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Ph.D. Thesis

Impact of DNA interhomolog polymorphism on meiotic crossover formation at the genome-wide and recombination hotspot scale in *Arabidopsis thaliana*

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Wpływ polimorfizmu DNA pomiędzy homologami na formowanie mejotycznych crossing-over w skali całogenomowej i na poziomie gorących miejsc rekombinacji u *Arabidopsis thaliana*

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Dedicated to my Husband, Adrian

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Contents

Streszczenie

Rekombinacja mejotyczna crossing-over jest kluczowym procesem polegającym na wzajemnej wymianie fragmentów chromosomów homologicznych, który prowadzi do tworzenia nowych kombinacji alleli w organizmach hybrydowych. Podczas rekombinacji mejotycznej, polimorfizm DNA pomiędzy homologami może mieć wpływ na częstość i rozkład crossing-over wzdłuż chromosomów. Jednak efekt ten pozostaje wciąż słabo poznany.

Aby zbadać związek między polimorfizmem, a rekombinacją w skali całego genomu zoptymalizowałam metodę genotypowania przez sekwencjonowanie (GBS), która umożliwia mapowanie zdarzeń crossing-over w różnych krzyżówkach *Arabidopsis thaliana*. Porównanie chromosomowego rozkładu crossing-over w liniach typu dzikiego i w tle mutantów *msh2*, które nie są zdolne do wykrywania błędnie sparowanych zasad w DNA, ujawniło znaczące różnice. Polegają one na tym, że miejsca bardziej polimorficzne, położone w regionach przycentromerowych są mniej aktywne rekombinacyjnie w mutancie *msh2*, natomiast crossing-over zachodzi z wyższą częstością w regionach subtelomerowych, które zawierają znacznie mniej polimorfizmów.

Ponadto, opracowałam nowe narzędzie do badania rozkładu crossing-over na poziomie gorących miejsc rekombinacji, które nazwaliśmy *typowaniem nasion* (ang. *seed-typing*). Metoda ta umożliwia zarówno pomiar częstości crossing-over, jak i precyzyjne mapowanie miejsc zajścia poszczególnych zdarzeń rekombinacyjnych. Korzystając z tego podejścia, zidentyfikowałam bardzo polimorficzny interwał ChP z trzema gorącymi miejscami rekombinacji: *Aro, Coco* i *Nala*. Nasze wyniki pokazują, że centra gorących miejsc rekombinacji są praktycznie pozbawione polimorfizmów pojedynczego nukleotydu (ang. SNP), ale obecność SNP w ich bezpośredniej bliskości stymuluje aktywność crossing-over w danym miejscu. Ponadto, zmiany strukturalne otaczające badany interwał chromosomowy nie mają wpływu na częstość rekombinacji, jeśli nie obejmują bezpośrednio gorących miejsc rekombinacji. Przy użyciu linii *A. thaliana* z naturalną delecją w *Coco* lub po wygenerowaniu sztucznej delecji przy pomocy CRSIPR/Cas9, potwierdziliśmy, że u Arabidopsis nie występuje współzawodnictwo między blisko zlokalizowanymi gorącymi miejscami rekombinacji.

Dodatkowo, zbadaliśmy związek pomiędzy SNP, MSH2 i rekombinacją poprzez mapowanie crossing-over w tle mutanta *msh2*. Ujawniło to interesującą tendencję polegającą na tym, że bardziej polimorficzne gorące miejsca rekombinacji są mniej aktywne po wyłączeniu systemu rozpoznawania błędnie sparowanych zasad (tj. w mutancie *msh2*). Bezpośredni wpływ polimorfizmów został sprawdzony w liniach, które są heterozygotyczne tylko w badanym interwale. Linie te, wykazywały wyższą częstość rekombinacji w badanym interwale w porównaniu z pełni homozygotycznymi liniami wsobnymi. Efekt ten nie był widoczny w roślinach posiadających mutację w genie *MSH2*, co sugeruje, że MSH2 promuje zachodzenie crossing-over w gorących miejscach rekombinacji otoczonych regionami o wysokiej gęstości SNP.

Słowa kluczowe: Mejoza, gorące miejsce rekombinacji, polimorfizm, MSH2, Arabidopsis

Abstract

Meiotic crossover recombination is a fundamental process relying on reciprocal exchanges of DNA fragments between homologous chromosomes, which enables to create novel allelic combinations in hybrid organisms. During meiotic recombination, the DNA polymorphism between parental chromosomes can be detected and influence the crossover frequency and distribution along chromosomes. However, this effect remains still poorly understood.

To investigate the relation between polymorphisms and recombination at the genome-wide scale, I optimized the genotyping-by-sequencing (GBS) method that enables crossover mapping in different *Arabidopsis thaliana* hybrids. Comparison of crossover distributions in wild type and in *msh2* mutants, defective in DNA mismatch recognition, revealed the remodeling of crossover landscapes from the diverse pericentromeres towards the less-polymorphic sub-telomeric regions in the *msh2* mutant.

Furthermore, I also developed a new tool to study crossover distribution at the hotspot level in Arabidopsis, called *seed-typing*. It provides both, a precise measurement of the crossover frequency and mapping of crossover breakpoints at the fine scale. Taking advantage of this approach, I identified highly polymorphic ChP interval with three recombination hotspots: *Aro, Coco* and *Nala*. Our results demonstrate that the hotspot centres are practically devoid of polymorphisms but the presence of SNPs around hotspots stimulate their crossover activity. We also show that structural changes surrounding the interval have no effect on the crossover frequency unless they directly involve recombination hotspots. We also confirmed lack of short-distance hotspot competition in Arabidopsis using a line with a natural deletion in *Coco* and after deleting a part of *Coco* by CRISPR/Cas9. The detailed protocol for efficient generation of desired mutations in different Arabidopsis accessions is also included in this dissertation.

The relation between polymorphisms, MSH2 and recombination was investigated by crossover mapping in the *msh2* background at the hotspots level. It revealed an interesting trend that more polymorphic hotspots are less active after disabling mismatch recognition system (i.e., in the *msh2* mutant). The direct effect of polymorphisms was examined in lines, which are heterozygous only within the tested interval. They showed higher crossover rates when compared to inbred lines. This effect was not observed in sibling plants carrying the *msh2* mutation, which highlights that MSH2 promotes crossover recombination in hotspot polymorphic regions.

Key words: Meiosis, meiotic hotspot, polymorphism, MSH2, Arabidopsis

List of scientific articles included in the dissertation

1. Dluzewska J[1], **Szymanska M[1]**, Ziolkowski PA. Where to cross over? Defining crossover sites in plants. Front Genet. 2018 Dec 12;9:609. Doi: 10.3389/fgene.2018.00609
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   [1]join-first author

2. Blackwell AR[1], Dluzewska J[1], **Szymanska-Lejman M**, Desjardins S, Tock AJ, Kbiri N, Lambing C, Lawrence EJ, Bieluszewski T, Rowan B, Higgins JD, Ziolkowski PA*, Henderson IR*. MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis. EMBO J. 2020 Nov 2;39(21):e104858. Doi: 10.15252/embj.2020104858
   PMID: 32935357; PMCID: PMC7604573
   Ministry points (MNiSW): 200
   Impact factor (2020): 11.598
   [1] - joint first authors
   * - joint corresponding authors

3. Bieluszewski T, **Szymanska-Lejman M**, Dziegielewski W, Zhu L, Ziolkowski PA. Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions. In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology, vol 2484. Humana, New York, NY. Doi: 10.1007/978-1-0716-2253-7_13
   PMID: 35461452
   Ministry points (MNiSW): 70

4. **Szymanska-Lejman M**, Dziegielewski W, Dluzewska J, Kbiri N, Bieluszewska A, Poethig RS, Ziolkowski PA. The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids. Nat Commun. 2023 Jan 3;14(1):33. Doi: 10.1038/s41467-022-35722-3.
   PMID: 36596804; PMCID: PMC9810609.
   Ministry points (MNiSW): 200
   Impact factor (2022): 17.694

Abbreviations

BT	Bow Tie interval
ChP	Chili Pepper interval
CO	Crossover
dHJ	Double Holliday junction
DSBs	Double strand breaks
ESIL	Extremely Short Interval Line
GBS	Genotyping-by-Sequencing
LR-PCR	Long-range PCR
MMR	Mismatch detection system
MSH2	MutS homolog 2
NCO	Non-crossover
NGS	Next generation sequencing
RF	Recombination frequency
SDSA	Synthesis-dependent strand annealing
SNP	Single nucleotide polymorphism
TIGER	Trained Individual GenomE Reconstruction

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Description of the results of the doctoral thesis

1. Scientific profile of the PhD candidate

I started Bachelor studies in Biotechnology at the Adam Mickiewicz University, Poznań, Poland in 2012. My Bachelor thesis titled "Studies of natural variability of genomic region spanning *MSH2*, *AT3G18530* and *AT3G18535* genes in *Arabidopsis thaliana*" was performed in the Department of Molecular and Systems Biology at the Institute of Bioorganic Chemistry (Polish Academy of Sciences) in Poznań. My results are included in the article (Doi: 10.1186/s12864-016-3221-1), published in BMC Genomics, in which I am a co-author. In 2015, I continued my education at the Adam Mickiewicz University and started my two-year Master studies in Biotechnology. My Master thesis was performed in the Department of Human Molecular Genetics in the Institute of Molecular Biology and Biotechnology at AMU. The title of my Master thesis was "In silico and in vitro identification and characterization of IRF1 inhibitors in vascular inflammation".

Furthermore, from 2014-2017 I have been an active member of Synthetic Biology Section at the Adam Mickiewicz University in Poznań. As a group of five students we developed a multipromoter expression system for an efficient production of proteins in *Escherichia coli.* We took part in iGEM (International Genetically Engineered Machine) competition organised by Massachusetts Institute of Technology and were awarded with the silver and gold medals (in 2015 and 2016, respectively) and with a special prize for The Best Manufacturing Project in 2016 (http://2016.igem.org/Team:UAM_Poznan).

In 2017, I started my PhD studies at the Adam Mickiewicz University under supervision of Prof. UAM Piotr Ziółkowski. I was involved in the projects covering the topics of understanding the mechanisms of crossover control and in identification of chromatin factors affecting meiotic crossover formation in plants. I also contributed to other projects carried out in the Genome Biology Lab, which resulted in being a co-author of six publications. I also had the opportunity to present my results as posters and oral presentations at nine international conferences.

2. Introduction

Meiosis underlies the heredity and genetic variation. It is a specialized cell division program which leads to production of gametes, which are crucial for sexual reproduction (Villeneuve and Hillers, 2001). Unlike mitosis, in meiosis the single round of DNA replication is followed by two rounds of cell divisions which give rise to haploid cells, with reduced number of chromosomes. Meiotic recombination takes place in the prophase of the first meiotic division and, apart from securing proper chromosome segregation, drives shuffling of existing genetic variants. This is due to crossovers (COs), reciprocal exchanges of DNA fragments between homologous chromosomes, which result in creation of novel haplotypes. The emergence of genetic diversity is crucial for the plant adaptation to changes in environmental conditions as well as crop improvement. Domestication and constant selective breeding contributed to the higher yields and more efficient harvesting but reduced the genetic variety. For instance, current tomato cultivars have lost 95% diversity of their wild relatives (Fuentes et al., 2022). Additionally, the majority of crops are self-pollinating, which leads to elimination of heterozygosity (Busch et al., 2022). Plant breeding programmes are pushed to introgress the desired alleles connected with disease resistance, stress tolerance or adaptation to changing environment by outcrossing. This can be achieved by crossovers during meiosis which allow shuffling of existing genetic variants and favour inheriting advantageous from the agronomical point of view combinations of traits. However, the crossover number and distribution along chromosomes are tightly regulated as in most species more than 80% of the crossover recombination events occur in less than a quarter of their genome (Mercier et al., 2015). One of the factors playing role in the crossover distribution is polymorphism between homologous chromosomes (Blackwell et al., 2020; Dluzewska et al., 2018; Szymanska-Lejman et al., 2023). In this Ph.D. dissertation, I provide a comprehensive analysis of the relation between interhomolog polymorphisms and meiotic recombination at the genome-wide scale and at the meiotic hotspot level.

Meiotic recombination

In all eukaryotes, meiotic recombination occurs in a similar way. It is initiated by the programmed DNA double-strand breaks (DSBs) formed by conserved topoisomerase VI-like complex (comprising SPO11 and MTOPVIB) (Fig. 1). DSBs are later resected to

generate 3' single stranded DNA ends (Keeney and Neale, 2006). Next, they are bound by the RecA-like recombinases (RAD51 and DMC1), which mediate invasion of either a sister chromatid or homologous chromosome forming an intermediate molecule called a displacement loop (D-loop) (Fig. 1) (Hunter, 2015). These joint molecules can be processed in numerous ways and can be resolved as crossover (CO) or non-crossover (NCO). CO is a reciprocal exchange between parental chromosomes, while NCO is a local and non-reciprocal recombination event as the change is observed only on one chromosome. The majority of the joint-molecules, which are formed after invading the homologous chromatid, are dissociated by DNA helicases and further repaired via synthesis-dependent strand annealing (SDSA) resulting in NCOs (Fig. 1). A subset of the recombination intermediates can undergo a second-end capture to form a double Holliday junction (dHJ) which can be further resolved as COs (Fig. 1).

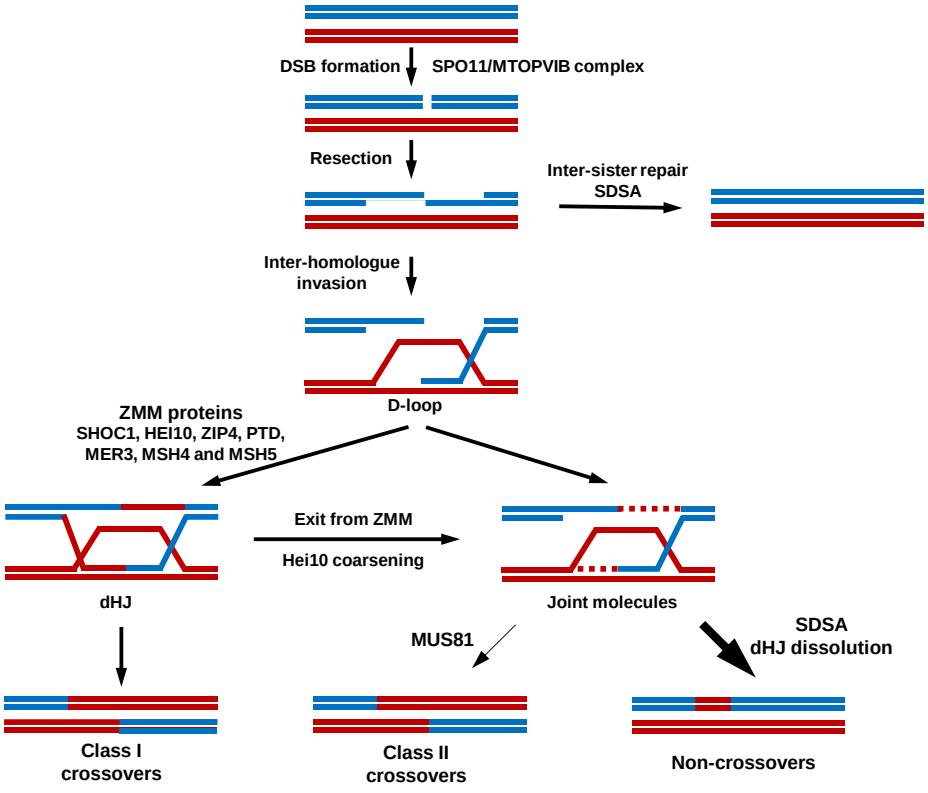

Fig. 1 Model of meiotic recombination. Meiotic recombination starts with the formation of DSBs. Next DNA ends are resected (blue chromosome) and DSBs can be repaired by sister chromatid or can invade the homologous chromosome (red chromosome) to form a displacement loop (D-loop). If the D-loop is stabilized by ZMM proteins it can form a double-Holliday junction (dHJ) and can be resolved as class I crossover. Even though, many recombination intermediates initially

enter the ZMM crossover pathway, they can exit the pathway. Alternatively, those recombination intermediates can be resolved as class II crossovers or non-crossovers by SDSA or dHJ dissolution. Based on the model described in Mercier et al. 2015.

In Arabidopsis, around 200 DSBs are generated per meiosis and only ~10 of them will be repaired as COs. Among them, 85-90% are class I COs, formed via ZMM pathway (Fig. 1) (Mercier et al., 2015). ZMM stands for a group of proteins that are involved in the pathway (Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, and Msh5) initially identified in yeast (Börner et al., 2004). In Arabidopsis, these are SHOC1, HEI10, ZIP4, PTD, MER3, MSH4 and MSH5 (Chelysheva et al., 2012; Higgins et al., 2004; Mercier et al., 2005). Those ZMM proteins play role in stabilizing and protection of recombination intermediates from dissolution, and further promote formation of class I crossovers (Lynn et al., 2007). In the absence of any of the ZMM proteins in Arabidopsis, COs are strongly reduced to 15% of the wild type level (Mercier et al., 2015). This remaining 10-15% of COs are class II COs. They are dependent on partially redundant pathways involving structure-specific endonucleases, mainly MUS81 (Fig. 1) (Berchowitz et al., 2007; Higgins et al., 2008; Kurzbauer et al., 2018).

The number of crossovers and the distances between them are tightly controlled. It is due to CO homeostasis, obligatory CO and CO interference, phenomena that are conserved among eukaryotes (Dluzewska et al., 2018; Hunter, 2015; Mercier et al., 2015). CO homeostasis describes that crossover number is maintained at the same level despite the changes in the number of DSBs (Martini et al., 2006; Varas et al., 2015). Obligatory CO defines that each homolog pair receives at least one CO to ensure accurate chromosome segregation (Mercier et al., 2015). CO interference prevents the formation of the two crossovers close to each other, as the occurrence of one crossover reduces the likelihood of a second one nearby. Class I COs are sensitive to interference, while the class II are not subjected to this phenomenon (Berchowitz and Copenhaver, 2010; Dluzewska et al., 2018).

HEI10, an E3 ligase, is a plant orthologue of yeast Zip3, which is one of the ZMM proteins (Chelysheva et al., 2012). HEI10 is highly dosage-sensitive as overexpression of HEI10 results in increases of crossover recombination (Ziolkowski et al., 2017). Initially, it is loaded onto synapsed chromosomes as many small foci in the prophase I but till pachytene, HEI10 is concentrated into approximately ten large foci defining the class I crossover sites (Morgan et al., 2021). HEI10 protein dynamics in pachytene and the

relationship between class I CO and HEI10 expression level allowed to propose a diffusion-mediated HEI10 coarsening model. This model explains that even though many recombination intermediates are initially stabilized by ZMM proteins and enter the class I crossover pathway, only a few of them will become class I crossovers (Fig. 1) (Lloyd, 2022; Morgan et al., 2021). This model supports the crossover interference as larger HEI10 foci tend to grow bigger at the expense of adjacent smaller foci which results in greater distance between final HEI10 foci (i.e. class I crossovers) than expected by random positioning (Morgan et al., 2021).

As only a small fraction of DSBs is repaired as COs, there are pathways to promote DSBs repair via NCOs. Repair of DSBs leading to NCO is usually performed using homologous chromosome as a template and can be sometimes visible as short gene conversions. Several helicases (FANCM, RECQ4A/B) prevent CO formation through dissembling the recombination intermediates to stimulate NCO formation via SDSA (Crismani et al., 2014; Séguéla-Arnaud et al., 2015) (Fig. 1). Additionally, FIGL1 counteracts DMC1/RAD51-mediated inter-homologue strand invasion (Girard et al., 2015). Mutants of any of those anti-crossover pathways demonstrate elevated levels of class II COs. For instance, CO frequency in *recq4a recq4b* double mutants increases sixfold (Séguéla-Arnaud et al., 2015). Furthermore, by combining increased HEI10 dosage and *recq4a recq4b* mutations, a massive, additive increase in crossover frequency was observed (Serra et al., 2018a).

Meiotic hotspots in plants

Meiotic recombination does not occur randomly across chromosomes. In eukaryotes crossovers occur in hotspots, in which recombination frequency is higher than the genome average (Wu and Lichten, 1995). There is a strong evidence for the existence of meiotic hotspots in plants (Mezard, 2006). It has been reported in maize that recombination across 140-kb of the *a1-sh2* region and 1.5-kb *bronze* locus is significantly higher than the genome average (Dluzewska et al., 2018; Dooner, 1986; Yao et al., 2002). Meiotic hotspots have been also identified in Arabidopsis: *3a* (Choi et al., 2013; Yelina et al., 2012), *3b* (Choi et al., 2013), *14a* and *130*× (Drouaud et al., 2013), hotspots within the *RAC1* and *RPP13* disease resistance genes (Choi et al., 2016; Serra et al., 2018b). Our analysis of the crossover distribution within highly polymorphic pericentromeric interval

revealed the presence of three neighbouring hotspots, called *Aro*, *Coco* and *Nala* (Szymanska-Lejman et al., 2023). *Coco* (62.14 cM/ Mb) is one of the strongest hotspots in Arabidopsis discovered so far (Szymanska-Lejman et al., 2023).

Crossovers are associated with the occurrence of meiotic DSBs, which are not evenly distributed within the genome. In mice and humans, the majority of DSB hotspots are determined by PRDM9, which binds to specific DNA sequence motifs (Baudat et al., 2010, 2013). Plants do not have PRDM9, therefore DSB formation is mainly associated with nucleosome occupancy (Choi et al., 2018). Purification and sequencing of SPO11-bound oligonucleotides provided nucleotide resolution maps of meiotic DSB distribution in *Arabidopsis thaliana* (Choi et al., 2018). As crossovers initiate from programmed DNA double-stranded breaks, the DSB sites are one of the factors determining crossover hotspots (Choi and Henderson, 2015).

In plants, the majority of crossover hotspots are observed in low nucleosome density regions (LNDs), which corresponds to gene promoters and 3' ends of the genes (Choi et al., 2018). This coincides with deposition of H2A.Z histone variant at transcription start sites (TSS) and transcription termination sites (TTS) (Choi et al., 2013). The chromatin accessibility in hotspots is also determined by DNA sequence motifs. Several motifs have been enriched within crossover hotspots including poly (A/T) motif, a CTT/GAA repeat (Choi et al., 2013; Shilo et al., 2015; Wijnker et al., 2013), and to lesser extent, the CCN (Shilo et al., 2015), and CT repeats (Rowan et al., 2019). It was also showed that Arabidopsis hotspots can be suppressed by CpG methylation via the RNA-dependent DNA methylation (RdDM) pathway (Yelina et al., 2015).

Hotspot competition

Hotspot competition is a phenomenon observed in yeasts and mammals (Billings et al., 2013; Fan et al., 1997; Fowler et al., 2018; Robine et al., 2007). It demonstrates compensation of the activity between adjacent hotspots on the same homolog (Fowler et al., 2018). In yeast, it was reported that due to DSB interference, DSB hotspots communicate along chromosomes to inhibit DSB formation over long distances (~200 kb) (Fowler et al., 2018). Thus, it was shown that generation of an artificial DSB hotspot in yeast decreases the activity of neighbouring hotspots (Robine et al., 2007). It is due to $TEL1^{ATM}$ and $MEC1^{ATR}$ which are DNA damage-response kinases, which play a crucial

role in controlling the DSB numbers. In Arabidopsis, mutants of homologs of these genes, *atm* and *atr,* show decreased fertility (Culligan et al., 2008; Garcia et al., 2003). It was also showed that ATM limits the number of non-interfering COs and affects chromatin loop size and SC length and width (Kurzbauer et al., 2021).

However, the phenomenon of hotspot competition was not observed in Arabidopsis (Yelina et al., 2015). The analysis performed in subtelomeric *3a* and *3b* hotspots, which are separated by 10 kb, showed that silencing one of the hotspot via RNA-dependent DNA methylation (RdDM) does not affect the activity of the neighbouring one (Yelina et al., 2015). We observed that partial inactivation of one of the hotspot in the interval resulted in decreased activity of this hotspot but had no impact on the activity of the adjacent hotspots in the interval. This further confirms lack of short-distance hotspot competition in Arabidopsis (Szymanska-Lejman et al., 2023).

Methods to detect crossover breakpoints at the fine scale in plants

The relatively low number of crossovers is the major limitation in crossover mapping with high resolution. The crossover frequencies of the known Arabidopsis hotspots are between 0.1-0.5 cM, which means that there is one to five crossovers in the particular hotspot per one thousand meiotic divisions (Choi et al., 2013; Drouaud et al., 2013; Yelina et al., 2012). Thus, identification of 100 crossovers at the analysed hotspot would require screening ~100 000 meioses, which is very challenging with the current methods (Choi and Henderson, 2015).

Till now, there was only one method allowing fine-scale characterization of the crossover pattern within a hotspot, called *pollen-typing* (Drouaud and Mézard, 2011). It is analogous to *sperm-typing* developed previously in humans (Jeffreys et al., 1998) and mice (Cole and Jasin, 2011) to analyse crossovers in hotspots. Briefly, the *pollen-typing* method relies on detection of SNPs between two haplotypes based on allele-specific amplification. Series of PCRs are performed using the highly diluted pollen DNA derived from F1 hybrids as a template (Drouaud and Mézard, 2011). The recombination rate is estimated based on the dilution of the gamete templates to obtain less than one putative recombinant molecule per PCR reaction (Drouaud and Mézard, 2011). It provides high resolution but diluting the gamete template in order to titrate parental and crossover molecules is challenging and amplifies the risk of errors (Choi et al., 2013; Drouaud et

al., 2013). Therefore, we described a novel approach for investigating the crossover distribution in *Arabidopsis thaliana*, called *seed-typing* (Szymanska-Lejman et al., 2023). The strategy is based on fluorescent reporter system to easily measure recombination frequency and further select recombinant seeds, which experienced CO event between two reporter genes (Szymanska-Lejman et al., 2023).

Detection of interhomolog polymorphisms

DNA polymorphism refers to the presence of ubiquitous genetic variants that can occur among different individuals or populations (Mitchell-Olds and Schmitt, 2006). It includes polymorphism in the nucleotide sequence (single nucleotide polymorphisms, SNPs), length (insertions and deletions – indels) and larger, structural rearrangements.

When an interhomolog joint molecule is formed during meiotic recombination, sequence polymorphisms between homologous chromosome (so called interhomolog polymorphisms) can be detected as mismatches (Chakraborty and Alani, 2016). The evolutionary conserved DNA mismatch repair (MMR) system is primarily responsible for detection the polymerase errors arising during replication. But it also plays a role in meiotic recombination since it recognizes the mismatches. MutS homologs form heterodimers, which can recognize different types of mismatches or small indels. MutSα (heterodimer of MSH2-MSH6) recognizes base-base mismatches and short insertion/deletion loops (IDLs) and MutSβ (heterodimer of MSH2-MSH3) binds only IDLs (Fukui, 2010). In plants, there is also the MSH2-MSH7 heterodimer, which preferentially recognizes some base–base mismatches (Culligan and Hays, 2000). Interestingly, MSH7 was recently validated as the causative gene for *Ph2* locus in wheat, thus it contributes to inhibition of homoeologous recombination (between chromosomes from different wheat genomes) (Serra et al., 2021). After the strand invasion during meiotic recombination, MutS heterodimers detect mismatches and recruit MutL heterodimers (MLH1-MLH3 or MLH-PMS1). MLH1-MLH3 (MutLγ) result in repair leading to class I crossovers. As MSH2 is present in all MutS heterodimers recognizing the mismatches, inactivation of this protein leads to inactivation of the whole MMR system.

Among the MutS homologs, there is also a MSH4-MSH5 heterodimer, which is meiosis-specific and is not involved in mismatch detection. MSH4-MSH5 complex

belongs to the ZMM proteins promoting class I crossovers. It is responsible for stabilizing the double Holliday Junctions (dHJs) (Higgins et al., 2004; Snowden et al., 2004). Additionally, at the hotspot level MSH4 was shown to play role in generation of NCOs but to a different extent depending on the hotspot (Drouaud et al., 2013).

Interhomolog polymorphism influence the crossover formation pathways

Many factors control the distribution of the crossovers along the chromosome. Due to genetic variation within species there are modifiers acting in cis or in trans (Yao and Schnable, 2005). Cis-modifications of the recombination is due to local genetic variation, for instance, SNPs between homologs or structural rearrangements such as inversions or translocations (Borts and Haber, 1987; Cole et al., 2010; Yao and Schnable, 2005). Trans modifiers are defined as loci encoding diffusible molecules that control recombination throughout the genome such as HEI10, FANCM, RECQ4, TAFVIB, HCR1 or SNI1 (Lawrence et al., 2019; Nageswaran et al., 2021; Séguéla-Arnaud et al., 2015; Zhu et al., 2021; Ziolkowski et al., 2017).

During meiotic recombination, heterozygosity refers to sequence polymorphisms between homologous chromosomes. Thus, in hybrids, generated by crossing two genetically different Arabidopsis accessions, for instance Col×L*er* or Col×Ct, the heterozygosity pattern is observed throughout the genome. In turn, in Col×Col inbred, the homologous chromosomes are identical, so no difference between parental chromosomes is observed. The genetic differences do not influence DSB formation, which is the initial step of meiotic recombination. The mismatches are formed at the stage of interhomolog strand invasion and can influence the choice of the repair pathway (Fig. 2).

In budding yeast, MSH2 heterodimers recognize the heteroduplex and result in limited extension and rejection of recombination intermediates via recruitment of helicases, which promote disassembly of the mismatched D-loops (Spies and Fishel, 2015; Sugawara et al., 2004). Studies in budding yeasts have demonstrated that elevated levels of polymorphism between two budding yeast species locally inhibit meiotic recombination at the *URA3* hotspot level (Borts and Haber, 1987). While budding yeast hybrids deficient in mismatch detection system (MMR) demonstrated an increased recombination frequency (Hunter et al., 1996). In mice, the inverted region of ~140 bp present in the highly polymorphic *A3* hotspot resulted in local increase of non-crossovers

at the expense of repair via crossovers (Cole et al., 2010). It was shown that in mammals, polymorphisms affect selection of the repair pathway and may alter CO placement directing their formation outside of the more polymorphic regions (Cole et al., 2010).

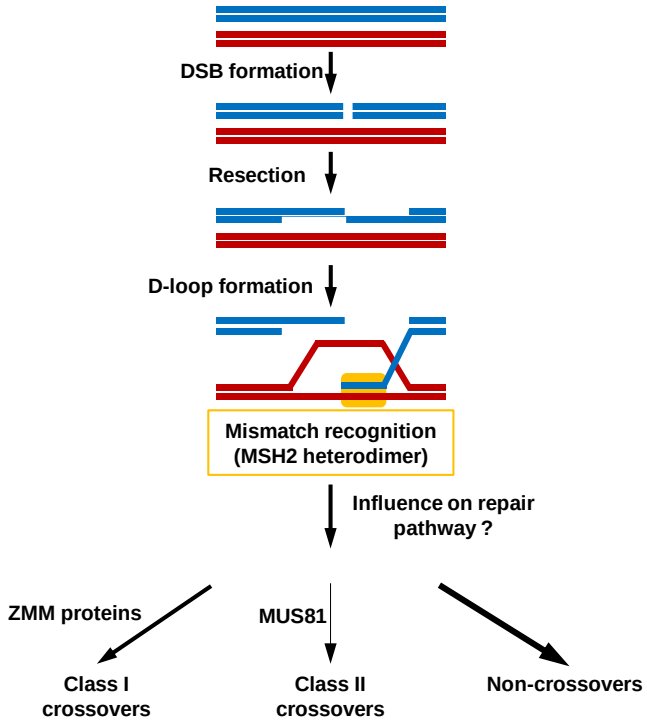

Fig. 2 Model of mismatch recognition during meiotic recombination in plant hybrids. Meiotic recombination is initiated by DSB formation followed by resection and providing a 3' single-stranded DNA (blue chromosome). Mismatches between parental chromosomes (marked in orange rectangle) are detected during the D-loop formation. Mismatch repair system detects the mismatches, however its influence on the repair pathway is currently not known. Parental chromosomes are presented in blue and red.

In Arabidopsis, class I and class II crossovers demonstrate distinct sensitivities to interhomolog polymorphisms (Ziolkowski, 2022). Indirect studies of non-interfering pathway, using *fancm* mutant, showed a very slight recombination increase in hybrid when compared to inbred Arabidopsis (Crismani et al., 2014; Girard et al., 2015). However, an elevated crossover frequency in hybrids was observed in *fancm figl1* double mutants when compared to either wild type or *figl1* (Girard et al., 2015). This suggests that in the absence of FIGL1, the FANCM-dependent pathway is able to repair

heterozygous chromosomal regions by crossover (Girard et al., 2015). Also combining *recq4a recq4b* and *figl1* mutations led to 7.8-fold increase in recombination in hybrid contexts (Fernandes et al., 2017). Studies in *A.thaliana* show that unleashing the non-interfering pathway by mutating anticrossover helicases, *recq4a recq4b*, did not result in increases of recombination in polymorphic intervals (Fernandes et al., 2017). The preference of class II crossovers towards homozygous regions was also confirmed at the megabase scale, in *fancm zip4* mutants where only class II is active (Ziolkowski et al., 2015). Finally, the analysis of crossover landscapes in a hybrid population suggested that class I, not class II, repair pathway mediates crossover association with elevated SNP density at the kilobase scale (Blackwell et al., 2020).

Impact of SNPs on crossover distribution

Analysis of crossover rate in Arabidopsis hotspots demonstrated a negative correlation between interhomolog polymorphism and recombination (Choi et al., 2013, 2016; Drouaud et al., 2013; Serra et al., 2018b; Yelina et al., 2013, 2015). Studies performed on *14a* hotspot using three crosses with different polymorphism levels showed that the hotspot almost disappeared in the most polymorphic hybrid Col×Pyl1 (Drouaud et al., 2013). The formation of crossovers at SNP-free sites is even more visible in highly polymorphic Arabidopsis hotspot located in *RAC1* disease resistance gene (Choi et al., 2018). Further analysis of the crossover distribution in different hybrids within the *RAC1* confirmed a negative relationship between polymorphism and crossover frequency (Serra et al., 2018b). What is more, increasing the number of both, class I crossovers by overexpression of HEI10, and class II crossovers by mutating anticrossover genes (*fancm recq4a recq4b*, *figl1)* resulted in unchanged or even decreased *RAC1* recombination rate (Serra et al., 2018b).

Genome-wide studies on crossover distribution were performed to compare crossover landscape between Arabidopsis quasi-inbred lines, in which a few hundred genetic markers were introduced through mutagenesis with hybrid line (Lian et al., 2022). The studies revealed no significant difference between the analysed lines suggesting that polymorphisms do not determine the megabase-scale crossover distribution (Lian et al., 2022).

In contrast, other studies in Arabidopsis, suggested a crossover preference for more polymorphic regions (Blackwell et al., 2020; Szymanska-Lejman et al., 2023; Ziolkowski et al., 2015). At the genome-wide scale, recombination is positively associated with SNP density in Arabidopsis hybrids (Blackwell et al., 2020). Crossover rates are increased in the polymorphic, pericentromeric regions. Furthermore, mapping crossovers in hybrids using *msh2* mutant, defective in mismatch recognition, demonstrates a significant redistribution of crossovers towards less polymorphic, subtelomeric regions (Blackwell et al., 2020).

Also, at the megabase scale, heterozygous regions receive more crossovers when they are juxtaposed to homozygous segments on the same chromosome (Ziolkowski et al., 2015). This phenomenon, called heterozygosity juxtaposition effect, depends on the mismatch detection factor – MSH2 (Blackwell et al., 2020).

Additionally, the direct effect of polymorphisms on CO formation at the hotspot scale was investigated in lines, in which only the hotspot-containing interval is heterozygous (Szymanska-Lejman et al., 2023). The increased recombination frequency in this interval when compared to inbred (homozygous) or hybrid (heterozygous) further suggests the positive correlation of COs and SNPs. This effect is associated with MSH2, as in *msh2* the crossover rate in the investigated interval was at the level of inbred (Szymanska-Lejman et al., 2023).

Taken together, there is a relationship between polymorphism, MSH2 and crossover events. But the question is the degree to which the polymorphisms and MSH2 affect the crossover distribution at the genome-wide and at the hotspot scale in plants.

Impact of structural rearrangements on crossover distribution

The first studies on the effects of chromosomal rearrangements on recombination were carried out in fruit fly, *Drosophila melanogaster*, in which the CO formation was suppressed in a region of chromosome III that contained an inversion in heterozygous state (Sturtevant 1926). It has been studied also in Arabidopsis that chromosomal rearrangements such as large deletions or inversions between two accessions are associated with local crossover suppression (Ederveen et al., 2015; Rowan et al., 2019; Salomé et al., 2012; Zapata et al., 2016). In Col×Sha hybrid a significantly lower

crossover frequency was observed in region overlapping the inversion (Loudet et al., 2002; Salomé et al., 2012; Ziolkowski et al., 2015). Similarly, in Col×L*er,* CO formation is suppressed in a 1.2-Mb inversion on the short arm of chromosome 4 (Rowan et al., 2019; Zapata et al., 2016). Consistent with that, reversing the inversion of 1.2 Mb in Col background by CRISPR/Cas9-engineering restored the meiotic crossovers in this region in Col×L*er* hybrids, in which recombination used to be inhibited due to the structural differences (Schmidt et al., 2020). The high-throughput sequencing of L*er*×Col hybrids provided information about more than 17,000 COs and further confirmed the trend of CO suppression within different types and lengths of structural variants (Rowan et al., 2019). However, this CO inhibition is limited only to the rearrangements, as the surrounding regions present even higher recombination frequency than genome average (Rowan et al., 2019). How the presence of structural rearrangements in the vicinity of recombination hotspots influences their activity has not been investigated. Thus, using highly polymorphic interval we showed that natural structural variation surrounding the recombination hotspots have no effect on crossover frequency in the investigated interval (Szymanska-Lejman et al., 2023).

3. Aims of the thesis

The principal goal of this dissertation is to investigate the impact of interhomolog polymorphisms on meiotic recombination at the genome-wide scale and at the level of a recombination hotspot in *Arabidopsis thaliana*. Achieving this goal required:

- Performing comprehensive analysis and summary of the most recent achievements connected with defining the crossovers in plants;
- Optimizing and validating the GBS (Genotyping-by-sequencing) method which enables analysis of the crossover distribution at the genome-wide scale;
- Preparing a detailed, CRISPR/Cas9-based protocol for efficient generation of mutants in different Arabidopsis accessions;
- Setting *seed-typing* method to study crossover distribution at the hotspot scale;
- Characterizing ChP and BT intervals in terms of polymorphism density and chromatin landscape;
- Investigating the effect of indels and structural variation on crossover frequency within the ChP interval;
- Deciphering the role of MSH2 on recombination in relation to SNP density at the hotspot scale;
- Generation lines for studying the direct effect of polymorphism on recombination at the hotspot scale.

4. The main theses and achievements of the article "Where to cross over? Defining crossover sites in plants", *Frontiers in Genetics, 2018*.

Despite the prominent role of crossovers in creating novel combinations of alleles, its number and distribution are subjected to a tight control by different genetic and non-genetic factors. The review of Dluzewska & Szymanska et al. (2018) summarizes the current knowledge about factors influencing the recombination initiation sites in plant genomes. We also discussed how crossover formation and distribution are affected by interhomolog polymorphisms at the level of a chromosome, at the hotspot scale and when heterozygous megabase regions are juxtaposed to homozygous segments. Moreover, we described the influence of heterozygosity on crossover formation pathways.

I contributed to this article by participating in the development of its concept as well as in the preparation of some subsections. Specifically, I was involved in writing "General information about crossover in plants" by describing the possible meiotic recombination pathways to repair DSBs. Next, I was also responsible for preparing "Impact of DSB on crossover distribution" section focussing on chromatin structure and DNA motifs within meiotic hotspots. Moreover, I briefly described the "hotspot paradox" hypothesis, which predicts fast erosion of hotspot sequence due to biased gene conversion. As a result, a DSB occurring at the hotspot side will be repaired using an uncut homolog which can lead to rapid hotspot extinction. Together with my colleague, Julia Dluzewska, I am the first author in this article.

The entire article presents a comprehensive analysis of factors characterizing meiotic hotspots emphasizing the role of interhomolog polymorphism on crossover distribution. Most of the aspects discussed in the article have already been briefly described in the introduction of this dissertation. Our review has more views than 99% of all Frontiers articles (https://www.frontiersin.org – data for January 2023) which proves its substantive usefulness among the meiotic community.

5. The main theses and achievements of the article "MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis", *The EMBO Journal, 2020*.

The main aim of this work was to investigate the relation between meiotic recombination, sequence polymorphisms and MSH2. It was done by analysing the CO landscapes in wild type hybrids and after inactivation of the mismatch detection system by mutating MSH2. Crossover distribution maps were generated based on the analysis of the genome-wide sequencing data that were either obtained specifically for this work or have already been published in the team of Prof. Henderson (Lawrence et al., 2019; Serra et al., 2018a).

The crossovers were identified based on Genotyping-by-sequencing (GBS) method (Rowan et al., 2015, 2019), which enables robust and cost-efficient fine-mapping of COs. This approach relies on next-generation sequencing (NGS) of large populations and further identification of crossover sites based on SNPs between parental genotypes (Rowan et al., 2015). GBS technique has been widely used in genetic diversity analysis, such as genome-wide association studies, QTL mapping or identification of crossover breakpoints (Blackwell et al., 2020; Wickland et al., 2017; Zhu et al., 2021; Ziolkowski et al., 2017).

I was responsible for setting and optimization this technique in the Laboratory of Genome Biology. I did a one-month internship in Prof. Ian Henderson Lab from the Department of Plant Sciences at the University of Cambridge who has an extensive experience in implementing the GBS method in meiotic recombination studies (Blackwell et al., 2020; Lawrence et al., 2019; Serra et al., 2018c; Underwood et al., 2018; Zhu et al., 2021). I learned how to analyse the NGS data and to identify CO breakpoints in a segregating population using Trained Individual GenomE Reconstruction (TIGER). TIGER is a probabilistic method which predicts the COs in recombinant individuals based on differences in single nucleotide polymorphisms (SNP) between parents (Rowan et al., 2015). Furthermore, I combined and modified three protocols to set the library preparation step in our laboratory (Hennig et al., 2018a; Rowan et al., 2015, 2019), which enables fast and low-cost library preparation of hundreds of samples in parallel. The libraries for sequencing are prepared using the genomic DNA from F_2 plants as a template. I validated the tagmentation-based library preparation using homemade Tn5 transposase

(Hennig et al., 2018b; Zhu et al., 2021). Tagmentation reaction is followed by DNA amplification with sample-specific pair of P5 and P7 indexing primers, which enable further demultiplexing process (Rowan et al., 2019; Zhu et al., 2021). As DNA from each sample is tagged with a unique pair of indices, it is possible to pool up to 576 samples on one sequencing lane (Zhu et al., 2021). Finally, using TIGER we are able to identify the CO breakpoints based on parental SNPs (Blackwell et al., 2020; Zhu et al., 2021).

In the article, the crossovers landscapes were generated based on GBS results from five crosses between Col reference with different Arabidopsis accessions: L*er*, Bur-0, Ws-4, Ct and CLC (a mosaic of Cvi-0, L*er* and Col). My role was to construct 305 libraries for Col×Ct hybrid and to perform the initial analysis of the sequencing results. Genome-wide data for Col×L*er* and Col×Bur-0 were reanalysed based on already published data (Lawrence et al., 2019; Serra et al., 2018a). Interestingly, in all populations there is a crossover promotion in the highly polymorphic pericentromeres. **We observed a parabolic relationship between crossover frequency and SNP density (Fig. 3A)**. It means that SNPs are positively associated with recombination but only to a certain threshold as elevated levels of SNPs cause the recombination decrease. This may suggest **the preference for crossovers to occur in more polymorphic regions, however these analyses were mainly based only on correlations between SNP density and crossover rates**. It has been widely elaborated that polymorphisms are not the major determinants of the recombination as there are other factors shaping the crossover landscapes, which are related to the chromatin structure (Choi et al., 2013, 2018; Hsu et al., 2022; Lian et al., 2022). Therefore, our analyses in this article rather explain the role of MSH2 in shaping the CO pattern than elucidate the impact of polymorphism on recombination.

MSH2 is a MutS homolog and is responsible for detection of mismatches between homologous chromosomes. In order to investigate the impact of MSH2 on meiotic recombination, the crossovers were mapped in the *msh2* background using GBS. The crossovers landscapes were generated for three hybrid populations: Ct×Col, L*er*×Col and CLC×Col in the mutant background. Together with Julia Dłużewska, the first author of the publication, we constructed 320 libraries for Ct×Col in *msh2* background. A significant redistribution of crossovers was observed as the crossovers occurred more often in less polymorphic regions when compared to wild type (Fig. 3B, C). As a result, **in *msh2* we observed a remodelling of the recombination at the chromosome scale by redistribution of the crossovers from polymorphic pericentromeric regions**

towards sub-telomeric and interstitial regions of chromosome arms (Fig. 3B, C). However, analysis of these results by opposing to those published by Lian et al. 2022 is challenging. Lian et al. 2022 showed genome-wide COs maps in quasi-absence of polymorphisms separate for male and female meiosis. The changes on crossover distribution are smaller than would be estimated based on our results for *msh2* mutant. Therefore, the observed redistribution suggests that MSH2 plays also additional roles in influencing the crossover distribution, apart from mismatch detection.

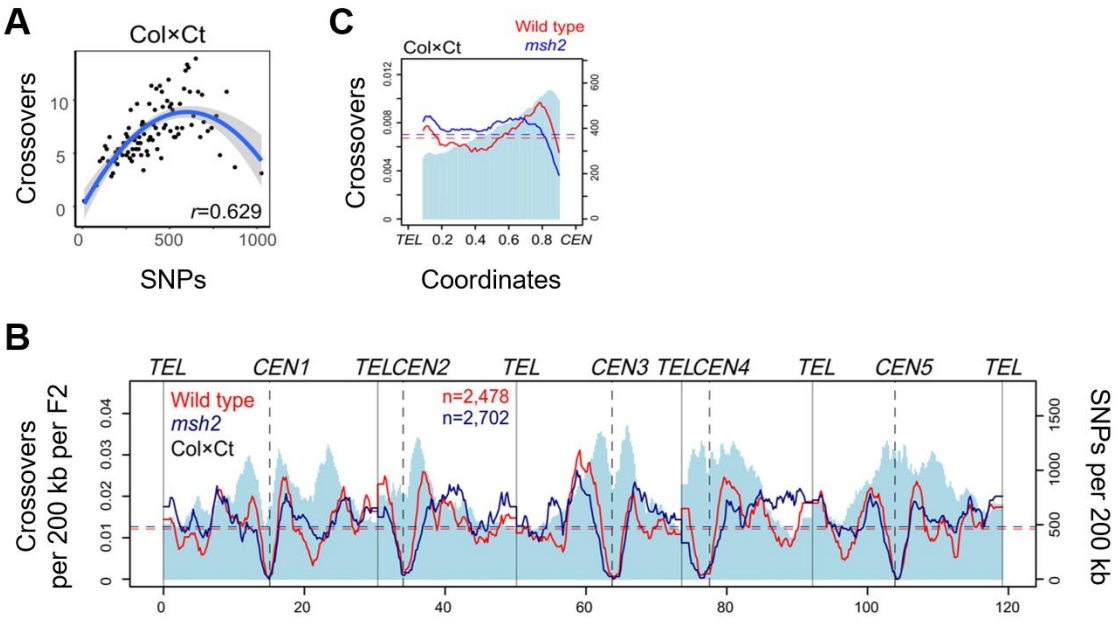

Fig. 3 Genome-wide relation between SNPs, MSH2 and recombination in Col×Ct. A) A parabolic relationship between SNPs and crossover frequency. Crossover frequency was normalized to the number of F_2 individuals and SNP density in 100 kilobase (kb) adjacent windows and ranked into percentiles according to SNP density. The Spearman's rank correlation coefficient (r=0.629) between SNP density and crossover frequency is printed inset. B) Genome-wide remodelling of the recombination landscape in *msh2*. Distribution of crossovers per 200 kb per F2 plotted along the five Arabidopsis chromosomes. Mean values are shown by horizontal dashed lines. Data are shown for wild type (red) and msh2 (blue) crossovers generated from Col × Ct hybrid. SNPs per 200 kb are shaded in blue colour. The positions of telomeres (TEL) and centromeres (CEN) are labelled. The number of crossovers analysed is printed inset. C) Data as for B, but analysing crossovers in wild type (red) and msh2 (blue), or SNPs along proportionally scaled chromosome arms orientated from telomeres (TEL) to centromeres (CEN). Modified from Blackwell et al. 2020.

Furthermore, at the megabase scale in Arabidopsis, a crossover preference was observed for more polymorphic regions (Ziolkowski et al., 2015). It was shown by measuring crossover frequency by seed-scoring system in a 420 reporter interval. The 420 interval is located in the subtelomeric region on chromosome three and is over 5 Mb in size. The recombination was measured in 420, in lines, which presented different heterozygosity patterns of chromosome three in Arabidopsis. The recombination frequency measured in 420 showed an increase when only the investigated interval was heterozygous while the remaining of the chromosome was homozygous. And reciprocally, when the 420 interval is in homozygous state while the rest of the chromosome is heterozygous, a decrease in recombination is observed. This phenomenon that heterozygous regions receive more crossovers when they are juxtaposed to the homozygous segments was called heterozygosity juxtaposition effect (Ziolkowski et al., 2015). To answer the question whether MSH2 plays role in the juxtaposition effect, Julia Dłużewska, the first author of the article, performed the crossover frequency measurements in those lines in *msh2* mutant background (Blackwell et al., 2020). The juxtaposition effect disappeared in the *msh2* mutant deficient in recognition of polymorphisms. This shows that **MSH2 has a pro-crossover role in the heterozygosity juxtaposition effect in Arabidopsis by promoting crossovers in heterozygous regions when they are juxtaposed with homozygous ones**.

6. **The main theses and achievements of the chapter "Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions"**, *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology, 2022*.

In this chapter, we provided a detailed protocol for efficient generation of CRISPR/Cas9-based mutants in different Arabidopsis accessions. Studies on plant's functional genetics require usage of different mutants. Although, there is a vast mutant collection in Arabidopsis, it is available only for Col reference. In meiotic recombination studies, DNA polymorphism between two parents are important markers to identify CO breakpoints in GBS analysis. Thus, mutants in other Arabidopsis accessions are required

for generation of CO landscapes. This protocol is not limited to be used only by the meiotic community, but it allows an easy implementation in any laboratory.

Dr Tomasz Bieluszewski created the vectors and optimized the method in our laboratory, although he was not directly involved in writing of the chapter. My role in this work was to accurately describe all the steps of the procedure, including critical technical details. The system combines efficient generation of knock-out mutants with an easy screen of the transformants by monitoring the seed fluorescence. The desired out-of-frame mutation is a result of deletion between two Cas9 cutting sites (Pauwels et al., 2018). First, a pair of 20-nt spacer sequences is designed. Spacer sequences are responsible for targeting the Cas9 to the gene of interest. The sgRNA cassettes with the spacer sequences are generated by cloning them to pJET with sgRNA scaffold under AtU3C or AtU6-26 promoters (available at Addgene #173156 and #173157). Later the final CRSIPR/Cas9 vector is obtained by introducing the amplified sgRNA cassettes to the binary vector (available at Addgene #173158). Our system allows cloning up to 6 different sgRNA cassettes to one vector. Additionally, in the vector there are two reporters: 1) coding sequence of dsRed under control of *NapA* promoter that provides expression in seeds, 2) coding sequence providing resistance to BASTA. The ready binary construct is introduced to Arabidopsis plants via Floral Dip. Selection of transformants can be performed using standard BASTA-selection or fast and easy method based on monitoring the seed-fluorescence. Screening T_1 seeds relies on selection those seeds showing a red fluorescence as they carry the binary construct. In the next generation, preselection of non-colour T_2 seeds enables faster segregating out the CRISPR construct from lines showing a desired mutation. All steps to generate a homozygous mutant are described in details in the protocol.

Within the articles forming part of my dissertation, I used CRISPR/Cas9 technique to generate:

- Col-pseudo-ChP (Col-ΨChP) line (Szymanska-Lejman et al., 2023),

- L*er*Δ lines (L*er*Δ#24 and L*er*Δ#76) carrying deletions within *Coco* in L*er* accession (Szymanska-Lejman et al., 2023).

The aim of the Col-pseudo-ChP (Col-ΨChP) line was to confirm that the presence of the fluorescent reporter cassettes on both ends of the ChP interval do not have an impact on recombination within the interval. Thus, I tried to generate Col-ΨChP by introducing

short deletions within coding sequences of both reporters, dsRed and eGFP. I designed two pairs of spacer sequences targeting coding sequences of dsRed or eGFP. Col-ChP plants were transformed with Agrobacterium carrying the binary vector with Cas9 and four sgRNAs. The vector was devoid of the fluorescent reporter due to transforming fluorescently-tagged Col-ChP line. In this case, the selection of transformants was also based on fluorescence but the transformants were associated with non-colour seeds. In order to segregate out the construct the preselected non-colour Col-ΨChP was backcrossed to Col-ChP. Unfortunately, the loss of fluorescence presented by the majority of lines was caused by silencing of the cassettes which prevented the recombination frequency measurements. Eventually, I obtained one line in which the fluorescent cassettes were CRISPR edited and were not silenced. Sequencing of the fluorescent reporters showed 8 bp frame-shift mutation in dsRed coding sequence while the eGFP cassette was largely removed.

The aim of L*er*Δ lines was to investigate the competition between neighbouring hotspots after deleting a part of the strongest hotspot in ChP. To do this, a pair of spacer sequences was designed to target Cas9 cutting to positions flanking the hyperactive part of the *Coco* hotspot in L*er* accession. The spacer sequences were first introduced to pJETs carrying sgRNA scaffold under the appropriate promoter. Next, the whole sgRNA cassettes were amplified and cloned to the final vector carrying Cas9 and dsRed reporter cassettes. Taking advantage of the fast screening of the seeds based on the fluorescence, it was possible to obtain the L*er*Δ lines with the desired deletions within merely two generation. The genotyping and sequencing revealed two independent lines with deletions of 714 bp (LerΔ#24) and 825 bp (LerΔ#76) within *Coco* hotspot.

7. **The main theses and achievements of the article "The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"**, *Nature Communications, 2023*.

Although, we observe the interconnection between sequence polymorphisms and meiotic recombination at the genome-wide scale, little is known about determinants of meiotic crossovers at the hotspot scale. In this work, which was my main Ph.D. project, we investigated the influence of various types of sequence polymorphisms between

homologous chromosomes on crossover distribution. I have performed all the experiments described in the article except for generation of the *msh2* mutant in L*er* accession and the bioinformatic analysis.

First, **I developed the *seed-typing* method which enables precise mapping of crossover breakpoints at the fine scale (Fig. 4)**. Currently, there was only one method to study distribution of the crossovers in plants with high resolution, called *pollen-typing* (Drouaud and Mézard, 2011). However, the amplification of the recombinants from pools containing a large excess of non-recombinant molecules amplifies the risk of errors and significantly reduces the robustness of this assay (Choi et al., 2013; Drouaud et al., 2013). To overcome those limitations, we described a novel approach for investigating the crossover distribution in *A. thaliana*. *Seed-typing* method is based on the fluorescent reporter system to easily monitor the segregation of the fluorescence in seeds and precisely measure CO frequency within the *seed-typing* interval (Fig. 4). I generated five lines in Col accession, carrying coding sequences of fluorescent proteins (eGFP and dsRed) separated from each other by no more than 50 kb; we called them extremely short interval lines (ESILs). If the crossover occurs within the interval (between two reporter genes), the recombinant seed obtained from such a gamete will be visible as single colour seed, either red, or green. This system allows both, the quick and accurate CO frequency measurements in the investigated interval (Kbiri et al., 2022) and an easy preselection of recombinant seeds based on fluorescence under stereomicroscope (Fig. 4). DNA extracted from the plants grown from those seeds is used as a template for long-range PCR (LR-PCR). Due to the fact that the intervals are relatively short (up to 50 kb), it is possible to amplify the interval within a few amplicons of 9-11 kb in size. Those amplicons are used for library preparation to perform the next generation sequencing (NGS). Identification of CO breakpoints is based on single nucleotide polymorphisms (SNPs) between the parental accessions (Fig. 4).

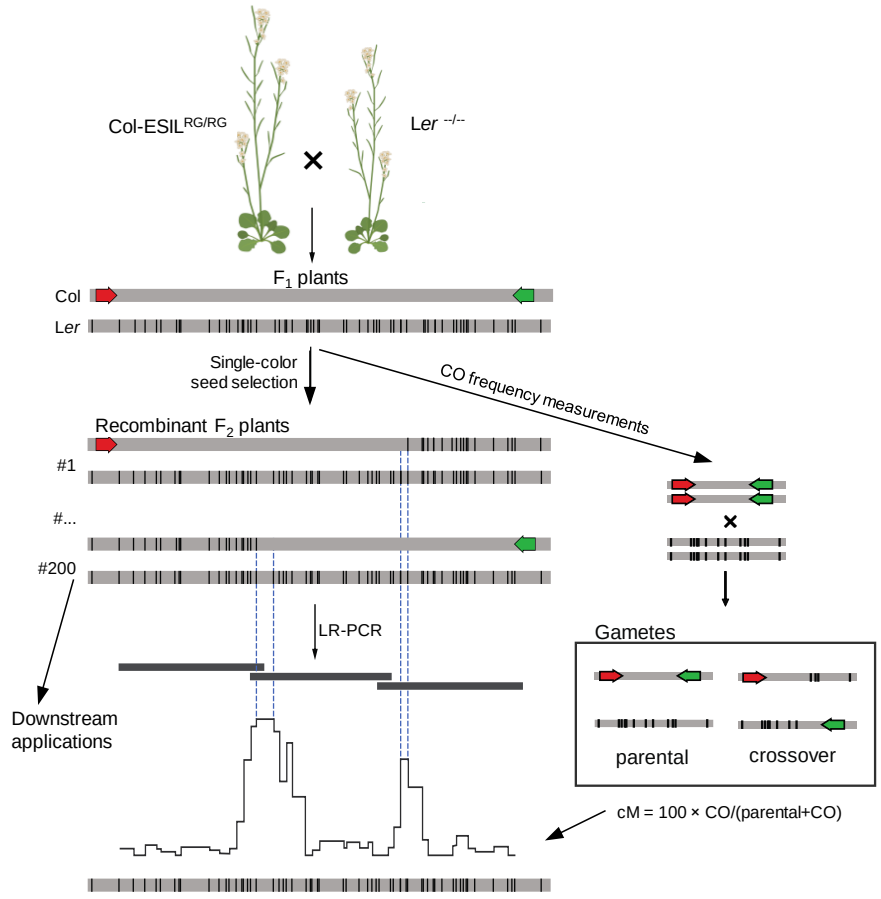

Fig. 4 The scheme of *seed-typing* method. The fluorescently tagged ESIL line crossed with a non-colour accession (e.g. L*er*) results in F_1 hybrid. Segregation of the fluorescent reporters in F_2 seeds obtained after self-pollination enables the CO frequency measurements in the interval and preselection of recombinant seeds. The DNA extracted from plants grown from those seeds is used to perform LR-PCR and construct libraries for sequencing. The identification of the crossover breakpoints is based on SNPs between parents. Additionally, progeny of the recombinants can be used for further experiments, for example generation of R^2 lines described in section 7. Modified from Szymanska-Lejman et al. 2023.

One of the generated ESILs, Chili Pepper (hereafter ChP), is 26.3 kb and is located in the pericentromeric region of chromosome 3 in Arabidopsis (Fig. 5A). Such a location of the interval is connected with high levels of interhomolog polymorphisms between Col and other Arabidopsis accessions (11.54 polymorphisms/kb between Col and Ct; 18.7 polymorphisms/kb between Col and L*er*). Recombination frequencies in the obtained ESILs were measured after crossing them with Col (inbred), Ct and L*er* (hybrids). Interestingly, **the recombination frequency is the highest for the most polymorphic**

ChP interval. Taking advantage of the *seed-typing* method we mapped the crossover breakpoints in 242 recombinants (preselected from F_2 Col-ChP×L*er* seeds) and identified three hotspots within ChP, which we called: *Aro*, *Coco* and *Nala* (Fig. 5B, C). They are clearly separated and located within genes. All three hotspots present higher recombination rate than the genome average. *Coco*, which is located in the middle, is the strongest (62.14 cM/Mb), being one of the strongest hotspots identified in Arabidopsis so far. **Although the hotspots are polymorphic, the crossovers are mostly located in the hotspots centres which are devoid of SNPs (Fig. 5C).** This is consistent with what has been observed in other Arabidopsis hotspots using *pollen-typing* method (Choi et al., 2016; Serra et al., 2018b). Although in Blackwell et al., (2020) we concluded that crossovers are associated with higher SNP density at the kilobase scale, the analysis were performed based on the genome-wide sequencing results, which significantly limits the resolution and introduces some averaging in the analysis of crossover breakpoints. The new *seed-typing* system allowed to explain this seeming contradiction between data obtained using the *seed-typing* and GBS methods (see below).

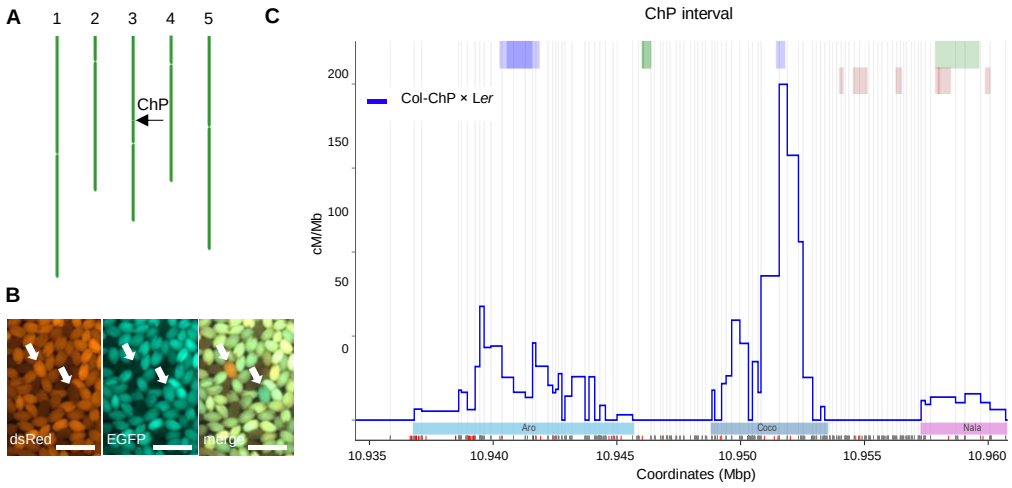

Fig. 5 Analysis of crossover distribution in ChP interval using *seed-typing*. A) Location of ChP in pericentromeric region on chromosome 3 in *A.thaliana*. B) Preselection of recombinant seeds from F_2 Col-ChP×L*er* seeds based on red and green fluorescence. White arrows indicates the single-colour recombinant seeds. C) High-resolution crossover mapping in Col-ChP×L*er* based on 242 recombinants. Polymorphisms (SNPs and small indels) are shown in gray, dashed lines, genes are represented in green and transposable elements in blue. Three separated crossover hotspots have been identified, *Aro, Coco and Nala*.

As ChP is located in the pericentromeric region, it exhibits not only high levels of SNPs but also structural polymorphisms between Col and other Arabidopsis accessions. In this work, we investigated how the structural rearrangements in the vicinity of the meiotic hotspots influence their activity. To analyse this phenomenon, I used two different approaches, natural variation within ChP region and Cas9-induced deletions. First, by crossing ChP (Col background) with seven different Arabidopsis accessions that show different patterns of interhomolog structural rearrangements surrounding ChP. I did not observe changes in the recombination frequency between hybrids with or without those structural changes. Similarly, only a slight decrease in recombination was observed in the recombination frequency in Col-ChP crossed with generated non-colour Col-ΨChP line (generation of Col-ΨChP line described in section 6). It suggests that reporter cassettes, which can be considered as big insertions on both ends of ChP, do not have an impact on recombination in ChP. Therefore, we concluded that s**tructural polymorphisms surrounding the interval do not affect crossover rate in hotspots.**

Among the recombination frequencies measured in analysed hybrid crosses of ChP with other Arabidopsis accessions, we observed a significantly lower values for C24×Col-ChP hybrid. Basically, the CO frequency in the hybrid cross (mean 8.73 cM/Mb) was at the level of inbred (8.54 cM/Mb). Sequence comparison between C24 line and Col reference showed the presence of a natural deletion of 1194 bp within the *Coco* hotspot. Using *seed-typing*, we proved that **the hotspots are highly conserved between Arabidopsis accessions** as the analysis of the crossover distribution in C24×Col-ChP revealed the presence of the same hotspots as in L*er*×Col-ChP. Hence, the relatively low ChP recombination frequency C24×Col-ChP when compared to other hybrids is due to significantly decreased activity of the *Coco* hotspot. We also took advantage of the presence of three adjacent hotspots in ChP to check the phenomenon of hotspot competition, whether partial deletion of *Coco* causes recombination changes in adjacent hotspots. Our results showed no alterations in the activity of *Aro* and *Nala*, located on both sides of *Coco*, suggesting that there is no short-distance hotspot competition in C24×Col-ChP. But comparing hotspot activities in two different hybrids C24×Col and L*er*×Col is challenging due to the possible influence of trans-acting modifiers in one of the accession. Thus, to eliminate possible effect of factors influencing recombination derived from the natural variability between C24 and L*er*, we used CRISPR-Cas9 to generate lines with the deletions within *Coco* in L*er* accession. CO frequency

measurements and analysis of the CO distributions showed similar changes in lines with natural deletion (C24) and Cas9-induced deletion in L*er*. **These results further demonstrate lack of short-distance hotspot competition in Arabidopsis**. However, this conclusion is based only on two closest hotspots located within a rather specific, pericentromeric region.

We sought to investigate the effect of SNPs on recombination at the hotspot scale by performing crossover frequency measurements in the *msh2* mutant background, in which the mismatch detection (MMR) system is not functional. The measurements were performed in inbreds and hybrids using two intervals located in interstitial (BT) or pericentromeric (ChP) chromosome regions. In inbreds, there aren't any interhomolog polymorphisms thus the crossover rate did not change after inactivation of MMR. In turn, in hybrids the effect of mutating the mismatch sensor, MSH2, depends on the interval location. Therefore, in interstitial L*er*×Col-BT, we observed significantly higher recombination frequency in *msh2*, while in pericentromeric L*er*×Col-ChP the recombination decreased even below the inbred level. These results suggest that **the observed increase or decrease of the CO rate is due to the chromosome-scale redistribution of the crossovers in *msh2* background** which was discussed in section 5 based on Blackwell et al., (2020).

To check, whether local polymorphisms have an impact on hotspot activity, we used *seed-typing* to check the CO distribution in BT and ChP in Col×L*er msh2* hybrids. We observed a similar trend in both intervals – **more polymorphic hotspots are less active after disabling MMR system while less polymorphic regions get relatively more COs**. It suggests that the presence of MSH2 redistributes crossovers from less polymorphic hotspots to more polymorphic ones. I am aware that the conclusions are drawn on the basis of only two intervals, although they show the same effect despite the difference in the location on the chromosome. Furthermore, we confirmed the direct effect of SNPs on crossover formation at the hotspot scale by generation R^2 lines. These lines demonstrate Col/L*er* heterozygous state only within ChP, or parts of ChP interval while the remaining of the genome is fixed for Col (Fig. 6). The R^2 lines (Recombinant×Recombinant) were obtained by crossing the progeny of two already sequenced recombinants to find a new recombinant in F_2, which was subsequently backcrossed three times to Col background. As a result, these lines enabled to exclude the effect of chromosome-level crossover redistribution. The CO frequency was measured in R^2 lines after crossing them to Col in

wild type and *msh2* backgrounds. Surprisingly, we observed that all lines that demonstrate heterozygous Col/L*er* pattern within the whole ChP interval show significantly higher crossover frequency in ChP than both inbreds and hybrids (Fig. 6). Thus, juxtaposition of even small patch of heterozygous region to the homozygous chromosome stimulates the recombination in the interval showing higher crossover rates (Fig. 6). This effect was not observed in the siblings of all R^2 plants in the *msh2* background as there was no change in recombination when compared to *msh2* inbreds and hybrids. This is the evidence that **locally occurring interhomolog polymorphisms detected by MSH2 promote crossovers at the hotspot scale**. Yet, the R^2 lines have been generated only for ChP interval, similar crossover rate pattern have been presented in the juxtaposition lines for subtelomeric 420 interval in Blackwell et al., 2020.

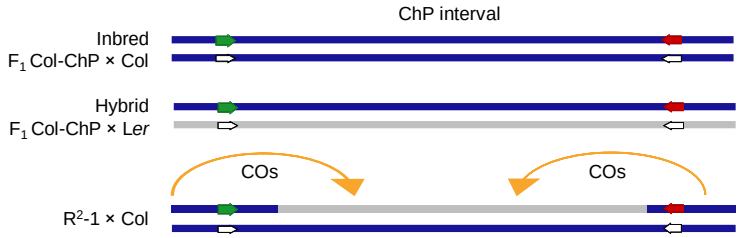

Fig. 6 Heterozygosity juxtaposition effect at the hotspot level. Polymorphism pattern for R^2_1 × Col line. The heterozygous Col/L*er* segment is only within ChP interval while the remaining of the chromosome is fixed for Col (blue). The F_1 crosses of inbred (Col-ChP × Col) and hybrid (Col-ChP × L*er*) are shown for comparison. Juxtaposition of the heterozygous ChP region to homozygous ones result in increased recombination frequency in ChP interval (orange arrows).

In this article, we showed that even though the crossover breakpoints tend to occur in the hotspot centres which are not polymorphic, the local SNPs in the proximity of the hotspots are detected by MSH2 to further trigger the crossovers within those hotspots (Fig. 7). This can explain the seeming inconsistency between the previous hotspot-scale *pollen-typing* data and the hotspot-averaged data from GBS analyses.

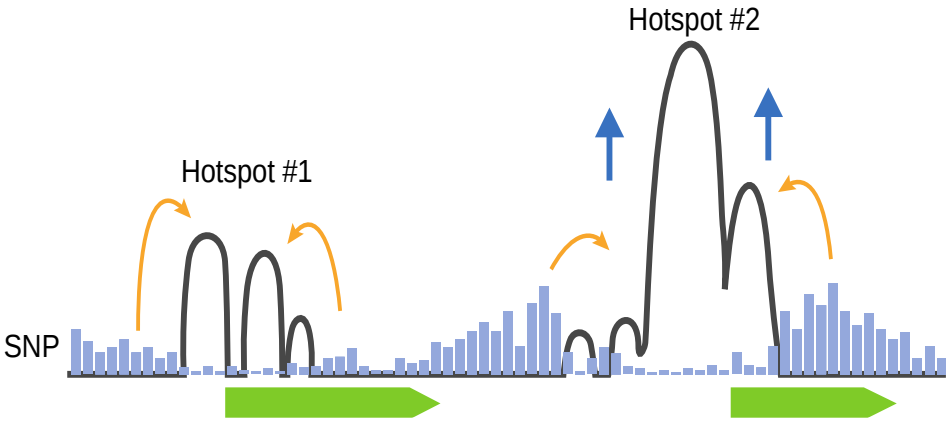

Fig. 7 Model showing the effect of MSH2 on Arabidopsis crossovers at the hotspot scale. Meiotic hotspots located in SNP-rich regions (blue bars) are more active than neighbouring hotspots at less polymorphic sites due to MSH2-dependent stimulation (blue arrows). However, COs occur mostly at polymorphism-free sites within each hotspot (orange arrows). Green horizontal arrows represent genes. Modified from Szymanska-Lejman et al. 2023.

8. Conclusions

The Ph.D. thesis focuses on the impact of interhomolog polymorphism on meiotic recombination in *A. thaliana*. It consists of four publications connected with the topic. The review (Dluzewska & Szymanska et al., 2018) provides the thorough analysis of the most recent articles about the influence of heterozygosity on recombination initiation sites in plant genomes. Two research articles (Blackwell et al., 2020 and Szymanska-Lejman et al., 2022) try to decipher the relation between crossover recombination, interhomolog polymorphism, and MSH2 mismatch detection protein. The protocol, (Bieluszewski et al., 2022), provides a detailed description of our method for efficient generation of Arabidopsis mutants using CRISPR/Cas9.

The main achievements and conclusions from the research articles:

1. I developed a novel tool to study crossover distribution at the hotspot scale in Arabidopsis, called *seed-typing*.

2. Polymorphic, pericentromeric regions in *A.thaliana* contain active crossover hotspots.

3. Structural changes have no effect on the crossover frequency unless they directly involve recombination hotspots.

4. Natural or induced inactivation of a hotspot does not trigger crossover stimulation in the neighboring hotspots suggesting the lack of short-distance hotspot competition.

5. Inactivation of *MSH2* mismatch detection gene triggers a chromosome-scale redistribution of the crossovers.

6. MSH2 promotes crossovers in hotspots based on locally occurring interhomolog polymorphisms.

7. The phenomenon of heterozygosity juxtaposition effect, which has been observed at the megabase scale in Arabidopsis (Blackwell et al., 2020; Ziolkowski et al., 2015) is also observed at the level of recombination hotspots.

8. Local SNP density affects crossover distribution in MSH2-dependent manner. This supports the role of meiotic recombination in genome evolution.

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10.Articles included in the dissertation

Dluzewska J[1], **Szymanska M[1]**, Ziolkowski PA.

Where to cross over? Defining crossover sites in plants.

Front Genet. 2018 Dec 12;9:609.

Doi: 10.3389/fgene.2018.00609

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Blackwell AR[1], Dluzewska J[1], **Szymanska-Lejman M**, Desjardins S, Tock AJ, Kbiri N, Lambing C, Lawrence EJ, Bieluszewski T, Rowan B, Higgins JD, Ziolkowski PA*, Henderson IR*.

MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis.

EMBO J. 2020 Nov 2;39(21):e104858.

Doi: 10.15252/embj.2020104858

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Bieluszewski T, **Szymanska-Lejman M**, Dziegielewski W, Zhu L, Ziolkowski PA.

Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions.

In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology, vol 2484. Humana, New York, NY.

Doi: 10.1007/978-1-0716-2253-7_13

Szymanska-Lejman M, Dziegielewski W, Dluzewska J, Kbiri N, Bieluszewska A, Poethig RS, Ziolkowski PA.

The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids.

Nat Commun. 2023 Jan 3;14(1):33.

Doi: 10.1038/s41467-022-35722-3.

11. Author’s statements

AUTHOR'S STATEMENT

I declare that the article "Where to cross over? Defining crossover sites in plants" Julia Dluzewska[1], **Maja Szymanska[1]**, Piotr A. Ziolkowski. *Front Genet.* 2018; 9:609; DOI: 10.3389/fgene.2018.00609 is a part of my PhD dissertation. I am the joint first author of this article. I participated in the development of its concept and contributed to the writing the manuscript by preparing "General information about crossover in plants" and "Impact of DSB on crossover distribution".

[1] - joint first authors

I declare that the article "MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis" Alexander R Blackwell[1], Julia Dluzewska[1], **Maja Szymanska-Lejman**, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 is a part of my PhD dissertation. I contributed by preparing the sequencing libraries for Col×Ct hybrid and performing the initial analysis of the sequencing results. My results are shown as parts of Fig. 1, 2A, 3A, 7A. I also helped in preparing the sequencing libraries for Col×Ct in *msh2* background. The results are shown as parts of Fig. 4 and 5.

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I declare that the article "Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions" Tomasz Bieluszewski, **Maja Szymanska-Lejman**, Wojciech Dziegielewski, Longfei Zhu, Piotr A. Ziolkowski. *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology*, vol 2484. Humana, New York, NY. DOI: 10.1007/978-1-0716-2253-7_13 is a part of my PhD dissertation. I contributed to writing the draft of the manuscript and preparing Fig. 2, Fig. 3c, Fig. 4b, Fig. 5 and Table 2.

I declare that the article "The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids" **Maja Szymanska-Lejman**, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski. *Nat Commun.* 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 is a part of my PhD dissertation. I contributed to research design and writing the manuscript. I performed the majority of the experiments. I set the seed-typing method and identified the CO breakpoints based on the list of SNPs generated by Wojciech Dziegielewski. I performed the recombination frequency measurements (Fig. 1b, 3b, 3d, 4e, 5a, 5b, 8c, 8d, 8e). I generated the pseudoreporter line for ChP (Supplementary Fig. 3), Cas9-generated deletion lines (LerΔ#24 andLerΔ#76) (Supplementary Fig. 6) and R^2 lines (Supplementary Table 5, Supplementary Table 6). My results contributed to all the figures presented in the article. I also shared funding.

Poznań, 23.02.2023

Maja Szymańska-Lejman

Prof. UAM, dr hab. Piotr Ziółkowski

Supervisor of the Ph.D. candidate

12. Co-author’s contribution statements

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Where to cross over? Defining crossover sites in plants"** Julia Dluzewska[1], Maja Szymanska[1], Piotr A. Ziolkowski.. *Front Genet.* 2018; 9:609; DOI: 10.3389/fgene.2018.00609 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Julia Dluzewska**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed to writing the manuscript and preparing the figures.
Date	20.02.2023
Signature	DTuzewska

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Where to cross over? Defining crossover sites in plants"** Julia Dluzewska[1], Maja Szymanska[1], Piotr A. Ziolkowski. *Front Genet.* 2018; 9:609; DOI: 10.3389/fgene.2018.00609 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Piotr A. Ziolkowski**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I created the concept of the work, contributed to writing and revising the manuscript and participated in preparation of figures.
Date	20/02/2023
Signature	P Ziolkowski

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis"** Alexander R Blackwell[1], Julia Dluzewska[1], Maja Szymanska-Lejman, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Julia Dluzewska**
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Contribution Description	I contributed to research design and writing the manuscript. I prepared the libraries for sequencing of Col×Ct in *msh2* background (Fig. 4, 5). I generated *msh2* mutants in juxtaposition lines and performed the measurements of the recombination frequency shown in Fig. 7C, 7D (Wild-type), Appendix Figure S5, Appendix Figure S11. I measured the CEN3 crossover frequency in wild type and *msh2* Col/Ct hybrids (Appendix Figure S8D).
Date	20.02.2023
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AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis"** Alexander R Blackwell[1], Julia Dluzewska[1], Maja Szymanska-Lejman, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Nadia Kbiri**
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AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis"** Alexander R Blackwell[1], Julia Dluzewska[1], Maja Szymanska-Lejman, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Tomasz Bieluszewski**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed by providing the vectors for the CRISPR/Cas9-mediated mutagenesis. I also shared the experimental details about the method which was used to generate *msh2* mutants in Ct and juxtaposition lines.
Date	2023-02-20
Signature	Tomasz Bieluszewski

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis"** Alexander R Blackwell[1], Julia Dluzewska[1], Maja Szymanska-Lejman, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Co-Author Name	**Piotr A. Ziolkowski**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I designed the research and supervised the experiments. I analysed the sequencing data and contributed to figures presented in the article. I wrote and revised the manuscript. I also shared funding.
Date	20/02/2023
Signature	

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"MSH2 shapes the meiotic crossover landscape in relation to interhomolog polymorphism in Arabidopsis"** Alexander R Blackwell[1], Julia Dluzewska[1], Maja Szymanska-Lejman, Stuart Desjardins, Andrew J. Tock, Nadia Kbiri, Christophe Lambing, Emma J. Lawrence, Tomasz Bieluszewski, Beth Rowan, James D. Higgins, Piotr A. Ziolkowski*, and Ian R. Henderson*. *The EMBO Journal*, 2020; 39:e104858; DOI:10.15252/embj.2020104858 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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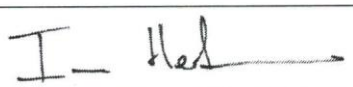
Co-Author Name	**Ian R. Henderson**
Affiliation	Department of Plant Sciences, University of Cambridge, Cambridge, UK
Contribution Description	I designed the research and supervised the experiments. I analysed the sequencing data and contributed to all figures presented in the article. I wrote and revised the manuscript. I also shared funding.
Date	20th Feb 2023
Signature	

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions"** Tomasz Bieluszewski, Maja Szymanska-Lejman, Wojciech Dziegielewski, Longfei Zhu, Piotr A. Ziolkowski. *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology*, vol 2484. Humana, New York, NY. DOI: 10.1007/978-1-0716-2253-7_13 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Tomasz Bieluszewski**
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Contribution Description	I generated the vectors and developed the protocols. I created graphics used in Fig. 1, Fig. 2, and Fig. 3a. I edited the subsequent drafts of the manuscript.
Date	2023-02-20
Signature	Tomasz Bieluszewski

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions"** Tomasz Bieluszewski, Maja Szymanska-Lejman, Wojciech Dziegielewski, Longfei Zhu, Piotr A. Ziolkowski. *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology*, vol 2484. Humana, New York, NY. DOI: 10.1007/978-1-0716-2253-7_13 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Contribution Description	I contributed to writing the manuscript and preparing the figures (Fig. 1, Table 1, Fig. 3).
Date	21/02/2023
Signature	Wojciech Dziegielewski

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions"** Tomasz Bieluszewski, Maja Szymanska-Lejman, Wojciech Dziegielewski, Longfei Zhu, Piotr A. Ziolkowski. *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology*, vol 2484. Humana, New York, NY. DOI: 10.1007/978-1-0716-2253-7_13 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Longfei Zhu**
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Contribution Description	I contributed by cloning the dsRed cassette to the binary vector.
Date	2023-02-22
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AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"Efficient generation of CRISPR/Cas9-based mutants supported by fluorescent seed selection in different Arabidopsis accessions"** Tomasz Bieluszewski, Maja Szymanska-Lejman, Wojciech Dziegielewski, Longfei Zhu, Piotr A. Ziolkowski. *In: Lambing, C (eds) Plant Gametogenesis. Methods in Molecular Biology*, vol 2484. Humana, New York, NY. DOI: 10.1007/978-1-0716-2253-7_13 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Piotr A. Ziolkowski**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed to the concept of the work and writing the manuscript.
Date	20/02/2023
Signature	

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"** Maja Szymanska-Lejman, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski.. *Nat Commun.* 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Wojciech Dziegielewski**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed by performing all the computational analysis. I analysed the sequencing data after seed-typing, which were further used to identify the CO breakpoints (Supplementary Fig. 2, Supplementary Table 8). I analysed the data of whole genome sequencing of R^2 lines. I also performed the verification of the *msh2* mutant in L*er* accession by RT-PCR (Supplementary Fig. 9).
Date	21/02/2023
Signature	Wojciech Dziegielewski

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"** Maja Szymanska-Lejman, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski.. *Nat Commun.* 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Julia Dluzewska**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed by collecting the material and counting the number of seeds for recombination frequency measurements in R^2 lines (Fig. 8c, d).
Date	20.02.2023
Signature	DTuiewska

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"** Maja Szymanska-Lejman, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski.. *Nat Commun*. 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Nadia Kbiri**
Affiliation	Laboratory of Genome Biology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland
Contribution Description	I contributed by generation *msh2* mutant in L*er* accession and preparing the Supplementary Fig. 8.
Date	23/02/23
Signature	Nadia KBiRi

AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"** Maja Szymanska-Lejman, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski.. *Nat Commun.* 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

Co-Author Name	**Anna Bieluszewska**
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Contribution Description	I contributed by providing Tn5 transposase, which was used for seed-typing.
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AUTHORSHIP CONTRIBUTION STATEMENT

I hereby declare that I am aware that the work in the article **"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"** Maja Szymanska-Lejman, Wojciech Dziegielewski, Julia Dluzewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig, Piotr A. Ziolkowski.. *Nat Commun.* 2023 Jan 3;14(1):33. DOI: 10.1038/s41467-022-35722-3 of which I am a co-author, has been included in the doctoral thesis of Maja Szymanska-Lejman.

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Contribution Description	I designed the research and supervised all experiments. I contributed to all figures presented in the article. I wrote and revised the manuscript. I also shared funding.
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