Defining meiotic recombination landscape in tomato

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Introduction

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Factors shaping the meiotic recombination landscape and tools to analyze crossovers

Meiosis is a conserved process in sexual reproduction in which cells undergo two successive and distinct cell divisions (Meiosis I and II) after a single round of DNA replication. During Meiosis I the homologous chromosomes segregate to daughter cells, while in Meiosis II the chromatids separate, finally resulting in two pairs of haploid gametes. Meiotic recombination occurs through the formation of crossovers (COs) between homologous chromosomes during the prophase of Meiosis I. COs denote biochemical events in which non-sister chromatids from the homologous chromosomes become physically linked and upon resolution exchange chromosomal segments. Thus, COs can uncouple the co-segregation of alleles within one chromosome during meiosis to generate recombinant chromosomes with new combinations of alleles in the gametes and eventually in the progeny. Consequently, a better understanding and control of meiotic recombination events can help to gain control over the genetic diversity generated and passed on to the next generation, which is essential in plant breeding.

Plant breeding can be improved by a better control of COs in two ways: First, to introduce an advantageous allele into an elite crop variety, breeders select for plants carrying an introgression with the desired alleles thereby limiting the amount of unwanted flanking regions ("linkage drag"). A small size of the introgression can be achieved by increasing CO frequencies nearby the gene of interest. Second, controlling the occurrence of CO events ensures that two desired alleles remain coupled during breeding, improving the predictability of the breeding process [1] . As the future of agriculture will remain based on classical breeding methods, speeding up or improving the process of recombination between traits of interest via crossovers can aid the production of new crop varieties that are adapted to modern environments and/or demands and thereby ensure food security in the future.

A brief overview of the meiotic pathways

Crossovers are the result of a tightly regulated recombination process that is initiated during early prophase I with the formation of programmed DNA Double Strand Breaks (DSBs) (Figure 2.1). The formation of DSBs during leptotene initiates homologous chromosomes to recognize each other and align along each other. In some species (flies and worms), however, homolog pairing is independent of the DSB formation [2, 3, 4, 5, 6, 7]. In mouse a significant proportion of homolog pairing precedes DSB formation [8].

DSBs are formed by the topoisomerase SPO11 within the SPO11-PRD1-MTOPVIB (SPOrulation-11-Putative Recombination initiation Defect 1-Meiotic TOPoisomerase VIB-LIKE) complex via a transesterification reaction by which SPO11 becomes covalently linked to the 5' ends of the broken DNA [9, 10]. The nucleolytic activity of the MRX/MRN complex (containing among others Mre11 and RAD50) cuts the DNA strand that is attached to SPO11 to release a short oligonucleotide linked to SPO11 ("spolligos"). The broken DNA is then resected in order to generate a 3'-OH single stranded DNA. The 3'-OH overhangs are loaded with the recombinase proteins RAD51 (RADIATION SENSITIVE 51) and DMC1 (DISRUPTED MEIOTIC CDNA 1) [11, 12] to form a nucleofilament capable of homology search and heteroduplex formation. The nucleofilament will search for homologous sequences by invading either the sister chromatid or a non-sister chromatid from the homologous chromosome. Only invasion of the homologous non-sister chromatid can lead to crossover formation via two pathways giving rise to either Class I or Class II crossovers. The Class I pathway accounts for 85-90% of COs and involves the group of ZMM proteins (including ZIP1, ZIP2, ZIP3, ZIP4, MER3 and MSH4 and MSH5), which were initially identified in *S. cerevisae* and were subsequently shown to be conserved in eukaryotes. The protein MLH1 is associated with the ZMM pathway. Much less is known about a second/additional, non-ZMM pathway producing so-called Class II crossovers, which accounts for 10-15% of crossovers and is known to include MUS81. The two pathways differ not only in the underlying molecular machinery, but also with regard to the distribution of the COs that are produced. Whereas the non-ZMM COs (Class II) are distributed independently from one another, the Class I COs are found further apart along the chromosome than expected by chance, as demonstrated by the γ-model [13, 14] , indicating that the presence of a crossover impedes the occurrence of another crossover nearby. This 'interference' phenomenon suggests that a signal is spread along the chromosome by epigenetic factors, which will be discussed later in this chapter.

Along with these two pathways, the vast majority of DNA DSBs are repaired by non-crossover (NCO) pathways. Most NCOs are products of synthesis-dependent strand annealing (SDSA), during which the invading DSB-end initiates DNA synthesis and is subsequently displaced and annealed to the other end of the DSB, resulting in DSB repair without physical DNA exchange. NCO repair can also result from dissolution of Holliday junction joint molecules by the combined activities of helicases and topoisomerase, such as the proteins RECQ4 and FIGL1 which are discussed in **chapter 3** of this thesis. The complexity of the meiotic recombination pathways is not fully understood yet. Hence the genome-wide distribution of the recombination events (DSBs and/or COs) is not yet predictable. The ability to predict CO events, or to steer the frequency and/or position where CO occur would provide powerful tools for improving classical breeding methods. The tight control of the meiotic recombination events is a multilevel process that involves many factors, ranging from chromosome architecture to the

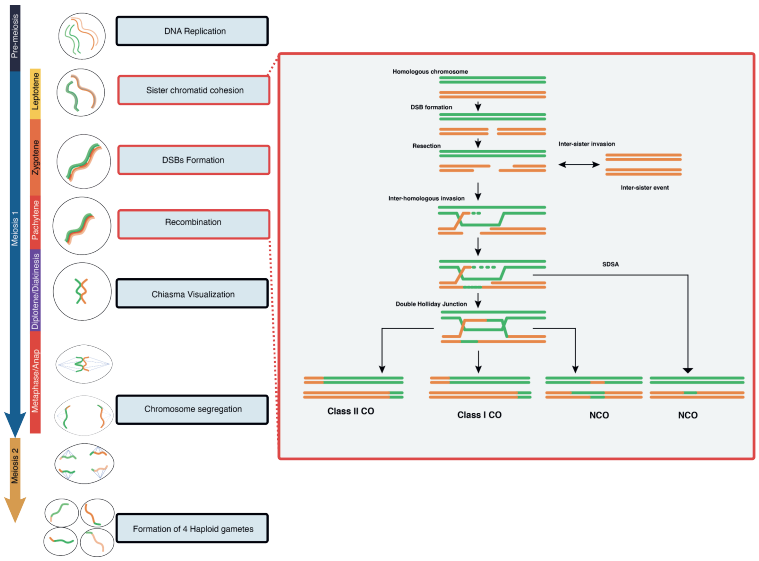

Figure 1.1: Overview of meiotic stages and recombination mechanisms during Prophase I. Meiosis is preceded here by pre-meiotic DNA replication and is presented by Meiosis 1 and Meiosis 2 with highlight on the Meiotic 1 stage. The scheme on the left shows the configuration of chromosomes throughout meiosis. The early Prophase I stages, DSB formation and recombination, are marked by red outlines and correspond with the biochemical steps as shown in the rightmost panel. A large number of double strand breaks (DSBs) are initiated during leptotene and subsequently processed via resection to generate 3'-OH single stranded DNA. The DSB is repaired via invading of the ssDNA into a homologous DNA template of either the sister chromatid (inter-sister invasion) or a non-sister chromatid (inter-homologous invasion). In the latter case, the structure created can either (1) lead to synthesis strand-dependent annealing (SDSA) inducing a non-crossover event (NCO), or (2) processed to the formation of a Double Holliday Junction (dHJ). The resolution of dHJ leads in a majority of cases to NCO events, or in the formation of Class I COs through the action of ZMM proteins or, less frequently, formation of Class II COs via the MUS81 dependent pathway.

DNA sequence. Many studies have tried to decipher the complexity of the meiotic regulation. New methods have been developed to identify factors shaping the landscape of recombination and to assess the chromatin profile. These methods have improved our understanding of the mechanisms that determine the distribution of meiotic recombination events. In this chapter we examine the state-of-the-art of the knowledge about the factors shaping the meiotic recombination landscape in eukaryotes and prokaryotes. We then discuss the advances in the methodology to study the chromatin landscape and the distribution of recombination events in meiotic cells and its potential applications.

Factors influencing the meiotic recombination landscape

Meiotic recombination events, DSBs and COs, are not evenly distributed along the genome, but tend to occur at genomic locations where the probability of an event is higher than at other genomic locations [15, 16, 10, 17, 18, 19]. Extensive analyses of meiotic recombination in mammals, yeast, and plants indicated that the recombination landscape and the position of recombination hotspots is determined by the hierarchical and combinatorial action of a multitude of factors that operate at different scales [20]. Meiotic recombination events are skewed within highly localized 1-2 kb long hotspots, whereas the bulk of the DNA is considered silent ('cold') for recombination. If recombination would occur randomly, then DSBs and COs would be equally likely to occur at any location along the chromosomes. This hypothesis has been refuted by many studies in budding yeast, Arabidopsis, wheat and humans where more than 80% of the recombination events occur in less than a quarter of the genome [21]. Based on observations in tomato, wheat, and barley, it appears that meiotic recombination events are avoided in heterochromatic pericentromeric regions at the chromosome level[22, 23, 24, 25, 26, 27, 28].

The distribution of meiotic recombination events is determined by a complex interplay of "intrinsic factors", such as chromatin structure, nucleosome distribution, loop-axis structure, which determine the accessibility or affinity for "extrinsic factors", such as the machineries involved in the formation of DSBs and or COs [29]. The combination of the intrinsic and extrinsic factors allows the tight regulation of the distribution of the recombination events along the chromosomes. In mammals, for example, crossover recombination hotspots have been correlated with the consensus target DNA sequence of the transcription factor PRDM9. This transcription factor induces meiotic recombination events by methylating H3K4 and recruiting DSB promoting proteins [30].

Several proteins involved in DSB formation are conserved across yeast, mammals and plants. This implies that the "extrinsic factors" shaping the DSB landscape are maintained in (nearly) all eukaryotes, at least in part [21]. Another layer of complexity for our understanding of the meiotic regulation, comes from the fact that the factors influencing meiotic DSBs and CO events are not necessarily all meiosis-specific proteins. They might be also involved in other cellular processes and/or biochemical pathways, such as DNA mismatch repair and chromatin accessibility remodeling [31]. Because of this it is extremely difficult to demonstrate a universal cross-species mechanism for the designation of DSBs and/or CO hotspots. In the next part I will summarize the knowledge about the diversity of factors regulating the meiotic recombination landscape in various species.

A chromosome-scale control of meiotic recombination events density

In order to promote the formation of interhomolog COs, chromosomes undergo massive large-scale structural changes during the meiotic prophase. At the onset of prophase I, in leptotene, chromatin gets compacted as loop arrays around the proteinaceous chromosome axis, which makes the chromosomes microscopically visible as thin fibers. The chromosome axis acts as a platform for recombination, gathering proteins that aid chromosome compaction. Zygotene is the next stage of meiosis, in which telomeres cluster together and attach to the nuclear envelope [32]. This clustering results in the formation of a 'bouquet' of chromosomes which helps the homologs to recognize each other, and to form pairs that can undergo synapsis. The synaptonemal complex (SC) is completed at the pachytene stage, during which homologous chromosomes are further compacted and processed into COs [33, 34, 35, 36]. All previously described morphological changes accompany the meiotic recombination events.

In contrast to mitosis prophase, where transcription is largely shut down [37], cells in meiotic prophase I remain transcriptionally active [38, 39, 40] and cope with two seemingly conflicting requirements: (1) the compaction and organization of DNA around the meiotic chromosome axis to ensure the homolog pairing and synapsis; (2) maintenance of transcription at various loci required for the meiotic process to continue. A recent Hi-C study in mouse spermatocytes demonstrated a dynamic reorganization of the chromosome at the onset of meiosis [41]. Topologically associated domains (TADs) were almost completely lost, but the A/B compartment structure, representing gene-dense domains (compartment A) and heterochromatin domains (compartment B), was maintained allowing the existence of highly transcribed "hubs" ensuring the expression of genes necessary to support later developmental stages. In addition, a comparison of meiotic recombination hotspots with Hi-C data, revealed that DSB hotspots highly correlate with compartment A. Evidently, the bias of meiotic recombination events is linked to chromosomal topology.

DSBs and COs are repressed around the centromeric regions in order to prevent deleterious recombination events. Centromeric regions are specialized regions where large protein complexes called kinetochores bind to chromosomes to ensure proper segregation during anaphase. Hence, the presence of DSBs and/or COs in these regions could potentially interfere with chromosome segregation and thereby induce aneuploidy and/or chromosome fragmentation. Meiotic recombination events are also suppressed in the 20kb flanking the telomeres in budding yeast [20]. Thus, the large-scale organization of chromosomes appears to have a high impact on the distribution of the recombination events.

Analyses of plant species with genomes widely varying in size and organization indicate that genome organization may also affect the frequency and patterns of COs. For example, in the relatively small genome of the model plant Arabidopsis *thaliana*, meiotic recombination events are distributed relatively homogenously over the genome, except for the centromeric regions where recombination is repressed. However, in organisms with larger genomes and a higher abundance and wider distribution of TEs such as maize, wheat and tomato, meiotic recombination events seem to be enriched in gene-rich euchromatin [42].

Another constraint imposed on the distribution of the meiotic recombination events at the chromosome scale is the size of the chromosomes on which the recombination takes place. Molecular and genetic studies of chromosome 1 in Saccharomyces cerevisae [43, 44, 45] showed that increases in chromosome size causes a reduction of the density of COs. This correlation was also observed for frequency of DSBs in yeast [20] and in mouse [46] . The inverse correlation is slightly stronger between chromosome size and the number of MLH1 foci (which mark COs) than with the number of Spo11 oligos [47], suggesting that chromosome size has a larger influence on the CO distribution than on the DSBs distribution [48] . It has been proposed that DSBs in smaller chromosomes invade their homologs on average more slowly than DSBs in larger chromosomes, thereby extending the time during which breaks can accumulate [45, 49, 50].

From these studies we can state that the chromosome-scale constraints imposed by the chromosome topology and size represent an important level affecting the distribution of the meiotic recombination events.

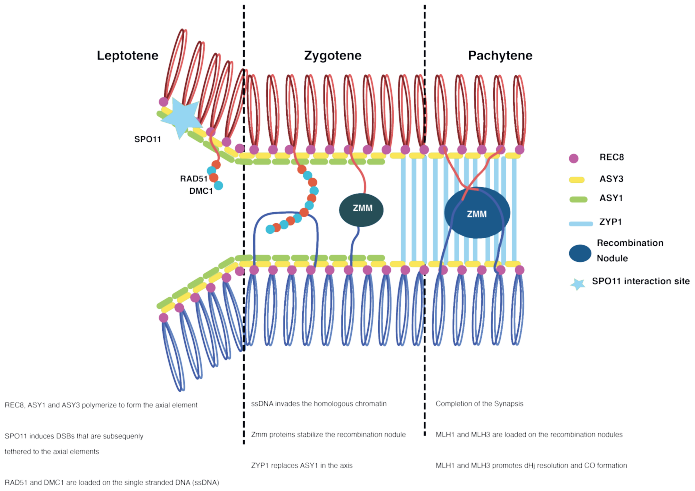

Figure 1.2: Overview of the localization of proteins shaping the axial element and the meiotic proteins inducing the formation of COs. Homologous chromosomes are represented in blue and red, with two sister chromatids of the same color (in bright and dark). At leptotene, SPO11 induces DSBs at specific loci. DMC1 and RAD51 are loaded until late zygotene and will help the single strand DNA to find and invade its homolog. ZMM proteins helps to stabilize the Double Holliday Junction (dHJ). At late zygotene and during whole pachytene, homologous chromosomes are fully synapsed. MLH1 and MLH3 promotes the CO formation by resolving the dHj during pachytene.

Chromosome axes shape the recombination landscape

During early meiosis the homologous chromosomes are brought in close proximity of one another by forming the synaptonemal complex (SC). Initiated during zygotene and completed during pachytene, the SC is a highly complex protein structure acting as a "zipper" between the two homologous chromosomes (Figure 2.2). The assembly of the SC occurs through the linkage of central elements and flanking transverse protein filaments that bridge the space between the axial elements. The components of the axial elements: Rec8 (meiotic cohesin subunit), Asy1 and Asy3 are important for the distribution of the DSBs. A reduction of DSB formation has been reported in mutant lines for the proteins that are involved in the formation of the axial elements. This highlights the central role of the axes in several steps of meiotic recombination [34, 51, 52].

The central element proteins are widely conserved and all share the structure of the yeast Zip1, having a coiled coil domain in the center and globular domains at both ends, while the primary sequence is poorly conserved [21]. Homologs of Zip1 have been identified in various plant species, however the role of these ZIP1 homologs differs between plant species. The putative ZIP1 homologs from Arabidopsis, AtZYP1A and AtZYP1B, are essential for formation of the SC [34]. Knockdown of these proteins decreased the number of chiasmata in bivalents only slightly, while it increased recombination between nonhomologous chromosomes. This suggests that AtZYP1A and AtZYP1B are not essential for crossing over but add to the faithfulness of CO events. In rice, a loss of function mutation of OsZEP1 leads to an increase of chiasmata formation between homologous chromosomes, suggesting that OsZEP1 acts as an anti-crossover molecule [53] . On the other hand in barley, ZYP1 [52] was identified, mutation of this protein induces an increase of chiasmata during diakinesis [53]. This suggests that ZYP1 homologs can also act as repressors of crossover events. The opposite effects of ZYP1 homologs in the different species implies species-specific differences in the mechanisms that regulate meiotic recombination through ZYP1 homologs.

In *S. cerevisae* meiotic DSBs are initiated before the formation of the SC [54, 55, 56], which was surprising because it was long believed that the synaptonemal complex was required for the initiation of DSBs. Later, it was shown that in Drosophila and *C. elegans* the SC is formed independently of the DSBs [57, 58, 59, 60]. The regulation was later subdivided into two categories of organisms, (1) organisms for which the DSB formation is synapsis dependent and (2) organisms for which the synapsis depends on the DSB formation [61]. The distribution of *bona fide* meiotic recombination events – that is COs between homologous chromosomes is also correlated with the physical length of the synaptonemal complex and anticorrelated with the chromosomes length [62, 63]. The SC length is not correlated with the chromosome length: human, Arabidopsis, and budding yeast have an SC length per chromosomes of approximately 10-25, 29.4, and 1-2 microns, respectively, while the CO numbers per chromosome in these species are respectively 1-3, 1-3, 2-11 [64, 65, 66, 67]. The ratio between the genome size and SC length is different between Arabidopsis (1Mb/μm) and human (11Mb/μm), and even smaller for budding yeast (0.5Mb/μm) [68]. This means that the DNA packed in chromatin loops is much longer in budding yeast than in Arabidopsis and human, which might explain the difference in number of COs per chromosomes [33, 69]. More recently, it was shown that the genome-wide distribution of the protein REC8 in Arabidopsis was inversely correlated with the presence of DSBs [70]. As REC8 is located at the SC, it suggests that the recombination is

initiated in the loop and then becomes tethered to the axis during the interhomolog repair [33, 29].

Nucleosome occupancy shapes the recombination landscape

A conserved feature of meiotic recombination hotspots in eukaryotes is their occurrence in the accessible chromatin regions of the genome. Accessible chromatin regions can be defined as nucleosome depleted regions (NDR). Nucleosomes represent the basic unit of the chromatin and consist of 146 bp of DNA wrapped around a protein complex containing 2 molecules each of histones H2A, H2B, H3, and H4. The packing of the DNA generally affects its accessibility to other proteins, including proteins involved in the formation of DSBs (SPO11) or other recombination steps. Genome-wide distribution of Spo-11 oligos in Saccharomyces species [20], Arabidopsis [42], and maize [71] revealed that DSB hotspots frequently correlate with nucleosome-depleted regions, suggesting that local chromatin accessibility contributes to DSB formation in these eukaryotes. Gene expression is often linked to chromatin accessibility through the activity of pioneer transcription factor complexes, which enable transcription by opening up closed chromatin regions [72]. This may explain why DSB hotspots are often associated with promoter regions of active genes in yeast and plants [20, 42] .

In Arabidopsis SPO11 oligos also cluster around the transcription termination sites [42], which also tend to have an open chromatin structure. It is most unlikely that the extensive reorganization of chromatin is due to spontaneous events [73, 24] . The most plausible explanation for the observed changes in chromatin accessibility during meiosis is the combination of histone modification and the action of nucleosome remodelers that regulate the turnover and mobility of nucleosomes positively or negatively [24] . Pan *et al.* (2011) proposed a combination of hierarchical factors shaping the map of DSBs during budding yeast meiosis. Moreover, it was shown that the genome-wide distribution of the meiosis-specific cohesin associated with the axis, REC8, was correlated with nucleosome dense regions in Arabidopsis. In accordance, the genome-wide distribution of REC8 is inversely correlated with DSBs distribution [70]. SPO11 is presented as an opportunistic cutter based on the spatial organization of the chromatin at the onset of meiosis. Indeed, other studies have shown dynamic reorganization of chromatin accessibility during meiosis. Using ATAC-seq, Maezawa *et al.* (2018) observed extensive changes in the chromatin accessibility accompanying the development from germ cells to functional sperm. During this transition, “mitotic type” open chromatin is closed while elsewhere new meiotic-specific accessible chromatin is formed. This observation is supported by information about the alteration of histone tail during the spermatogenesis, which supports the extensive transcriptional changes during spermatogenesis. Genes active during late spermatogenesis are covered by the histone marks H3K4me3 and H3K27me3 on their promoter [74]. More recently it has been shown that the HELLS/PRDM9 pioneer complex actively opens chromatin at meiotic recombination hotspots [31]. In this complex, PRDM9 recognizes potential DSB sites based on sequence specificity and recruits the helicase HELLS to promote chromatin accessibility at the designated DSBs sites. In the absence of HELLS, DSBs hotspots are retargeted at already accessible chromatin regions. This can be deleterious for the cells because DSBs could arise within exons of essential genes [31].

Overall it appears that an accessible chromatin state is required for DSB formation at meiotic recombination hotspots. DSBs are then a consequence of chromatin remodeling caused by pioneering remodeling complex(es), such as PRDM9-HELLS in mice, which may act before the breaks are induced by SPO11. Hence more knowledge of the accessible chromatin regions could help us predict the potential meiotic recombination hotspots. The accessible chromatin landscape of the tomato meiocytes will be analyzed in **chapter 4** of this thesis.

The DNA methylation pattern shapes the recombination landscape

DNA methylation at a specific genomic region represents an important factor affecting meiotic recombination. This is consistent with the fact that low levels of DNA methylation are observed at DSBs and CO hotspots in Arabidopsis and maize [75, 76, 77, 42, 78] and the widespread suppression of recombination in the highly methylated pericentromeric heterochromatin, which is a cold spot. For long, it was believed that hypermethylation of pericentromeric heterochromatin was the reason for the suppression of meiotic recombination events in these regions. However, several studies have shown that the loss of DNA methylation does not induce an elevation of the meiotic recombination events around the centromere [79, 80, 81, 82, 83, 84, 77]. It has been proposed more recently that repression of recombination at pericentromeric regions is under control of non-CG methylation and/or H3K9me2, at least in Arabidopsis, since DSBs and COs increased in pericentromeric regions upon disruption of the H3K9me2 and non-CG methylation pathways [85]. Hence, the role of DNA methylation in meiotic recombination repression in centromere regions remains poorly understood. There is, on the other hand, ample evidence for the repressive effect of DNA methylation on recombination in euchromatin regions. For example, a mutation in the SWI/SNF chromatin remodeler DDM1 (Decrease DNA Methylation 1) did not increase the CO rate in the densely methylated pericentromeric regions, but increased CO rates significantly in euchromatic regions [86]. Furthermore, insertions of hypermethylated transposable elements into euchromatic regions of maize suppress meiotic recombination events locally [87, 88]. In addition, the epigenetic process of RNA-directed DNA methylation (RdDM) appears responsible for the suppression of meiotic recombination events in euchromatic regions in Arabidopsis [89]. It is possible that DNA methylation acts as a safeguarding mechanism, suppressing meiotic recombination events in pericentromeric regions, as recombination in these regions can cause chromosome mis-segregation and aneuploidy. DNA methylation imposes constraints on meiotic recombination in the euchromatic regions while DNA methylation participates together with other mechanisms to prevent the occurrence of meiotic recombination events in pericentromeric regions.

Protein complexes control the meiotic recombination pathways

Meiosis is a tightly regulated process involving a multitude of protein complexes in the pathways leading to formation of crossovers and non-crossovers [15, 90, 21]. CO events are actually rare with usually one to three events per chromosome. Different pathways limiting meiotic crossover have been recently proposed in Arabidopsis. They rely on the activity of anti-crossover factors including the ATPases RECQ4 [91]and FANCM [92], and the helicases FIGL1, FIDGETIN-Like-1 [93], and XRCC1 [94]. Modulation of the expression of these non-CO proteins affects the CO rates [95, 96]. In **chapter 3** of this thesis, we analyze the effect of the disruption of the proteins RECQ4 and FIGL1 on the recombination rates in tomato.

RECQ4 is an evolutionary conserved helicase, which is duplicated in Brassicaceae. Hence, Arabidopsis contains two orthologs: RECQ4A and RECQ4B. RECQ4 acts in the NCO pathway as part of the RTR (RECQ4/TOP3α/RMI) complex to promote non-crossover outcomes of meiotic DSBs [97]. Mutation of RECQ4AB increased the CO frequency six-fold in intraspecific Arabidopsis crosses [91], by blocking the anti-crossover pathway (NCO pathways). The

AAA-ATPase FIGL1 interacts with the recombinases RAD51 and DMC1 to promote the repair of DSBs using the sister chromatid as a template instead of the inter-homolog strand. Mutation of FIGL1 leads to a 1.7-fold increase in CO rates [93, 95]. Finally, FANCM (FANCONI ANEMIA COMPLEMENTATION GROUP M-LIKE PROTEIN stimulates SDSA (single-dependent strand annealing) and thereby favors the resolution of intermediates as NCOs. In the absence of FANCM, these intermediates are resolved by MUS81, leading to an increase of the total class II COs [93]. In Arabidopsis it has been possible to increase the crossover frequencies by upregulating class I and/or class II pathways [95]. The combination of some of the mutations in FIGL1, RECQ4 and FANCM leads to an additive increase of crossovers frequencies. In chapter 3 of this thesis we study the effect of RECQ4 and FIGL1 on crossover frequency in the interspecific F1 hybrid of *S. lycopersicum* and *S. pimpinefllifolium*, and found that depletion of RECQ4 increases the crossover frequency [98], whereas depletion of FIGL1 reduces the crossover frequency .

Histone modification shapes the recombination landscape

Histone modifications participate in the positioning of potential meiotic recombination events. Chromatin-immunoprecipitation combined with high-throughput sequencing (ChIP-seq) has greatly increased our understanding of the genome-wide correlation between meiotic recombination events and the histone modification landscape. For example, dimethylation of H3K9 over pericentromeric regions of the Arabidopsis genome represses undesirable meiotic recombination events [85], whereas histone modifications associated with chromatin accessibility, such as H3K4me3, have been correlated to DSBs hotspots [99]. The mechanism by which H3K4me3 is linked to DSB formation was further highlighted by the discovery that H3K4me3 facilitates the meiotic DSB protein complex to bring chromatin loops into close contact with the chromosome axis for Spo11 processing [100]. The promotion of this axis-loop contact appears to bring more flexibility to regions where meiotic recombination events can take place [24].

PRDM9 is a histone lysine trimethyltransferase that determines the position of DSB hotspots in mammals by binding to a specific sequence motif and trimethylating histone H3 (H3K4me3) [101, 102, 103, 104, 105] and H3K36 [106]. In mammals, PRDM9 binding sites enriched for H3K4me3 correlate with DSBs [107]. Upon PRDM9 mutation in mouse, DSBs no longer occur in regions containing PRDM9 bindings sites, and now occur 'ectopically' in other H3K4me3-marked regions that lack PRDM9 binding sites, such as active gene promoters(Brick *et al.* 2012). This indicates that PRDM9 designates specific loci for meiotic recombination [108, 31] . This was further validated by ChIP-seq of DMC1 in a mouse mutant for PRDM9 and/or HELLS [31], which revealed that DMC1 peaks were also shifted to gene promoters upon depletion of PRDM9. These studies suggest that PRMD9 diverts DSBs away from functionally conserved genomic elements. Integration of multiple histone modifications identified by ChIP-seq performed on meiocytes have allowed the identification of epigenetic signatures for the DBS hotspots [109, 31]. These signatures revealed that DSBs hotspots are positioned towards loci marked by H3K4me3 and H3K36me3.

In plants the importance of H3K4me3 is less obvious, which may be related to the absence of PRDM9. Spo11-oligo sequencing in Arabidopsis showed that the enrichment of DSBs at gene

promoters is not correlated with H3K4me3 on the 1st nucleosome immediately downstream of the transcription start site TSS [42]. This is also observed in maize, where only a minority of the DSB hotspots overlapped with regions containing H3K4me3 [71]. In Arabidopsis, the histone variant H2A.Z appears to promote the formation or the processing of DNA DSBs, as in a mutant, arp6, lacking the deposition of the H2A.Zmark, the number of DMC1 foci is reduced [110]. H2A.Z follows the same pattern of deposition as H3K4me3 and seems to increase the displacement of histones to promote the chromatin accessibility at its deposition sites. A similar observation was made in fission yeast [111, 112], highlighting the role of H2A.Z in the initiation of the meiotic recombination by modulating the chromosome architecture to favor the binding of the DSB machinery. Other chromatin studies show that disruption of H2AK5 acetylation induces a significant change in the meiotic DSB and CO landscape in *C.elegans*, while in fission yeast H3K9ac is found at recombination hotspots and possibly facilitate recombination [113].These data show that chromatin modifications influence the meiotic recombination landscape. The precise regulation of meiotic recombination events by epigenetic mechanisms, however, is not yet fully understood and may vary between species.

The DNA sequence shapes the recombination landscape

In human and mouse, binding of the PRDM9 proteins is a major factor that determines the location of recombination hotspots. PRDM9 contains both a methyltransferase domain and a sequence-specific DNA-binding domain composed of several C2H2 zinc fingers. The most common variant of this protein in human recognizes the 13bp consensus sequence, NCCNCCNTNNCCNCN-, induces the trimethylation of the lysine 4 on the nucleosomes [101, 107, 104], and potentially recruits via its Krüppel associated box (KRAB) the SPO11 machinery. PDRM9 is an extremely fast evolving protein, which is reflected by the numerous variants of its repetitive zinc finger array, of which 30 have been identified producing unique DSB distributions [103, 104, 114, 17]. The action of PRDM9 tends to place hotspots in intergenic regions, in order to avoid meiotic recombination within genes. Orthologues of PRDM9 have so far not been identified in plants or fungi. Over the past years, several studies have investigated a possible role of certain DNA sequences on the formation of meiotic recombination events. In plants, Demirci *et al.* 2018 observed a bias of CO occurrence towards the dinucleotide sequence AT/TA in both Arabidopsis and tomato [115]. As the DNA sequence affects nucleosome stability, some sequences are more favorable to nucleosome displacement than others [116]. Demirci *et al.* also highlighted the relationship between DNA sequence and DNA structures that seem to be more attractive to the meiotic recombination machinery. DNA structures such as propeller and helical twist appear to correlate with recruitment of the meiotic recombination machinery more than other DNA structures in Arabidopsis and tomato [116]. Hence it seems that in Arabidopsis dA:dT sequence repeat compensate for the lack of consensus sequence designating the meiotic recombination site [110, 76]. In yeast AT-rich sequences are more favorable to nucleosome displacement and/or exclusion [117]. This corroborates the fact that meiotic recombination events take place at AT-rich accessible regions upstream of the gene body. However in contrast to Arabidopsis, in some other plant species, such as rice and maize, other DNA features, respectively high roll DNA and gene rich features, are more prone to recruit the recombination machinery [116]. Hence, the correlation between typical nucleotide tracks and meiotic recombination seems to be species-specific.

Concluding remarks on the factors determining the meiotic recombination landscape

The occurrence of meiotic recombination hotspots is conserved within eukaryotes. Yet, the multiple layers of factors, specifying position and restraining their numbers activity, are not universal and seem to be species-specific. The development of methods to study and analyze the meiotic recombination landscape will increase our understanding of these "gate-keepers" of meiotic recombination. These gatekeepers do not operate in an isolated manner but rather coalesce into a complex control system. The next part of this chapter will be dedicated to review the methods of identification of recombination hotspots and the meiotic recombination landscape that guides them to take place at those specific locations.

Methods in chromatin profiling and crossover distribution analysis

In recent years, the development of techniques that provide a better understanding of the "gatekeepers" of meiotic recombination has made much progress. Those methods allow to investigate meiosis from chromosome to nucleotide level. In this part of the chapter we will provide an overview of the tools and approaches available to study meiosis.

Chromosomes conformation methods

In order to promote the formation of accurate interhomolog crossovers during meiosis, chromosomes undergo important morphological changes. At the onset of the meiotic prophase 1, homologous paternal and maternal chromosomes interact via a series of highly controlled events involving homolog alignment, pairing and synapsis. This is accompanied by chromatin condensation and the formation of arrays of chromatin loops that are anchored at a semi-rigid axis composed of various proteins including cohesins. The movement and structure changes of meiotic chromosomes were mostly described by microscopical methods or biochemical assays [33, 118]. Recent development of omics technology enabled the analysis of spatial chromosome organization and chromatin landscapes during interphase and meiosis at a much more detailed level.

Hi-C is a method of chromosome conformation capture that quantifies the number of interactions between genomic loci in close 3D proximity [119]. Hi-C relies on the crosslinking of interacting genomic loci that may reside far apart (megabases) in the linear chromosome, or even in distinct chromosomes. The crosslinked genome is digested with a restriction enzyme and DNA fragments subsequently biotinylated and ligated. The re-ligated fragments containing the biotinylated nucleotides are end-sequenced, to unveil the long-range interactions from which they derived. From the quantification, the average intra- and inter-chromosomal organization of the genome can be drawn [120]. Unlike in mitosis where chromosome folding is well described [121], the asynchrony of the meiocytes could hinder the study of the chromosome conformation during meiosis. Modification or complete loss of long-range contacts, as it occurs in mouse spermatocytes [41] between early and late meiotic prophase,

might be problematic for the identification of phase-specific structures in organisms where synchronization of meiosis is not possible.

Based on Hi-C, several studies have been recently published providing a better understanding of the chromosome reorganization throughout meiosis in mouse and *S.cerevisae* [122, 123, 124, 124, 125, 126] . Syn-HiC is a method that allows the identification of low levels of interhomolog contacts in S. cerevisae [122]. Syn-HiC relies on the insertion of regularly interspaced 144 kb regions containing restriction sites on a particular region of the S. cerevisae chromosome 4. Application of Syn-HiC revealed an increase of interhomologous contacts between centromeres during pachytene and increased insulation at Rec8 binding sites [123].

The development of single-cell Hi-C (Sci-Hi-C) methods [127] might provide chromosome conformation information for lowly abundant cell types or cells that are difficult to isolate, such as meiocytes. Sci-Hi-C relies on the same principle as regular Hi-C with ligation of barcoded sequences at different steps of the procedure, in order to obtain single-cell information [128]. Sci-Hi-C has yet to be applied for the investigation of meiosis.

Cytological methods

A useful and effective approach for the characterization of the subnuclear distribution of meiotic proteins and thereby localization of recombination events, is chromosome surface spreading. This cytological method has long been indispensable to analyze chromosome configurations and the subnuclear distribution of proteins as well as to quantify DSBs and CO events. It is generally based on the preservation of morphological structures by fixation with either cross-linking or denaturing fixatives. To maintain the epitope structure for immunodetection, the spread chromosomes have to be cross-linked with paraformaldehyde fixative. To visualize individual chromosomes during meiosis, chromosomes need to be separated by spreading. This method gives reproducible results and has been widely used to analyze the spatial and temporal binding of proteins throughout prophase of meiosis I.

An alternative approach to study chromosomes is based on denaturation fixation using a mixture of ethanol and acetic acid. This has the advantage of preserving the chromosome structure but may disrupt the epitopes of proteins due to the alcohol-acid denaturation. Yet successful immunodetection of ASY1 and MLH1 in Arabidopsis chromosome spreads with this method has been reported [129].

Using the two chromosome fixation methods, DSBs and COs can be quantified by counting the number of foci formed by fluorescent antibodies against meiotic proteins involved in these recombination processes. For example, the number of DSBs has been precisely estimated by counting DMC1 foci, while the number of COs was determined by the number of MLH1 foci [130]. Alongside protein immunolabeling, chromosome spreads have been widely used in combination with fluorescence in-situ hybridization to analyze in detail the meiotic organization of telomeres and centromeric organization.

COs can also be detected cytologically as chiasmata, which are microscopically visible structures, where the homologous chromosomes are physically connected during diakinesis (see **chapter 3** of this thesis). Cytological methods allow to quickly monitor the effect of specific mutations on the formation of DSBs and COs.

By combining these cytological methods with Super-resolution microscopy, resolution can be pushed beyond the limits of fluorescence microscopy (200 nm) to observe cellular structures at resolutions up to 1nm. To date, PALM (Photoactivated Localization Microscopy) or STORM (Stochastic Optical Reconstruction Microscopy) have only been implemented in non-meiotic plant cells [131]. Development of super-resolution microscopy to study plant meiotic cells will give further insight into the importance of the chromatin organization, as it did in C. elegans [132]. Recently, live cell imaging of meiosis in Arabidopsis *thaliana* has been developed [133]. The live imaging relies on the visualization fusions of fluorescent proteins to cohesin (REC8) and microtubule proteins to monitor parameters like cell shape, microtubule array, nucleus position, nucleolus position and chromatin condensation. By analyzing the states of these parameters throughout meiosis, they found that these parameters are often associated, as landmarks, during a particular point of the meiotic progression. The definition of these landmarks allows quantitative and qualitative analyses of the role of particular meiotic proteins in the establishment of these landmarks.

Methods based on molecular markers and genotyping

Meiotic crossover events recombine parent-specific traits, which can be monitored in their progeny, for example by genotyping molecular markers. The resolution of the linkage map depends on the number and position of the markers used for genotyping and on the sample size of the genotyped progeny. Genetic mapping is the most common method of assessing genetic variation. Sperm-typing simplified and improved the mapping of recombination events in mammals, as the numbers of sperms cells that can be obtained is manifold larger than the number of progenies. This method relies on PCR amplification of DNA from either single sperm cells or pooled sperm and has been used in mice to characterize around thirty recombination hotspots [134, 135, 136].

In plants and especially in the model plant Arabidopsis, the detection of meiotic recombination events is simpler than in most animals due to the possibility of making inbred lines via crossing and backcrossing. Also the relative “ease” of mutant production has greatly facilitated meiotic recombination studies. Inspired by the sperm-typing methods, Francis *et al.* (2007) developed a tetrad-based visual assay in pollen for detecting COs and gene conversion in Arabidopsis. This system combines the quartet mutant background, which yields tetrads of meiotically related pollen grains with transgenes that encode for red, yellow or cyan fluorescent proteins. The COs can be detected in pollen of the F1 by crossing plants with differently colored markers on the same chromosome. The F1 plants carry both markers in *cis* (same chromosome) and possess a genetic interval between two fluorescent markers genes. It is possible to infer a CO by scoring the segregation of the two fluorescent protein [137]. Tetrad analysis combined with fluorescence markers is a very powerful high-throughput approach for the detection of COs across the Arabidopsis genome. This method is still popular and provides a basic idea of the recombination frequency before initiating a high-throughput

sequencing approach. The drawbacks of methods using molecular markers is the necessity of transgenic plants with appropriately located marker transgenes. In addition, the number of recombination events to analyze might limit the resolution of the recombination events mapped.

High-throughput genome sequencing methods

Measuring recombination by conventional methods is limited by the number and density of genetic markers [138, 139]. Using whole-genome sequencing methods, one can identify SNPs between two genomes of the same species and obtain the average distance between them. In principle, high-throughput methods with high-coverage DNA sequencing can be applied to compare the combinations of single nucleotide polymorphism (SNPs) in parents and progeny in order to characterize meiotic crossover and chromosome segregation profiles in an unbiased manner.

By crossing the Arabidopsis accessions Columbia-0 and Landsberg *erecta*, Wijnker et al (2013) were able to identify recombination events by using high-quality SNP markers [76]. Similarly, Demirci *et al.* (2016) identified the distribution and genomic characteristics of crossovers in recombinant inbred lines (RILS) of F6 crosses between *S. lycopersicum* and *S. pimpenifollium*. In *S. cerevisae*, a nucleotide resolution map of 91 COs and NCO events was obtained by using 46000 SNPs between two budding yeast strains. Indeed, the identification of COs is based on the presence of sufficient SNPs between the parental lines in order to assess CO location with sufficient resolution using high throughput sequencing methods.

Another method, Kompetitive allele specific PCR (KASP) allows the identification of SNPs using competitive PCR between two sets of fluorescently marked primers, one containing the complementary nucleotide to the SNP and another without the SNP. In the presence of an SNP, a specific fluorescence signal is emitted [140].

Chromatin immunoprecipitation (ChIP) followed by high throughput sequencing (ChIP-seq) is a powerful tool to study the landscape of the meiotic recombination events. This technique involves the immunoprecipitation of chromatin fragments of interest and high throughput sequencing the associated genomic regions. By targeting proteins involved in meiotic recombination or the histone modifications expected to be correlated with recombination events our knowledge of DSB and CO distribution over the genome has significantly increased and improved the resolution of recombination maps. For example, the SPO11 ChIP-seq was successfully used to identify DSB hotspots at nucleotide resolution in budding yeast [20] and mouse [46], which helped to identify the combination of factors that initiate meiotic recombination in budding yeast. The ChIP technique has been optimized over the years and it is now possible to identify the chromatin binding sites using limited cell numbers. For instance, Cao et al (2015) developed a ChIP strategy using an extremely small (710 nl) microfluidic chamber filled with a dense pack of microbeads coated with the desired antibodies to determine the epigenomic profile of only 100 cells. ChIP has also been improved to detect rare protein binding sites. For example, the 3' single stranded overhangs that are produced at DSBs and which are covered by the strand exchange proteins RAD51 and DMC1 and can be recovered by a pull down of RAD15 or DCM1 and sequenced using a specific library preparation method

[141]. The reduction of the dsDNA background and the enrichment of the ssDNA of this method relied on the ability of the microhomology found in the ssDNA sequence to form hairpin-loop like structure [141]. The remaining challenges of ChIP applications for meiosis are: (1) the low frequency of recombination events, (2) the heterogeneity of the meiocyte population, (3) the relatively low efficiency of ChIP which depends on high quality antibodies. Furthermore, the large number of cells, usually necessary for ChIP-seq, is a problem when studying meiosis in organisms where cell cycle synchronization is not possible. Therefore, it is necessary to optimize the ChIP protocol/efficiency for each species of interest. Indeed, protocols for sequencing libraries require relatively large amounts of DNA because of the inherent material loss during purification steps and the several enzymatic reactions in the protocol. This holds in particular for plant cells, where digestion of the cell wall requires an extra step for chromatin isolation. An example of optimization of the procedure for analyzing tomato meiocytes is described in **chapter 2** of this thesis.

Genome-wide maps of histone marks allowed researchers to assess the correlation between histone marks and DSBs and/or COs events. Understanding this correlation helps to unveil the interactions between chromatin modifiers and meiotic proteins. Several methods derived from ChIP-seq improved the resolution by which chromatin-protein interactions can be mapped. ChIP-exo initial steps are the same as for ChIP. However after immunoprecipitation unbound ds-DNA is digested from 5' to 3' by a lambda exonuclease from one end [142]. The digestion is blocked at the border of the protein-DNA covalent interaction. DNA sequences at the 3' of the exonuclease remain intact and are then sequenced. This step greatly increases the map resolution of the binding location because the non-protein binding fragments are decreased. Another method, called ChIPmentation, exploits a hyperactive Tn5 transposase – which is also used in ATACseq, see below – to insert sequencing primers in the pulled-down chromatin that is bound to the beads [143]. By simplifying preparation of the sequencing library, it allows the genome-wide analysis of bound proteins at high resolution, with only 10000 cells. However, these two last methods have never been proven to work in meiosis research so far.

The genome-wide distribution of proteins is often assessed along with analyses of the accessibility of DNA within the chromatin. Currently, several methods to study chromatin accessibility are available, such as DNase-seq [144], MNase-seq [145, 146], ATAC-seq [147, 148] and ATAC-see [149]. These methods are frequently utilized for capturing gene regulatory elements which often reside in open chromatin. In ATAC-seq, open chromatin regions are targeted by a hyperactive Tn5 transposase, that inserts sequencing adapters at all accessible DNA locations. DNase-seq relies on the sensitivity of open chromatin regions to DNase I activity. The chromatin targeted by DNAse I is subsequently purified, sequenced and mapped to the genome of interest, resulting in a genome-wide map of the chromatin accessible regions [150]. MNase-seq was developed to identify nucleosome occupancy and hence infer chromatin accessible regions. The micrococcal nuclease digests all accessible sequences, only DNA fragments wrapped around nucleosomes are recovered and mapped to the genome [146]. Both DNAse-seq and MNase-seq require millions of cells as input material to obtain a sufficiently high genome coverage and might therefore not be possible to adapt for all species. On the other hand, ATAC-seq can be used with as few as 500 cells in several species, which makes it suitable for application in meiocytes whose isolation is difficult and tedious. Despite the recent advances in high-throughput technology, most amplification methods are PCR-based and thus suffer the exponential amplification bias. In addition, they require well-sequenced genomes and clearly identified SNPs that might not fit every species.

Aim and outline of this thesis

Meiosis is a highly complex process, due to the number of biochemical factors involved and the changes in chromatin landscape and the chromosomal conformation, all needed for DNA repair, chromatin condensation and homolog pairing, synapsis and recombination. Over the past decades several methods have been developed in order to get a better insight of meiosis and to understand the meiotic recombination landscape. This study aims to understand the factors that shape the crossover landscape during meiosis in tomato using genome-wide sequence analysis techniques.

Chapter 2 of this thesis presents the adaptation of the ChIPseq method on tomato meiocytes. The improved ChIPseq protocol involves the isolation of chromatin from stage-specific anthers of tomato flowerbuds, immunoprecipitation of chromatin fragments with antiserum of interest. This protocol allows the analysis of the binding sites of meiotic proteins in tomato meiocytes. In addition, a bioinformatic pipeline was developed using Snakemake to analyze ChIP-seq data in a reproducible manner.

Chapter 3 presents the effects of CRISPR-Cas generated mutations of two anti-crossover factors, RECQ4 and FIGL1, on the frequency of COs in the interspecific tomato hybrid S. lycopersicum x S. pimpinellifolium. The cytogenetic analysis of chiasmata at diakinesis reveal a significant increase in COs in the absence of RECQ4 and a significant decrease in the absence of FIGL1.

Chapter 4 describes the distribution of accessible chromatin sites in tomato prophase I meiocytes using the ATAC-seq technique. The protocol is adapted for the purification of meiocytes and includes FANS step to obtain high-quality ATAC-seq DNA libraries. The sequence data analysis shows that meiotic samples have a higher chromatin accessibility over promoter regions than somatic samples. Also, the meiocytes have more accessible genes than leaf cells. The somatic samples, on the other hand, have a higher chromatin accessibility in the distal intergenic regions, which is in agreement with DNase-seq results. A comparison with chromatin datasets from other labs shows that the ATAC results for open chromatin in meiocytes in our study correspond with H3K4me3 patterns (Fuentes pers. Comm.).

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