, in most of the traditional literature on plant cytogenetics, the term chromosome pairing was used as the equivalent of chromosome associations mediated by chiasmata at metaphase I.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Diploid plants of Columbia (Col-0) and Landsberg *erecta* (L*er*-1) accessions ($2n = 2x = 10$), and also Col-0/L*er*-1 hybrid plants were treated with colchicine in order to obtain the corresponding autotetraploids ($2n = 4x = 20$) (Santos et al., 2003). This treatment consists in applying a 10 μL drop of colchicine at a 0.25% w/v concentration on the center of the plant rosette prior to the first flowering. Seeds from these plants were sown on a mixture of 3 parts of soil and 1 part of vermiculite and grown under constant conditions of 16h day-length, 70% relative humidity and 19°C.

Cytological Analyses

Fixation of flower buds, slide preparations of pollen mother cells (PMCs), and fluorescence *in situ* hybridization (FISH) were conducted according to Sanchez-Moran et al. (2001), with minor modifications due to the polyploid samples. The DNA probes used comprise ribosomal DNA 45S and 5S loci (Gerlach and Bedbrook, 1979; Campell et al., 1992). The existence of changes in the localization of the 5S rDNA locus belonging to chromosome 3 (Fransz et al., 1998; Sanchez-Moran et al., 2002), and variations in the size of the 45S rDNA locus located at chromosome 2 (this work) made possible the differentiation of the parental origin of these chromosomes in the diploid and tetraploid hybrid plants analyzed (**Figure 1**). Images were captured using an Olympus BX-60 microscope with an Olympus DP71 camera and processed with Adobe Photoshop CS5 software.

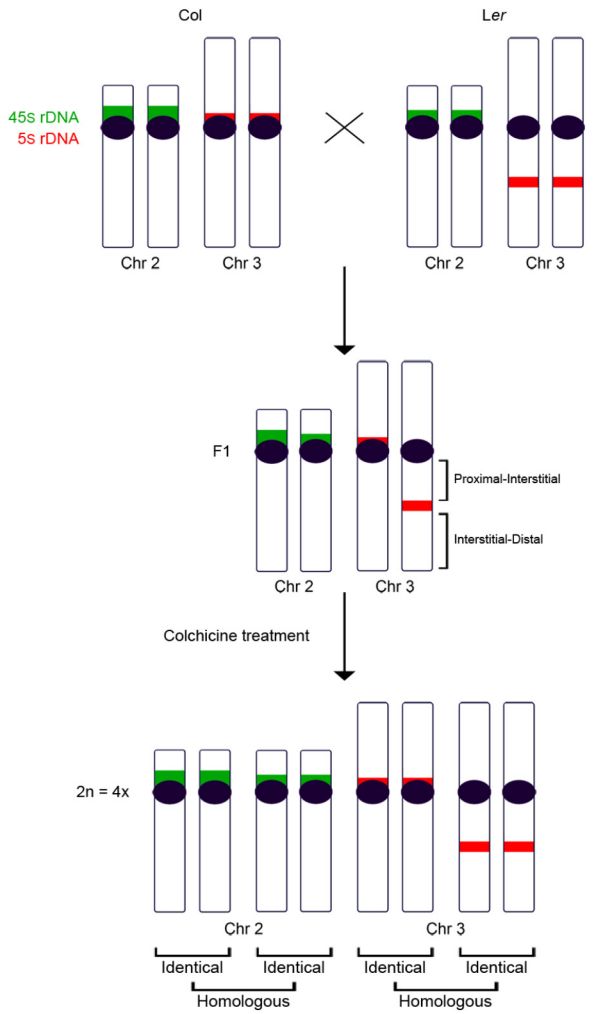

FIGURE 1 | Ideogram of chromosomes 2 and 3 in Col, L*er* and in the hybrid progeny obtained from the cross between both accessions before and after the chromosome duplication. Col and L*er* chromosomes 2 and 3 can be distinguished after applying FISH with 5S (red) and 45S (green) rDNA probes. These differences allow the identification of the parental origin of each chromosome in the hybrid, before and after the colchicine treatment. In the tetraploid hybrid, each chromosome is accompanied by one identical chromosome and two homologous chromosomes.

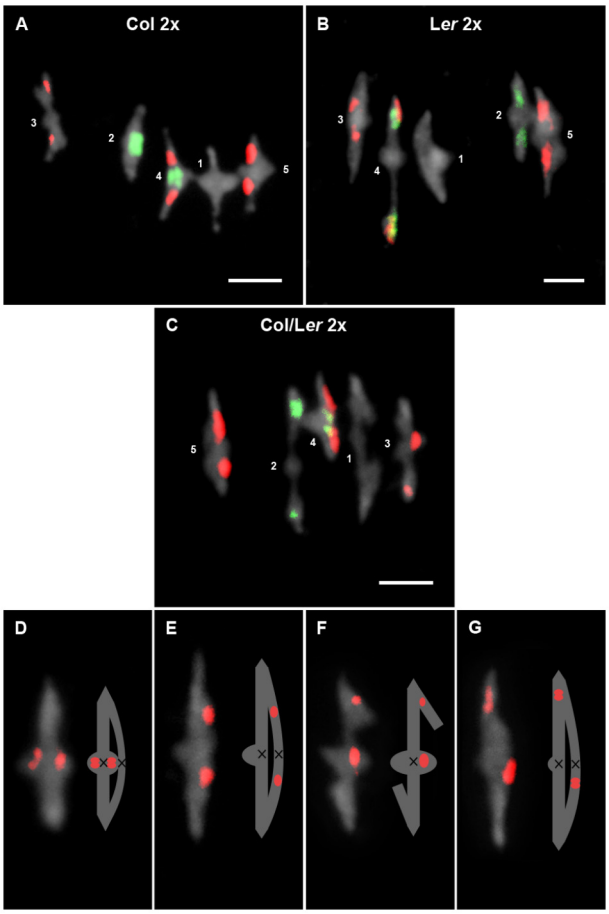

FIGURE 2 | Cytological analysis of diploid cells at metaphase I. Representative examples of metaphases I from Col 2x **(A)**, L*er* 2x **(B)**, and Col/L*er* 2x **(C)**. Chromosomes are identified by FISH with 5S (red) and 45S rDNA (green) probes. Images and representations of bivalents formed by chromosomes 3 in which the chiasma (black cross) has been formed before (**D,F**) or after (**E,G**) the position of the 5S rDNA in a L*er* (**D,E**) and in a Col/L*er* (**F,G**) background. Bars represent 5 μm.

RESULTS

Chiasma Analyses in Diploid Plants of Col, L*er* and Col/L*er* Hybrids

Chromosome morphology together with 45S (NOR) and 5S rDNA FISH probes allow the identification of the whole complement set of *Arabidopsis* in some accessions (Fransz et al., 1998; Sanchez-Moran et al., 2002). Chromosomes 1, 3 and 5 are submetacentric/metacentric, while chromosomes 2 and 4 are acrocentric. Chromosomes 1 do not possess any rRNA genes. Chromosomes 2 are characterized by the presence of 45S rDNA sequences distally on their short arms. Chromosomes 3 and 5 bear 5S rRNA genes and chromosomes 4 have both 45S and 5S rDNA sequences also located on the short arm. Col and L*er* are distinguished because 5S rRNA genes are located on a different arm at chromosome 3 (short arm in Col and long arm in L*er*). In this study, we have also detected another difference between both accessions. The NOR region belonging to chromosome 2 is bigger in Col than in L*er* (**Figures 1**, **2A–C**).

Chiasma scoring was conducted in PMCs at metaphase I. Three plants were analyzed in each accession and also in the hybrid progeny obtained from the cross between both accessions. Since there were no significant differences in the mean chiasma frequencies per cell among them, individual plant data were grouped. In all of the cells assessed in this study, the five chromosome pairs invariably formed five bivalents that could be classified into two categories: rod and ring. A rod (open) bivalent has a single chiasma, whereas in a ring (close) bivalent both chromosome arms are bound by chiasmata.

TABLE 1 | Chiasma frequencies observed for the different chromosomes (1–5) in PMCs from Col, L*er,* and Col/L*er* diploid plants.

	Chromosomes						
	1	2	3	4	5	$\bar{X}$	*N*
Col 2x	2.55 (25.0)	1.73 (17.0)	2.16 (21.2)	1.50 (14.7)	2.28 (22.4)	10.20	70
L*er* 2x	2.09 (22.9)	1.74 (19.1)	1.79 (19.6)	1.61 (17.6)	1.90 (20.8)	9.13	158
Col/L*er* 2x	2.14 (22.6)	1.76 (18.6)	1.85 (19.5)	1.70 (17.9)	2.04 (21.5)	9.48	120

Values showed in parentheses represent the contribution in percentage of each chromosome to the total mean chiasma frequency ($\bar{X}$). Number of cells analyzed (N).

TABLE 2 | Multivalents (M), bivalent pairs (II) and chiasma frequency (Xta) observed for the different chromosomes (1–5) in PMCs from Col, L*er,* and Col/L*er* autotetraploid plants.

		Chromosomes							
		1	2	3	4	5	Total	$\bar{X}$	*N*
Col 4x	M	153 (82.3)	129 (69.4)	150 (80.6)	122 (65.9)	151 (81.2)	705 (75.9)	3.79	186
	II	33 (17.7)	57 (30.6)	36 (19.4)	63 (34.1)	35 (18.8)	224 (24.1)	1.20	
	Xta	4.19 (21.0)	4.02 (20.1)	3.91 (19.6)	3.82 (19.1)	4.05 (20.2)		19.99	
L*er* 4x	M	40 (80)	31 (64.6)	36 (73.5)	33 (70.2)	39 (73.6)	179 (72.5)	3.58	53
	II	10 (20)	17 (35.4)	13 (26.5)	14 (29.8)	14 (26.4)	68 (27.5)	1.36	
	Xta	4.00 (21.6)	3.60 (19.5)	3.64 (19.7)	3.54 (19.1)	3.72 (20.1)		18.50	
Col/L*er* 4x	M	97 (69.8)	70 (51.5)	70 (50.7)	89 (64)	99 (71.2)	425 (61.5)	3.06	139
	II	42 (30.2)	66 (48.5)	68 (49.3)	50 (36)	40 (28.8)	266 (38.5)	1.91	
	Xta	4.07 (21.4)	3.78 (19.9)	3.60 (18.9)	3.59 (18.9)	3.99 (20.9)		19.03	

Values showed in parentheses represent the percentage of multivalents and pairs of bivalents to the total cells (N), and the contribution of each chromosome to the total mean chiasma frequency ($\bar{X}$). Number of cells analyzed (N).

The mean chiasma frequencies per bivalent and per cell are summarized in **Table 1**. Col showed a significantly higher mean chiasma frequency per cell (10.20 ± 0.14) than L*er* (9.13 ± 0.10; $t = 6.2$, $p < 0.001$). The value for the Col/L*er* hybrid was intermediate to the previous ones (9.48 ± 0.11; $n = 120$), and it was statistically significant respect to both Col ($t = 4.14$, $p < 0.001$) and L*er* ($t = 2.39$, $p < 0.05$). In all the backgrounds analyzed, individual bivalent chiasma frequencies changed according to the chromosome size (the chromosome 1 had the highest mean chiasma frequency while the short acrocentric chromosomes, 2 and 4, presented the lowest frequencies).

In L*er*, the interstitial 5S rDNA region on chromosome 3 divides the long arm of this chromosome in two regions: a proximal region between the centromere and the 5S rRNA genes, and a distal region from these genes to the telomere. This feature has allowed a more accurate analysis of chiasma distribution on this arm not only in this accession but also in the Col/L*er* hybrid (**Figures 2D–G**). In both backgrounds, about 50% of chiasmata were located in each region (L*er*: $\chi^2_1 = 1.58$, $p > 0.05$; Col/L*er*: $\chi^2_1 = 1.09$, $p > 0.05$). Therefore, chiasma localization on this chromosome arm do not change in the hybrid.

Multivalent and Chiasma Analyses in Autotetraploid Plants of Col, L*er* and Col/L*er* Hybrids

Frequencies for the different configurations observed at metaphase I were recorded for each chromosome in three plants of each genotype (**Table 2**). Data from plants sharing the same background were grouped since there were no significant differences in multivalent and chiasma frequencies among them. Chromosomes were predominantly associated as bivalents, quadrivalents, and trivalent + univalent (**Figures 3A–C**). Since the frequency of the latter was very low (16/186 = 9% in Col 4x; 8/50 = 16% in L*er*; 11/139 = 8% in Col/L*er* 4x), no distinction was made between quadrivalents and trivalents and they were simply grouped as multivalents (**Table 2**).

Synaptic configurations in autotetraploids with metacentric chromosomes have usually been estimated under the following premises (for review see Sybenga, 1975): (i) the presence of two independent synapsis initiation points per chromosome, one at each end; (ii) the absence of synapsis preferences; (iii) the existence of same probabilities for chiasma formation in all meiotic configurations; and (iv) the possibility of free partner switches between the two synapsis initiation points at each chromosome. In this context, there are nine possible configurations to be formed among each group of homologous chromosomes (tetrasome), of which six are quadrivalents (2/3) and the remaining three are pairs of bivalents (1/3), i.e., the ratio of multivalents to bivalent pairs at prophase I would be 2:1.

The observed ratios of multivalents to bivalent pairs were tested for the agreement with the theoretical 2:1 ratio for each chromosome in the three autotetraploid genotypes analyzed (**Tables 2**, **3**). The level of multivalent formation over the five chromosomes (705 multivalents: 224 bivalent pairs) displayed by Col 4x significantly excess the 2:1 ratio (66.66% multivalents) [$\chi^2_{(1)} = 35.55$; $p < 0.001$]. In this accession, at the chromosomal level, only the three largest chromosomes of the complement (1, 3 and 5) showed multivalent frequencies consistently in excess of the 66.66%. L*er* 4x also presented an excess of multivalents (72.5%), but with a value that is at the limit of the significance level [$\chi^2_{(1)} = 3.74$; $p = 0.053$]. In this case, only chromosome 1 showed a significant excess of multivalents, while the other four chromosomes fitted to the random theoretical expectations. Conversely, there was a significant excess of bivalent pairs in the Col/L*er* 4x hybrid (38.5%) [$\chi^2_{(1)} = 8.28$; $p < 0.01$], mainly due to the behavior of chromosomes 2 and 3 (**Table 3**).

Col 4x showed the highest mean cell chiasma frequency (19.99 ± 0.11) followed by the hybrid Col/L*er* 4x (19.03 ± 0.15). L*er* 4x presented the lowest frequency (18.5 ± 0.27) (**Table 2**). These means are about twofold the corresponding mean cell

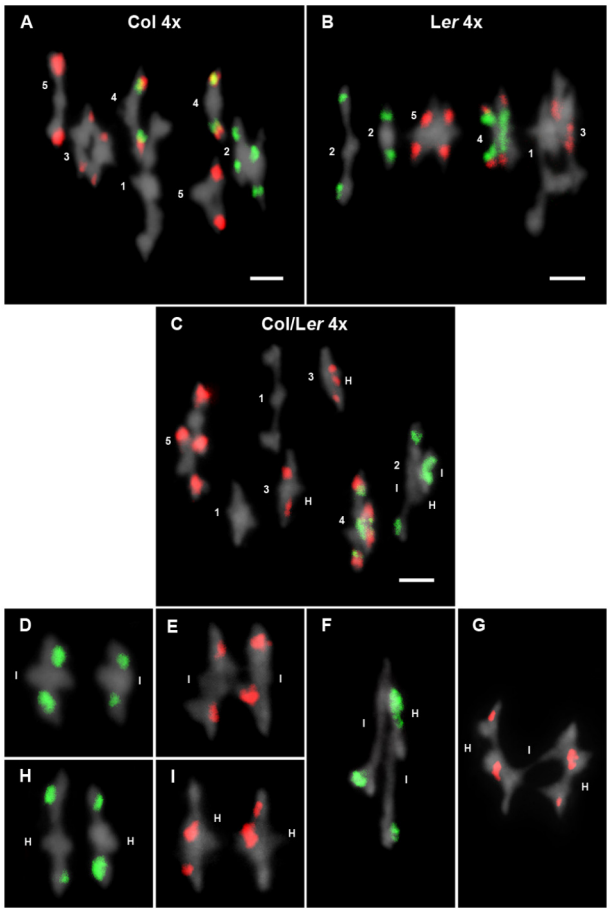

FIGURE 3 | Cytological analysis of autotetraploid cells at metaphase I. Representative examples of metaphases I from Col 4x **(A)**, L*er* 4x **(B)**, and Col/L*er* 4x **(C)**. Chromosomes are identified by FISH with 5S (red) and 45S rDNA (green) probes. In Col 4x, tetrasomes 1, 2, and 3 appear as quadrivalents, whereas two bivalents are formed in tetrasomes of chromosomes 4 and 5. In L*er* 4x, only the tetrasome of chromosomes 2 appears as two bivalents. In the hybrid, two bivalents are formed in tetrasomes of chromosomes 1 and 3, whereas the remaining tetrasomes appear as quadrivalents. The figure also includes examples of associations between identical chromosomes in bivalents formed by chromosomes 2 **(D)** and chromosomes 3 **(E)**. Examples of associations between homologous chromosomes in bivalents formed by chromosomes 2 **(H)** and chromosomes 3 **(I)**. Examples of quadrivalents formed by chromosomes 2 **(F)** and 3 **(G)**. Capital letters I and H represent identical and homologous association, respectively. Bars represent 5 μm.

chiasma frequencies of the diploid counterparts. There were significant differences between the means of Col 4x and L*er* 4x ($t = 5.19$, $p < 0.001$), and also between Col 4x and the hybrid 4x ($t = 3.24$, $p < 0.001$), but not between L*er* 4x and the hybrid 4x ($t = 1.19$, $p = 0.08$). The relative contribution of each chromosome to the total mean cell chiasma frequency was similar in these three backgrounds, with the exception of a slightly lower contribution of chromosome 3 in the hybrid (**Table 2**). In addition, chiasma localization was analyzed in the long arm of chromosome 3 in L*er* 4x and also in the hybrid 4x, but only in cells in which chromosomes 3 did not form a multivalent. As well as in diploid cells, about 50% of chiasmata were located in the proximal region (centromere – 5S rDNA) and the remaining 50% in the distal region (5S rDNA – telomere).

Competition for Chiasma Formation Between Identical and Homologous Chromosomes in the Hybrid Col/L*er* 4x

One of the main objectives of this study was to analyze whether chromosome intraspecific differences in autotetraploid plants are enough to determine preferences in terms of chiasma formation. Taking into account the cytological differences between Col and L*er* accessions, namely: variations in the size of the NOR region located on the short arm of chromosome 2 and changes in the localization of the 5S rDNA at chromosome 3, Col/L*er* diploid hybrids were treated with colchicine to obtain autotetraploid plants. In these hybrids, there is one pair of identical chromosomes from Col and another identical pair from L*er*. These two pairs of identical chromosomes are homologous, but non-identical with each other (**Figure 1**). Then, two types of two-by-two metaphase I associations are possible for any chromosome arm: between identical chromosomes or between homologous chromosomes (**Figures 3D–I**). Assuming that chiasma formation takes place randomly among the four members of each tetrasome, homologous associations will be twice as common as identical ones.

Following the criteria established by Benavente and Orellana (1991), in this analysis we have included cells with at least one chiasma between identical or homologous chromosomes (regardless of the chromosome configuration adopted by the corresponding tetrasome) to test randomness (**Figures 3D–I** and **Table 4**). When data from multivalents and bivalent pairs were grouped, we detected a different behavior of chromosomes 2 and 3. We observed random chiasma formation between identical and homologous arms of chromosome 2, and homologous preferences for chiasma formation in both arms of chromosome 3. This tendency was also maintained when only data from bivalent pairs were considered, although in this case the excess of chiasmata between homologous short arms of chromosome 3 was at the limit of the significance level.

DISCUSSION

Multivalent and Chiasma Frequencies at Metaphase I

The frequencies of multivalents observed in Col 4x plants significantly exceed the 2:1 ratio (66.66% multivalents) expected on the random-end pairing model (**Table 3**), which means that, despite their small size, at least in this accession chromosomes have more than two autonomous synaptic initiation sites (López et al., 2008), and more than one synaptic partner switch per tetrasome. In addition, there was a significant excess of bivalent pairs in the autotetraploid Col/L*er* hybrid (**Table 2**; $\chi^2_1 = 8.30$, $p < 0.01$). It might be produced, at least partially, as a consequence of the heterozygosity.

TABLE 3 | Chi-square test values (χ_1^2) testing goodness of fit to 2:1 ratio of multivalents: bivalent pairs for the different chromosomes (1–5) in PMCs from Col, L*er* and Col/L*er* autotetraploid plants.

	Chromosomes					
	1	**2**	**3**	**4**	**5**	**Total**
Col 4x	> 20.35***	= 0.60^{NS}	> 16.35***	= 0.04^{NS}	> 17.64***	> 35.55***
L*er* 4x	> 4.00*	= 0.09^{NS}	= 1.02^{NS}	= 0.64^{NS}	= 0.64^{NS}	= 3.74^{NS}
Col/L*er* 4x	= 0.61^{NS}	< 14.13***	< 15.78***	= 0.44^{NS}	= 1.30^{NS}	< 8.28**

*Less than and greater than symbols indicate direction of deviation: < (< 2:1), > (> 2:1). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; NS, not significant.*

TABLE 4 | Number of Col/L*er* 4x chromosome configurations with at least one chiasma between identical (I) or homologous (H) chromosomes in bivalent pairs (II) and multivalents + bivalent pairs (M+II) and the goodness of fit to the expected ratio 1I:2H.

	Chromosome 2						Chromosome 3					
	Short arm			Long arm			Short arm			Long arm		
	I	**H**	χ_1^2	**I**	**H**	χ_1^2	**I**	**H**	χ_1^2	**I**	**H**	χ_1^2
M+II	36	63	0.41	34	67	0.01	21	78 ↑	6.58*	20	85 ↑	9.64*
II	24	42	0.27	22	44	0.00	15	51	3.34	14	54 ↑	4.97*

*Chi-square test values obtained (χ_1^2). Values that exceed the 1:2 (I:H) ratio expected at random (↑). Chi-square test values statistically significant, *p ≤ 0.05.*

Col and L*er* diverged ∼200,000 years ago (Koch et al., 2000). Around 16,000 single feature polymorphisms between Col and *Ler* accessions were detected in ∼8,000 of the ∼26,000 genes represented in a 44,000 feature exon-specific oligonucleotide array (Singer et al., 2006). Furthermore, more than 6,000 insertions or deletions distinguish both accessions, which differ in 564 transpositions and 47 inversions that comprise around 3.6 Mb (Ziolkowski et al., 2009; Zapata et al., 2016). Increases in bivalent frequency are strongly chromosome dependent and are generally ascribed to overall decreases in chiasma frequency and/or changes in chiasma distribution, with a more rapid response of the shortest chromosomes to these alterations. The behavior of chromosome 3 in L*er* can shed some light to this issue since it carries a 170 kb inversion on the short arm (Zapata et al., 2016). Hence, Col/L*er* hybrid is heterozygous for such inversion, and it is well known that the heterozygosity for inversions suppresses meiotic recombination.

The mean cell chiasma frequencies of chromosome 3 in L*er* 4x and Col/L*er* 4x are similar (3.64 vs. 3.60; $t = 0.31$, $p = 0.76$), but there are significant differences between the means of bivalent pairs (0.26 vs. 0.49; $t = 3.03$, $p < 0.001$) (**Table 2**). Hence, other factors in addition to chromosome rearrangements, such as genotypic, epigenetic or cryptic structural differences along chromosomes, may be involved in the increase of bivalent frequency observed not only in this hybrid but also, for instance, in established autotetraploid lines of *Arabidopsis* (Santos et al., 2003). On these grounds, Zhang et al. (2015) reported that, after 49 self-pollinated generations, autotetraploid rice showed a significant increase in the methylation of class II transposons in relation to its diploid donor that may affect gene expression. Also, Dar et al. (2017) observed differences between the frequency of both quadrivalents and bivalents from C_0 to C_2 synthetic autotetraploids of *Phlox drummondii,* associated with changes in both repetitive and non-repetitive regions.

Polyploidy is a major process in plant speciation. The potential evolutionary success of polyploids has been linked, among other hypotheses, to the buffering of mutations and sub- and neo-functionalization of duplicated genes (see for reviews, Otto, 2007; Soltis and Soltis, 2009; Parisod et al., 2010; Zielinski and Mittelsten Scheid, 2012). It has been reported that polyploids of *Gossypium* and *Arabidopsis* enhance meiotic recombination compared with diploids (Desai et al., 2006; Pecinka et al., 2011). Increases in chiasma frequency could help to the establishment of new polyploid species by rapid creation of genetic diversity when population sizes are small. The data reported in the present work unfit to this proposal since the autotetraploids showed chiasma frequencies about twofold in comparison with their diploid counterparts (**Tables 1**, **2**). However, the possibility to obtain an increase in recombination in certain chromosome regions cannot be ruled out. It would be interesting to test this hypothesis by examining chiasma frequencies not only in other *Arabidopsis* accessions but also in other non-related species.

Competition for Chiasma Formation Among Identical and Homologous Chromosomes

Benavente and Orellana (1991) analyzed preferences for chiasma formation in synthetic autotetraploids of *Secale cereale* obtained from heterozygous hybrids for telomeric C-bands at chromosome 1R. They found a clear tendency for preferences between identical partners in inter-subspecific hybrids. This tendency increased in inter-specific hybrids with a higher chromosomal divergence between homologous chromosomes. These results reflect the potential effect of chromosomal differentiation on chiasma preferences in polyploids (see also Jenkins and Chatterjee, 1994). However, the hybrids resulting from crosses between inbred lines showed a wide range of

preferential associations. Therefore, chiasmata between identical partners are not always favored.

In this study, we have observed that although chromosomes 2 and 3 exhibited similar frequencies of bivalent pairs (0.47 and 0.49, respectively) and chiasmata (3.78 and 3.60, respectively) (**Table 2**), they presented different preferences in chiasma formation in the hybrid Col/L*er* 4x. Chiasmata were randomly formed between identical and homologous chromosomes 2, but preferentially established between homologous chromosomes 3 (**Table 4**). These results indicate that although chromosome differentiation between related genomes may be the main cause of the excess of bivalents in the hybrid, bivalent formation between identical chromosomes is not necessarily favored (Sybenga, 1992, 1994; Bourke et al., 2015). In this regard, random chiasma formation among identical and homologous chromosomes 2 could be related to their close spatial nuclear location as a consequence of bearing the NOR region on the short arm, since differences in the number of 45S tandem repeats (Rabanal et al., 2017) do not seem to have an influence. On the other hand, preferences for chiasma formation between homologous chromosomes 3 could be more related to specific features of particular chromosome regions. Actually, in L*er* the 5S rDNA region on the long arm of this chromosome is close (∼6 Mb) to the 170 kb inversion mentioned before (Simon et al., 2018). This means that the genomes of the two accessions are different in a large region, which would have important consequences for meiotic recombination. However, recent meiotic recombination analysis suggests that high levels of sequence divergence are not necessarily inhibitors of meiotic recombination (Barth et al., 2001; Singer et al., 2006; Salomé et al., 2012). This idea is in agreement with a positive correlation of ancestral recombination frequencies and regions with high sequence divergence (Kim et al., 2007). In addition, heterozygous regions increase chiasma formation when are juxtaposed with homozygous regions, which reciprocally decrease (Ziolkowski et al., 2015). In relation to chromosome 3, Barth et al. (2001) found a strong negative correlation between genetic similarities of ecotypes and recombination frequencies for two adjacent markers located on the long arm of this chromosome, but not for other genomic regions. In general, there are difficulties in mapping and sequencing this chromosome, consequently this fact suggests the existence of unusual chromatin-related features respect to the other chromosomes of the complement (Schmidt, 2018).

Taking into account the information compiled in this work, it is evident that when the chromosome complement of a diploid individual is duplicated, the degree of relationship between two chromosomes within each tetrasome may be greater than mere homology. In this situation, there is a competition for chiasma formation between identical and homologous chromosomes that can be resolved through different ways depending on the chromosome. Accordingly, identical and homologous chromosome regions will persist in each tetrasome in a differential pattern throughout generations. This chromosomal genetic variation has not be considered in current models about tetrasomic and disomic inheritance and it could produce a relevant impact on haplotypes.

AUTHOR CONTRIBUTIONS

PP-N completed the experiments and performed the data analyses. All authors conceived and designed the experiments, wrote and reviewed the manuscript.

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Chapter 3

How to Perform an Accurate Analysis of Metaphase I Chromosome Configurations in Autopolyploids of *Arabidopsis thaliana*

Pablo Parra-Nunez, Mónica Pradillo, and Juan Luis Santos

Abstract

During meiosis, accurate segregation of chromosomes requires the formation of bivalents at metaphase I. In autopolyploids, there are more than two copies of each chromosome with the same chance to form chiasmata at meiosis. This leads to the formation of multivalent configurations in which chiasma quantification is rather complicated. Here, we present an improved cytological protocol, including fluorescence in situ hybridization, to obtain high quality spreads of metaphase I chromosomes from *Arabidopsis thaliana* autotetraploids. This method allows an accurate analysis of the different meiotic configurations and enables the assessment of the number of chiasmata formed by each tetrasome (group of four homologs).

Key words Arabidopsis, Chiasma, FISH, Meiosis, Polyploidy

1 Introduction

Polyploidy is a key feature in the evolution of eukaryotes, especially in plants. Estimates suggest that between 47% and 70% of flowering plants have polyploid ancestors [1]. Polyploidy can confer several advantages such as drought and salinity tolerance, but the presence of more than two sets of chromosomes can also generate unbalanced gametes [2–4]. During polyploid meiosis, each chromosome has more than one potential partner to pair, recombine, and synapse. This can result in the presence of multivalents, homologous chromosome missegregations and reduced fertility [5]. Hence, the analysis of meiotic chromosome behavior in polyploids has a considerable practical value in terms of understanding the undesirable effects of chromosome duplication on fertility. To date, fluorescence in situ hybridization (FISH) is probably the best tool to perform this analysis. This technique was previously applied to study the partial diploidization of meiosis in successive generations of different autotetraploid lines of *Arabidopsis thaliana* [6].

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As early as 1907, Laibach [7] determined the correct chromosome number of *A. thaliana* as $2n = 10$. He found 10 chromocenters in interphase nuclei of somatic cells, corresponding to the number of chromosomes observed during meiosis. However, detailed cytological studies on chromosome morphology were hampered during most of the twentieth century. This was due to the small size of the chromosomes and the difficulty of getting good spreads that allow accurate light microscopic observations of both mitotic and meiotic cells [8]. This situation changed drastically with the improvement of cytological techniques, including the application of FISH [9, 10]. 45S and 5S rDNA FISH probes, combined with chromosome morphology, uniquely identify each of the Arabidopsis chromosomes and chromosome arms. The short acrocentric chromosomes 2 and 4 bear 45S rDNA sequences on their short arms in all accessions that have been examined to date, coinciding with the locations of the nucleolus organizing regions (NORs). In addition, a 5S rDNA site on the short arm of chromosome 4 is always present. This sequence helps to distinguish the two acrocentric chromosomes. A further invariant large 5S rDNA site occurs on the shorter arm of the submetacentric chromosome 5, which serves to distinguish this chromosome from chromosome 1. Chromosome 3 is the smallest of the submetacentric/metacentric group of chromosomes (1, 3, and 5) and is variable with respect to possession, location, and size of a third 5S rDNA site. Application of FISH with these DNA probes allows to identify and record the chiasma frequency of each chromosome and chromosome arm in pollen mother cells (PMCs) at metaphase I [11, 12].

In *Arabidopsis*, there are natural autopolyploid accessions as well as synthetic autotetraploid plants obtained after colchicine treatment [13–15]. In addition, polyploidy may be induced during tissue culture or may even occur spontaneously in certain accessions [16, 17]. Bivalent configurations at metaphase I can fall into two categories, rods and rings. Rods are bound by chiasmata only in one arm, whereas rings have both arms bound by chiasmata (Fig. 1a). However, as mentioned above, this situation turns out to be more complicated in autopolyploids due to the presence of multivalents [18]. Here, we describe an improved cytological protocol to obtain proper spreads of metaphase I chromosomes in autotetraploid PMCs (*see* Fig. 1b as a representative example of a conventional protocol, and Fig. 1c, d as examples of improved chromosome spreads). This protocol allows an accurate analysis of the different meiotic configurations and, with the required expertise, the assessment of the number of chiasmata formed in each tetrasome (group of four homologs). We also provide several examples of the different quadrivalent configurations that can be observed with the number of chiasmata necessary for their formation.

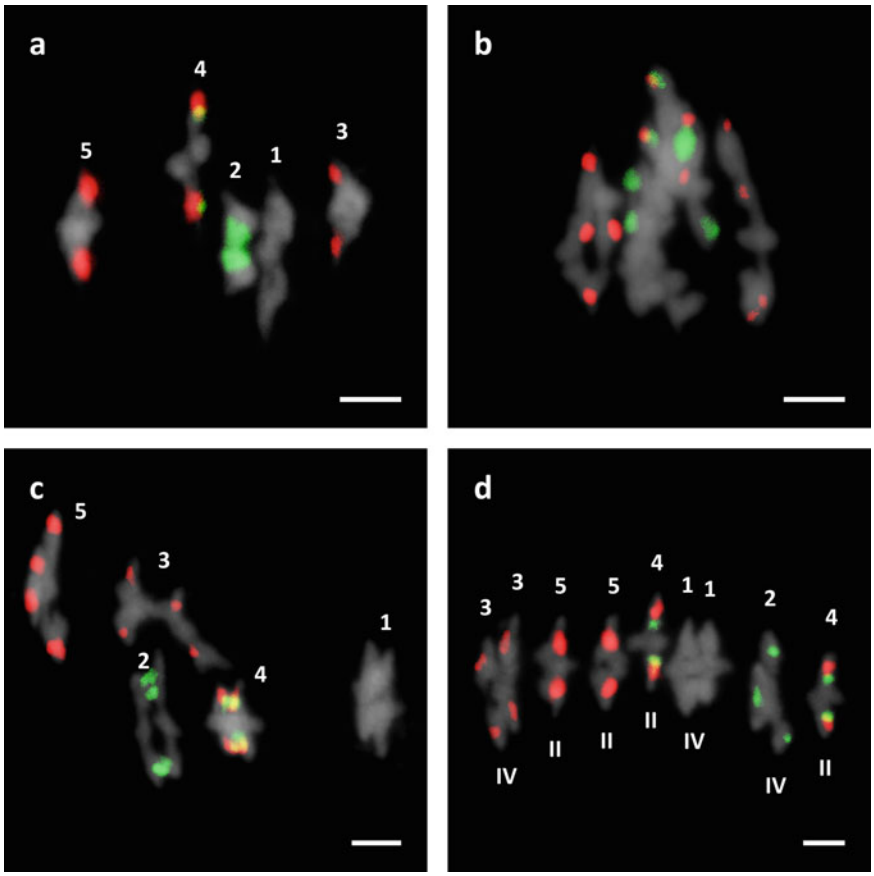

Fig. 1 Representative examples of FISH analysis of metaphase I cells. Arabic numerals (1–5) identify the different chromosomes according to their morphology and distribution of rDNA signals. Green signals identify 45S rDNA loci and red signals identify 5S rDNA loci. (**a**) Diploid cell. Individual bivalents are indicated. This cell has three ring bivalents (1, 3, and 5) and two rod bivalents (2 and 4). (**b**) Tetraploid cell obtained with the same protocol applied in (**a**). (**c**, **d**) Tetraploid cells obtained with the protocol described in this chapter. Five quadrivalents are observed in (**c**), whereas in (**d**) there are three quadrivalents (IV) and four bivalents (II). Bars represent 5 μm

2 Materials

2.1 Plants

Sow Arabidopsis Columbia-0 accession seeds in sterilized substrate with commercial soil and vermiculite (3:1), and grow plants until flowering in a constant environment chamber under the following conditions: 19 °C; photoperiod of 16 h light–8 h dark.

2.2 Colchicine

Prepare a colchicine solution of 0.25% w/v by diluting the colchicine stock with sterilized deionized water (SDW). Store solution at 4 °C.

2.3 Reagents and Equipment Required for Chromosome Spreads

1. Carnoy's fixative solution: 6 volumes of absolute ethanol, 3 volumes of chloroform, and 1 volume of glacial acetic acid.
2. 3:1 fixative solution: 3 volumes of absolute ethanol and 1 volume of glacial acetic acid. Store at 4 °C.
3. 1× citrate buffer. Prepare from a 10× stock solution: 40 mL of 0.1 M sodium citrate and 60 mL of 0.1 M citric acid, pH 4.6. Store at 4 °C.
4. Digestion enzyme mixture: 0.3% (w/v) cellulase, 0.3% (w/v) pectolyase, and 0.3% (w/v) cytohelicase in 1× citrate buffer. Store at −20 °C (*see* **Note 1**).
5. 60% glacial acetic solution diluted with SDW. Store at 4 °C.
6. Staining solution: 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/mL in antifade mounting medium Vectashield®. Store at 4 °C.
7. Watch glasses, Pasteur pipettes, dissecting needles, and fine forceps. Microscope slides and coverslips. Diamond pen.
8. Binocular magnifier.
9. Incubator (37 °C).
10. Heating block (42 °C).
11. Hair dryer.

2.4 Preparation of DNA Probes

1. DNA probes: Clone pTa71 containing a 9-kb *Eco*RI fragment from *Triticum aestivum* consisting of the 18S–5.8S-25S rDNA genes and the spacer regions [19]. Plasmid pCT4.2 containing the 5S rDNA gene from *A. thaliana* as a 500 bp insert cloned in pBlu [20].
2. Label probes with digoxigenin-dUTP or biotin-dUTP (*see* **Note 2**) using a nick translation labeling kit and follow the manufacturer's instructions. Store the labelled probes at −20 °C until required.
3. Thermocycler (for nick translation reaction).

2.5 Hybridization and Washing

1. 20× SSC buffer (saline sodium citrate): 0.3 M NaCl, 0.03 M sodium citrate, pH 7. Store at room temperature (RT). Prepare a working dilution (2×) with SDW (*see* **Note 3**).
2. Pepsin solution: mix 90 mL of SDW and 0.01 g of pepsin powder (0.01% w/v). Later add 1 mL of 1 M HCl and SDW up to 100 mL.
3. Paraformaldehyde: solution of 4% (w/v), pH 8 (adjust with 1 M NaOH). Shake a minimum of 20 min at 60 °C. Store at 4 °C (*see* **Note 4**).

4. Alcohol series: Prepare 70% and 90% solutions with absolute ethanol and SDW. For the 100% solution use absolute ethanol. Store at 4 °C (*see* **Note 5**).
5. Hybridization mixture: 5 mL of deionized formamide, 1 mL of 20× SSC buffer, and 1 g of dextran sulfate. Dissolve at 65 °C and adjust pH to 7. Store at −20 °C.
6. 50% Formamide–2× SSC: Deionize formamide for a minimum of 1 h using ion-exchange resin beads (Amberlite). For 200 mL of solution: 100 mL of deionized formamide, 20 mL of 20× SSC, make up to 200 mL with SDW. Store at RT (*see* **Note 6**).
7. 4T buffer: Obtain 4× SSC buffer from the 20× SSC stock, 0.05% (v/v) of Tween 20 detergent, pH 7. Store at RT (*see* **Note 7**).
8. TNB: 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% (w/v) of a blocking reagent (e.g., Boehringer). Store at −20 °C.
9. Antibodies: Cy3-streptavidin and FITC-anti-digoxigenin. Dilute to the concentration of 5 ng/μL in TNB buffer (*see* **Note 8**).
10. Staining solution (*see* **step 6** in Subheading 2.3).
11. Coplin jars, coverslips and Parafilm, Eppendorf tubes.
12. Incubator (37 °C, 45 °C).
13. Heating block (72 °C, 80 °C).
14. Fluorescence microscope equipped with optical filters for DAPI, FITC, and Cy3 fluorochromes and image acquisition software.

3 Methods

3.1 Polyploidy Induction

Synthetic autotetraploids are obtained by a colchicine treatment applied to diploid plants. For that purpose, treat young plants at the preflowering rosette stage by placing 1 drop (about 7–10 μL) of 0.25% colchicine solution on the apical meristem (*see* **Note 9**).

3.2 Fixation

Fix closed flower buds in fresh Carnoy's fixative solution at RT. Exchange this solution after 24 h as many times as needed until the fixative solution remains clear. Store fixed samples at 4 °C. In polyploids, use material fixed for no more than 15 days as a crucial step for obtaining metaphase I cells in which chromosomes are not clumped (Fig. 1c, d) (*see* **Note 10**).

3.3 Flower Bud Digestion

1. Place several inflorescences on a glass well and add enough volume of 3:1 fixative to cover the samples (at least 1 mL).
2. Individualize flower buds and discard those that are not at the appropriate meiotic stage (*see* **Note 11**).

3. Wash flower buds three times (at least 5 min each) in 3:1 fixative solution.
4. Wash three times in 1× citrate buffer (at least 5 min each) to prepare flower buds for the enzyme digestion.
5. Remove the citrate buffer completely and add the enzyme mix (1 mL). Beware that the buds are submerged.
6. Cover the glass well and place it in a moist chamber to avoid evaporation. Digest flower buds in the enzyme mixture for 2.5 h at 37 °C (*see* **Note 12**).
7. Stop enzymatic digestion by replacing the enzyme mix with ice-cold SDW (also ice-cold 1× citrate buffer can be used) (*see* **Note 13**).

3.4 Chromosome Spreads

1. Place a digested flower bud on a slide with a small volume of buffer using a Pasteur pipette (*see* **Note 14**).
2. Squeeze the flower bud with a needle until the anthers are completely shattered obtaining a cell suspension (*see* **Note 15**).
3. Add 15 μL of ice-cold 60% acetic acid to the cell suspension and place the slide on a hot plate for 1 min at 42 °C (*see* **Note 16**). To fix more cells on the slide, especially in polyploids, move the cell suspension making circles with a needle without touching the surface of the slide. Then, add another 15 μL of ice-cold acetic acid to the suspension.
4. Add 200 μL of ice-cold 3:1 fixative solution making a circle around the cell suspension. Next, add further 200 μL of ice-cold 3:1 fixative solution to the center of the circle. Discard the fixative by tilting the slide. Wash again with 200 μL of ice-cold 3:1 fixative solution and dry the slide using a hair dryer.
5. Mount the slides adding 10 μL of staining solution per slide and covering with a 24 × 24 mm cover slip (*see* **Note 17**).
6. Check the slides under a microscope to select those containing metaphase I meiocytes to perform the FISH protocol (*see* **Note 18**).

3.5 DNA Hybridization

1. Remove the coverslips from the slides by washing them in a Coplin jar with 4T buffer at RT until the coverslip slips off. If the coverslip has immersion oil, remove it by washing the slides in a Coplin jar with absolute ethanol at RT. Afterward, in both cases, wash the slides in 4T buffer for a minimum of 1 h, but overnight (ON) is recommended. Skip this step when the slides were not stained before the FISH protocol.
2. Preheat 100 mL of the pepsin solution in a Coplin jar at 37 °C.
3. Wash the slides in 2× SSC buffer for 10 min at RT.

4. Incubate the slides in the pepsin solution for 90 s at 37 °C (*see* **Note 19**).
5. Wash the slides in 2× SSC buffer for 10 min at RT.
6. Fix the material by incubating the slides in 4% paraformaldehyde for 10 min at RT.
7. Rinse the slides in SDW twice for 5 min at RT.
8. Dehydrate the material in an ethanol series of 70%, 90% and 100% of absolute ethanol. Wash the slides for 2 min in each solution at RT. After the last wash, let the slides dry for a minimum of 30 min at RT in darkness.
9. To prepare the probe mixture, mix for each slide 3 μL of each probe and 14 μL of the hybridization mixture. Denature the probe mixture for 10 min at 80 °C and place the mixture on ice for 5 min.
10. Add 20 μL of the probe mixture on a coverslip. Invert the slide and touch gently the liquid to mount the coverslip on the slide. Place the slides on a hot plate for 4 min at 72 °C.
11. Incubate the slides ON at 37 °C (*see* **Note 20**).
12. Preheat four Coplin jars at 45 °C: two with 50% formamide–2× SSC, one with 2× SSC buffer and one with 4T buffer.
13. Remove the coverslip from the slides (*see* **Note 21**).
14. Wash the slides twice in 50% formamide–2× SSC buffer for 7 min at 45 °C.
15. Wash the slides for 5 min in 2× SSC buffer and for 5 min in 4T buffer at 45 °C.
16. Wash in 4T buffer for 5 min at RT.
17. Prepare the solution containing fluorescent antibodies (50 μL per slide).
18. Add 50 μL of this solution to each slide and cover with Parafilm (*see* **Note 22**).
19. Incubate the slides for 1 h in a humidified atmosphere at 37 °C in darkness.
20. Remove carefully the Parafilm using forceps and wash the slides three times in 4T buffer for 5 min at RT (*see* **Note 23**).
21. Finally, mount the slides by adding 10 μL of the staining solution on coverslips. Invert the slides and mount the coverslips upside down. Carefully, apply gentle pressure. Store slides for up to 2 weeks at 4 °C in darkness.

3.6 Microscope Captures of Cells

1. Use an epifluorescence microscope equipped with a camera and the appropriate filters to detect DAPI, FITC, and Cy3 signals.

2. Merge the three pictures obtained from each metaphase I cell (corresponding to DAPI, FITC, and Cy3). Adjust contrast and brightness using a photo editing software such as Adobe Photoshop®.

3.7 Interpretation of Meiotic Configurations

After applying the protocol mentioned above, the most difficult part to be conducted is the interpretation of the different meiotic configurations that can be observed. They are mainly conditioned by the morphology of the chromosomes involved: the appearance is different depending on whether the chromosome is acrocentric (chromosomes 2 and 4) or submetacentric (chromosomes 1, 3, and 5). The number and location of chiasmata formed also influence the configurations. FISH signals and the observation of the chromatin in grey, without the blue color of the DAPI, can help to identify the chromatids bond by chiasmata. Representative examples of the most common configurations for quadrivalents with three (Fig. 2) or more than three chiasmata (Fig. 3) are included. These images can serve as a first approximation in studies that require a record of chiasmata in tetraploids of *Arabidopsis* and species with similar chromosomal morphologies.

4 Notes

1. Alternatively, prepare the digestion enzyme mixture with 1% (w/v) of each enzyme (3×). Freshly prepare working dilution (1×) with citrate buffer (1×).
2. We traditionally apply digoxigenin-dUTP for 45S rDNA sequences and biotin-dUTP for 5S rDNA sequences, but it works just as well if it is done the other way.
3. To avoid bacterial growth in 2× SSC buffer prepare it fresh each time the protocol is conducted.
4. Paraformaldehyde solution can be reused several times.
5. Ethanol dilutions (70%, 90% and 100%) can be reused sealing the Coplin jar to avoid evaporation.
6. Deionized formamide can be stored at RT until needed.
7. 4T buffer is contaminated when the solution starts to look cloudy (in a couple of weeks). Check it before its use.
8. Usually red (Cy3) is used for detecting 5S rDNA because these signals are smaller and red is usually more clearly distinguished than green (FITC).
9. Approximately 10–20% of plants survive the colchicine treatment. The appearance of the first flowering depends on the accession used. It is typical to obtain mixoploid individuals. As a first indication for plants producing polyploid tissues, look for abnormal leaf trichomes with more than three branches [21].

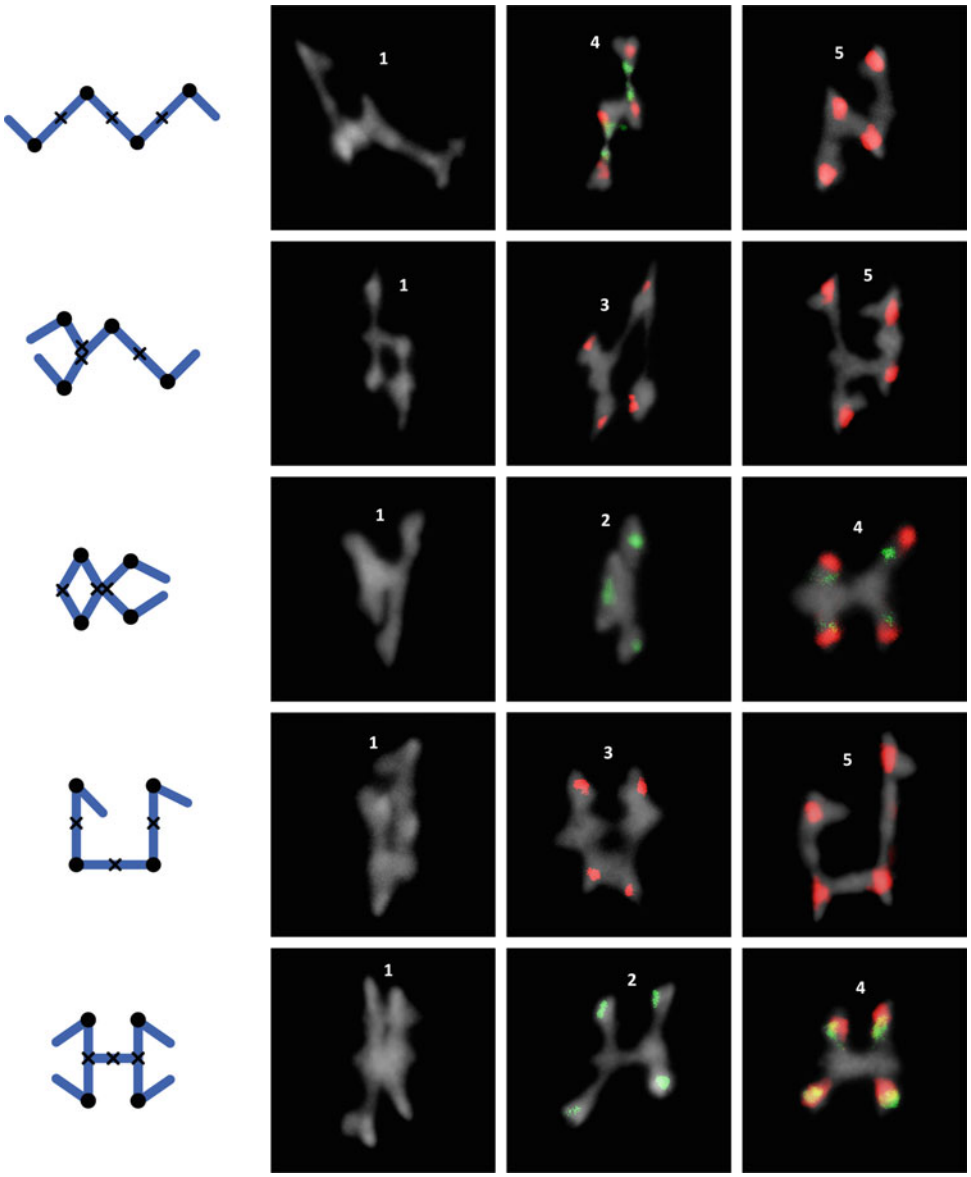

Fig. 2 Examples of quadrivalents with three chiasmata. Images of the same row show the same quadrivalent configuration formed by different chromosomes. On the left, there is a scheme of the quadrivalent indicating the number of chiasmata necessary for its formation (black crosses). For simplicity, chiasmata are considered distally located

10. The less time flower buds are in fixative solution, the better spreads are obtained. However, less than 5 days of fixation might lead to lose cells during the FISH protocol. Additionally, it is important to exchange the fixative solution after the first 24 h until it remains clear. This is essential in polyploids to obtain proper spreading.

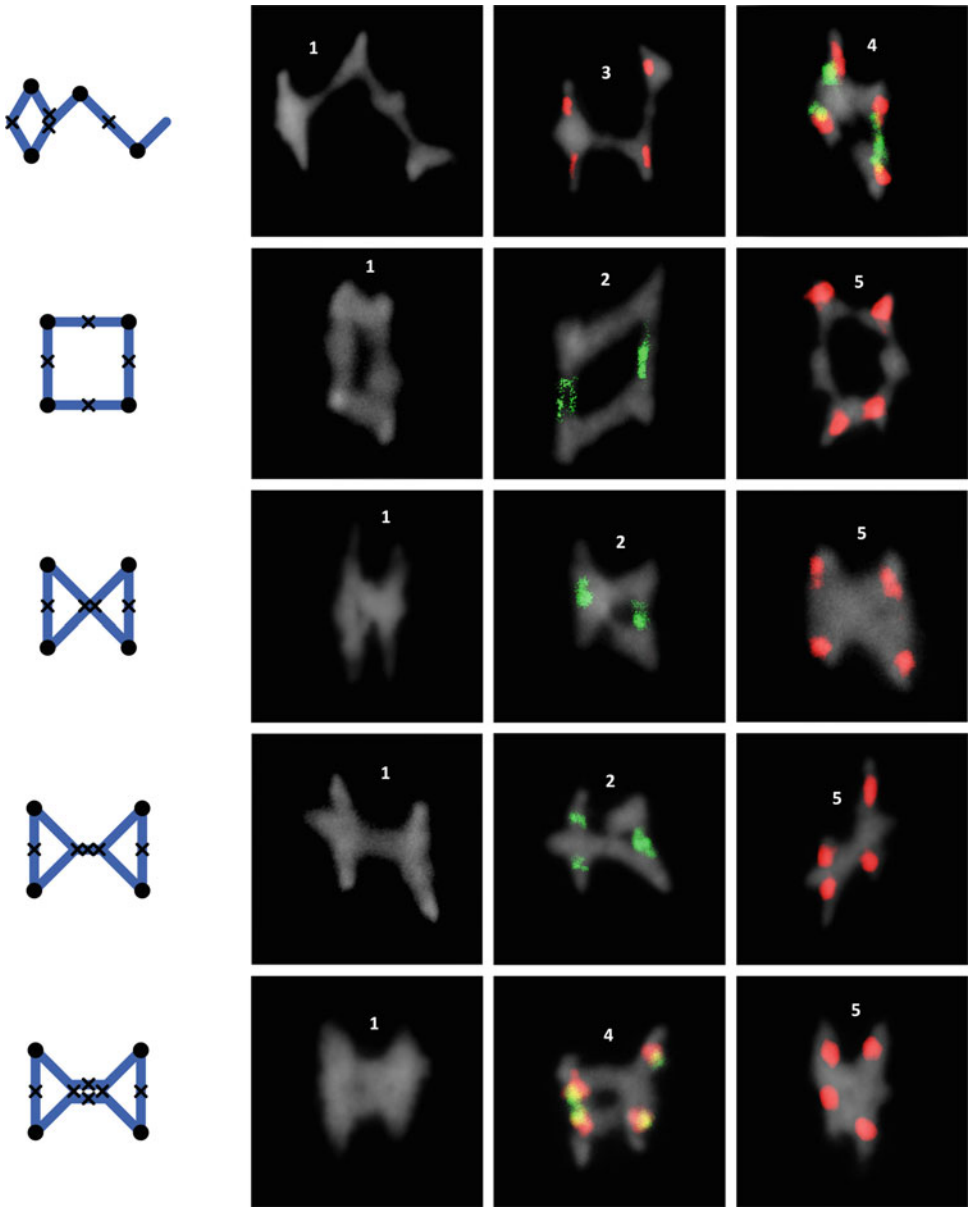

Fig. 3 Examples of quadrivalents with four or more chiasmata. Images of the same row show the same quadrivalent configuration formed by different chromosomes. On the left, there is a scheme of the quadrivalent indicating the number of chiasmata necessary for its formation (black crosses). For simplicity, chiasmata are considered distally located

11. There is a correlation between meiotic stages and bud size. Flower buds with yellow anthers contain pollen grains.
12. In the protocol designed for diploid plants, the digestion time is 2 h. For polyploids, it is necessary to increase the digestion time to get a better spread of the chromosomes.

13. Digested flower buds can be used immediately to continue the protocol or stored up to 2 days at 4 °C in a moist chamber.
14. Do not add too much buffer to be able to squeeze the flower bud easily, but at the same time be careful to ensure that the suspension does not dry out.
15. It is useful to make a hook bending a needle to squeeze better the flower buds.
16. Mark the region of the slide that contains the cell suspension using a diamond pen.
17. This is an optional step because the slides with metaphase I meiocytes can also be selected using a phase contrast microscope prior to the staining step.
18. Write down the microscope coordinates of metaphase I cells to find them easily after the FISH protocol.
19. Increase the time if chromosomes appear to be covered by cytoplasm.
20. Place the slides in a moist chamber to prevent evaporation.
21. Tilt slides to let the coverslip fall. Place the slides immediately into the 50% formamide–2× SSC to avoid getting the material dry.
22. It is important to remove air bubbles as much as possible.
23. To avoid the presence of background (nonspecific binding of the antibody), it is advisable to extend this time, as well as to shake the Coplin jars during the washes.

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