Generation And Analysis Of Pro- And Anti-Crossover Meiotic Mutants In Tomato

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Abstract

One of the unique characteristics of meiosis is the formation of meiotic crossovers (CO) between homologous chromosomes that leads to the reciprocal exchange of DNA and eventually diverse haploid gametes. Meiotic CO leads to generation of genetic variation and therefore creates diversity at the level of the individual organism and population. Research and knowledge on the fundamental mechanisms of meiotic CO could ultimately expedite the improvement of crop plant varieties and animal breeds. As has been reported previously in many types of organisms, the class I CO pathway is usually responsible for the majority of CO events, while the class II CO pathway is the minor CO pathway. The genetic knock-out of some factors that facilitate non-crossover (NCO) repair have been found to lead to increased activity of the class II CO pathway. Information on the regulation of meiotic CO for both class I CO and NCO are previously extensively described in the model plant, *A. thaliana*. However, there is a lack of information available in other dicot plants especially those used as a food crops. Therefore, this thesis described the generation of mutants in the class I CO (*SlMLH1* and *SlZIP4*) and NCO repair (*SlRECQ4*) genes, the role of those genes and their products in promoting and regulating meiotic CO, and also the importance of those genes for the fertility in tomato.

CRISPR/Cas9 mutagenesis was applied to produce three different mutants in the dwarf tomato variety, Micro-Tom. *Slmlh1* and *Slzip4* mutants are associated with reduced activity of the class I CO pathway, while the *Slrecq4* mutant is a NCO repair gene. All three mutants exhibited a significant reduction in plant fertility. The average percentage of viable pollen is 1.49%, 13.4% and 37.3% for *Slzip4*, *Slmlh1* and *Slrecq4* mutants respectively, when compared to wildtype Micro-Tom (97.6%). This showed that both class I CO genes and NCO genes are essential to ensure tomato fertility probably due to their important functions in DNA repair during meiosis. A lack of meiotic CO between homologous chromosomes leads to univalent formation and unequal segregation at the end of the first meiotic division. Cytological analysis of meiotic chromosomes spreads indicated that reduced fertility in all three mutants was associated with univalent formation, and other meiotic defects. A higher percentage of univalent were observed in the most infertile *Slzip4* mutant, whilst the *Slrecq4* had the least number of univalent. However, *Slrecq4* mutants also exhibit DNA fragmentation during meiosis leading to a further reduction in fertility rate. This fragmentation in *Slrecq4* suggests that either too much CO has occurred or the intermediate for Holiday Junction (HJ) is not being fully repaired.

Despite the reduced fertility rate of *Slrecq4,* it can still partially supress class I CO infertility as double mutant of *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* had a significant improvement on the percentage of viable pollen, fruit number, fruit size and seed set. The number of univalent during diakinesis stage was also reduced significantly in the *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants. As similarly observed in *A. thaliana recq4* mutant, the *Slrecq4* mutant can supress both tomato class I CO mutants suggesting that the elevated CO in *Slrecq4* occurs via the class II CO pathway. Another interesting observation is the introgression of the *mlh1* mutation in the *S. lycopersicum* background into the wild tomato species, *Solanum pimpinellifolium* can significantly increase meiotic CO number and also the percentage of viable pollen. This suggests that there is a potential modifier of meiotic CO rate between the two accessions studied.

Overall, it is suggested that meiotic CO and NCO mutants can have different phenotypes depending on the context of other genomic factors. For example, the *SlRECQ4* gene was found to be very important not only to regulate meiotic CO but also to ensure normal DNA repair, correct chromosome segregation and also to prevent abnormal gametes formation. In the future, it will be of interest to identify the parity value between CO and NCO regulation in tomato which could be used to stimulate more CO without have negative side effects on the fertility rate. Another interesting point of view is to look further into the genetic control of meiotic CO in wild tomato species which might have interesting genetic modifiers to stimulate higher meiotic CO in tomato breeding.

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%	Percentage
˚C	Degree Celsius
µL	Microliter
AtCAS9	*A. thaliana Cas9*
BC	Back-crossing
Cas9	CRISPR-associated protein 9
cm	Centimeter
CmYLCV	Cestrum yellow leaf curling virus
CO	Crossover
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4’,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSB	Double strand break
F_1	First generation
F_2	Second generation
g	Gram
gDNA	Genomic DNA
HJ	Holliday junction
HR	Homologous recombination
HRM	High Resolution Melting
IAA	Indole-3-acetic acid
L	Liter
LB	Luria Bertani
mL	Milliliter
mm	Milimeter
mM	Millimolar
MOPS	3-morpholinopropane-1-sulfonic acid
NCO	Non-crossover/anti-crossover

NGS	Next generation sequencing
NPT	*Neomycin phosphotransferase*
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
PMC	Pollen mother cell
qPCR	Quantitative PCR
rpm	Revolutions per minute
RTR	RECQ4-TOP3α-RMI1 protein complex
SC	Synaptonemal Complex
SDSA	Synthesis-dependent strand-annealing
sgRNA	Single guide RNA
SlACT	*S. lycopersicum Actin*
SlMLH1	*S. lycopersicum MutL homolog 1*
SlRECQ4	*S. lycopersicum ATP-dependent DNA helicase Q4*
SlZIP4	*S. lycopersicum ZIP4*
Taq	*Thermus aquaticus*
TE	Tris-EDTA
Tm	Temperature melting
v/v	Volume per volume
w/v	Weight per volume
WT	Wildtype
YEP	Yeast extract peptone dextrose media
ZMM	Synapsis initiation complex protein

List of publications

1. Wang Y, van Rengs WMJ, **Zaidan MWAM**, Underwood CJ. Meiosis in crops: from genes to genomes. *J Exp Bot*. 2021 Sep 30;72(18):6091-6109. doi: 10.1093/jxb/erab217. PMID: 34009331; PMCID: PMC8483783.

2. van Rengs WMJ, Schmidt MH, Effgen S, Le DB, Wang Y, **Zaidan MWAM**, Huettel B, Schouten HJ, Usadel B, Underwood CJ. A chromosome scale tomato genome built from complementary PacBio and Nanopore sequences alone reveals extensive linkage drag during breeding. Plant J. 2022 Apr;110(2):572-588. doi: 10.1111/tpj.15690. Epub 2022 Mar 7. PMID: 35106855.

Manuscripts in progress:

1. **Mohd Waznul Adly Mohd Zaidan**, Roven Rommel Fuentes, Yazhong Wang, Sieglinde Effgen, Marianne Harperscheidt, Christine Sänger, Mijael Alejandro Torres-Mendoza, Willem M. J. van Rengs, Joiselle Fernandes, Tamara Susanto and Charles J. Underwood. Pivotal role of *SlMLH1* and *SlZIP4* genes for the formation of class I meiotic crossover and fertility in tomato (In preparation).

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3. Yazhong Wang, Roven Rommel Fuentes, Sieglinde Effgen, Willem M. J. van Rengs, **Mohd Waznul Adly Mohd Zaidan**, Joiselle Fernandes, Raphael Mercier and Charles J. Underwood. Skipping meiosis facilitates clonal reproduction and Polyploid Genome Design in tomato hybrids (Submitted).

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Introduction

1. Introduction

1.1. Genetic diversity ensures species survival

Every species has a unique DNA code, a genome, that contains genes that give instructions on how to develop and function properly. Notably, the DNA code in different individuals from the same species is not identical even though it is derived from the same original progenitor line. This condition, known as genetic diversity, is the variation of the genetic make-up among individuals of a population, a species, an assemblage or a community. Genetic diversity allows species to adapt to the fluctuations in the environment in the long term and to secure the survival of the species and avoid extinction (Ellegren & Galtier, 2016; Fu, 2015). Only species that can adapt to the environmental changes and other selection pressures with the right traits will be able to survive and sustain (Tigano & Friesen, 2016).

The main importance of genetic diversity is to help species adaptability and survival towards different abiotic and biotic stresses. Species with high genetic diversity will likely have higher survival rate towards the changes of major abiotic factor such as water, air, soil, sunlight and temperature. This is exemplified in *Arabidopsis thaliana* recombinant inbred line (RILs) where adaptation to different environments can be achieved by changing a few genomic regions where the fitness trade-offs are common and lack of genetic variation can limit adaptation (Ågren et al., 2013). A study conducted on European plant species revealed that plants survive in Alpine zone exhibit a lower genetic diversity than lowland regions suggesting smaller population size which has been selected for high altitude adaptation (Reisch & Rosbakh, 2021). With regards to biotic stress, genetic diversity provides a greater number of genetic solutions for the organism/species to counter pathogens and herbivores. As an example, high genetic diversity in wild *Lactuca* L. species leads to a wide variety of traits with a great potential for resistance against various biotic stresses and can be exploited to the improvement of domesticated Lettuce (*Lactuca*

sativa) (Lebeda et al., 2014). Another example is by taking advantage of diversity in 12 wild tomato relatives under Lycopersicon clade where there are many traits of interest for biotic and abiotic stresses from wild species that can be introgressed into domesticated tomato (Bauchet & Causse, 2012).

Genetic diversity also can affect the geographical distribution of a species and allow habitat expansion by adapting to various environmental conditions and increase their survival rate. Evidence for this is found in the invasive wetland grass, *Phalaris arundinacea* L. where high genetic variation allows it to adapt to the new environment by evolving to overcome the differences from its native habitat (Lavergne & Molofsky, 2007). Hence, species survival will be secured with genetic diversity providing high evolutionary potential for rapid selection of genotype with higher vegetative colonization ability and phenotypic plasticity (Ellegren & Galtier, 2016; Lavergne & Molofsky, 2007). Moreover, habitat fragmentation by habitat loss or by biotic and abiotic stresses can also shape genetic diversity for certain species (Wilson et al., 2016). Low rates of genetic diversity can decrease the fitness of population resulting from intraspecific hybridization and loss of neutral genetic variation (Ellegren & Galtier, 2016; Fu, 2015). Lastly, genetic diversity is responsible for the morphological, physiological and behavioural variation between individuals in the same species as response to the adaption and evolution to the new environment (Johnson & Munshi-South, 2017).

Factors such as mutation, genetic drift, gene flow and natural selection play an important role in controlling the development, sustainability and evolution of genetic diversity (Ellegren & Galtier, 2016). Mutation is the fundamental cause of all genetic diversity and can be defined as a change in nucleotide composition of the genome. Apart from natural genetic mutation, induced mutation also plays an important role for creating new genetic diversity and is very useful for the improvement of crop species with limited diversity (Smith et al., 2015). Secondly, genetic drift is the

change in frequency of an existing gene variant in the population due to random chance and creates regions with low genetic diversity at the expansion front. Genetic drift resulted in opposes mutualism during spatial population expansion causing less co-existence and genetic diversity (Müller et al., 2014). Thirdly, gene flow is the movement of genes into or out of a population that promotes diversity of alleles and known to be involved in the adaptation to local environment (Tigano & Friesen, 2016). Finally, natural selection can lead to the removal of genetic variation at linked neutral sites, even in species with large populations, suggesting that natural selection can constrain the level of neutral genetic diversity across many species (Corbett-Detig et al., 2015).

As genetic diversity is essential for the survival of organism, the key mechanism that responsible to regulate and manipulate genetic composition in eukaryotes through sexual reproduction is a special process known as meiosis. Meiosis responsible for the production of reduced ploidy number of haploid gamete cell before fertilization that contains diverse combination of the next progeny lines. Meiotic crossover (CO), also known as "crossing over" is an important process that occurs during meiosis and plays a crucial role in genetic diversity. The mechanism of meiotic CO that occurred during prophase I is a special event that play an important element during meiosis (Grelon, 2016).

1.2. Meiotic crossing over shapes genetic diversity

Meiosis is a specialized cell division that results in the production of genetically diverse haploid cells from a diploid parent cell. The process includes one round of DNA replication followed by two rounds of division, resulting in four daughter cells, each with half of the number of chromosomes as the parent cell (Mercier et al., 2015). Meiosis plays a crucial role in sexual reproduction, allowing for the mixing of genetic material between two individuals and producing offspring with unique combinations of traits. During meiosis, each of homologous chromosomes

will be duplicated into two sister chromatids that are held together by a tripartite proteinaceous cohesin ring (Nasmyth, 2005). Later, bivalents will be formed when homologous chromosomes become physically linked during recombination and crossover formation. After chromosome replication, through Anaphase I and II stages where two rounds of chromosome segregation will be occurred (Grelon, 2016). The separation of homologous chromosomes happens during meiosis I while separation of sister chromatids will occur during meiosis II (Lambing et al., 2017; Mercier et al., 2015).

In general, meiotic recombination start with the formation of large amount of DNA double strand breaks (DSBs). Resection of 5' end of the DSB to produce 3' single stranded DNA molecules will therefore allow homology search and invasion of intact DNA duplex from the sister chromatid or homologous chromosome. Subsequently, the recombination intermediates that are formed after invasion will be stabilised by the ZMM proteins (ZIP1, ZIP2, ZIP3, MSH4, MSH5, MER3, SPO16, and SPO22/ZIP4) and this leads to the double holiday junctions (dHJs) formation (Pyatnitskaya et al., 2019). A small fraction of dHJs will be resolved as a CO while the majority of these joint molecules will be repaired as a non-crossover (NCOs). Meiosis through CO formation helps to ensure genetic diversity in sexually reproducing organisms by shuffling and combining genetic information and also through random segregation of homologous chromosomes (Lambing et al., 2017)

The main evolutionary benefit of meiotic COs is that chromosomal recombination enables the reciprocal exchange of genetic material between homologous chromosomes and this creates diverse genetic diversity through generating new combination of genes (Blary & Jenczewski, 2019). As described in section 1.1, meiotic CO can assist the improvement of adaptation in the way the progeny produced from the combination of different traits will have better adaption to stresses, improved fitness and ensuring sustainability of the population (Fayos et al., 2022). Another

benefit of meiotic CO is it play as a chromosomal aberrations corrector by repairing recombining segments of chromosome that contains deletion or duplication (Mercier et al., 2015; Wang & Copenhaver, 2018). Thus, it is also can prevent the occurrence of abnormal numbers of chromosome which usually can lead to aneuploidy and genetic disorder. Lastly, meiotic CO is capable to separate the formation of linked genes which usually occurred on the same chromosome and this will allow greater segregation of the traits. Separation of linked genes can help to eliminate the deleterious trait and boosting the expression of beneficial traits (Blary & Jenczewski, 2019; Fayos et al., 2022; Li et al., 2021). In general, meiotic crossover is regulated by two different mechanism which are pro-crossover pathway (class I and class II CO) that promote crossover and anti-crossover pathway that consequential to gene conversion and non-crossover event (Figure 1.0) (Mercier et al., 2015; Youds & Boulton, 2011).

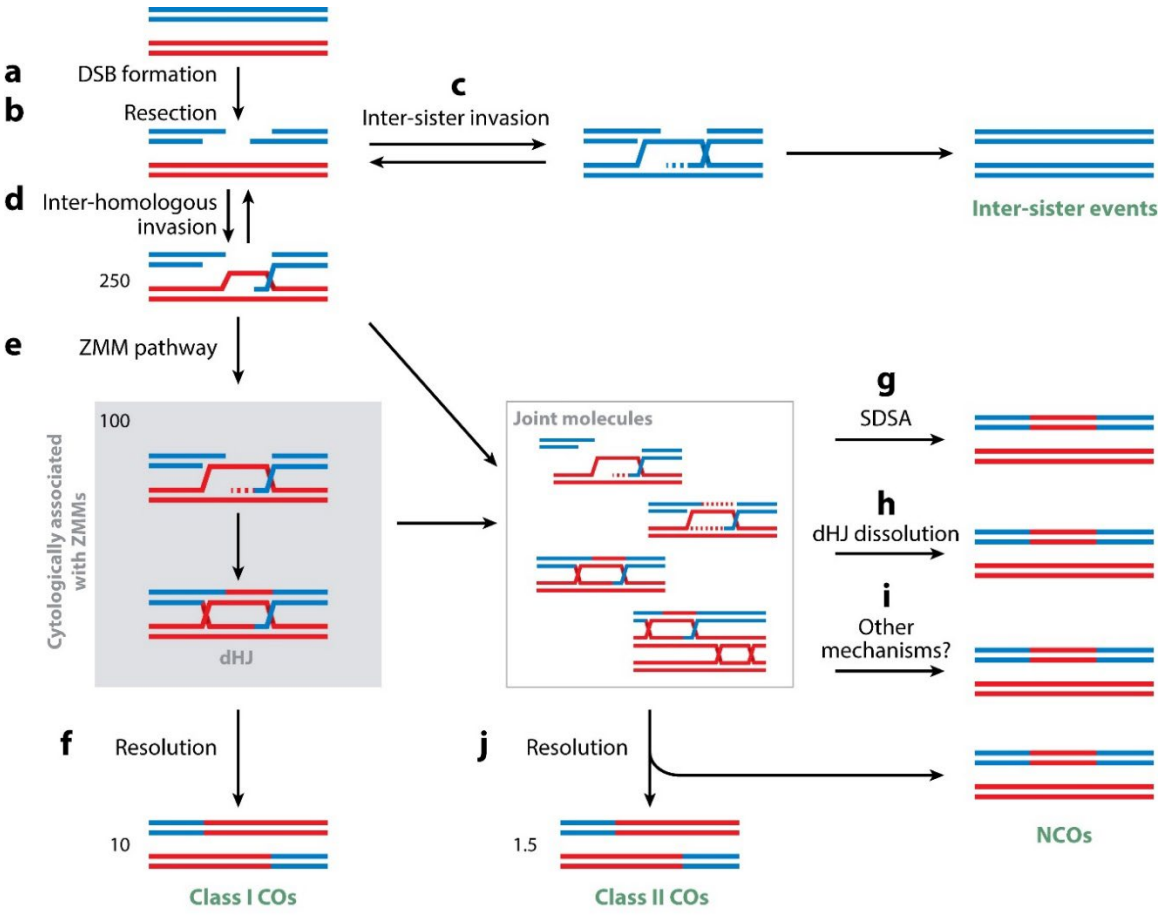

Figure 1.0: Model of meiotic recombination mechanisms (Mercier et al., 2015). Meiotic recombination is initiated by the formation of a large number of double-strand breaks (DSBs) **(a)** that are processed **(b)** to yield 3′-OH single-stranded DNA. This DNA can then invade either the intact sister chromatid **(c)** or one of the two homologous chromatids, forming a D loop **(d)**. Inter-homologous intermediates can be protected by components of the ZMM pathway **(e)**, generating double Holliday junction (dHJ) intermediates that can be resolved into class I crossovers (COs) **(f).** Alternatively, the intermediates can be matured into non-crossovers (NCOs) through different mechanisms, including synthesis-dependent strand annealing (SDSA) **(g)**, dHJ dissolution (h), and possibly other mechanisms **(i)**. In addition, a ZMM-independent pathway produces class II COs **(j)**. The estimated number of each intermediate in *Arabidopsis thaliana* is indicated.

1.2.1. Pro-Crossover

After formation of DSBs, later it will be nicked and resected to 3’ ssDNA and further associated with DMC1 and RAD51 to search for homologous sequences either from sister chromatid or homologous chromatid (Lambing et al., 2017). Strand invasion to homologous sequence will result in the formation of dHJs and further will be repaired either by COs pathway (class I and/or class II) or NCO pathway. The mechanism that decide for either CO or NCO is remain unclear but many factors as described in section 1.3 contributed to the CO/NCO fate (Pyatnitskaya et al., 2019).

Class I CO pathway are known to be the major pathway accounted for overall 85-90% of CO event in plant (Mercier et al., 2015). CO that depend on dHJs formation is known as class I CO and it is CO interference sensitive and are controlled by a group of protein known as ZMM protein (Karthika et al., 2020; Manhart & Alani, 2016). ZMM proteins involved in class I CO detected during early recombination intermediates and only few matured in CO at late pachytene stage (Lambing et al., 2017). While, HEI10 and MLH1/MLH3 protein is also play an important role in class I CO especially in late stage pachytene although these proteins are not a part of ZMM protein (Chelysheva et al., 2012; Karthika et al., 2020; Manhart & Alani, 2016).

Another pathway that promote meiotic CO is class II CO pathways and it is non interreference sensitive (Youds & Boulton, 2011). In Arabidopsis, this class II CO pathways accounted for 10 to 20% of meiotic CO. Class II CO pathway do not involved ZMM protein and to date in MUS81-

EME1 protein complex has being characterized in Arabidopsis cleave Holliday Junctions (Geuting et al., 2009). It is suggested in yeast, MUS81 protein act as endonuclease to resolve aberrant join molecule that maybe formed during class I CO pathway (Hollingsworth et al., 2007). Class I and class II CO pathways have different molecular regulation mechanism and also differ in the distribution of CO formation (Lambing et al., 2017; Li et al., 2021; Mercier et al., 2015; Youds & Boulton, 2011).

1.2.2. Non-crossover/Anti-Crossover factors

Additional to two CO pathways, there is also an anti-crossover pathway which is also contributed to the DSBs repair. There are a greater number of DSBs when compared to the COs formation and majority of DSBs are repaired through NCO pathways. In Arabidopsis, approximately 250 DSBs were generated and only 10 DSBs are repaired through class I CO pathways, 1 to 2 DSBs are repaired by class II CO pathway and the rest are fixed via NCO pathways (Mercier et al., 2015). NCO formation always associated with the present of heteroduplex formation from paternal and maternal strands and this need to be corrected which eventually lead to the gene conversion (Lambing et al., 2017).

In *S. cerevisiae*, NCO formation is derived from the dissolution of ssDNA invasion intermediate in the Synthesis-dependent Strand Annealing (SDSA) pathway (McMahill et al., 2007). At least in Arabidopsis, three different anti-crossover pathways have been identified to be involved in non-crossover repair, namely; (1) FANCM helicase and DNA binding co-factors, (2) FIGL1-FLIP complex and (3) BTR (BLM-TOP3α-RMI1) anti-CO pathways (W Crismani et al., 2012; Fernandes et al., 2018; Séguéla-Arnaud et al., 2015). Loss-of-function mutations in these genes/pathways is associated with increased meiotic CO particularly through class II crossover pathway (Blary & Jenczewski, 2019; Li et al., 2021).

1.3. Factors influencing meiotic crossover

In general, meiotic CO rate varied between organisms with most organisms on average have 1-3 COs per chromosome. As described in figure 1.1, some organisms can have more than 10 COs such as fission yeast and honey bee with majority have less than three CO (Mercier et al., 2015). There are many factors that may influence the CO rate and it can come directly from the properties of organism's genome or can be caused by environment factors.

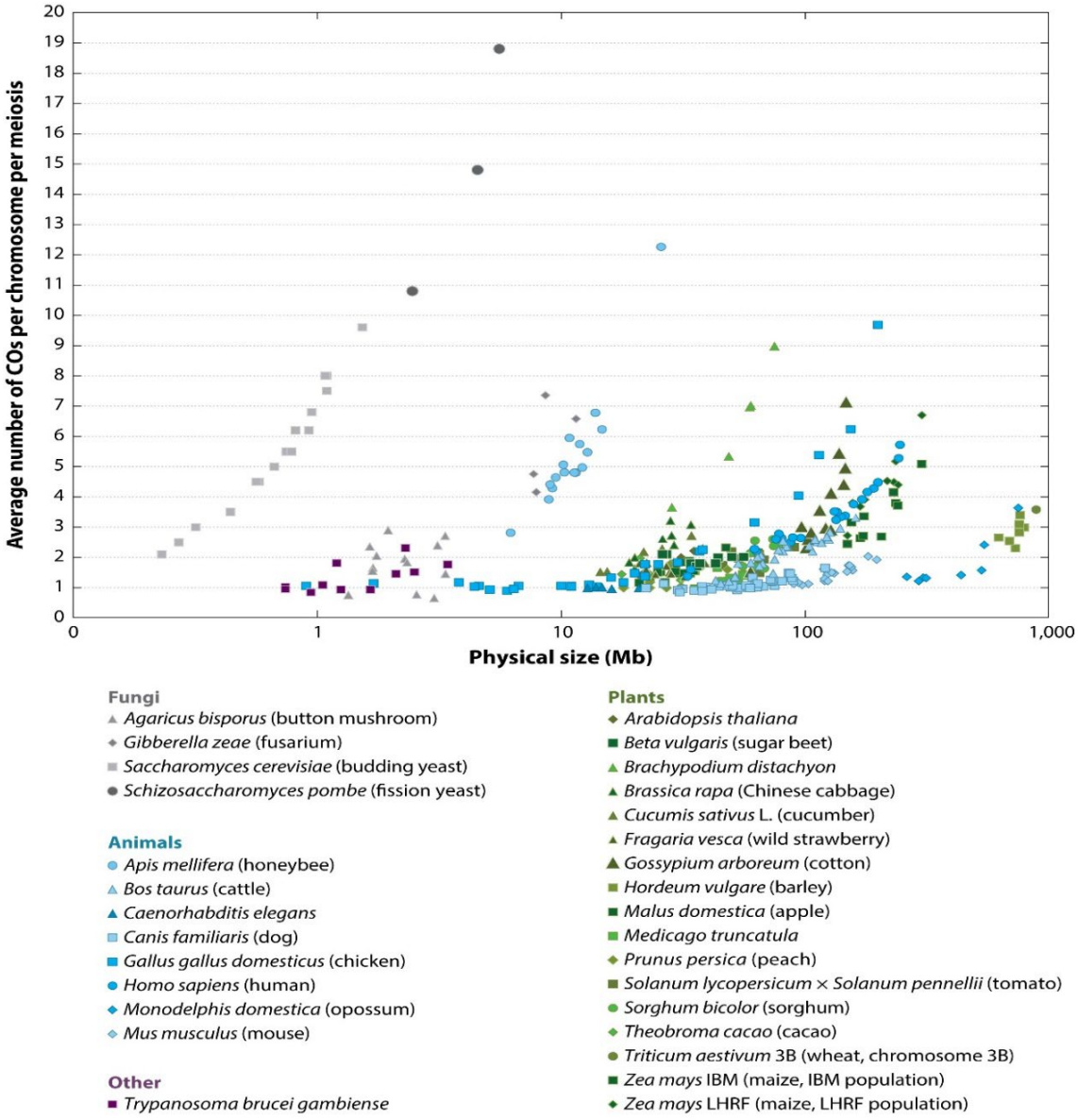

Figure 1.1: Number of crossovers (COs) per chromosome per meiosis in a variety of eukaryotes (Mercier et al., 2015). The number of COs, deduced from male/female-average genetic maps, is plotted against the physical size of each autosomal chromosome (Mb, log scale).

The first factor that influence meiotic crossover frequency is the **size of the genome or chromosome**. Larger genomes may have a lower recombination frequency due to the increase of physical distance between genes and thus making it more difficult for genetic material to be exchanged during meiosis. In various angiosperms species, it is observed that recombination rate is increasing toward smaller genome size as it is probably caused by removal of LTR retrotransposons (Tiley & Burleigh, 2015). In different genome species, the correlation between recombination rate and the distance from centromere is varied based on genome size with smaller plant genome such as Arabidopsis (Horton et al., 2012)showed lower correlation while plant with bigger genomes like barley and wheat have higher correlation (Dreissig et al., 2020; Jordan et al., 2018). However, another study has suggested that larger genomes in angiosperm species have a higher recombination frequency when it compared to the size of the euchromatic or non-repetitive fraction of the genome (Ross-Ibarra, 2007). In order to achieve a higher genome-wide crossover rate, an organism evolved to have smaller size genome as short chromosome has more homogenous crossover rate when compared to long chromosome which has low crossover rate in the centre (Haenel et al., 2018). Another observation made by Stapley et al., (2017) suggesting that larger plant genome has lower recombination rate but there is no evidence to suggest recombination rate declines with genome size in animals and fungi.

Interhomolog polymorphism is referred as a genetic variation that occurs within the same chromosome pair or also known as homologs in diploid organism. This type of polymorphism can derive from differences in the number of copies of a particular genetic element such as a tandem repeat or from point mutations in a specific gene (Ziolkowski, 2022). Meanwhile, heterozygosity is referring to the presence of different alleles at a particular locus or chromosome position in individual (Dluzewska et al., 2018). Both **interhomolog polymorphism and heterozygosity** can influence the frequency or location of crossover events in the region that involved in crossover. In Arabidopsis, a negative non-linear relationship was

observed in *RAC1* and *RPP13* disease resistance genes between interhomolog divergence and crossover frequency within the hotspots and it is consistent with polymorphism locally suppressing crossover repair of DSBs (Serra et al., 2018). Interhomolog polymorphism do not change crossover frequency unless it is located directly within crossover hotspots and analysis using mismatch detection-deficient *msh2* mutants found there is an increase of crossover in hotspot region of heterozygous lines when compared to homozygous inbred line (Szymanska-Lejman et al., 2023). Moreover, DNA mismatches recombining molecules caused by interhomolog polymorphism in Arabidopsis was recognised by MSH2 mismatch repair protein suggesting its role as pro-crossover in region with higher sequence diversity (Blackwell et al., 2020). In Arabidopsis hybrid, CO are observed to occur more common between heterozygous regions that are close to homozygous regions on the same chromosome indicating that boundaries between the identical and non-identical region influence the location of the CO (Ziolkowski et al., 2015). The recombination landscape is altered by heterozygous inversion with the changing of the CO and NCO gene conversion proportions suggesting that DSB fate will be hold until a CO assurance checkpoint has been satisfied (Crown et al., 2018). Heterozygosity is also suggested to have stronger level of interference by preventing another CO to happen nearby on the same chromosome (Ziolkowski et al., 2015).

Heterochromatin is a tightly packed and condensed form of chromatin, which is the material that makes up chromosomes and its influences the frequency and location of meiotic CO. Plants with large genome and extensive repeat-rich pericentromeric region such as barley, wheat, maize, cotton and tomato have uneven distribution of meiotic crossover where high recombination rates were observed in distal regions while large pericentromeric regions have little to no recombination (Lloyd, 2022). This is reported for maize knob heterochromatin located on telomere region where it has suppression of crossover but not totally eliminated based on the signal from MLH1 foci (Stack et al., 2017). Less crossover

occurs in heterochromatin due to its compact and tightly packed structure, which limits access to DNA for recombination and repair processes. Additionally, heterochromatin often contains repetitive DNA sequences, which can lead to the formation of double-stranded DNA breaks during meiosis, reducing the frequency of successful crossover events. Connected to heterochromatin structure is **DNA methylation** where is often associated with gene repression and the formation of heterochromatin. Effect of DNA methylation on crossover was reported using *Decreased DNA Methylation 1* (*ddm1*) mutant that play a role in CG and non-CG DNA methylation and heterochromatin maintenance (Melamed-Bessudo & Levy, 2012). Higher crossover in *ddm1* mutant for both heterozygous and homozygous genotype on euchromatic regions but has no effect on heterochromatic pericentromeric regions that underwent demethylation (Melamed-Bessudo & Levy, 2012). Interestingly in *drm1 drm2 cmt3* mutant which removes most of non-CG methylation while CG methylation remains intact, there is a strong increase in meiotic crossover in heterochromatic pericentromeric regions (Underwood et al., 2018).

Crossover interference is the mechanism of the presence of one meiotic CO reducing the likelihood of another crossover nearby resulting in crossover spacing which is more uniform (Wayne Crismani et al., 2021). Class I CO is associated with the CO interference as class I CO crossovers is non-randomly distributed along chromosomes (Lloyd, 2022). This interference resulted in the reduction of the overall frequency of crossovers and ensures that crossovers are distributed evenly along the chromosomes, avoiding clustering or gaps and this help maintaining the stability of the genome and promotes the formation of genetically diverse offspring. ZYP1 protein which is a transverse filament in Arabidopsis was reported to play role in regulating the number and distribution of CO, imposing CO interference and heterochiasmy (Capilla-Pérez et al., 2021).

Generally, **ploidy level** can affect meiotic recombination as the frequency and pattern of meiotic recombination is influenced by the number of homologous chromosomes in the cell. In autotetraploid and allotetraploid Arabidopsis, the rates of meiotic recombination for reciprocal crosses identified using seed-specific fluorescence were significantly higher when compared to genetically identical diploid (Pecinka et al., 2012). This is because in diploid organism, the recombination is occurred between two copies of homologous recombination while in polyploid organism has multiple set of chromosomes and therefore the number of potential recombination partner increases and affect the frequency and pattern of meiotic recombination. Similar pattern was also observed in *Brassica* species where the number of crossovers is highest in allotriploid AAC hybrid and followed by allotetraploid AACC hybrid when those compared to diploid AA suggesting this increase is associated with hybrid karyotype composition and interference maintenance in the AAC hybrids (Leflon et al., 2010).

Temperature is another factor that influences meiotic CO in two different ways which is first by altering the frequency and placement of crossover events and second by causing the disruptions of core structures of the axis and synaptonemal complex that lead to failures in chromosome pairing, synapsis, recombination and segregation (Morgan et al., 2017). In Arabidopsis, elevated temperature from 20°C to 28°C induces higher meiotic recombination and this extra CO derived from class I CO interference sensitive pathway (Modliszewski et al., 2018). However, Arabidopsis growth under 30-32°C for 24 hours showed defects in SC formation, presence of chromosome bridges at anaphase I, occasional display of univalent chromosomes and eventually lead to bi- or polynuclear microspore formation (De Storme & Geelen, 2020).

1.4. Importance of Meiotic Crossover in Plant Breeding

Meiotic crossover is crucial in plant breeding as it allows breeders to generate novel genotypic combinations and improve elite lines (Youds & Boulton, 2011). Genetic exchange between homologous chromosomes during meiosis results in the shuffling of genes and the formation of new combinations of alleles (Mercier et al., 2015). Thus, this creates genetic diversity that can be used in plant breeding programs for the production of new cultivars with desired traits such as improved yield, disease resistance or enhanced nutritional content (Li et al., 2021). By controlling the frequency and location of meiotic crossover events, it could be possible in future for breeders to target specific genes or traits leading to the development of more efficient and effective breeding strategies (Blary & Jenczewski, 2019).

Manipulating meiotic crossover is a promising strategy to improve the resilience of crops to abiotic and biotic stress. Abiotic stress factors such as drought, salinity, and extreme temperatures can limit crop productivity, and biotic stress factors such as pests and diseases can cause significant yield losses (De Storme & Geelen, 2014). Nevertheless, manipulating the frequency and location of meiotic crossover events can enhance the expression of genes involved in stress tolerance and further improve the overall stress tolerance of crops (Wang & Copenhaver, 2018). For example, an increase in the frequency of crossover events in the specific regions of the genome can enhance the expression of stress-tolerant related genes and improve crop toward abiotic stresses. Similarly, decreasing the frequency of crossover events in specific regions of the crop genome that contain stress-susceptible genes can reduce the expression of these genes and improve stress tolerance (Fayos et al., 2022).

Therefore, manipulation of meiotic crossover is a valuable tool in plant breeding as it allows for the construction of new genetic combinations and also control the regulation of specific genes or traits for crops improvement (Blary & Jenczewski, 2019; Li et al., 2021). This allow to the development

of crops with improved abiotic and biotic stress and subsequently produce high-yield crops to cater challenging environment changes.

1.5. Tomato as a crop meiotic model

Tomato is the most cultivated vegetable in the world with 182.86 million metric tons of tomato fruit being produced in 2020 (FAO, 2020). It is also an emerging dicot plant model system for functional genetics research with an increase number of mutant collection available for research (Shikata & Ezura, 2016). Peters & Underwood (2022) have stated that cultivated tomato and its related wild species are highly suitable model for plant meiosis research particularly in understanding the recombination suppression, genetic heterozygosity on CO behaviour and the effect of meiosis on abiotic stress. As an important crop, there are plenty of tomato genome sequences available on different kind of domesticated cultivars and related wild species (Su et al., 2021; van Rengs et al., 2022; Zhou et al., 2022). There is also high-continuity and annotated tomato reference genome available for Heinz 1706 variety with high resolution and accuracy for high-density genetic map (Su et al., 2021).

The recent development of long read sequencing platforms such as PacBio sequel II and ONT Promethion are accelerating the generation of near-complete genome sequences for tomato and increase the number of tomato genomes that are available (van Rengs et al., 2022). Moreover, 838 different tomatoes lines have been re-sequenced for the construction of the tomato graph pangenome and this will facilitate the improvement of tomato by marker-assisted breeding and genomic selection (Zhou et al., 2022). Tomato has 12 chromosome pairs with total genome size which is approximately around 800 Mbp and this medium genome size make tomato as an ideal model to be used as a dicots plant model. As tomato has diploid chromosome with no recent history of polyploids, study meiosis through cytology and genomic will be easier as it has less complicated genome with low number of gene duplications (Peters & Underwood, 2022).

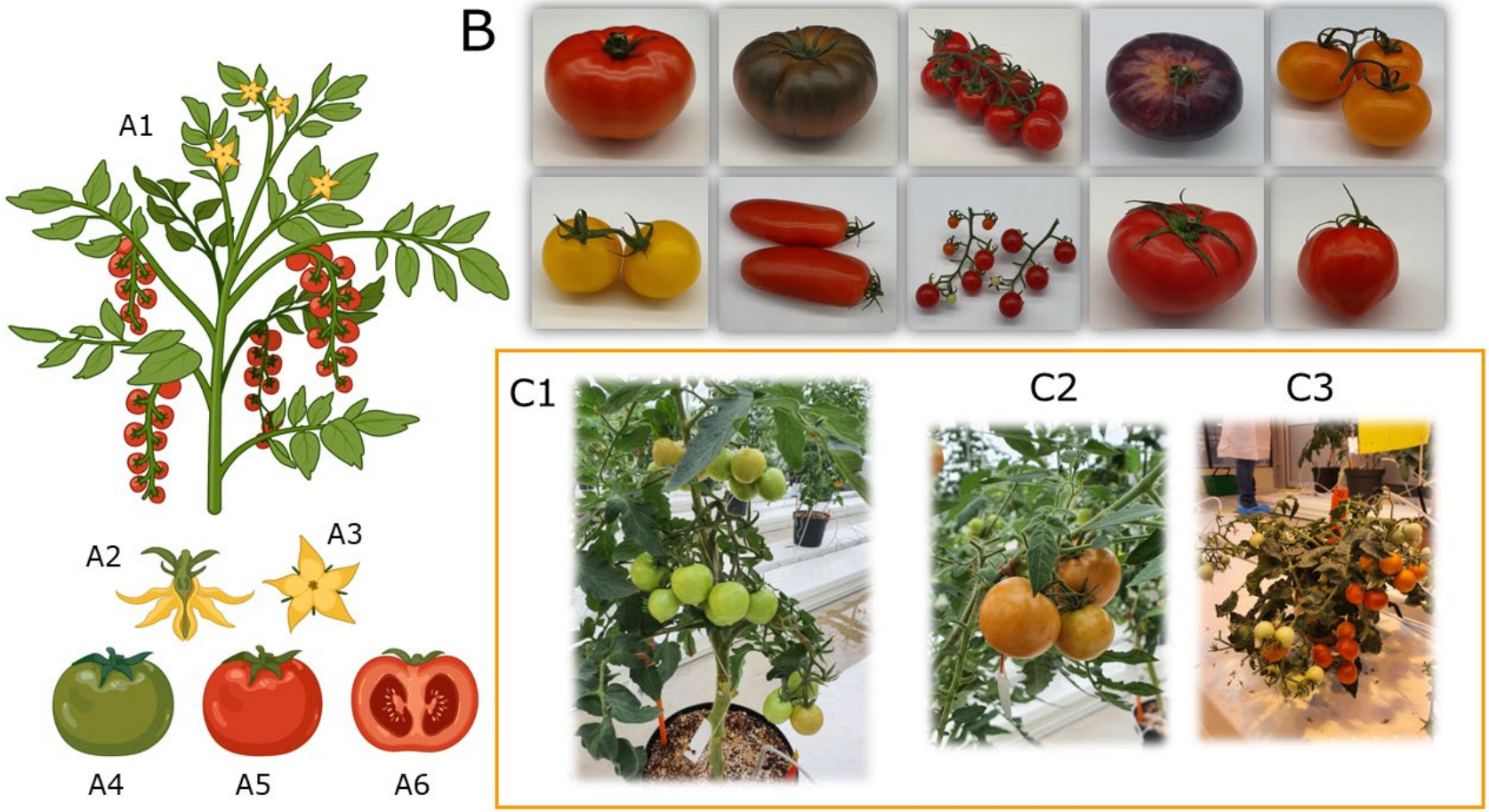

Figure 1.2: Illustration of tomato (*Solanum lycopercisum*) plant with the diversity of fruit phenotypic and tomato cultivar as a plant model system for the dicot crop species. **A1):** Mature tomato plant with ripe fruit bunches and yellow flowers, **A2):** Cross-section of tomato flower, **A3):** Full bloom of tomato flower, **A4):** Green unripe tomato fruit, **A5):** Red fully ripe tomato fruit, **A6):** Cross-section of fully ripe tomato with two locus. **B):** Diversity of cultivated tomato varieties/hybrids with diverse fruit phenotypes with different colour, size and shape. **C1):** Greenhouse indeterminate tomato cv. Moneyberg, **C2):** Tomato cv. Moneyberg fruit start to ripe, **C3)** Dwarf determine tomato cultivar, Micro-Tom used widely as ornamental plant and also as plant model systems for dicot species.

Tomato also offer an extra advantage as a model system since it can be crossed with other Solanum species particularly in Lycopersicon clade (Zeist et al., 2020). Another advantage of tomato is there are numerous establish genetic transformation and *in vitro* regeneration protocol and this allow production of tomato mutant to study any function of genes or pathways (Khuong et al., 2013; Sun et al., 2006). As tomato is widely grown as a crop, some of cultivar especially ornamental dwarf variety like Micro-Tom has rapid growth rate, early flowering, easy to handle and thus can speed up research work and reduced working space usage (Just et al., 2013).

1.6. Objectives of the research

As taking advantages of tomato as an emerging dicots model system and also an important crop worldwide, this research aims to:

I. To develop pro- and anti-crossover meiotic mutants in tomato using genetic transformation of gene editing constructs

II. To observed the physiological characteristics of the pro- and anti-crossover meiotic mutants

III. To characterize the chromosomal behaviour of pro- and anti-crossover meiotic mutants using via cytology

IV. To study the behaviour of meiotic crossover mutant in interspecific tomato hybrid

1.7. References

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Pivotal roles of SlMLH1 and SlZIP4 in the formation of class I meiotic crossovers and fertility in tomato

2.1 Abstract

Meiotic crossover (CO) plays a key role in the balanced segregation of homologous chromosomes during the first meiotic division and increases genetic diversity in natural populations. The Class I CO pathway is the major pathway for the formation of meiotic CO. This pathway has been described in many organisms and involves conserved "ZMM" proteins that co-operate to stabilize recombination intermediates that are finally resolved as COs in late prophase of the first meiotic division. In Arabidopsis, the ZMM mutant *zip4* has a stronger reduction in CO rate and fertility than *mlh1* mutants that are defective in the biased resolution of recombination intermediates as COs. There is lack of information for the behaviour *mlh1* and *zip4* mutants in other dicot species. Therefore, the tomato *MutL homolog 1* gene (*SLMLH1*) and tomato *ZIP4* (*SlZIP4*) were identified by bioinformatic analysis and targeted by CRISPR/Cas9 mutagenesis in the dwarf tomato cultivar, Micro-Tom. Many independent lines were regenerated for both *Slmlh1* and *Slzip4,* and two representative stable lines for each mutant were further characterized. *Slmlh1* mutants had reduced fertility as only 13.4% of pollen is normal and viable, leading to reduced fruit set, fruit size, and extremely few seeds by selfing. In contrast, *Slzip4* mutants produce just 1.49% viable pollen which is very significantly reduced when compared to wildtype Micro-Tom (97.6%) and were completely sterile without any seed being produced by selfing. Observation of *Slmlh1* and *Slzip4* meiocytes showed abnormal chromosome behaviour such as formation of univalent, aberrant homologous chromosome segregation and micronuclei formation at the tetrad stage, which explain the respective reduction in fertility and sterility. Significant reduction of CO number were observed in *Slmlh1* (8.2 crossovers) with *Slzip4* (5.2 crossovers) had the lowest CO number when compared to wildtype Micro-Tom (17.8 crossovers). Formation of univalent chromosome in *Slmlh1* meiocytes cell resulted in aneuploidy in some progeny. Remarkably, hybridization of *Slmlh1* with wild *S. pimpinellifolium* and introgression, caused an increase of CO number and pollen viability of *mlh1* mutants (10 crossovers), suggesting the presence of a natural genetic modifier of CO rate in the progenitor species of the domesticated tomato. Tomato has more chromosomes, a larger total genome size and more heterochromatin component than Arabidopsis. We conclude that loss of MLH1 and ZIP4 in different genomic settings can ultimately lead to different effects on plant fertility.

2.2 Introduction and research background

The Class I CO pathway is responsible for the majority meiotic COs in sexually reproducing organisms (Lambing et al., 2017; Y. Wang & Copenhaver, 2018). This type of CO is dependent on the meiosis-specific protein known as a ZMM proteins (ZIP1, ZIP2, ZIP3, MSH4, MSH5, MER3, SPO16, and SPO22/ZIP4) (Pyatnitskaya et al., 2019). It is known that the main role of the ZMM protein is to stabilize recombination intermediates that formed between homologous chromosomes (Lloyd, 2022). Another specific characteristic of class I CO pathway is the involvement of another two conserved proteins, MLH1 and MLH3 that help regulate CO in ZMM pathway (Cannavo et al., 2020; Manhart & Alani, 2016). MLH1-MLH3 heterodimer are not classified as ZMM proteins but they have direct involvement with ZMM proteins in CO formation and bias the resolution of recombination intermediates as COs during late prophase I stage (Cannavo et al., 2020; Manhart & Alani, 2016).

In budding yeast, double mutation of *mlh1* and *mms4* caused largest decrease in crossover up to 15-fold but still retain 42% of spore viability (Argueso et al., 2004). A similar picture is observed in plants, for example in Arabidopsis *Atmsh4,* a zmm mutant, there is a great reduction of CO formation to just 15% when compared to wildtype Arabidopsis indicating the crucial role of ZMM protein in CO formation (Higgins et al., 2004). In addition it was also observed that the frequency CO in *mlh3 zmm* and *mlh1 zmm* double mutants are indistinguishable from *zmm* single mutants (Mercier et al., 2015). Those CO that are formed through the ZMM pathway are always regulated by controlled mechanism known as "CO interference" which means that CO number and also distribution along chromosomes is in a non-random manner whereby CO events are spread apart from one another (Crismani et al., 2021). However, non-zmm class I CO genes displayed milder effect in term of CO reduction as this can be seen in Arabidopsis and rice *mlh1* mutant (Dion et al., 2007; Mao et al., 2021; Xin et al., 2021).

In order to have proper meiotic chromosome segregation, homologs chromosome must be linked with at least one CO formation. Formation of at least one obligatory crossover per bivalent, a pair of homologous chromosome during meiosis ensures accurate segregation so that each chromosome will remains as "a part of the permanent chromosome complement" and this phenomenon known as CO assurance (Lloyd, 2022; Y. Wang & Copenhaver, 2018). As meiotic crossover is not only involve in the mechanism for the formation of CO but it is also required to ensure all DSBs in meiosis is repaired (Gray & Cohen, 2016; Pazhayam et al., 2021). Failure to perform DNA repair and to form meiotic crossover or chromosome cannot be separated equally resulted to unequal segregation leading to aneuploidy (Luo et al., 2013). This aberrant chromosome resulted from abnormal meiotic crossover will reflect the fertility rate of haploid gamete cell that being produced in the final process of meiosis (Dion et al., 2007; Higgins et al., 2004; Luo et al., 2013; Mao et al., 2021).

As in class I CO pathway, mutation of ZMM proteins and other genes related to the class I CO pathway during late prophase I stage influence meiotic CO rate and fertility differently (Argueso et al., 2004). This is depending on the mode of the action of the protein to regulate class I meiotic CO and involvement of cofactor that may regulate CO dependently or independently (Pannafino & Alani, 2021). As example, the ZMM proteins induced obligate CO formation as the binding of ZMM protein complex into DNA strand is crucial during strand invasion in order to prevent dissolution of D-loop that eventually prevent DNA repair through NCO pathways (Pyatnitskaya et al., 2019). However, mutation of certain component of ZMM protein like ZYP1 do not give strong effect to fertility and surprisingly abolish crossover interference and can increase CO frequency (Capilla-Pérez et al., 2021). In general, it was reported in many researches that abnormal function of ZMM protein during class I meiotic CO usually will lead to massive reduction of

fertility when compared to those non-ZMM genes that regulate crossover in the late stage of class I CO pathway (Chelysheva et al., 2007; Dion et al., 2007; Higgins et al., 2004; Mao et al., 2021; Shen et al., 2012; Zhang et al., 2014). As this can be seen in Arabidopsis for *zmm* mutant, *Atmsh4* which showed high percentage of infertility when compared to *Atmlh1* mutant with milder effect in fertility rate (Dion et al., 2007; Higgins et al., 2004).

Nevertheless, there are also other factors that may influence the occurrence of meiotic CO such as the size and length of chromosomes, CO interference, ploidy number and the location of CO hotspots (Haenel et al., 2018; Suay et al., 2014; Tock & Henderson, 2018; P. Wang et al., 2019). Variation in recombination rate is also diverge between related taxa, populations and individual which are associated to genome architecture, genetic and epigenetic mechanisms, sex, environment perturbations and variable selective pressure (Stapley et al., 2017). Study on CO interference in entire genome of *Populus euphratica* has found that the CO interference is depends on the length of chromosomes and the genomic locations within the chromosome (P. Wang et al., 2019). It is described that long chromosome has low crossover rate in their centre while short chromosomes show a quite homogeneous crossover rate suggesting that organism have higher genome-wide crossover rate by evolving to have smaller chromosomes (Haenel et al., 2018). Therefore, homolog gene for meiotic CO between different species may regulate CO rate and recombination frequency differently as many factors influence its mechanism in different genomic background.

As stated above, contribution and involvement of different genes in regulating class I CO mechanism is depending on the various factor such as hierarchy of class I CO gene in the pathway, species, genetic properties and also various environment factors. In case of tomato, contribution of ZMM proteins and other proteins in late prophase I during meiotic CO may not showing similar behaviour and may not reflect to those reported in

Arabidopsis. Taking advantage of numerous genome databases of cultivated and wild tomato species, we aim to study the behaviour of class I CO gene, *MLH1* and *ZIP4* in tomato (Fernandez-Pozo et al., 2015; Kobayashi et al., 2014; X. Wang et al., 2020). Using the dwarf tomato variety, Micro-Tom, with short lifespan cycle, rapid flowering onset and an established genetic transformation protocol, we have developed tomato *mlh1* and *zip4* mutants, using CRISPR-Cas9 technology (Čermák et al., 2017a; Khuong et al., 2013; Martí et al., 2006; Sun et al., 2006). Thus, in this research we extensively characterized the behaviour of meiotic chromosomes and fertility of *Slmlh1* and *Slzip4* mutants.

2.3 Results

2.3.1 Identification of a *MLH1* and *ZIP4* ortholog in tomato

Based on alignment and comparison with *AtMLH1*, *SlMLH1* gene was found to have 72% similarity with *AtMLH1* with 92% of query cover. *SlMLH1* gene was located on chromosome 4 of the tomato genome and has high similarity with the *MLH1* gene from the tomato wild relative *Solanum pennellii* (99.2% homology). The sequence of the SIMLH1 protein was found to contain MLH1 conserved domains such as Mlh1C, DNA mismatch repair and HATPase C3. These motifs are similar to those identified in the *A. thaliana* MLH1 protein indicating a high similarity of protein domains despite the SlMLH1 protein being slightly shorter than AtMLH1 protein (**Figure 2.0A**). Phylogenetic analysis of SlMLH1 protein sequence and other selected MLH1 proteins from different taxa, group and species of organisms revealed, as expected, that SlMLH1 groups together with MLH1 proteins from other eudicot species (*Glycine Max*, *Arabidopsis thaliana* and *Brassica rapa*)(**Figure 2.1A**). This result indicated that more conserved and similarity of SlMLH1 with MLH1 protein in the dicots plant species when compared to other group of organisms.

Meanwhile for *SlZIP4* gene, it was found that this gene has 66.6% homology with *AtZIP4* with 62% of query cover. Located on chromosome 9 in the tomato genome, blast analysis for *SlZIP4* gene to NCBI database showed that it has the highest similarity to *ZIP4* gene from *S. pennellii* with 98.8% homology for 100% query cover confirming its significant similarity in the Lycopersicon clade. Motif sequence analysis using HMMER software indicated that SlZIP4 protein contain conserved motif for meiosis protein SPO22/ZIP4 which similarly present in AtZIP4. However, Tetratricopeptide repeat (TPR_8) motif was not identified in SlZIP4 protein but was presence in AtZIP4 protein. Similarly observed like in SlMLH1 protein, SlZIP4 protein is slightly smaller in size when compared to AtZIP4 protein (**Figure 2.0B**). Phylogenetic analysis on SlZIP4 protein revealed that it has closest similarity with *G. max* ZIP4

protein. Overall, SlZIP4 protein is closer to other dicots ZIP4 protein when compared to ZIP4 protein in monocot plants and other organisms taxa (**Figure 2.1B**).

2.3.2 Regeneration and characterization of tomato *mlh1* and *zip4* stable mutant lines

In order to generate *Slmlh1* mutant, the plant CRISPR/Cas9 mutagenesis construct pDIRECT_22C was modified to generate a construct containing 2 sgRNAs targeting the *SlMLH1 gene* (hereafter called pWZ1) **(Appendix 1.0)** (Čermák et al., 2017). Genetic transformation of pWZ1 into *S. lycopersicum* cv. Micro-Tom led to the regeneration of 85 T_0 plants from eight different lines **(Appendix 4.0)**. Lines 2 (pWZ1-A-2) and line 5 (pWZ1-A-5) were most prolific with more than 25 and 20 plantlets transferred to soil respectively. Positive transformant plants (T_0) were validated by amplification of *AtCAS9* and *NPT* genes that are present on the pDIRECT22C construct. These T_0 plants were further evaluated with flow cytometer to determine the ploidy level as the use of phytohormone, Zeatin in transformation and regeneration protocol can lead to tetraploid plants. In total, 79 of the 85 were shown to be normal diploid plants. PCR amplification for *SlMLH1* gene in regenerated mutant plants showed various sizes of genetic deletions in the *SlMLH1* gene in the independent lines. The largest *SlMLH1* deletion was observed in line 1 (pWZ1-A-1) with an approximate 71 bp deletion while for line 2 and line 5 exhibiting smaller deletions **(Appendix 5.0)**. Some of *Slmlh1* mutant lines also potentially contained bi-allelic deletions such as for line 2 (pWZ1-A-2) and line 6 (pWZ1-A-6) for having 2 bands when observed on 3% (w/w) agarose gel electrophoresis. Selected lines of *Slmlh1* were backcrossed with wildtype Micro-Tom and BC_1F_1 plants heterozygous for mlh1 mutation had normal fertility. Two stable lines of *Slmlh1* mutant (*Slmlh1-1* coming from pWZ1-A-4 and *Slmlh1-2* coming from pWZ1-A-1) were successfully isolated in BC_1F_2

population and these two lines did not contain the unwanted transgene, validated by absent of *AtCAS9* and *NPT* markers (**Figure 2.2**). Based on Sanger sequencing, we found 32 bp and 71 bp deletions in *Slmlh1-1* and *Slmlh1-2*, respectively. Both deletions cause frame shifts in the first exon of the *SlMLH1* gene and were found to alter the overall protein codon for *Slmlh1* gene **(Appendix 5.0 and 6.0)**. *Slmlh1-1* and *Slmlh1-2* behave similarly based on the percentage of pollen viability and fruit and seed setting observed in both lines. However, *Slmlh1* mutant from backcrossed of line 4 (*Slmlh1-1*) with 32 bp deletion was selected to be used in phenotypic and cytology experiments.

As also described in chapter 3, *Slzip4* mutant was isolated from 2 stable lines of double mutant *Slzip4 Slrecq4*. Similar approaches as described for *Slmlh1* mutant, backcross with wildtype Micro-Tom was carried out on double mutant *Slzip4 Slrecq4*. Segregation BC_1F_2 population was screened for the single mutant of *Slzip4* without any presence of *NPT* and *AtCAS9* transgenes. Two stable lines of *Slzip4* mutant named as *Slzip4-1* and *Slzip4-2* were successfully generated (**Figure 2.3**). The first line, *Slzip4-1* was identified to have 8 bp frame shift deletion on exon 1 while second *Slzip4-2* was identified to have 25 bp frame shift deletion on exon 5. As predicted, both lines were sterile with less than 2% viable pollen and without any seed produced from fruit. *Slzip4-1* with 8 bp deletion was selected to be used in the next experiment and HRM screening was used to genotype *Slzip4-1* as 8 bp deletion cannot be identified easily using conventional PCR and agarose gel electrophoresis **(Appendix 7.0).**

2.3.3 Phenotypic observation of *Slmlh1* and *Slzip4* mutant

In order to understand how the loss of *MLH1* and *ZIP4* affect vegetative and reproductive development in tomato, we performed extensive phenotyping evaluation. Phenotyping of both class I CO mutants was conducted for 90 days but the physiology images of wildtype, heterozygous and homozygous for both *MLH1* (**Figure 2.4**) and *ZIP4* (**Figure 2.5**) deletion were captured until day 75. All wild-type, heterozygous and homozygous *Slmlh1* plants were derived from the same BC_1F_1 heterozygous *Slmlh1* plants as it has validated 32 bp frame shift deletion that is predicted to ablate the whole function of *SLMLH1*. For the *Slzip4* mutant we also utilized plants derived from the BC_1F_1 heterozygous parent plant. In general, there were no differences between wild-type, heterozygous and homozygous *Slmlh1* in term of growth rate and general traits such as size, height and flowering time. As similarly observed in the regenerated T_0 plants, a severe reduction in pollen viability was observed in homozygous *Slmlh1* mutant (13.4% viable pollen) compared with normal pollen behaviour and viability observed in wild-type (>90% viable pollen) and heterozygous *Slmlh1* sister plant (>90% viable pollen). This result indicated that *SlMLH1* is homozygous recessive where the existence of one normal allele is enough for the function of *SlMLH1* gene. The same is true for the *Slzip4* mutant which is also homozygous recessive with normal pollen behaviour was observed for wildtype and heterozygous *Slzip4* sister plants. However, *Slzip4* has an enormous reduction of viable pollen with an average of 1.49% viability. Pollen staining to determine pollen viability in both *Slmlh1* and *Slzip4* mutant has given the first indicator for the important role of *SlMLH1* and *SlZIP4* in tomato meiosis and fertility rate (**Figure 2.6**).

Slmlh1 homozygous mutants were observed to have more inflorescence branches at 50 days old when compared to wild-type and heterozygous *Slmlh1* mutant (**Figure 2.7A**). *Slmlh1* homozygous mutants exhibited a two-fold increase in average inflorescence number with 30 ± 3.5 inflorescences per plant ($p<0.0001$) when compared to wild-type plants (13.1 ± 1.8) and

heterozygous *Slmlh1* mutant (14.5 ± 1.9). This phenotypic trait in *Slmlh1* mutant may be associated with lower fruit and seed set, which we decided to explore in more detail that eventually induces plant to produce more inflorescence branches and subsequently increase the number of flowers. As for the fruit production, at 45 days after sowing there is very low fruit set observed in homozygous *Slmlh1* with 1.0 ± 1.1 of fruit per plant ($p<0.0001$) when compared to wild-type (19.6 ± 4.4) and heterozygous *Slmlh1* mutant (18.2 ± 4.8) (**Figure 2.7C**). At 60 days, the average number of fruit set in *mlh1* increased to 11.9 ± 7.9 per plant but this was still significantly less ($p<0.0001$) than wild-type (36.2 ± 4.1) and heterozygous *Slmlh1* mutant (37.2 ± 6.5) (**Figure 2.7D**). Reflecting the low number and slower fruit setting, the average day for the first fruit starting to ripe is also delayed in homozygous *Slmlh1* mutant which only started at 81 days ($p<0.0001$) while 70 days needed for both wild-type and heterozygous *Slmlh1* sister plants (**Figure 2.7B**).

A significant increase of inflorescence number is not only observed in *Slmlh1* mutant but the same characteristic was also observed in the *Slzip4* mutant. *Slzip4* mutant which is having more issue in fertility rate than *Slmlh1* was found to have significant increase of inflorescence branches with an average of 40.5 ± 3.9 inflorescence ($p<0.0001$) when compared to wildtype (27.6 ± 3.0) and heterozygous *Slzip4* (28.6 ± 3.4) sister plants (**Figure 2.8A**). Very low fruit set by homozygous *Slzip4* mutant without any fruit being produced at day 45 and only 1.4 ± 1.1 fruit ($p<0.0001$) was produced on day 60 which is very significantly reduction when compared to wildtype (40.8 ± 6.6) and heterozygous *Slzip4* (35.9 ± 10.1) sister plants (**Figure 2.8C** and **Figure 2.8D**). The average day taken by wildtype and heterozygous *Slzip4* sister plants to have ripe fruit is on 76.8 ± 5.2 and 76.1 ± 4.0 days respectively and as delay in fruit set has caused significant delay in fruit ripening for homozygous *Slzip4* mutant with 90.6 ± 1.6 days ($p<0.0001$) needed for fruit to start ripe (**Figure 2.8B**).

The fruit setting and number is not the only fruit related trait observed in both *Slmlh1* and *Slzip4* mutants but it is also reflect to the size of the fruit and seed number (**Figure 2.9**). Significantly smaller fruit was produced from *Slmlh1* mutant with the average weight for the ripe fruit is 1.65 ± 0.32 g ($p<0.0001$). The fruits produced by *Slmlh1* mutant are only 34% from the average fruit weight of wild-type (4.82 ± 0.99 g) and heterozygous *Slmlh1-1* mutant (4.81 ± 1.12 g) (**Figure 2.10A**). The smaller fruit in *Slmlh1* is likely linked to the low seed set where we found most selfing fruits were completely without seeds and in total we found only 1 seed obtained from 70 *Slmlh1-1* homozygous fruits (**Figure 2.10B**). This is an extreme reduction of seed set in *Slmlh1* compared with wild-type (35.7 ± 8.2) and heterozygous *Slmlh1* (34.9 ± 9.3) sister plants (**Figure 2.10B**). However, we found that by assisting the pollination of the *Slmlh1* flower manually, it is possible to increase seed number in *Slmlh1* to a maximum of 10-12 seeds per fruit.

Similar observation was also observed in *Slzip4* mutant as this sterile plant produced parthenocarpic fruit that do not contain any seed. The significantly small fruit obtained from homozygous *Slzip4* mutant weighed on average 1.4 ± 0.5 g ($p<0.0001$) when compared to wild-type (5.7 ± 1.1 g) and heterozygous *Slzip4* (5.6 ± 1.0 g) sister plants (**Figure 2.10C**). *Slzip4* mutant was observed to be sterile as no seed formation was ever being identified in the 50 fruits of homozygous *Slzip4* mutant. Meanwhile, wild-type and heterozygous *Slzip4* sister plants produced on average 40.9 ± 14.6 and 40.7 ± 16.6 seeds respectively (**Figure 2.10D**). Unlike *Slmlh1* mutants, assisting the pollination of the *Slzip4* flower manually did not lead to an increased seed set. Observations on the reproductive performance of *Slmlh1* and *Slzip4* mutants in terms of fruit and seed set behaviour is another parameter confirming the mutation of both *SlMLH1* and *SlZIP4* affect the fertility of tomato.

2.3.4 Chromosome behaviour of *Slmlh1 and Slzip4* mutants

Reduction in pollen formation and fertility for *Slmlh1* and *Slzip4* mutant is most likely associated with defects in meiotic chromosome behaviour in the pollen mother cells (PMCs). Therefore, a comparison of meiotic chromosome behaviour was made between wild-type, *Slmlh1* mutant and *Slzip4* mutant. In wild-type, at leptotene the chromosomes were condensed and become clearly visible. Later at zygotene, the homologous chromosomes were partially synapsed and concentrated and the Synaptonemal Complex (SC) formation was completed at pachytene. SC started to disassemble with the 12 pairs of homologous chromosomes maintain and further 12 bivalents were highly condensed at diakinesis. 12 pairs of homologous chromosomes were then aligned on the equatorial plate during metaphase I. After that, equal segregation of homologous chromosomes toward poles of the cell at anaphase I with the chromosome number reduced by a half. After that, the segregated chromosome will enter meiosis II stage where sister chromatids started to separate and tetrads was produced with each cell containing 12 chromosomes.

In general, there is no abnormal observation for the chromosome behaviour in *Slmlh1* mutant during early prophase I **(Figure 2.11)**. There is a similarity between *Slmlh1* and wild-type sample from leptotene to pachytene suggesting that normal chromosome pairing and full synapsis occurred in *Slmlh1* mutant. It was observed in *Slmlh1* mutant that some homologous chromosome separated from each other and condensed chromosome was abnormal at diakinesis with some normal bivalents and a few univalent. Those bivalents observed to align on the equatorial and univalent were scattered randomly during metaphase I. This led to the imbalanced distribution of chromosomes during anaphase I. Abnormal dyads were observed with microsatellite chromosomes during the beginning of second meiotic division. This is also then reflected in anaphase II stage with the unequal formation of 4 haploid cells with few chromosomes randomly distributed. Uneven tetrad

with microsatellites chromosomes was formed in *Slmlh1* sample. Abnormal behaviour of chromosome in *Slmlh1* PMCs suggesting to the aberration in microspore development which is contributed to the pollen sterility in *Slmlh1* mutants. Hence, this finding demonstrated that *SlMLH1* gene is essential for the regulation of fertility and also involved as an important meiosis protein for tomato.

The behaviour of meiotic chromosomes in *Slzip4* was found to be similar to wild type from leptotene to pachytene stages, as was the case in *Slmlh1* meiotic cells. This indicated that chromosome pairing and full synapsis occurred normally in *Slzip4* mutant. During diakinesis in *Slzip4* mutant homologous chromosomes were later separated from each other and chromosome condensation was abnormal with few normal bivalents and many univalent. Bivalents observed to align on the equatorial and univalent were scattered randomly during metaphase I. Further, the presence of univalent caused imbalanced distribution of chromosome during anaphase I. In *Slzip4* mutant, abnormal dyad was observed with the formation of microsatellite chromosomes during the beginning of second meiotic division. This is further reflected to the abnormal formation of anaphase II with the unequal formation of 4 haploid cells with few chromosomes randomly distributed. Tetrad with microsatellites chromosomes was formed in *Slzip4* sample (**Figure 2.12**). Similarly as observed in *Slmlh1*, abnormal behaviour of chromosome in *Slzip4* is also interrupt microspore development which lead to the formation of unviable pollen. As being observed for the chromosome behaviour of both *Slmlh1* and *Slzip4*, formation of univalent as a result of disturbance or abnormal crossover has caused delayed, unequal and mis-segregation leading to abnormal separation in both meiosis I and II stages. This could cause the formation of aneuploid from unequal segregation event. Since *Slzip4* do not produced any seed, further observation was made to *Slmlh1* mutant selfing seed population to determine any aneuploidy formation.

2.3.5 Comparison of crossover number for wildtype Micro-Tom, *Slmlh1* and *Slzip4*

Next, we set out to quantify the crossover number for *Slmlh1* and *Slzip4* mutants based on the cytological work carried out above. Crossover number can be predicted by counting the number of univalent (no crossover), rod shape bivalent (1 crossover) or ring shape bivalent (2 crossover) during diakinesis stage. As an example in **Figure 2.13A**, univalent chromosome as marked with blue arrow, rod shape bivalent marked with yellow arrow while the green arrow indicates a ring shape bivalent. Based on univalent/bivalent counting, the average number of crossover in wildtype Micro-Tom (n=44) is 17.8 ± 2.1 which is significantly higher than both crossover number for *Slmlh1* (8.2 ± 1.8, n=45) and *Slzip4* (5.2 ± 2.3, n=66) mutant ($p<0.0001$) (**Figure 2.13B**). In wildtype Micro-Tom, 51.3% of chromosomes in diakinesis stage are rod shape bivalent while 48.7% were identified as a ring shape bivalent. As for *Slmlh1* mutant, the percentage of rod and ring shape bivalent were 58.3% and 5.0% respectively with 36.7% of univalent chromosomes. Higher amounts of univalent chromosomes (60%) were counted in *Slzip4* mutant while only 36.7% and 3.4% rod and ring shape bivalent were respectively observed (**Figure 2.13C**). Based on this observation, mutation of *SlMLH1* and *SlZIP4* genes reduced the formation of ring shape bivalent when compared to the percentage observed in wildtype Micro-Tom, consistent with an overall reduction in meiotic CO in both mutant backgrounds

2.3.6 Aneuploidy of the progeny of self-pollinated seed from *Slmlh1*

As there is univalent formation and unequal chromosome formation in the *Slmlh1 mutant* we wanted to explore if we could observe aneuploidy in the selfing offspring. A total of 20 homozygous *Slmlh1* mutant plants derived from self-pollinated seed of homozygous *Slmlh1* mutant were observed for any abnormal phenotype. Among 20 plants observed, 3 homozygous *Slmlh1* plants

showed abnormal phenotypes such as having abnormal inflorescence branches, abnormal flower shape, abnormal leaf shape and pattern, thick leaf, big sterile fruit, elongated fruit shape and darker green colour. Therefore, further observation using cytology was made in order to confirm the number of chromosome and to detect any aneuploidy event resulted from *SlMLH1* mutation (**Figure 2.14**). As observation on dicot-like *Slmlh1* mutant plant **(A)**, chromosome number during pro-metaphase stage was counted to have 24 chromosomes which indicating normal chromosome number for a diploid tomato **(B)**. As for aneuploid-like *Slmlh1* mutant, abnormal phenotype which is previously observed were matched with the indication for the incident of aneuploidy in *Slmlh1* mutant with plants in **(C)**, **(E)** and **(G)** were observed to have 25 **(D)**, 54 **(F)** and 27 **(H)** chromosomes respectively. This result confirming that unequal segregation occurred in *Slmlh1* mutant resulting to aneuploidy of selfing seed from *Slmlh1* mutant.

2.3.7 Phenotypic behaviour of *mlh1* mutation in tomato interspecific hybrid (*S. lycopersicum* x *S. pimpinellifolium*)

We introgressed the *mlh1* mutation into wild tomato species, *S. pimpinellifolium* because we were interested to observe the behaviour of the *mlh1* mutation in the wild ancestor of tomato. The *mlh1* mutation was introgressed into the wild tomato species, *S. pimpinellifolium* (LA2102) and found to have a higher percentage of pollen viability when compared to those *Slmlh1* mutant in Micro-Tom background (**Figure 2.15B** and **Figure 2.15C**). F_2 generation of LA2102 x *Slmlh1* hybrid were found to have higher pollen viability with 34.1 ± 10.0% of viable pollen and significantly higher than *Slmlh1* mutant ($p<0.0001$). Similar value for the BC_1F_2 and BC_2F_2 population of LA2102 x *Slmlh1* hybrid with percentage of viable pollen recorded as 31.1 ± 12.3% and 29.48 ± 8.6% respectively (BC_1F_2:$p=0.0002$, BC_2F_2:$p=0.0004$) increase when compared to *Slmlh1* mutant in The Micro-Tom background.

Next we used our *mlh1* mutant in the *S. pimpinellifolium* (LA2102) background to cross back to *Slmlh1* in Micro-Tom background to have a more consistent interspecific hybrid background for detailed phenotyping **(Appendix 8.0)**. The cross of heterozygous *Slmlh1* (Micro-Tom) with BC_2F_1 (LA2102 x *Slmlh1*^{het}) were segregated into wildtype, heterozygous and homozygous plant for *mlh1* deletion (**Figure 2.16B** and **Figure 2.16D**). We checked pollen viability for the F1 hybrid both on wildtype and *mlh1* deletion plants and was observed in reciprocal cross for F_1 *Slmlh1* x LA2102 hybrid background where the percentage of pollen viability was 30.71 ± 4.8% ($p<0.0001$) while for the Micro-Tom x LA2102 hybrid showed 90.6 ± 6.0% of viable pollen (**Figure 2.15A**).

It was also observed that fruit for *mlh1* mutant from F_1 generation of *Slmlh1* x LA2102 hybrid is significantly smaller than those from F_1 Micro-Tom x LA2102 hybrid ($p<0.0001$) (**Figure 2.16D**). Similarly as observed in Micro-Tom background, the average of fruit weight for *mlh1* mutant from F_1 *Slmlh1* x LA2102 hybrid is 0.8 ± 0.2 g which is smaller than F_1 Micro-Tom x LA2102 hybrid with 3.4 ± 0.8 g for an average fruit weight (**Figure 2.16A**). Moreover, reduction of seed number from F_1 *Slmlh1* x LA2102 hybrid was also observed with an average 0.3 ± 0.7 seed ($p<0.0001$) was produced with most fruit do not have any seed available when compared to wildtype F_1 Micro-Tom x LA2102 hybrid (30.1 ± 12.0 g). However, the number of seed was increase when assisted pollination for F_1 *Slmlh1* x LA2102 hybrid pollen was carried out on wildtype Micro-Tom plant used as female recipient with an average 5.0 ± 3.3 seeds were produced and 31.8 ± 9.9 seeds were obtained from pollination of F_1 Micro-Tom x LA2102 hybrid pollen to Micro-Tom as female recipient (**Figure 2.16C**). Even though *mlh1* mutant from Micro-Tom x LA2102 showed an increase in pollen viability, however the number of seed set remained low. This suggests that the mutation of *mlh1* in interspecific hybrid has a more severe effect on the female meiocyte rather than male meiocyte.

2.3.8 Crossover number for wildtype and *mlh1* mutant of Micro-Tom x *S. pimpinellifolium* hybrid

Based on univalent and bivalent (rod or ring) counting (n=150), there is no significant differences of crossover number for F_1 Micro-Tom x LA2102 hybrid with 17.6 ± 2.2 crossover were counted when compared to Micro-Tom (17.8 ± 2.1). In other hand, crossover number for F_1 *Slmlh1* x LA2102 hybrid (n=126) is significantly lower than F_1 Micro-Tom x LA2102 hybrid with 10.0 ± 2.8 crossover were counted ($P<0.0001$). However, crossover number in F_1 *Slmlh1* x LA2102 hybrid is significantly higher ($p<0.0001$) than crossover number in *Slmlh1* mutant (Micro-Tom background) having an average of 8.2 ± 1.8 crossovers (**Figure 2.17**). This result indicates that an increase in crossover for F_1 Micro-Tom x LA2102 hybrid resulted to the increase of pollen viability percentage as described in (g).

As shown in **Figure 2.18**, there is almost an equal percentage of rod and ring bivalent in F_1 Micro-Tom x LA2102 hybrid during diakinesis stage with 53.6% rod shape bivalent, 46.3% ring shape bivalent and very small univalent with 0.1% of total chromosome. This rod/ring bivalent percentage observed to be almost similar like those observed in wildtype Micro-Tom. Nevertheless, there is an increase of rod shape bivalent in F_1 *Slmlh1* x LA2102 hybrid with an average of 64.6%. F_1 *Slmlh1* x LA2102 hybrid only consist of 9.4% of ring shape bivalent and 26.0% of univalent. As observed in *Slmlh1* mutant, 36.7% of chromosome during diakinesis stage is univalent and there is an reduction of univalent formation by 10.7% in F_1 *Slmlh1* x LA2102 hybrid. As for rod and ring shape bivalent formation, there is an increase of 6.3% and 4.4% respectively for F_1 *Slmlh1* x LA2102 hybrid. Therefore, an increase of crossover and also pollen viability in F_1 *Slmlh1* x LA2102 hybrid associated with the reduction of univalent formation.

2.4 Discussion

Here I generated *Slmlh1* and *Slzip4* mutants in tomato and found that their general characteristics are consistent with similar mutants in other organisms yet the strength of phenotypes can be different when compared with the model plant *Arabidopsis thaliana*. The relative importance of class I CO genes in meiotic CO and fertility can be different in each organism and findings in plants with more complex genomes, such as tomato, can be distinct from Arabidopsis.

2.4.1 Class I CO is important for fertility in tomato

We found a severe reduction in fertility in the absence of the *SlMLH1* gene which encodes an important component for DNA repair and also assisting CO resolution in tomato. We found that *Slzip4* mutants are fully sterile, which suggests that SlZIP4 protein is required to stabilize key recombination intermediates during tomato meiotic prophase I. As a downstream consequence it is likely that a mature synaptonemal complex does not form properly. Unlike *Slzip4* mutant, mutation of *SlMLH1* gene leads to major compromise of fertility but *Slmlh1* mutants are still capable to produce viable pollen and seed indicating that absence of *MLH1* has a less strong meiotic defect. This could be derived from class II crossover pathway that may help in the resolution of the CO junctions (Hartung et al., 2006). This is can be seen in rice *MUS81* gene that contributes little to crossover designation but plays a crucial role in the resolution of atypical meiotic intermediates (Mu et al., 2023). The MUS81 activity is considered as a back-up mechanism for class I CO pathway (Girard et al., 2023). Another possibility for the mechanism to repair HR is also can derived from PMS1/2-MLH3 heterodimer that have a minor role in meiosis CO for yeast and mice (Pannafino & Alani, 2021; Qin et al., 2002).

It is also observed that the role of NCO pathway to repair the majority of DSB to NCO event is not very clear in *Slmlh1*. As being reported, NCO

pathways contributed to the major DSB repair in many organisms resulted to NCO formation during meiosis (Gray & Cohen, 2016; Youds & Boulton, 2011). As the *SlMLH1* protein regulating CO in the late prophase I, theoretically, NCO formation can be triggered to function as the binding of the NCO protein occurred after resection of double strand break in early prophase I (Lloyd, 2022). Therefore, this could suggest that with the absence of class I CO, NCO pathways are not capable to repair all intermediate fragment into NCO and this indicates that class I CO not only responsible to ensure there is obligate crossover but also have some dedicated role to help in DSB repair during meiosis.

2.4.2 Univalent formation caused aberrant chromosome behaviour in tomato *mlh1* and *zip4* mutant

In both *Slmlh1* and *Slzip4* mutants, we found that univalent chromosome formation occurred as a result of reduced in meiotic crossover during meiosis. In both mutants we found there is a significant percentage of univalent formation confirming that meiotic CO formation by the class I CO pathway is highly disturbed. Formation of univalent during meiosis not only caused reduction in meiotic recombination, it is also responsible for the meiotic non-disjunction, gamete unviability and genomic instability (Guo et al., 2010; Souza et al., 2012; Uroz & Templado, 2012; C.-J. Wang et al., 2010). The deficiency of univalent formed during meiosis caused an error for the process of equal segregation. Non-disjunction lead to an unequal distribution of chromosome in the gametes and further caused aneuploidy (Uroz & Templado, 2012). This can be seen in progenies of *Slmlh1* mutant where some of the plants were identified as aneuploid. As aneuploidy contains abnormal number of chromosome, severe consequences such as genetic disorders or developmental abnormalities will be formed. As a result of that, univalent will produce abnormal gametes that are not viable or have reduced fertility. This

abnormal gamete may not capable to participate in fertilization or can lead to the formation of embryos with chromosomal abnormalities. There is also another consequences of univalent formation which is the chromosome is more susceptible to structural changes such as deletions, insertions, or rearrangements caused by lack of proper pairing and recombination (De Storme & Mason, 2014). This can lead to further genomic instability and increase the risk of genetic mutations or chromosomal aberrations. Therefore, this univalent formation observed in *Slmlh1* and *Slzip4* explained why there is a severe reduction in fertility. The percentage of univalent were bigger in *Slzip4* than *Slmlh1* and this explained the sterility condition of *Slzip4* as it contain large proportion of abnormal univalent.

2.4.3 Species-specific genomic shape CO formation

The behaviour of *Slmlh1* and *Slzip4* observed in tomato were more severe when compared to those described in Arabidopsis. This could happen as a result of species-specific genomic characteristic. Usually, the larger and more complex of the genome will caused more abnormality if mutations of CO related gene occurred. As tomato has 12 pairs of chromosomes, there are higher possibilities of mis-segregation due to univalent formation when compared to Arabidopsis with only 5 pairs of chromosomes. This can be seen in the Arabidopsis *Atmlh1* mutant that have milder effect in term of univalent formation and also fertility (unpublished observations from Mercier's group, MPIPZ) when compared to the stronger reduction of fertility in the tomato *Slmlh1* mutant. Another report for *Atmlh1* mutant showed reduction in seed set and produced shorter siliques compared to a wild-type plant but retain both male and female fertility (Dion et al., 2007). Meanwhile Arabidopsis *Atzip4* mutant have severe reduction in crossover and fertility but still capable to produce seed by selfing (Chelysheva et al., 2007). This is not the case for tomato *Slzip4 mutants* where plant sterility occurs suggesting that SlZIP4 is

essential for normal stabilization of recombination intermediates and downstream meiotic processes in tomato. Thus, this could suggest that the number of chromosome pairs influences the class I CO behaviour in plants.

However, number of chromosome pair is not the only factor contributed to the behaviour of class I CO genes. Rice with 12 pairs of chromosomes but mutation of *MLH1* gene is less severe than those observed in tomato. In rice, *Osmlh1* mutant show milder effect on seed set and crossover rate with 14% and 70% respectively when compared to the wild type (Mao et al., 2021). As for *Oszip4* mutant, the plant is almost sterile with 70% of CO reduction when compared to wild type and similar to those observed in *Slzip4* mutant indicating that important role of ZIP4 protein (Shen et al., 2012). As comparison, rice have smaller genome size (400 Mb) when compared to tomato (900 Mb). Here we can see that tomato has the average size of chromosome which is around 75 Mb per chromosome. Therefore, average size per chromosome for tomato with 3 and 2.5 times bigger than those in Arabidopsis and rice respectively can influence the behaviour of the class I CO genes.

Another differences that may influences the behaviour of *Slmlh1* and *Slzip4* mutant is the proportion of heterochromatin. Heterochromatin is a tightly packed and condensed form of chromatin often contain repetitive DNA sequences and are associated with genomic regions prone to structural rearrangements (Mehrotra & Goyal, 2014). Heterochromatin properties can influence the destiny of meiotic crossovers. In term of DNA properties, tomato also have 77% of heterochromatin make up which is known as a cold spot for the CO (Peterson et al., 1996). Higher heterochromatin in tomato may have influence in the landscape of meiotic crossover and recombination as the proportion of heterochromatin in Arabidopsis and rice is much lower around less than 20% and 10% respectively (Espinas et al., 2020; Koornneef et al., 2003).

2.4.4 Potential of new meiotic CO gene in wild *S. pimpinellifolium*

Hybridization of *Smlh1* mutant with wild *S. pimpinellifolium* improved CO number and increased pollen viability. This indicates that there could be a genetic modifier of CO rate in wild *S. pimpinellifolium* which is absent in domesticated tomato (*S. lycopersium*). This significant increase in both CO number and viable pollen could appears to be derived from wild *S. pimpinellifolium* that caused higher crossover rate when compared to domesticated tomato. Previous study on crossover rate reported that there is higher crossover rate in wild *S. pimpinellifolium* when compared to partially domesticated tomato, *S. lycopersicum* var. *cerasiforme* and fully domesticated tomato, *S. lycopersicum* var. *lycopersicum*, most notably in the pericentromeric heterochromatin (Fuentes et al., 2022). Study of CO frequency for the introgression of wild *Solanum lycopersicoides* into cultivated tomato by mutating the DNA mismatch repair system (MSH2 and MSH7) resulted to nearly 18% increase in CO frequency suggesting that mutation induced homoeologous recombination (Tam et al., 2011). As *MLH1* gene is also MMR gene, this could suggest that mutation of *mlh1* gene in *S. lycopersicum* x *S. pimpinellifolium* hybrid induced homoeologous recombination which is not happening in inbred tomato mutant, *Slmlh1* (Micro-Tom background). In order to know the CO distribution of *mlh1* mutant in tomato, we are in the latter stages of analysing two *S. lycopersicum* x *S. pimpinellifolium* populations that have been characterized by high throughput sequencing to map CO number and distribution, in the presence and absence of MLH1.

2.5 Conclusion

A severe reduction of fertility was observed for *Slmlh1* mutant with massive reduction of viable pollen, fruit size and seed number. *Slzip4* was identified as a sterile plant as SlZIP4 is a mandatory element for meiotic crossover in tomato. Both mutants exhibited abnormal chromosome behaviour with the formation of a significant amount of univalents that resulted to reduce number of meiotic crossover. Univalent formation in *Slmlh1* mutant is responsible for the unequal segregation leading to aneuploidy. It is proposed that the severe defects in tomato after loss of class I CO factors genes may be associated with the larger number of chromosome pairs and the size of the tomato genome. However, mutation of *mlh1* mutant in interspecific hybrid between *S. lycopersicum* and *S. pimpinellifolium* has a higher crossover rate (compared to *mlh1* mutant in inbred *S. lycopersicum*) and also an increased percentage of viable pollen. This could suggest that there is another player regulating CO rate in wild *S. pimpinellifolium*.

2.6 Materials and methods

2.6.1 Plant materials and plant growth

Solanum lycopersicum cv. Micro-Tom used in this study was obtained from the Tomato Growers Supply Company (Florida, USA). Meanwhile, for interspecific tomato hybrid experiment, wild *Solanum pimpinellifolium* (LA 2102) was obtained from TGRC University of California, Davis (California, USA) and was used in the hybrid crosses for *mlh1* mutant cross distribution experiment. As an initial treatment, the seeds were soaked in the clean water and later was treated with saturated Na_3PO_4 and was rinsed with water for few times. Later, the treated seed was placed on wet tissue paper in petri dish to assist germination and germinated seed was transferred to germination pots containing peat moss. After 3 weeks, Micro-Tom seedlings were transferred to potting soil in growth chamber with 25°C, 16 hours light and around 60-70% of humidity. Micro-Tom plants were fertilized weekly using 0.4%(v/v) Wuxal® Super (Precision Laboratories, Germany) and watering every 2 days interval. Wild *S. pimpinellifolium* seedling was transferred into 8 litres pot with cocopeat as media and was irrigated using automatic fertigation system.

2.6.2 *SlMLH1* and *SlZIP4* ortholog identification

The tomato *MLH1* and *ZIP4* genes were identified based on the reference sequence of *MLH1* and *ZIP4* genes from *Arabidopsis thaliana*. The full sequence of *AtMLH1* and *AtZIP4* were obtained from 'The Arabidopsis Information Resource' (TAIR) (https://www.arabidopsis.org/index.jsp). The gene sequence of *AtMLH1* (Accession no: NM_116983.3) and *AtZIP4* genes (Accession no: NM_124214.7) was used to query a tomato genome (Heinz 1706 reference cultivar) BLAST database using Sol Genomics Network (https://solgenomics.net/tools/blast/) and also Phytozome v12.1 (https://phytozome.jgi.doe.gov). The identified *MLH1* and *ZIP4* genes in tomato were single copy and further characterized for its intron and exon

sequence. The sequence motif for SlMLH1 and SlZIP4 proteins were also characterized by using HMMER software (http://hmmer.org/). Further analysis was carried out to determine the similarity of the *SlMLH1* sequence between different types of tomato cultivars and related wild species. Therefore, analysis that was carried using our custom UCSC Genome Browser (https://ucscbrowser.mpipz.mpg.de/). Using MEGA 11.0 software (Tamura et al., 2021), the phylogenetic analysis of SlMLH1 and SlZIP4 proteins were generated by comparing protein sequence of orthologs protein from various organisms to determine the evolutionary relationship of SlMLH1 and SlZIP4.

2.6.3 Construction of CRISPR/Cas9 construct for the development of *Slmlh1* and *Slzip4* mutant

Then, sgRNAs targeting *SlMLH*1 and *SlZIP4* genes were designed using 2 types of online software which are CHOPCHOP (https://chopchop.cbu.uib.no/) and CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR). The best sgRNA was selected by the ranking suggested by the software and was further validated using Cas-Offinder (http://www.rgenome.net/cas-offinder/) in order to determine the potential off-target sites of Cas9 RNA-guided endonucleases. The sgRNAs for the development of null mutation of *SlMLH1* were listed in the Table 2.0. The selected two sgRNAs were cloned into pDIRECT 22C backbone vector using Golden Gate assembly method (Čermák et al., 2017). Successful construct containing two *MLH1* sgRNAs was name as pWZ1 **(Appendix 1.1)**. Meanwhile, early expectation predicted that mutation of *SlZIP4* gene will resulted to sterility in tomato. Therefore, 3 sgRNAs to target *SlZIP4* gene was constructed together with *SlRECQ4* gene in order to produce double mutants with mutation of *SlRECQ4* will supress the infertility in *Slzip4* mutant. Success insertion of 3 sgRNAs of *SlZIP4* gene and 2 sgRNAs of *SlRECQ4* gene in pDIRECT 22c was later named as pWZ3 **(Appendix 1.2)**. The sgRNAs sequence and related primers for Golden Gate cloning for *SlZIP4* gene was described in the chapter 3 (Table 3.0).

2.6.4 Transformation and transgenic line regeneration

One colony of *Agrobacterium* containing pWZ1/pWZ3 was inoculated in 150 mL of YEP medium containing 50 mg/L Kanamycin, 30 mg/L Gentamicin and 10 mg/L Rifampicin and was incubated at 28°C with 220 rpm shaking for 48 hours. The culture was used as an inoculum for further transformation experiment where 0.5-1 mL of culture was inoculated into new YEP medium and was incubated for overnight. On the same day, 3-4 weeks old of Micro-Tom seedling grown in the ½ MS was used as an explant where the leaves were cut in 6 x 6 mm size on sterile glass petri dish. The leaf explants were cut one by one and the bottom side were placed on MS media supplemented with 2 mg/L Zeatin Riboside (Duchefa Biochemie, Netherland), 0.1 mg/L Indole-3-acetic acid (IAA) (Merck, Germany) and 73 mg/L of Acetosyringone (Merck, Germany) in the sterile petri dish. The explants were placed at 25°C in the dark room or growth chamber at least for overnight before co-cultivated with *Agrobacterium*. 5 ml of overnight *Agrobacterium* culture with OD_{600} around 800 were diluted with 100 mL of LB without antibiotic at 1:20 ratio and the incubated leaf explants were infected with diluted culture for 15 minutes with mild shaking. The leaf explants were then placed on sterile dry Whatmann filter paper to remove excessive liquid and further placed in the same MS media plate for 48 hours in the dark. After that, the co-cultivated explants were transferred to new MS media containing 1.5 mg/L Zeatin Riboside, 100 mg/L Kanamycin and 500 mg/L Carbenicilin and were incubated for 1 weeks in the 25°C with 16 hours day light. The explants were then sub-cultured into new MS media and placed in the same condition and were sub-cultured every 2 weeks. After several sub-cultures, any green and resistant embryoid or shoot emerged from the explant was further transferred to the MS supplemented with 1.0 mg/L Zeatin Riboside, 100 mg/L Kanamycin and 250 mg/L Carbenicilin. Developed shoot from resistant embryoid with at least 20 mm length size was further transferred to rooting media, MS supplemented with 1

mg/L IAA and cultured for at least 3 weeks until enough number of roots formed before being transferred into soil **(Appendix 2.0)**.

2.6.5 Tomato meiotic mutant genotyping

Genomic DNA (gDNA) from mutant and wild type tomato plants were isolated using BioSprint 96 DNA Plant Kit (Qiagen, Germany). Isolated gDNA was further used as a template for the amplification of DNA fragment for various genes/transgenes using primers as described in the Table 2.1. Amplification of DNA fragment by PCR was carried out using Mango*Taq*™ DNA Polymerase (Meridian Bioscience, United Kingdoms). The cycle of the PCR used are: Initial denaturation; 5 minutes, denaturation; 30 seconds, annealing: 30 seconds, extension; 30 seconds and final extension; 3 minutes. Amplified PCR product was observed using electrophoresis 1% (w/v) agarose gel for *ACT*, *NPT* and *AtCAS9* and 3% (w/v) agarose gel for *SlMLH1* genes. MLH1-pWZ1-F and MLH1-pWZ1-R primers was also used for the identification of *SlMLH1* gene sequence in *Slmlh1* mutant and also wild-type using Sanger sequencing. The genotyping of *Slzip4-1* mutant with 8 bp deletion utilized High Resolution Melting (HRM) to differentiate wildtype, heterozygous and homozygous *Slzip4* mutant. Meanwhile, *Slzip4-2* line used conventional PCR and 3% (w/v) agarose gel as the 25 bp deletion is easily identified on agarose gel. HRM is also used to genotype homozygous, heterozygous and wild-type *SlMLH1* gene for lines 4 (*Slmlh1-1*) that was used for further phenotyping and EMS-treated experiment. HRM analysis also using the same type of polymerase that was used for PCR amplification with addition of 0.25 µL of LCGreen® Plus (BioFire Defence, LLC, USA) for every 12.5 µL reaction. The thermal cycles for HRM-qPCR are: Initial denaturation; 3 minutes, denaturation; 30 seconds, extension; 30 seconds. After complete 40 cycles, HRM was performed by temperature ramping from 65˚C to 95˚C with increment of 0.2˚C for every 5 seconds. HRM-qPCR analysis was performed using CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA). The melting curve plot for HRM to

differentiate homozygous, heterozygous and wild-type for *SlMLH1* gene was performed using Bio-Rad Precision Melt Analysis Software (Bio-Rad, USA).

2.6.6 Identification of ploidy level of mutant regenerated from *in vitro* transformation

The ploidy level of tomato plant regenerated from *in vitro* culture was analysed using CytoFLEX flow cytometer (Beckman Coulter, USA). Tomato leaf with approximately 2cm x 2cm in diameter was finely chopped in 0.5 mL Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0). The mixture was then filtered using green filter (30 µM) and 20 µL of 100 µg/mL DAPI was then added. The mixture was incubated on ice for 15 minutes and further analysed using flow cytometer. Leaf sample from wildtype Micro-Tom plant germinated from seed was used as a diploid control.

2.6.7 Phenotyping experiment

Slmlh1 mutants which are regenerated from the genetic transformation and *in vitro* selection and successfully transferred to soil was further crossed with wild type Micro-Tom. This aim to remove the presence of unwanted *NPT* and *AtCAS9* transgenes. The BC_1F_1 without those transgenes was selected for phenotypic experiment. This experiment was conducted on the population of BC_1F_1 from line 4 named as a *Slmlh1-1*. This line has frame shift mutation of *SlMLH1* gene with 32 bp deletion started from 102 nucleotide bases of the upstream region. Similarly conducted for *Slzip4* mutant, segregation population from double mutant *Slzip4 Slrecq4* identified as *Slzip4-1* with 8 bp deletion was used in phenotypic experiment. This line was obtained from the backcross work with wildtype Micro-Tom and absent with unwanted transgenes, *NPT* and *AtCAS9* transgenes. Phenotypic experiment compared the morphology of homozygous, heterozygous and wild-type plants that segregate from the same parent of heterozygous BC_1F_1 *Slmlh1-1* mutants. Similarly conducted for *Slzip4-1*, plants germinated from parent of

heterozygous BC_1F_2 *Slzip4-1* mutants seed were used. Protocol to grow and the condition of the phenotypic experiment as described in the section a. The growth parameters such as vegetative, inflorescence and fruit data were observed from day 22 to day 90 after seed germination and comparison was made between homozygous, heterozygous and wild-type for *Slmlh1* and *Slzip4* genotypes. Phenotypic characteristics that associated with meiosis and reproduction systems was mainly focus such as the flowering times, number of inflorescence and flower, pollen viability, fruit set and size and also seed number.

2.6.8 Pollen viability staining

The percentage of pollen viability was carried using Alexander Staining protocol (Alexander, 1969). The secretion of pollen from full bloom flower was assisted using pollination vibrator tool and secreted pollen was collected in the glass staining cavity block. After that, 20 µL of Alexander staining solution was added, mixed evenly with the pollen, covered with glass lid and leaved for at least one minute in the fume hood. Then, 10 µL of the stained pollens were placed on the glass slide and further covered with glass lid. The stained pollen slide can be observed immediately or leaved overnight. The image of the stained pollen was captured using Axioplan 2 Imaging Microscope (Zeiss, Germany) and was further processed using Labscope software and Adobe Photoshop.

2.6.9 Cytological analysis

Classic chromosome spread protocol for observation of chromosome behaviour in tomato was carried out using SteamDrop method with modification (Kirov et al., 2014). *S. lycopersicum* cv. Micro-Tom flower bud with size approximately around 2-2.5 mm was collected fresh and immediately soaked into fresh fixative solution, Ethanol: Acetic acid (3:1). The fixative solution was

refreshed 3 times in 1 hour and ideally the fixed flower bud was leaved in the fixation solution at least for 1 day before being used in cytology experiment. The fixed flower buds can be stored in 4°C or -20°C up to 6 months. 6-8 flower buds were taken out from the fixation solution and were soaked for 10 minutes in the sterile water. The flower buds were the rinsed with the sterile water twice and further soaked in 1 mL of 10 mM citrate buffer, pH 4.5. The flower buds were dissected on glass slide with few drops of citric buffer to keep it moist. The out layer of immature petal, calyx, stigma and ovary will be removed. The anthers were separated individually and were transfer into 1.5 mL microcentrifuge tube containing 200 µL 10 mM citrate buffer. Citrate buffer was gently discarded using micropipette and the same volume of enzyme mixture containing 0.3% (w/v) cellulase, 0.3% (w/v) pectolyase Y23, 0.3% (w/v) driselase in citrate buffer were added. The digestion of flower buds by the enzyme mixture was carried out for 3 hours in 37°C incubator. After 3 hours, the digestion mixture tube was plugged on ice and 600 µL of TE buffer, pH 8 was added. Then, the solution mixture was gently push up and down using micropipette to separate the digested cell and create the suspension cell of meiocytes. After that, the mixture was centrifuged at 10,000 rpm, 4°C for 45 second and supernatant was gently discarded using micropipette. The pellet was resuspended in 96% (v/v) ethanol and was mix thoroughly using micropipette. Again, the resuspended cells were centrifuged at 10,000 rpm, 4°C for 45 second and the pellet was finally resuspended in 400-500 µL of in 96% (v/v) ethanol and was mix thoroughly using micropipette. Obtained suspension cell can directly be used for the preparation of chromosome spread slide or it can be stored in -20°C up to 6 months. In order to prepare chromosome spread, 10 µL of well mixed suspension cell were dropped onto the slide and were leaved until the surface become granule-like as the ethanol meniscus occurred on the top of the cell within 10-15 seconds. Then, 20 µL of fixative solution (Ethanol:acetid acid=3:1) were added onto the slide and leaved until the surface become granule-like and the layer of fixation become

thin within 25-35 seconds. The slide was then turn upside down toward steam for approximately 8 seconds at 10-15 cm distance from water surface of water bath with 55°C temperature. Immediately after that, the slide was air dried toward the airflow of a table fan for 15-20 seconds. Finally, 10 µL of DAPI (2 µg/mL) were added on the meiocytes slide after it has dried completely and the meiocytes slide was covered with glass cover. The meiocytes images from the slide were acquired and processed using a ZEISS microscope (AXIO-Imager.Z2) under 100× of oil immersion objective and further analysed using ZEN software. Figures prepared from images were prepared using Adobe Photoshop. The meiocytes slide prepared as above mentioned can also be stored in 4°C or -20°C until further used.

2.6.10 Introgression of the *mlh1* mutation into *S. pimpinellifolium*

Slmlh1-1 mutant was crossed to the wild *Solanum pimpinellifolium* (LA 2102) with the wild species used as a female. F_1 hybrid plant was screened for the heterozygous deletion of *SlMLH1* gene and also marker specific for *S. pimpinellifolium*. Selected F_1 hybrid plants were again crossed with *S. pimpinellifolium* using the same approach as previous cross and the BC_1F_1 was also screened for the present of heterozygous *SlMLH1* deletion and *S. pimpinellifolium* marker. Another round of crossing with *S. pimpinellifolium* was carried out to produce BC_2F_1 before being crossed with heterozygous *Slmlh1-1* to produce approximately 50-50 genetic make-up from both *Slmlh1* mutant and *S. pimpinellifolium*. The F_1 from this cross were screened for homozygous deletion and wild-type *SlMLH1* genes and each of these genotypes were then crossed again to the Micro-Tom which is used as a female. 96 F_1 plants from each cross between homozygous deletion and wild-type *SlMLH1* hybrid with Micro-Tom was selected from same population and was used to study male crossover distribution using Next Generation Sequencing (NGS). The overall flow for the crossing experiment were described in **(Appendix 8.0)**.

2.6.11 Statistical analysis

Data visualisation and statistical calculations were conducted using GraphPad Prism 9.0.0 software. P value was calculated using Kruskal-Wallis one-way ANOVA, a non-parametric method for multiple comparisons. Meanwhile, T-Test was used to compare the means of two groups.

2.7 References

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2.8 Tables

Table 2.0 SgRNAs and primers used in Golden Gate Assembly cloning sgRNAs for editing *SlMLH1* gene in tomato, colony PCR screening and DNA sequencing validation.

Promoter Primer	Primer Sequence
CmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC

SgRNA	SgRNA Sequence	Primer	Primer Sequence
***SlMLH1* -1**	AGGAGCCACCGAAGATTCA GCGG	**MLH1_1R_gRNA1**	TGGTCTCCTCGGTGGCTCCTCTGCCTATACGG CAGTGAACCTG
		MLH1_2F_gRNA1	TGGTCTCACCGAAGATTCAGGTTTTAGAGCTAG AAATAGC
***SlMLH1* -2**	ATGTGTGGTGAACAGAATAG CGG	**MLH1_2R_gRNA1**	TGGTCTCCTTCACCACACATCTGCCTATACGGC AGTGAAC
		MLH1_3F_gRNA1	TGGTCTCATGAACAGAATAGGTTTTAGAGCTAG AAATAGC

Terminator Primer	Primer Sequence
CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Colony PCR and Sequencing Primers	Primer Sequence
TC320	CTAGAAGTAGTCAAGGCGGC
M13F	GTAAAACGACGGCCAGT

Table 2.1: Primers used for PCR amplification of *SlACT* housekeeping gene, *SlMLH1*, *SlZIP4*, *NPT* and *AtCAS9* transgenes and HRM analysis for *SlMLH1* gene.

Genes	Primers Name	Primers Sequence	Amplicon Size (bp)
***S. lycopersicum Actin* (*SlACT*)**	ACTF ACTR	AGCAGGAACTTGAAACCGCT ACAAAAGCTCACCTGCTGGA	404
***Neomycin phospotransferase* (*NPT*)**	NPT-35sF NPT-35sR	TTCAGTGACAACGTCGAGCA GACGTAAGGGATGACGCACA	413
***A. thaliana CRISPR associated protein 9* (*AtCAS9*)**	AtCAS9-F AtCAS9-R	ATGCCACAGGTGAACATCGT GAGAGCAAGCTCGTTTCCCT	420
***S. lycopersicum MutL homolog 1* (*SlMLH1*)**	MLH1-pWZ1-F MLH1-pWZ1-R HRM1-MLH1-F1 HRM1-MLH1-R1	AAGCCATTCCAGTGCCGATT GACAGTGACGTGACCCACAT GAAGCCATTCCAGTGCCGAT ATGAGCTCTTTCACGGCAGA	462 125
S. lycopersicum(SlZIP4/SlSPO22)	ZIP4-HRMSg1-F2 ZIP4-HRMSg1-R2 ZIP4-sg2F (line 2) ZIP4-sg2R (line 2)	GCTTCGAGAAAGCTTCCGATCT TGTCCGCAAAAGCGAAGAGCA AAGGCTTGATGACACGGGCA TGCTTCATCCATATGCGCCA	186 412

(Appendix 5.0 & 7.0)

2.9 Figures

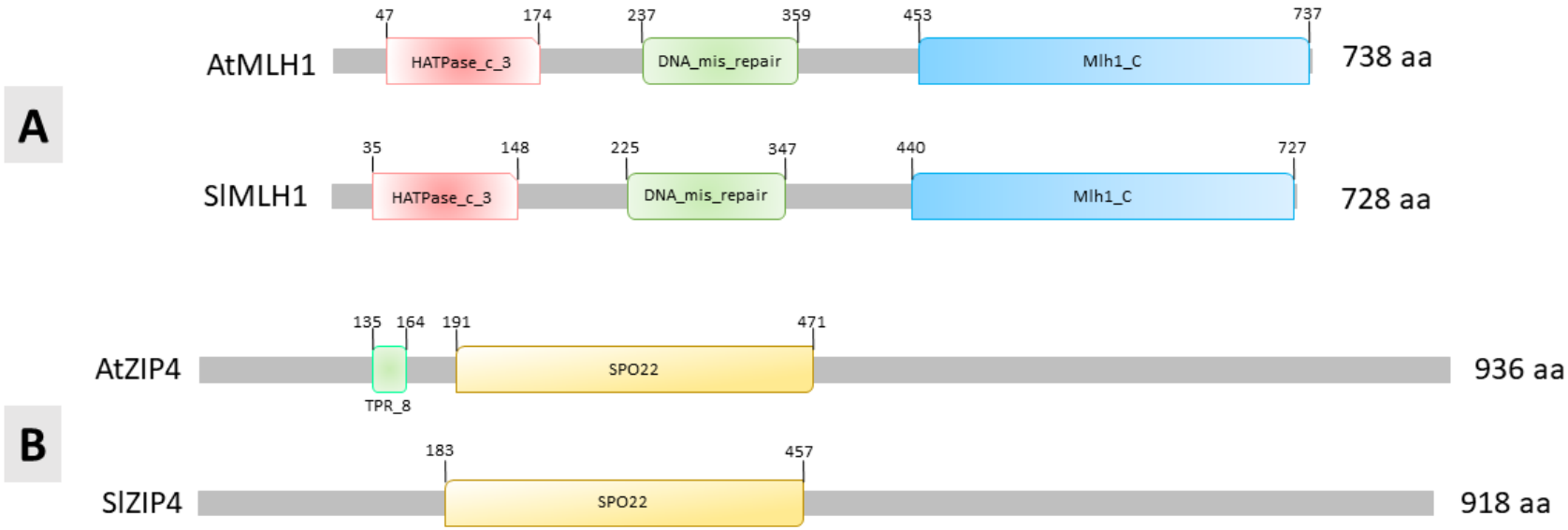
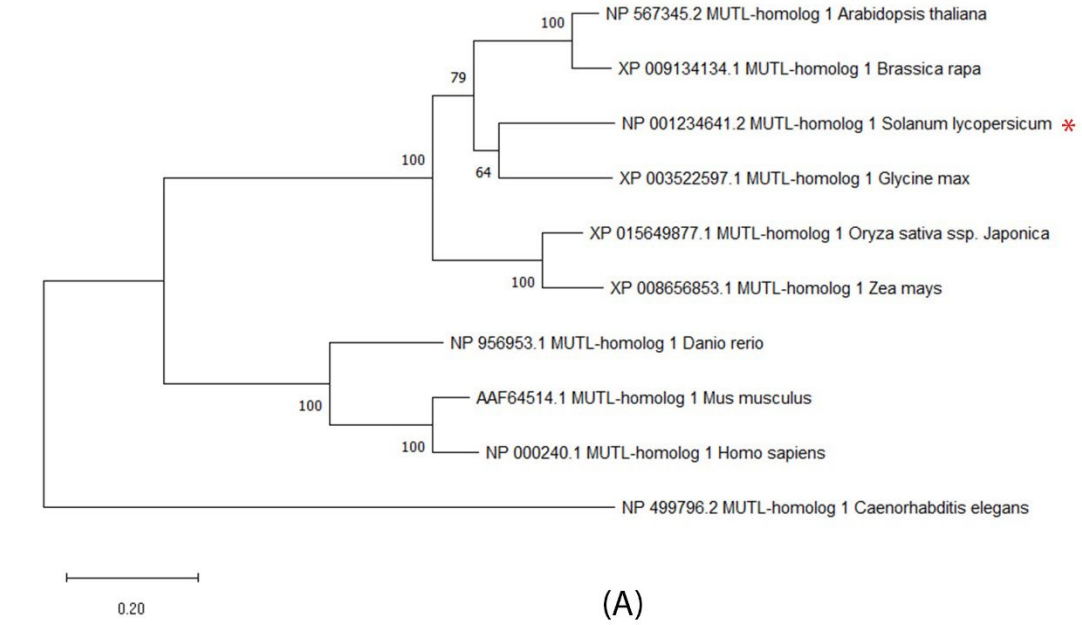
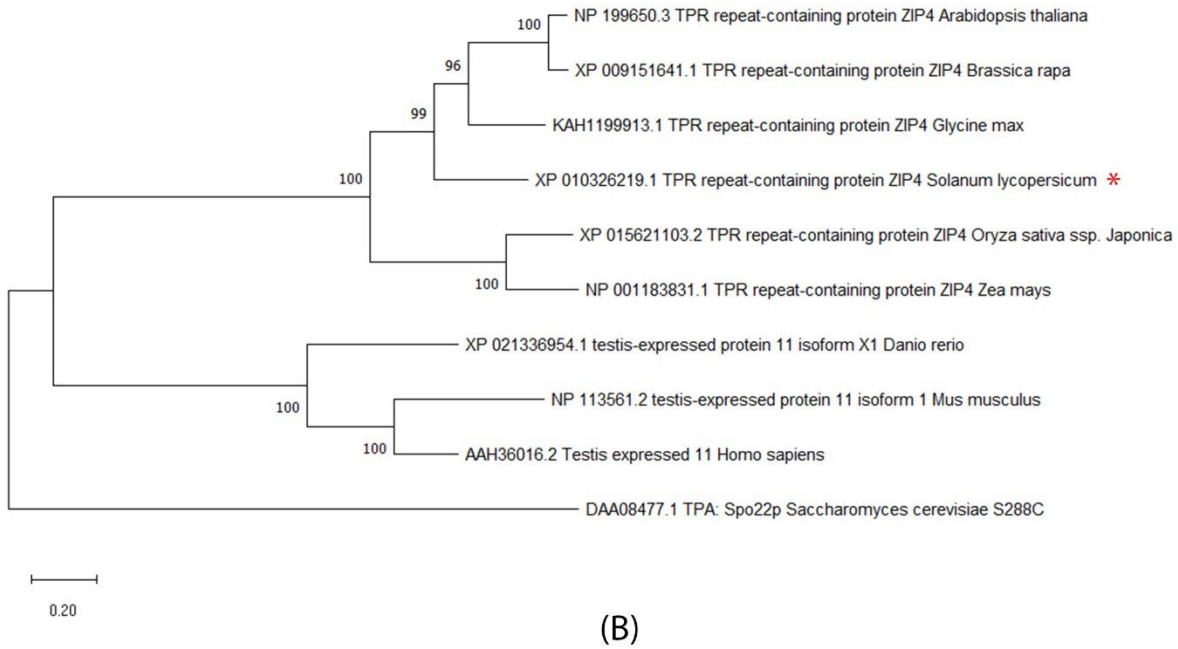


Figure 2.0: Domain composition of the tomato MLH1 and ZIP4 protein and its ortholog protein in *A. thaliana*. **(A):** Comparison of protein domain sequence between AtMLH1 and SlMLH1 protein. The domains are, in red (**HATPase_c_3**): Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase, in green (**DNA_mis_repair**): DNA mismatch repair protein, C-terminal domain and in blue (**Mlh1_C**): DNA mismatch repair protein Mlh1 C-terminus. **(B):** Comparison of protein domain sequence between AtZIP4 and SlZIP4 protein. The domains are, in green (**TPR_8**): Tetratricopeptide repeat and in yellow (**SPO22**): Meiosis protein SPO22/ZIP4 like.



(A)



(B)

Figure 2.1: Phylogenetic tree of MLH1 and ZIP4 orthologs protein using the maximum likelihood method in MEGA 11.0 software with SlMLH1 and SlZIP4 protein marked with asterisk (*) symbol. **(A):** Phylogenetic tree of MLH1 ortholog protein in different type of organisms. **(B):** Phylogenetic tree of ZIP4 ortholog protein in different type of organisms. Species and NCBI protein accession number as described in the tree graph. The scale bar represents 0.2 substitutions per nucleotide position.

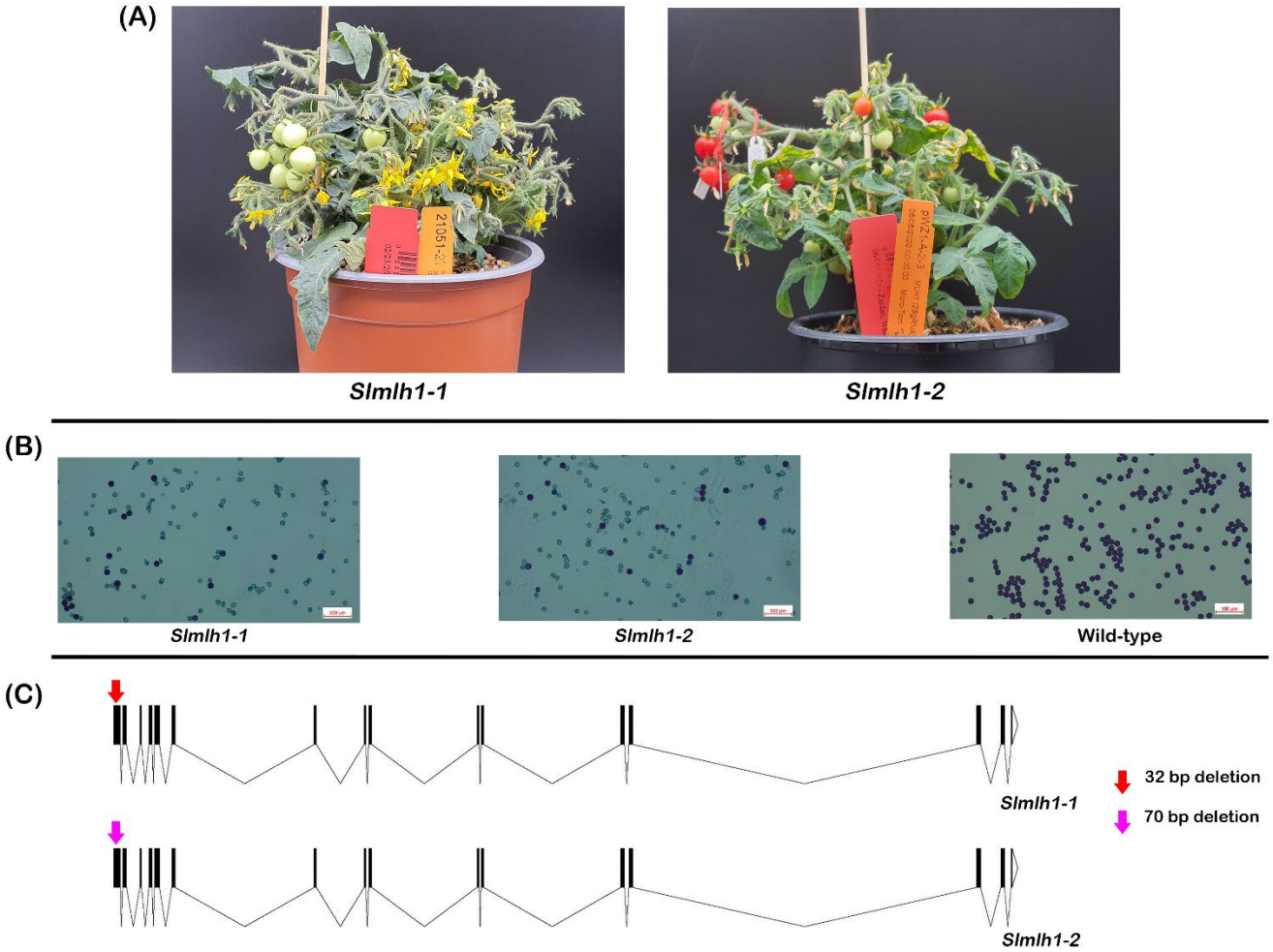


Figure 2.2: Stable lines of *Slmlh1* mutant from CRISPR/cas9 mutagenesis in Micro-Tom background. **(A):** Two different lines of *Slmlh1* mutants, *Slmlh1-1* and *Slmlh1-2*. **(B):** Pollen viability test by Alexander staining on *Slmlh1-1*, *Slmlh1-2* and wildtype Micro-Tom. **(C):** Location and size of the DNA fragment deletion on *SlMLH1* gene for each *Slmlh1-1* and *Slmlh1-2*.

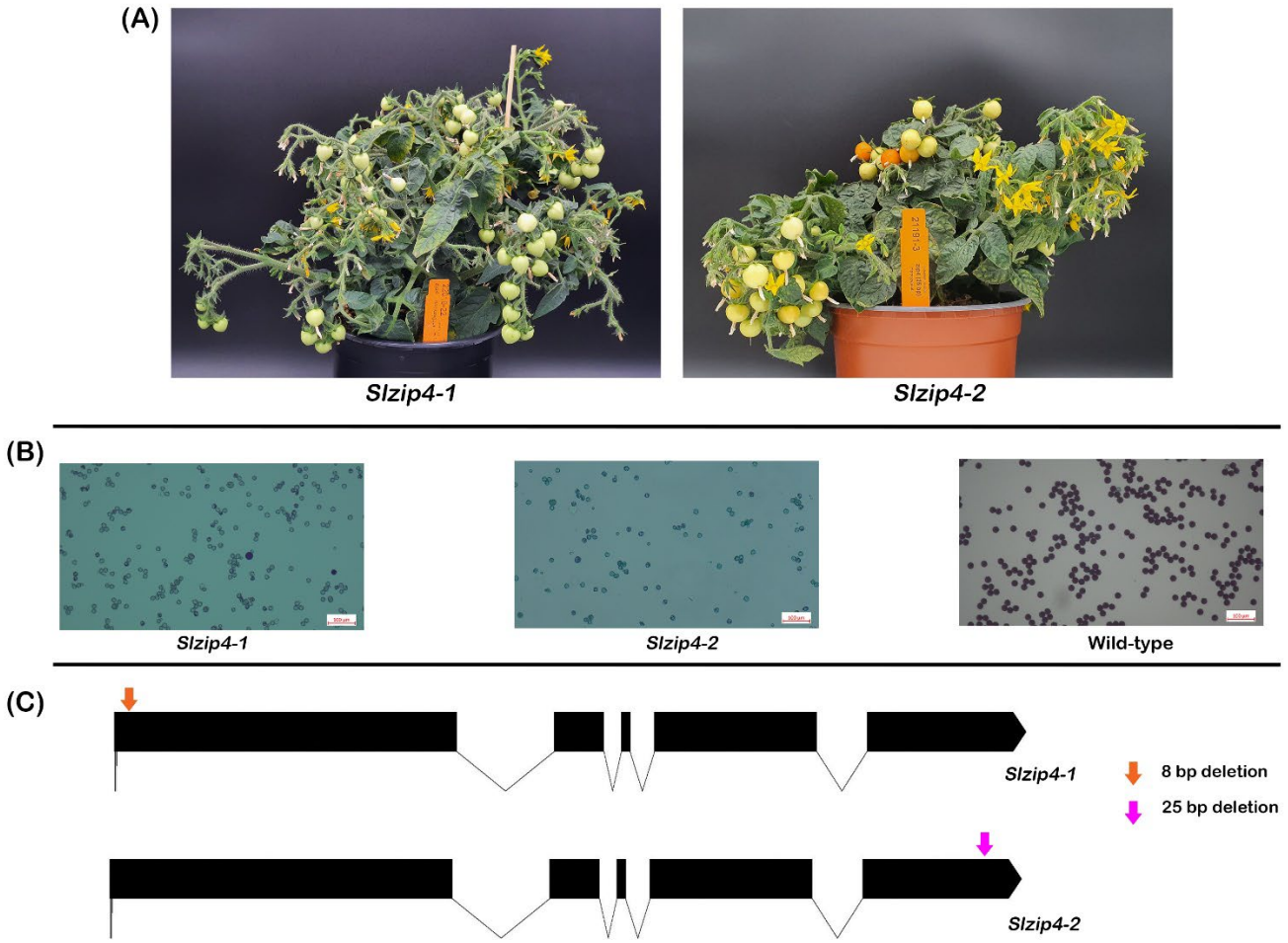


Figure 2.3: Stable lines of *Slzip4* mutant from CRISPR/cas9 mutagenesis in Micro-Tom background. **(A):** Two different lines of *Slzip4* mutants, *Slzip4-1* and *Slzip4-2*. **(B):** Pollen viability test by Alexander staining on *Slzip4-1*, *Slzip4-2* and wildtype Micro-Tom. **(C):** Location and size of the DNA fragment deletion on *SlZIP4* gene for each *Slzip4-1* and *Slzip4-2*.

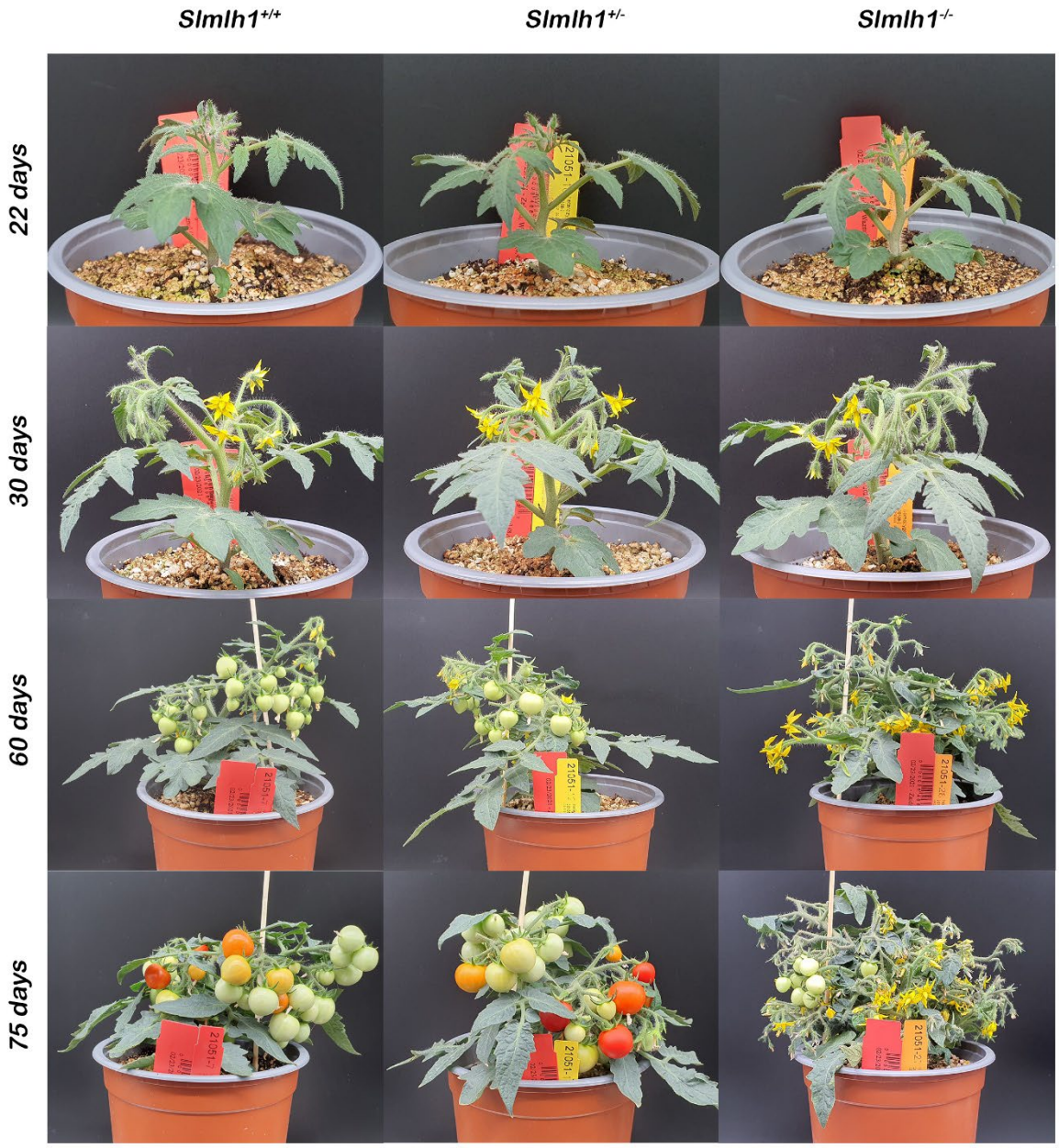


Figure 2.4: Phenotypic evaluation and comparison of wildtype, heterozygous and homozygous sister plants for *Slmlh1* single mutant at 22 days, 30 days, 60 days and 75 days after germination.

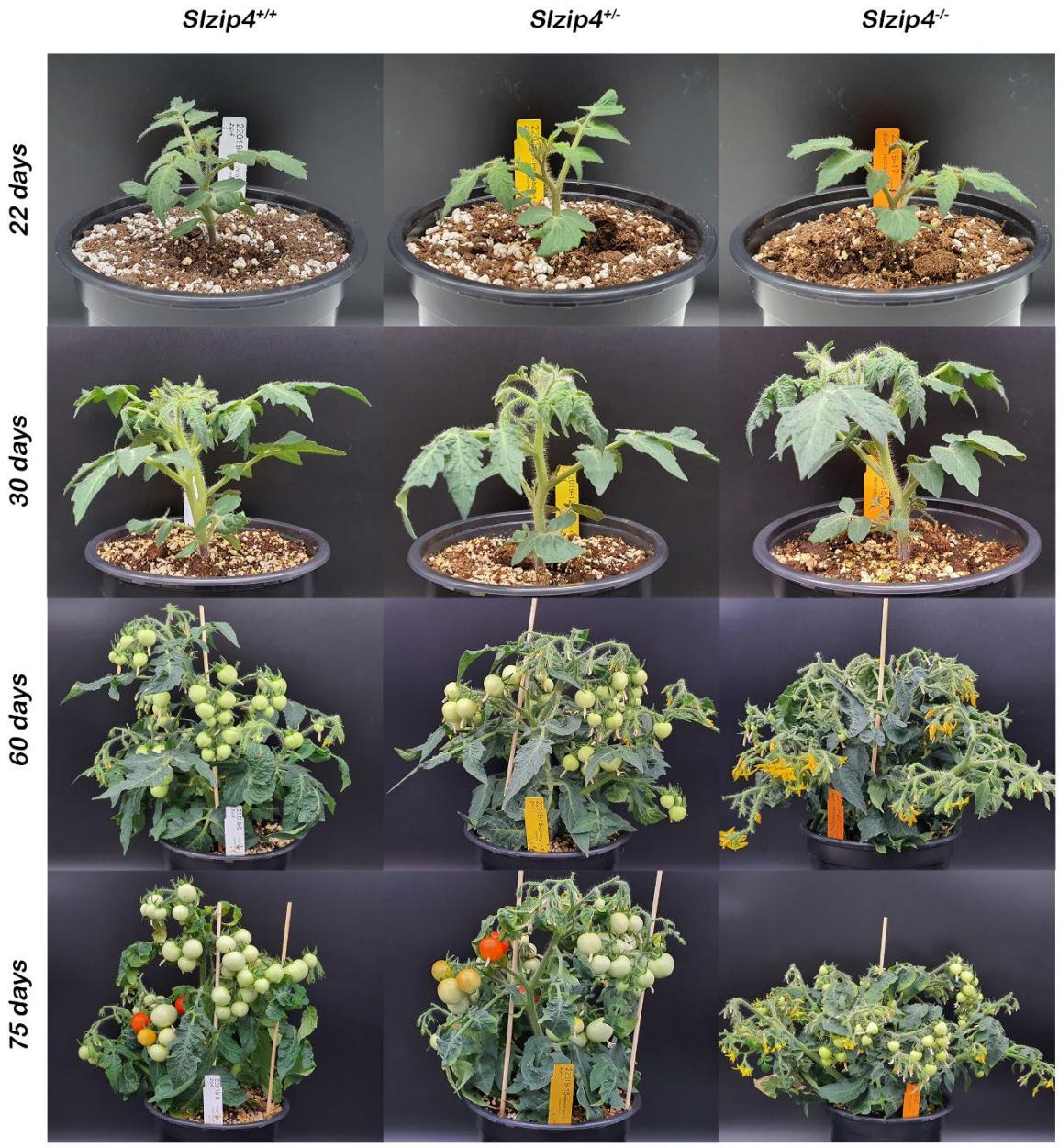


Figure 2.5: Phenotypic evaluation and comparison of wildtype, heterozygous and homozygous sister plants for *Slzip4* single mutant at 22 days, 30 days, 60 days and 75 days after germination.

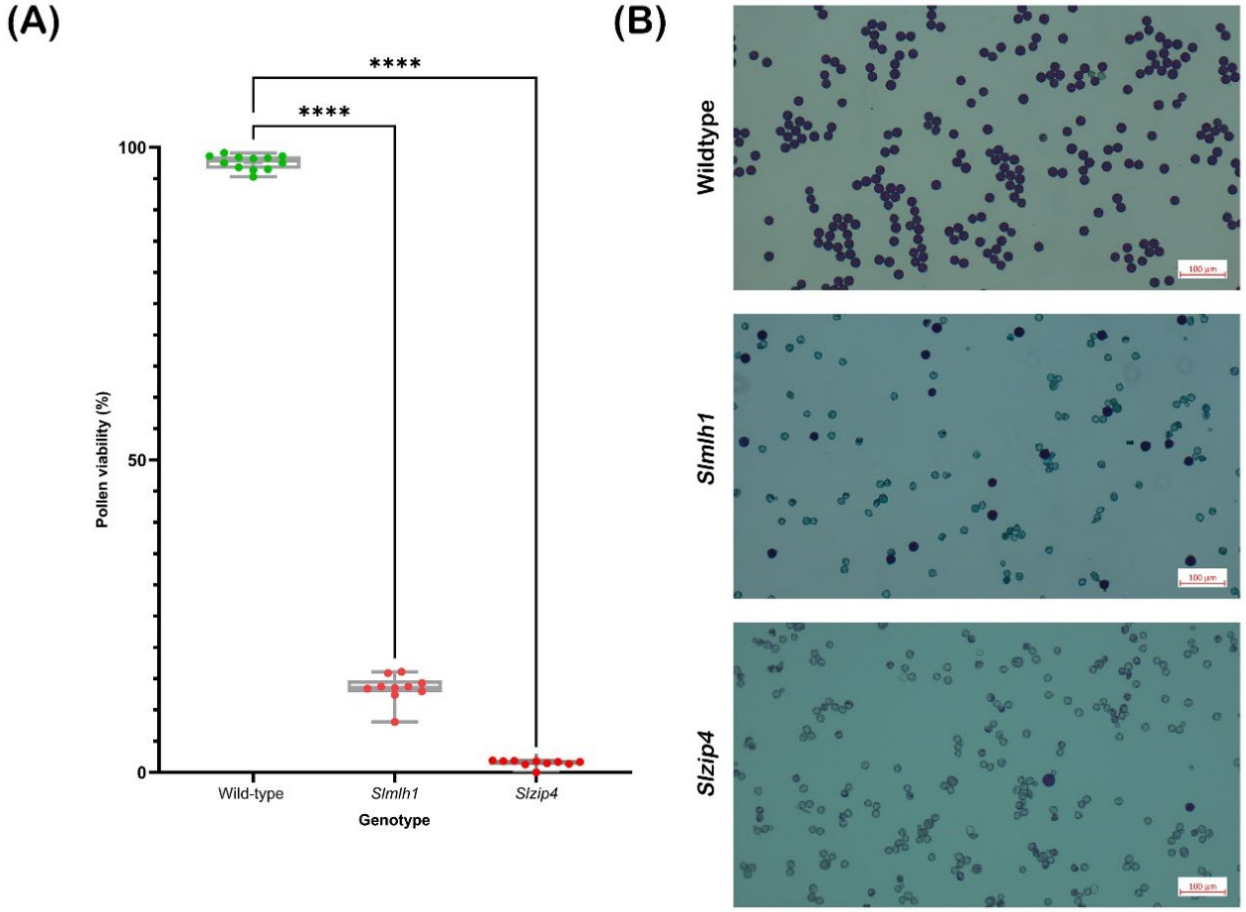


Figure 2.6: Behaviour of viable pollen for wildtype Micro-Tom, *Slmlh1* and *Slzip4* mutants. **(A):** Box and whisker plot graph of viable pollen for Micro-Tom, *Slmlh1* and *Slzip4* mutants. **(B):** Stained pollen image of wildtype Micro-Tom, *Slmlh1* and *Slzip4* mutants at 10x magnification with dark purple indicated viable pollen and light green indicated unviable pollen.

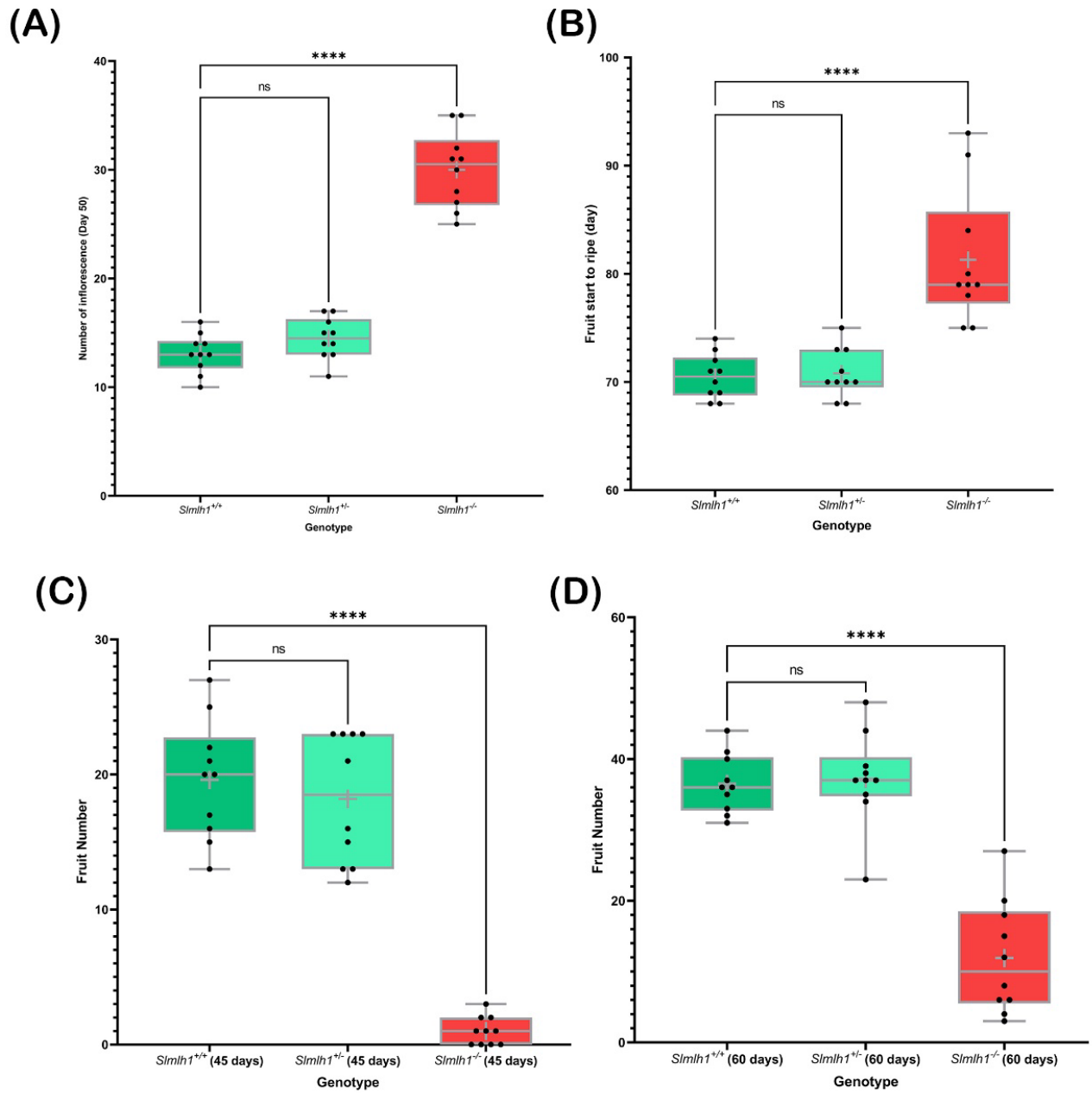


Figure 2.7: Box and whisker plot graph for the number of inflorescences, day for fruit to start ripe and the number of fruits at 45 and 60 days for wildtype, heterozygous and homozygous sister plant of *Slmlh1* mutant. **(A):** Number of inflorescences at day 50 **(B):** Day for the plant to produce ripe fruit **(C):** The number of fruits setting at day 45 and **(D):** The number of fruits setting at day 60.

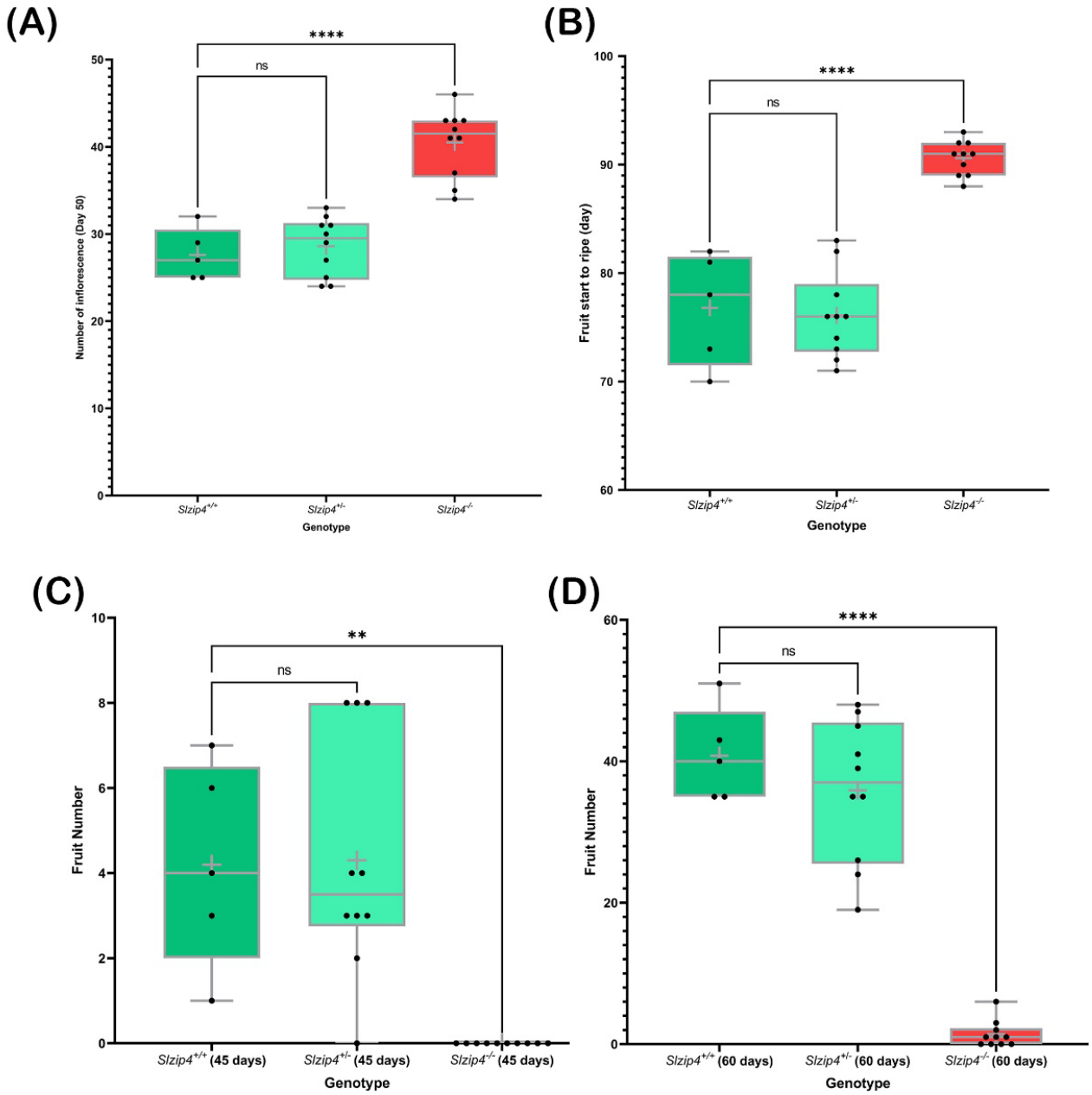


Figure 2.8: Box and whisker plot graph for the number of inflorescences, day for fruit to start ripe and the number of fruits at 45 and 60 days for wildtype, heterozygous and homozygous sister plant of *Slzip4* mutant. **(A):** Number of inflorescences at day 50 **(B):** Day for the plant to produce ripe fruit **(C):** The number of fruits setting at day 45 and **(D):** The number of fruits setting at day 60.

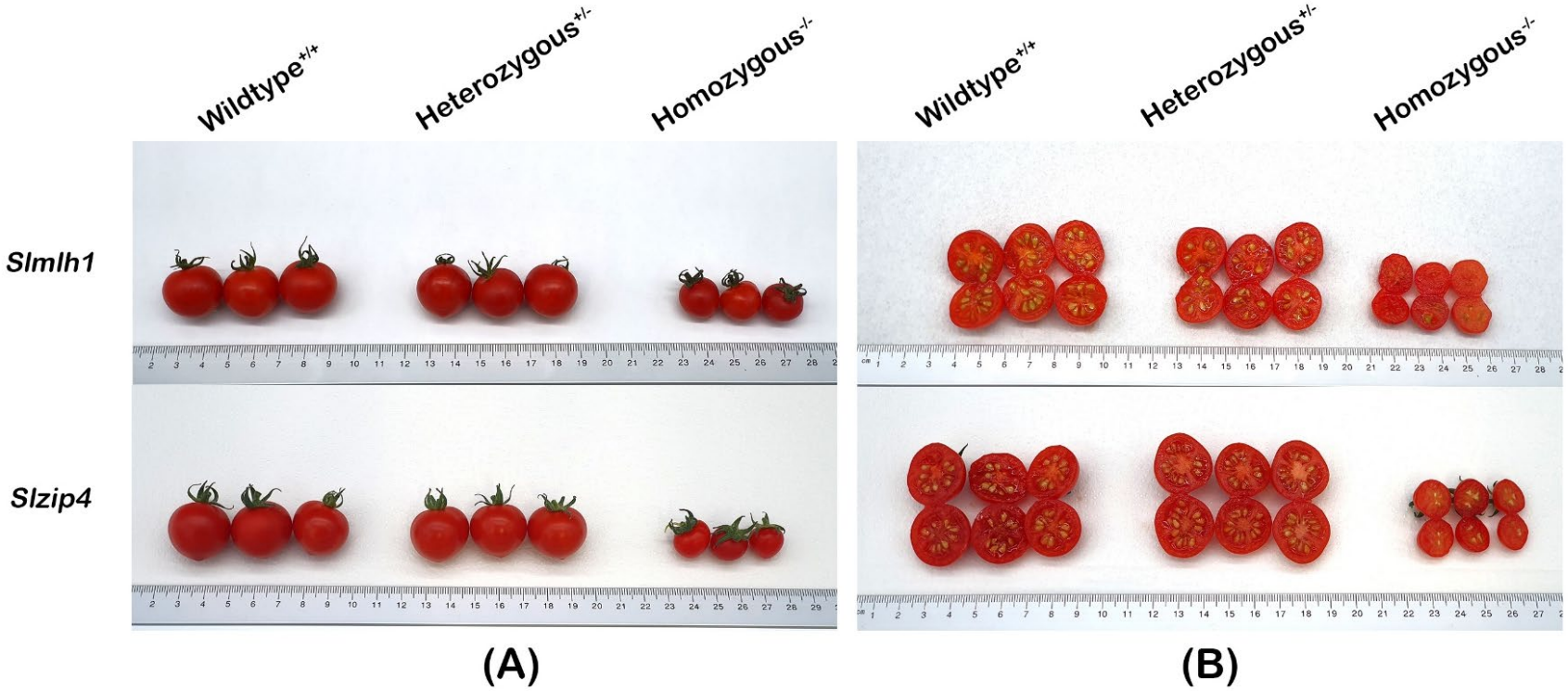


Figure 2.9: Fruit evaluation and comparison from wildtype, heterozygous and homozygous sister plant of *Slmlh1* and *Slzip4* mutants. **(A):** Phenotypic of full ripe fruit. **(B):** Cross-section of full-ripe fruit.

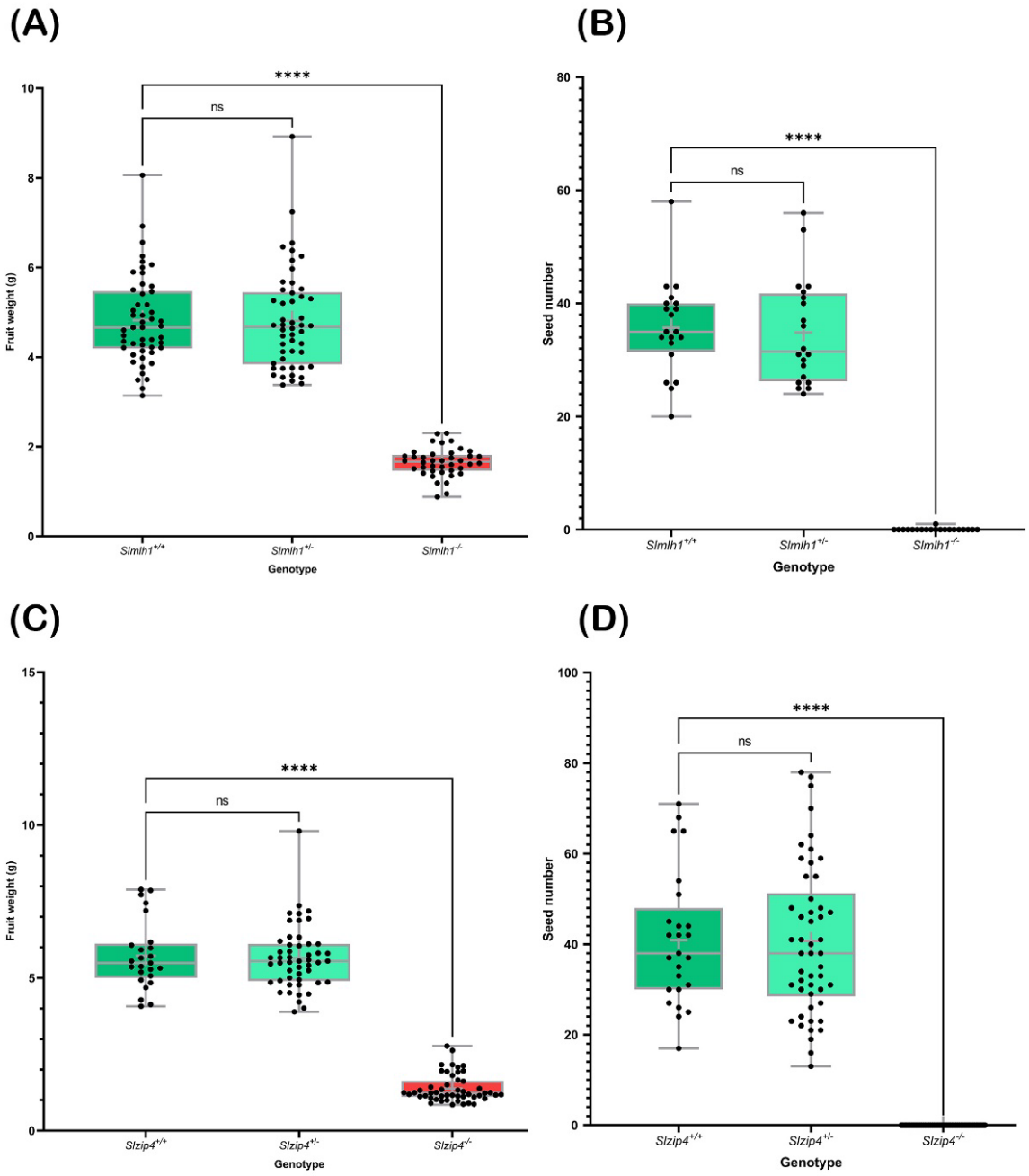


Figure 2.10: Fruit weight and seed number of *Slmlh1* and *Slzip4* mutants for wildtype, heterozygous and homozygous sister. **(A):** Fruit weight of full ripe fruit from *Slmlh1* mutant. **(B):** Average seed number per fruit in *Slmlh1* mutant. **(D):** Fruit weight of full ripe fruit from *Slzip4* mutant. **(B):** Average seed number per fruit in *Slzip4* mutant.

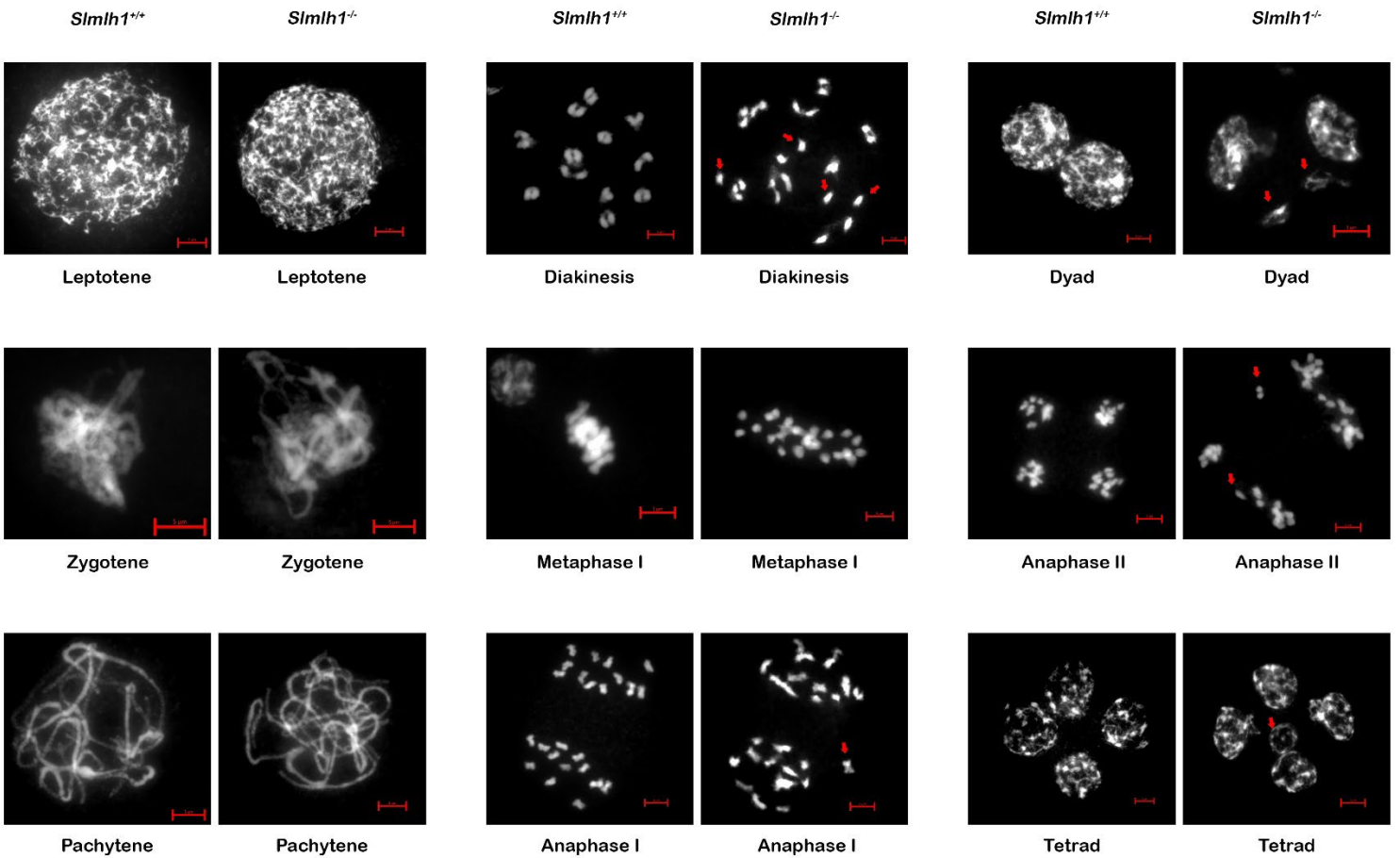


Figure 2.11: Comparison of chromosome behaviour between wildtype and *Slmlh1* mutant by chromosome spread and DAPI staining during meiosis I and II stages. **Meiosis I stages:** Leptotene, Zygotene, Pachytene, Diakinesis, Metaphase I, Anaphase I and Dyad. **Meiosis II stages:** Anaphase II & Tetrad/Polyad. Scale bar= 5 µm

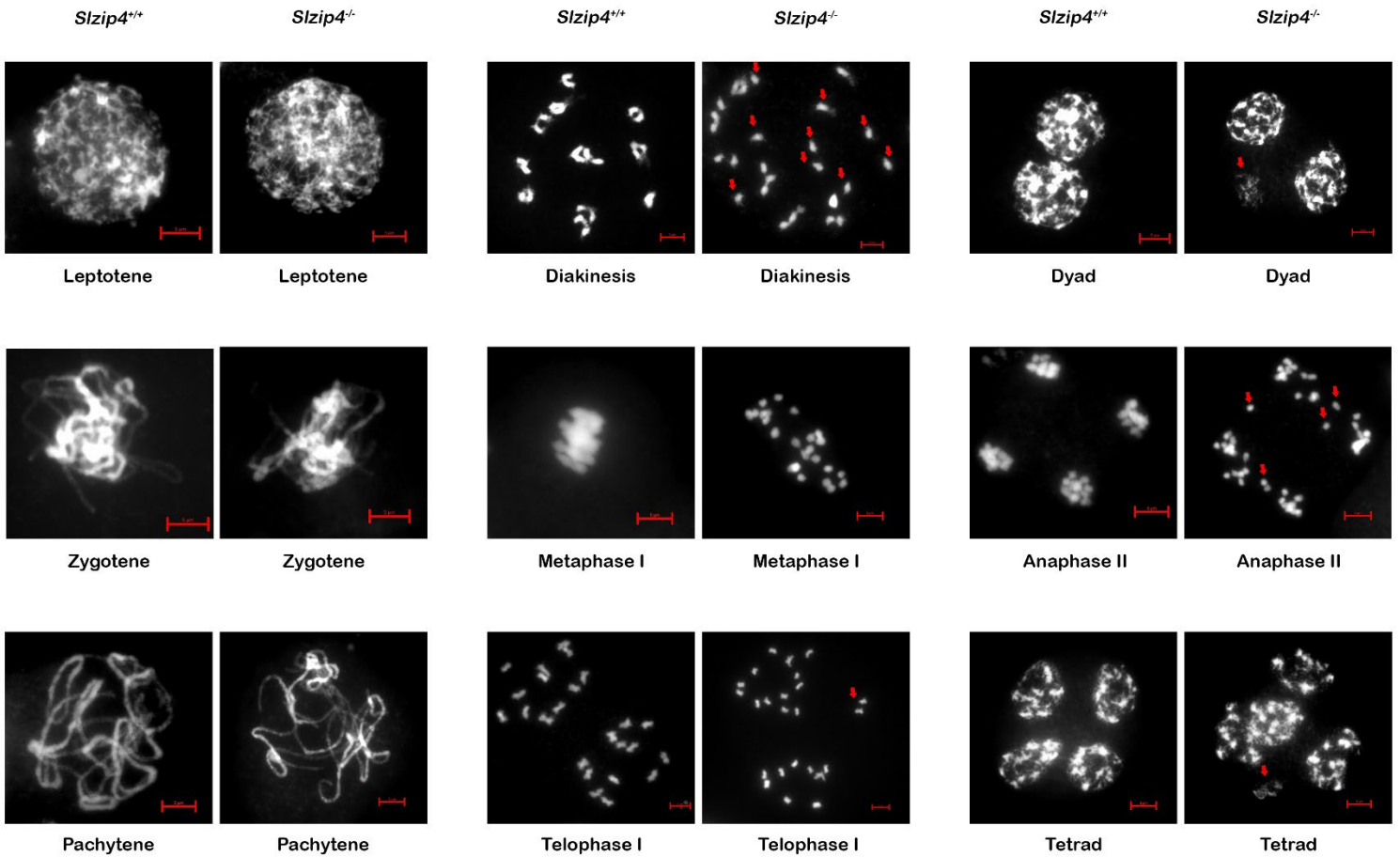


Figure 2.12: Comparison of chromosome behaviour between wildtype and *Slzip4* mutant by chromosome spread and DAPI staining during meiosis I and II stages. **Meiosis I stages:** Leptotene, Zygotene, Pachytene, Diakinesis, Metaphase I, Anaphase I and Dyad. **Meiosis II stages:** Anaphase II & Tetrad/Polyad. Scale bar= 5 µm

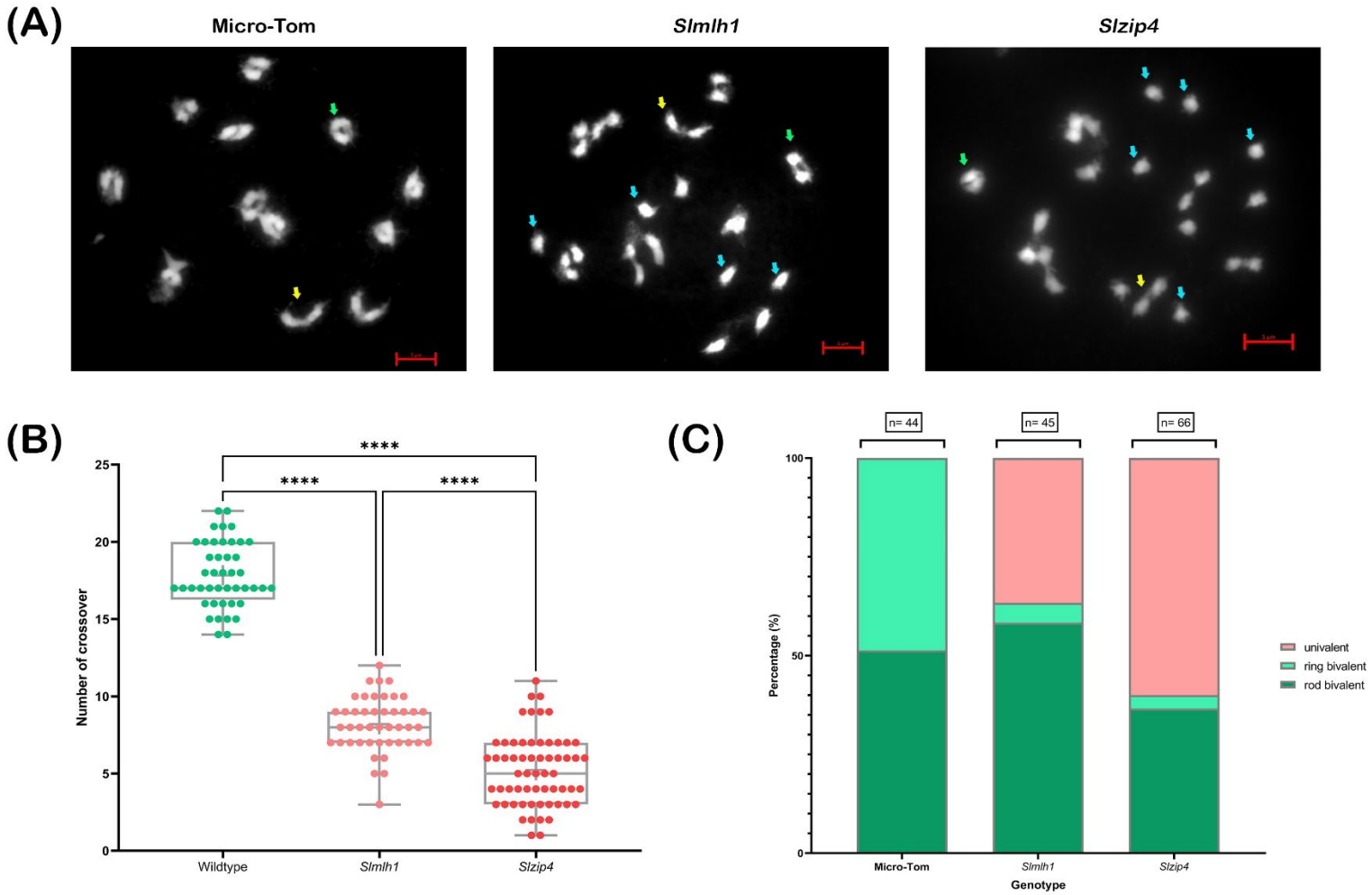


Figure 2.13: Formation of univalent and bivalent and estimate number of meiotic crossover in wildtype Micro-Tom, *Slmlh1* and *Slzip4* mutants during diakinesis stage. **(A)** Diakinesis stage of meiocytes cell for Micro-Tom, *Slmlh1* and *Slzip4* with **green arrow** (ring bivalent= 2 crossover), **yellow arrow** (rod bivalent= 1 crossover) and **blue arrow** (univalent= no crossover). **(B)** Box and whisker plot graph for the number of crossover based on univalent and bivalent counting (ring and rod). **(C)** Bar graph for the percentage of univalent, rod bivalent and ring bivalent during diakinesis stage of meiocyte cell. Scale bar= 5 µm

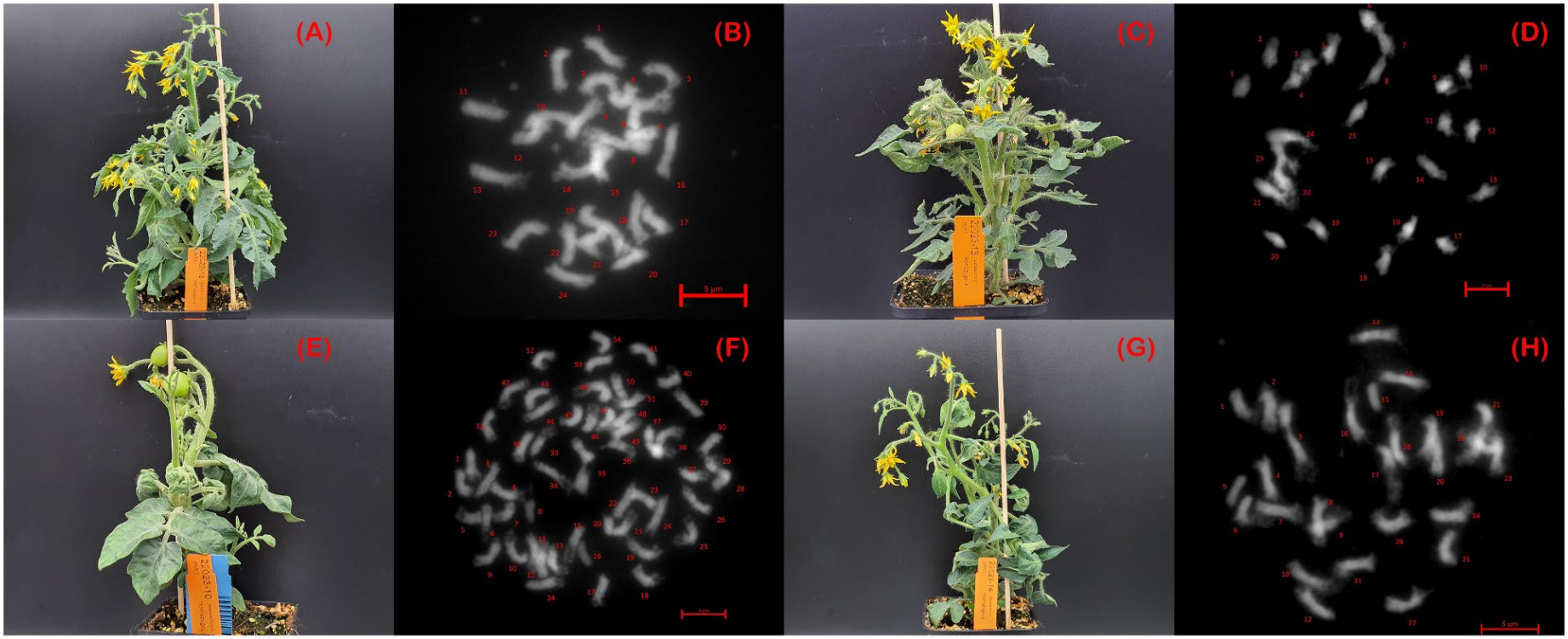


Figure 2.14: Chromosome spread of pro-metaphase stage of meiocytes cell in plants derived from selfing seed of homozygous *Slmlh1* mutant **(A):** Diploid-like phenotype of *Slmlh1* mutant. **(B):** Chromosome spread of **(A)** with normal 24 chromosomes. **(C):** Abnormal/aneuploidy-like phenotype of *Slmlh1* mutant. **(D):** Chromosome spread of **(C)** with abnormal 25 chromosomes. **(E):** Abnormal/aneuploidy-like phenotype of *Slmlh1* mutant. **(F):** Chromosome spread of **(E)** with abnormal 54 chromosomes. **(G):** Abnormal/aneuploidy-like phenotype of *Slmlh1* mutant. **(H):** Chromosome spread of **(G)** with abnormal 27 chromosomes. Scale bar= 5 µm

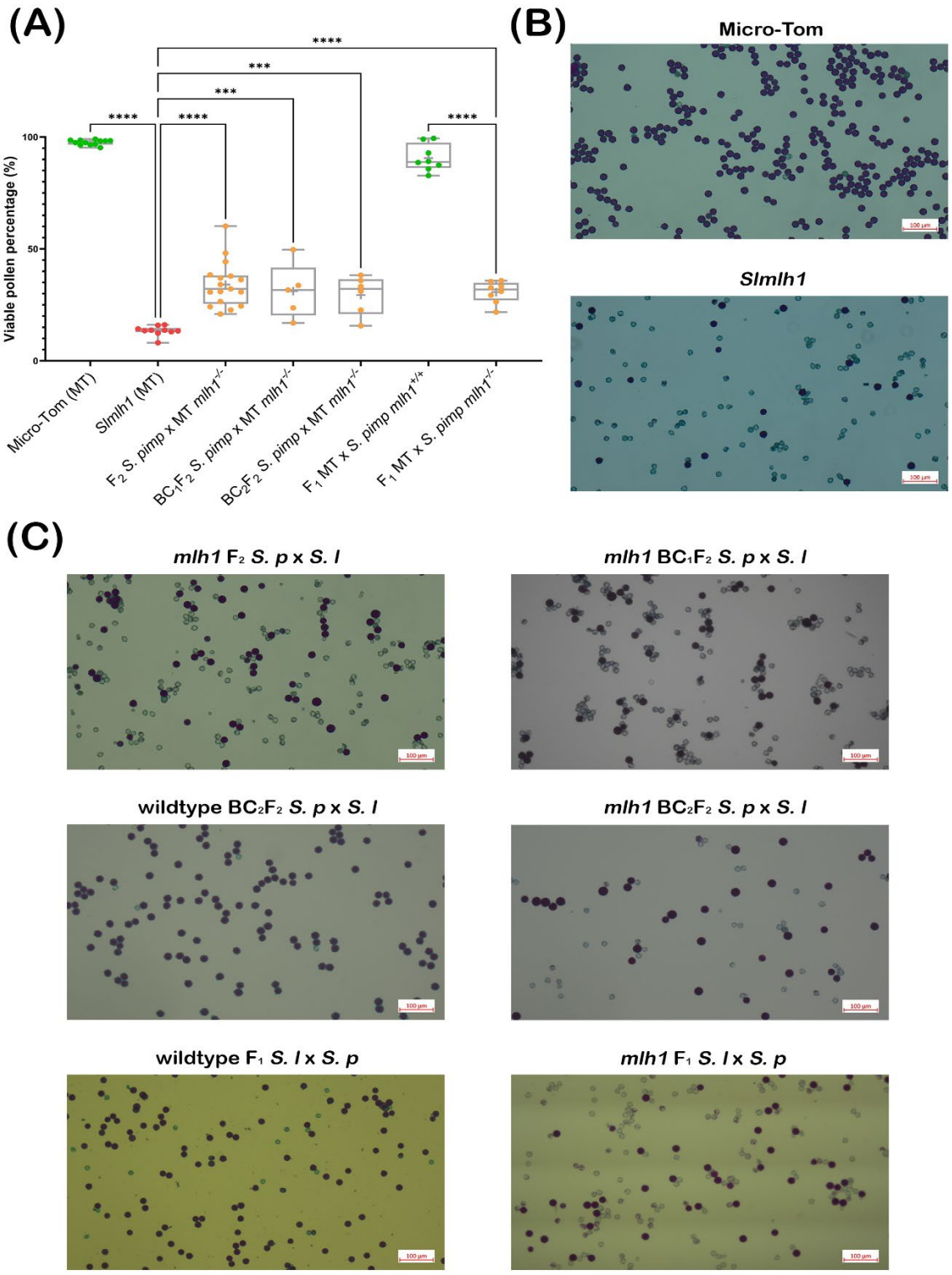


Figure 2.15: Percentage and behaviour of pollen viability for *mlh1* mutant in interspecific hybrid of *S. lycopersicum (S. l)* x S. *pimpinellifolium (S. p)* or *S. pimpinellifolium (S. p)* x *S. lycopersicum (S. l)* crosses or backcrosses background **(A):** Box and whisker plot graph for the percentage of pollen viability in wildtype Micro-Tom, *Slmlh1* and wildtype or *mlh1* mutant in interspecific hybrid *S. lycopersicum (S. l)* x S. *pimpinellifolium (S. p)* or *S. pimpinellifolium (S. p)* x *S. lycopersicum (S. l)* crosses and backcrosses background. **(B):** Stained pollen image of wildtype Micro-Tom and *Slmlh1*. **(C):** Stained pollen image of *mlh1* mutant for F_2, BC_1F_2 generation, wildtype and *mlh1* mutant of BC_2F_2 generation (*S. p x S. l*) and wildtype and *mlh1* F_1 generation (*S. l x S. p*). *S. pimpinellifolium* accession= LA2102.

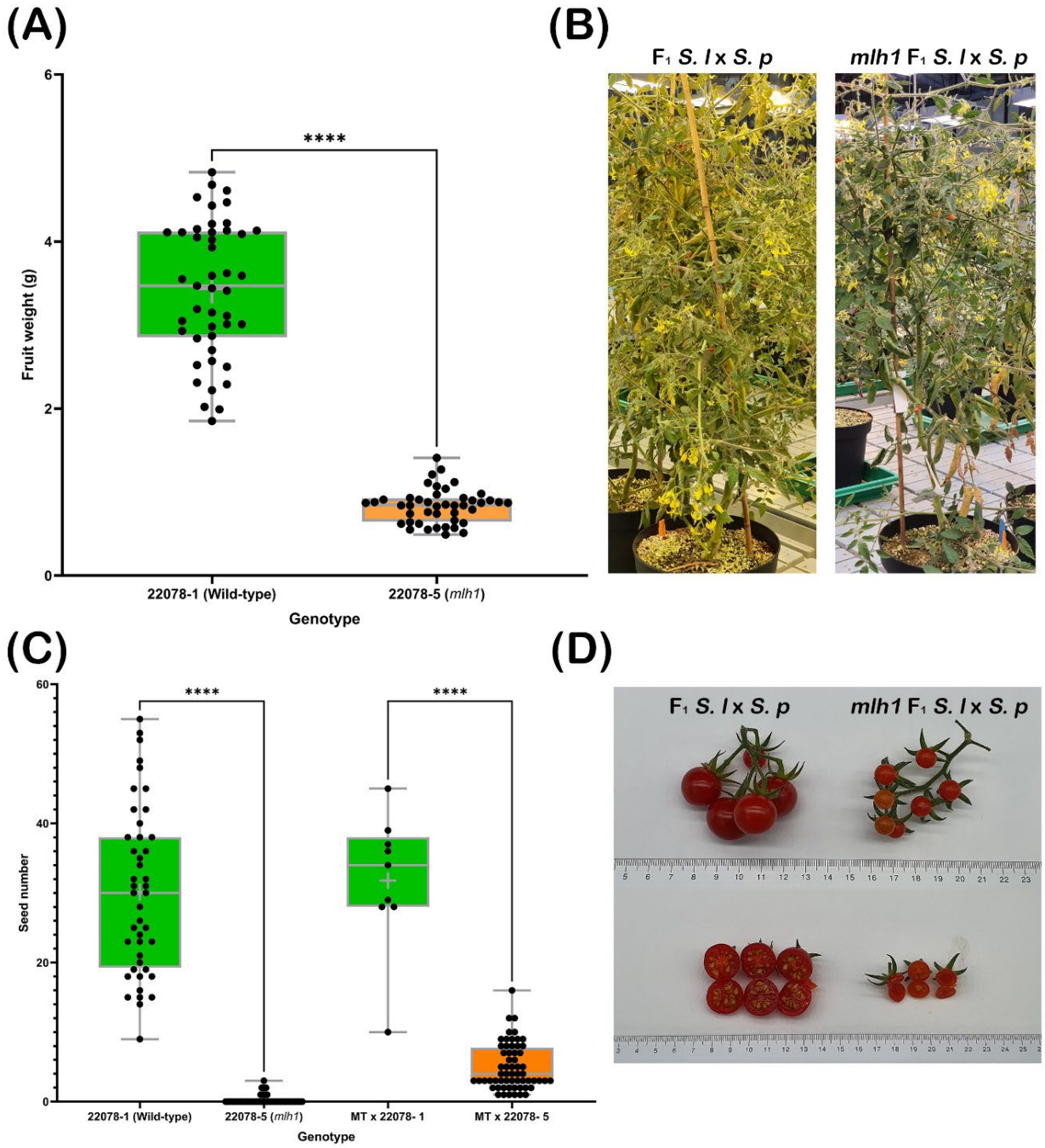


Figure 2.16: Fruit weight and seed number of wildtype and *mlh1* mutant of *S. lycopersicum* (Micro-Tom) x *S. pimpinellifolium* (LA 2102) interspecific hybrid. **(A):** Box and whisker plot graph of fruit weigh for wildtype and *mlh1* mutant of F_1 Micro-Tom x LA 2102 hybrid. **(B):** Wildtype and *mlh1* mutant of F_1 Micro-Tom x LA 2102 hybrid sister plants. **(C):** Seed number of wildtype and *mlh1* mutant of F_1 Micro-Tom x LA 2102 hybrid and seed from cross fruit using Micro-Tom as a female & wildtype or *mlh1* F_1 hybrid as a male. **(D):** Phenotype of fully ripe fruit and it cross-section.

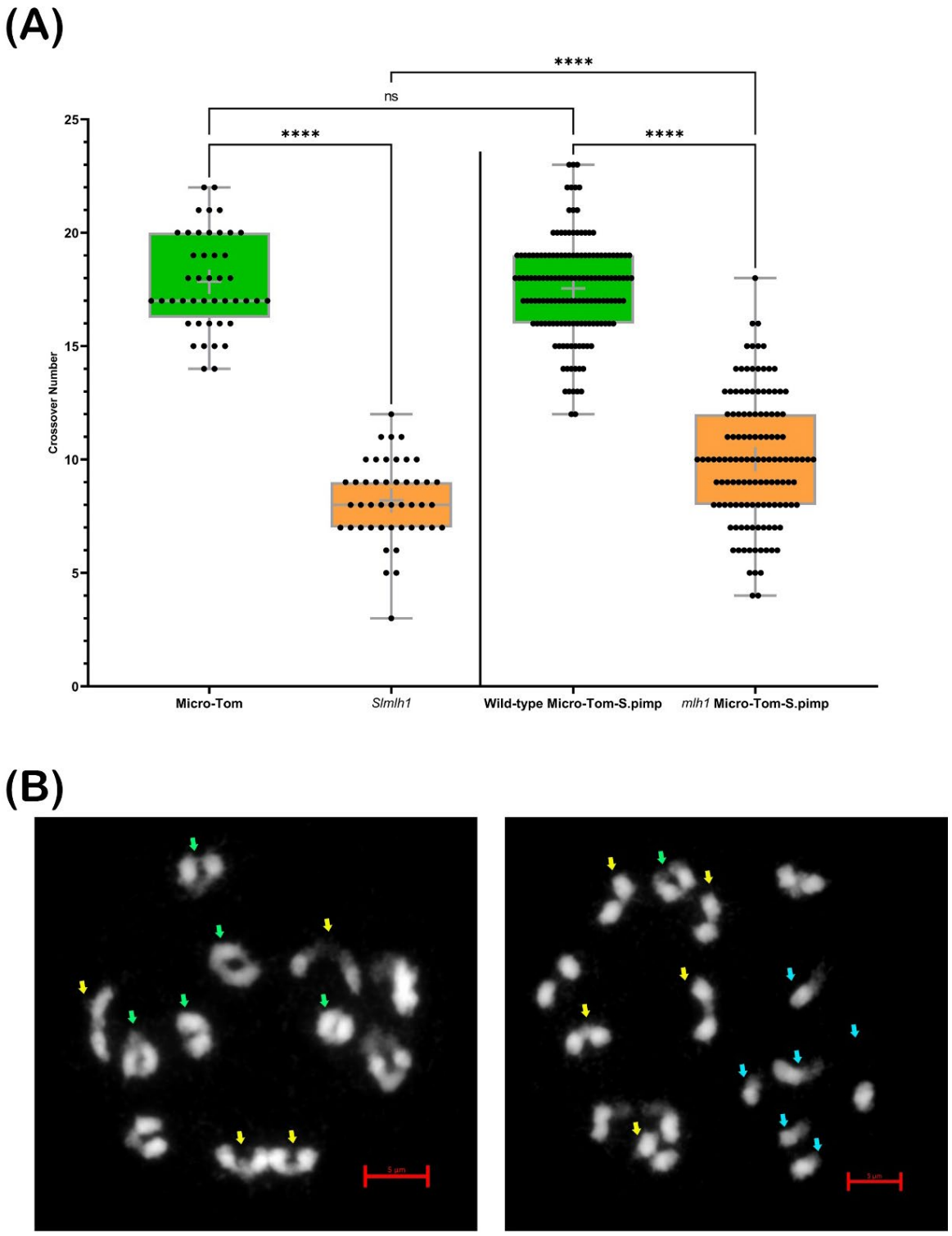


Figure 2.17: Comparison of crossover number between wildtype and *mlh1* mutant for inbred tomato (Micro-Tom) and interspecific tomato hybrid (Micro-Tom x *S. pimpinellifolium*). **(A):** Box and whisker plot graph of crossover number based on univalent and bivalent formation (rod or ring) for wildtype Micro-Tom, *Slmlh1* mutant, wildtype (22078-1) and *mlh1* mutant (22078-5) of interspecific hybrid (Micro-Tom x *S. pimpinellifolium*). **(B):** Chromosome spread of diakinesis stage of meiocyte cell for wildtype and *mlh1* mutant of interspecific hybrid (Micro-Tom x *S. pimpinellifolium*): **green arrow** (ring bivalent= 2 crossovers), **yellow arrow** (rod bivalent= 1 crossover) and **blue arrow** (univalent= no crossover). *S. pimpinellifolium* accession= LA2102. Scale bar= 5 µm

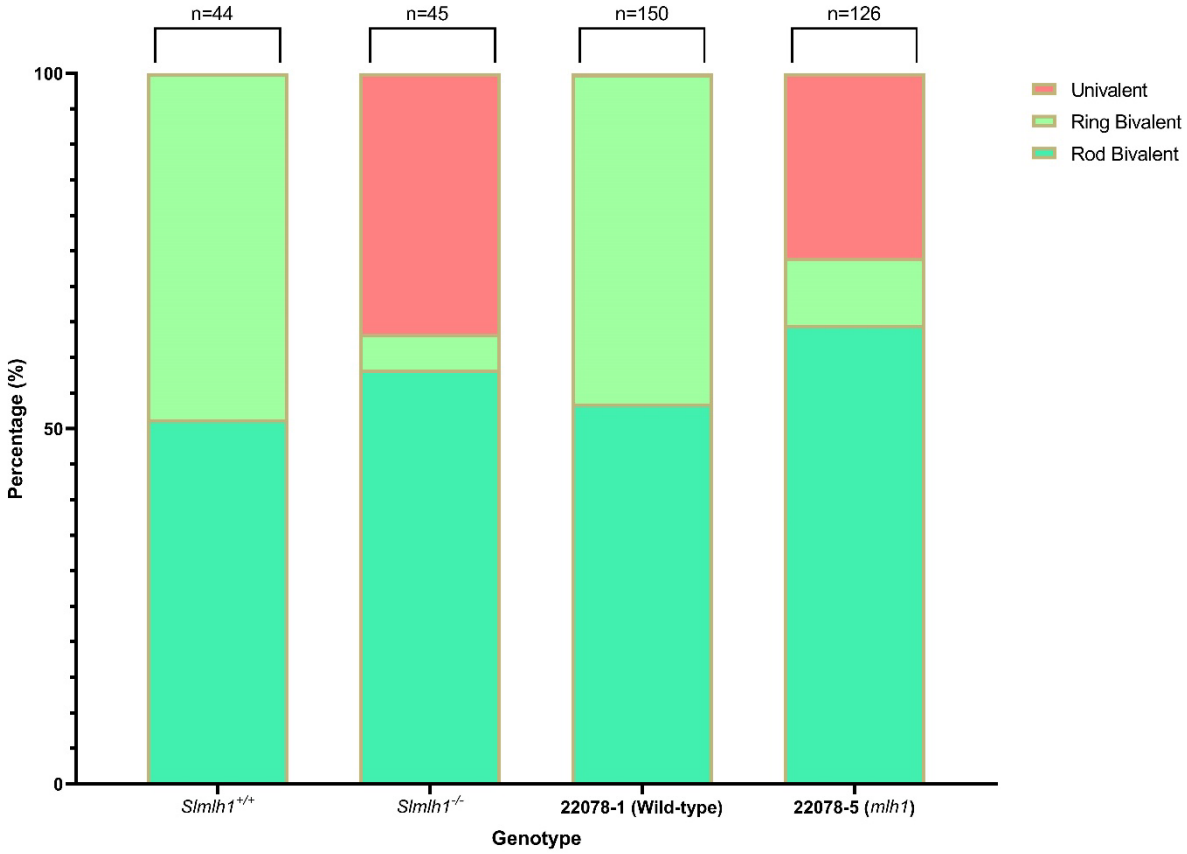


Figure 2.18: Bar graph for the percentage of univalent, rod bivalent and ring bivalent in wildtype Micro-Tom, *Slmlh1* mutant, wildtype (22078-1) and *mlh1* mutant (22078-5) of interspecific hybrid (Micro-Tom x *S. pimpinellifolium*). **Univalent** (no crossover), **rod bivalent** (1 crossover) and **ring bivalent** (2 crossovers). *S. pimpinellifolium* accession= LA2102.

Pro- and anti-crossover functions of RECQ4 helicase in inbred tomato

3.1 Abstract

Programmed meiotic DNA double strand breaks are repaired by recombination with the sister chromatid or homologous chromosome. Repair of invasion events on the homologous chromosome can result in large reciprocal exchanges of genetic material either by forming meiotic crossovers (CO) or can proceed via non-crossover (NCO) repair. The RECQ4-TOP3α-RMI1 (RTR) complex is required for a NCO pathway that functions via Synthesis-dependent strand annealing (SDSA) and has been reported in yeast, mouse and Arabidopsis among other species. In Arabidopsis, loss of RECQ4, and thereby loss of the NCO pathway in which it functions, leads to a concomitant increase in CO frequency without any defect in plant performance and fertility. Meiotic CO also increases in tomato *recq4* mutants however the fertility and chromosome behaviour were not fully characterized. Here, stable null tomato *recq4* mutants were developed via CRISPR/Cas9 gene editing using the inbred Micro-Tom variety. *Slrecq4* displayed a significance reduction in fertility with reduced percentage of viable pollen, reduction of average fruit size and also seed number. Chromosome spreading showed fragmentation during diakinesis, anaphase I, metaphase II and anaphase II stages suggesting that SlRECQ4 is important for DNA repair during tomato meiosis. Moreover, at least 1 univalent pair was identified in 43.5% of diakinesis cells indicating that SlRECQ4 was also required for normal CO formation. Despite the reduction of fertility in *slrecq4* mutants it is, however, capable of partially supressing the much higher infertility of two class I CO mutants - *Slmlh1* and *Slzip4*. This suggests that the increase of CO in *Slrecq4* mutant occurs via the class II CO pathway. Based on these multiple lines of evidence, we conclude that SlRECQ4 is required for normal function of at least two DNA repair pathways during meiosis in tomato. We propose a role for SlRECQ4 in a shared precursor pathway for both CO- and NCO-based repair.

3.2 Introduction and research background

The class I and class II meiotic crossover (CO) pathways are the two key pathways that contribute to meiotic CO formation (Mercier et al., 2015). In addition, anti-crossover (NCO) factors have been identified to play an important role in controlling CO formation in many eukaryotes species by acting to restrain the occurrence of meiotic CO (F. Li et al., 2021; Mercier et al., 2015). Defects in NCO factor were found to increase the frequency of meiotic COs in eukaryotes including plant species (Crismani et al., 2012; Fernandes et al., 2018; Séguéla-Arnaud et al., 2015). Many genes associated with NCO have been identified and these genes were reported to be involved in the regulation of recombination intermediates at different stages. Some of the NCO genes also have functions in somatic DNA repair (Hartung et al., 2007).

NCO factors can influence both class I and class II COs pathway. In plant species, the majority of meiotic CO events (~85% in Arabidopsis) are via the class I CO pathway. In the class I CO loss of function background (e.g. *msh4*, *zip4*, *hei10*), very low fertility is observed due to the requirement of one obligate CO per pair of homologous chromosomes for accurate chromosome segregation in meiosis I (F. Li et al., 2021; Mercier et al., 2015). Through forward mutagenesis screens in class I CO mutant backgrounds, novel mutants that restore fertility were found. Many anti-CO factors that reduce class II COs were found and these mutants channel more recombination intermediates via the class II CO pathway instead of the NCO repair pathway (Arrieta et al., 2021; Crismani et al., 2012; Séguéla-Arnaud et al., 2015). In plants, genes such as *FANCC*, *FANCM*, *FIGL1*, *FLIP*, *MHF1*, *MHF2*, *RECQ4*, *RMI1*, *RPA1a* and *TOP3a* were among those reported to be NCO factors that suppress class II CO formation pathway during strand invasion or D-loop formation (Crismani et al., 2012; Fernandes et al., 2018; Girard et al., 2014; Hartung et al., 2007; Séguéla-Arnaud et al., 2015, 2017; Singh et al., 2023). Based on evidence

found in Arabidopsis, these NCOs factor that limiting the class II CO formation can be divided into 3 different pathways which is FANCM helicase and its two DNA binding co-factors, FIGL1-FLIP complex and BTR (BLM-TOP3α-RMI1) anti-CO pathways (A. Blary & Jenczewski, 2019; F. Li et al., 2021). In addition, HCR1 (HIGH CROSSOVER RATE1), a phosphatase, was recently identified as a NCO factor that represses class I CO by antagonizing a pro-recombination kinase in Arabidopsis (Nageswaran et al., 2021). Identified by forward genetics screening using fluorescent reporters, *hcr1* mutant was found to have significant increase in class I CO based on MLH1 foci number with an average of 12 foci when compared to 10 foci in wildtype (Nageswaran et al., 2021).

Crismani et al., 2012 has identified FANCM helicase as a NCO factor that can limit class II CO formation by promoting NCO formation through the SDSA pathway and work by disassembling the D-loop during strand invasion. A three-fold increase in recombination frequency in inbred *Atfancm* mutants was observed while growth and fertility remained normal (Crismani et al., 2012). It is also reported that FANCM in Arabidopsis is stimulated by two other co-factors namely MHF1 and MHF2, and these co-factors showed weaker NCO capacity based on genetic distance and meiotic recombination frequency (Girard et al., 2014). Genetic analysis suggests that MHF1 and MHF2 suppress CO formation through the same pathway as FANCM (Girard et al., 2014). The capacity of FANCM to act as a NCO factor was found to be conserved across *Brassica* species because CO frequency in *fancm* mutants is increased by 3-fold and 1.3-fold in diploid *Brassica rapa* and allotetraploid *Brassica napus*, respectively (Aurélien Blary et al., 2018). In rice and pea, the mutation of *FANCM* also enhanced CO frequency with respective increases by 2.3-fold and 2.0-fold (Mieulet et al., 2018). However, a lettuce *fancm* mutant exhibited reduced fertility where 77.8% of *Lsfancm* meiocytes exhibited univalent formation suggesting that *LsFANCM* might be required for normal synapsis and CO formation (X. Li et al., 2021). Moreover, LsFANCM appears important for shaping the distribution of meiotic class I CO based on LsHEI10 foci and

chiasmata in *Lsfancm* meiotic chromosomes which was markedly different from the wildtype (X. Li et al., 2021). Similarly tetraploid and hexaploid wheat *fancm* mutants have reduced pollen viability and seed set which was associated with a significant reduction of HEI10 foci in late prophase I (Desjardins et al., 2022). FANCM in wheat also showed its involvement in class II COs as *fancm msh5* exhibited a 2.6 fold increase in COs when compared to wheat *msh5* single mutant (Desjardins et al., 2022). Another Fanconi Anemia (FA) complex protein, FANCC, was recently shown to play a role in NCO in Arabidopsis together with its subcomplex partners FANCE and FANCF (Singh et al., 2023).

The second NCO factor in plants is the FIGL1-FLIP complex which forms a conserved complex that regulates the crucial step of strand invasion in homologous recombination (Fernandes et al., 2018). The FIGL1-FLIP complex was found to interact with the recombinases RAD51 and DMC1 and further limit the class II CO (Fernandes et al., 2018; Girard et al., 2015). In Arabidopsis, both *figl1* and *flip* single mutant were able to suppress infertility in class I CO mutant background due to increased CO formation. Mutation of *FIGL1* in Arabidopsis can restore meiotic CO in the *Atzip4* mutant and *Atfigl1* single mutant increases CO frequency by an average of 72% when compared to wildtype (Girard et al., 2015). Similarly, FLIP mutation in Arabidopsis was able to restore infertility in two class I CO mutants (*AtHei10* and *Atmsh5*) and *Atflip* single mutant produced around 14% of univalent that was observed at metaphase I stage meiocytes cell (Fernandes et al., 2018). Combination of FIGL1-FLIP mutation with FANCM mutation can further elevate the crossover frequency suggesting that they act in two distinct pathways that limit crossover formation in meiosis (Girard et al., 2015). It was later reported that *FIGL1* mutation in rice, pea and tomato leads to sterility indicating that it may have an important role in fertility, less important in DNA double-strand break repair and also may not have a repair capacity for higher number of aberrant recombination intermediates in a large genome (Mieulet et al., 2018)

The third complex identified as an anti-CO/NCO factor is the BTR/RTR complex (BLM-TOP3α-RMI1 or RECQ4-TOP3α-RMI1 in plant species). The BTR complex is the most influential NCO factor for limiting class II crossover in Arabidopsis and acts by unwinding D-loops through the SDSA pathway (Séguéla-Arnaud et al., 2015, 2017). The BTR complex is mostly conserved in eukaryotic species and it has multiple roles in meiotic recombination independently or together with other protein or cofactor (F. Li et al., 2021). In inbred Arabidopsis (Col-0), *AtRECQ4A* and *AtRECQ4B* genes encode two copies of BLM (Bloom syndrome protein) and the double mutant of these two genes exhibits a 6-fold increase in CO in Arabidopsis (Séguéla-Arnaud et al., 2015). Hartung et al., 2007 reported that *AtRECQ4A* has a similar function to its homologues from yeast and mammals, as *recq4a* mutants have enhanced frequency of homologous recombination, are lethal in *Atmus81* background and can partially suppress lethal phenotype in *top3α* background. Recently the effect of *recq4* mutation was also demonstrated to increase COs in pea, rice and tomato resulting to 4.7-fold, 3.2-fold and 2.7-fold increase of CO formation respectively (Mieulet et al., 2018). Other components in BTR complex which are TOP3α and RMI1 were also validated to act as Arabidopsis NCO factor wherein 1.5-fold of increase in CO was observed in *top3α-R640* mutant (Séguéla-Arnaud et al., 2015) and 5.0-fold increase in *rmi1?-G592* mutant with no chromosome entanglement or fragmentation defects were detected (Séguéla-Arnaud et al., 2017). Specific roles of the RMI1 and TOP3α C-terminal domains were suggested to prevent extra COs as they contain an Oligo Binding domain (OB2) and ZINC finger motifs which is sub-domain of the BTR complex that essential to limit extra COs but not compulsory for the resolution of recombination intermediates (Séguéla-Arnaud et al., 2017).

As previously mentioned for the behaviour of anti-CO/NCO factor, there is an interest in the manipulation of such factors in crop breeding as it is predicted to boost CO frequency, alter the CO distribution region in genome and reduce sensitivity to sequence homology (A. Blary & Jenczewski, 2019).

A *recq4* mutant in a close interspecific tomato hybrid between *S. lycopersium* and *S. pimpinellifolium* has a significant increase in CO with 1.53-fold increase by bivalent counting and 1.8-fold increase when measured by analysing SNP markers in the F_2 progeny (de Maagd et al., 2020). Yet meiotic defects including anaphase bridges were observed but not fully explored. In addition, it was identified that the homolog of RECQ4 in barley, *HvRECQL4* has a significant reduction in percentage of fertile florets per ear, seed per ear, number of floret and ear weight even though it shows a significant increase in crossover rate and capable to restore infertility of class I CO mutant (Arrieta et al., 2021). The details of *recq4* mutant behaviour in tomato in terms of yield and fertility performance is not fully explored to date. Here, we developed *recq4* mutants in the dwarf tomato variety, Micro-Tom and explored meiotic defects through comprehensive analysis of every meiotic stage in more details on inbreed tomato line whether that it can increase the CO rate, suppress the infertility in class I mutant and also the overall view of the chromosome behaviour in term of defect and abnormal orientation using gene editing and cytology. This finding will perhaps can be used as guide in making decision whether the mutation of *SlRECQ4* can be applied for manipulation of CO frequency and distribution for future tomato breeding.

3.3 Results

3.3.1 CRISPR/Cas9-mutagenesis and generation of stable line of *Slrecq4* mutant

We set out to generate *Slrecq4* mutants in the cultivated tomato (*S. lycopersicum*) variety Micro-Tom using CRISPR/Cas9 using the pDIRECT22c genome editing system (Čermák et al., 2017). We introduced two single guide RNAs (sgRNAs) that target *SLRECQ4* in exon 1 and exon 3 that were previously validated in tomato (de Maagd et al., 2020) into the pDIRECT22C construct (Čermák et al., 2017) along with 3 sgRNAs targeting *ZIP4* (see chapter 2). *Agrobacterium*-mediated genetic transformation was performed **(Appendix 2.0)** and regenerated plants were further screened for the presence of *AtCAS9*, *NPT* transgenes and *SlACT* (housekeeping gene control) **(Appendix 5.0)**. In total 33 regenerated plants were positive for *AtCAS9* and *NPT* genes, and these plants were further screened for gene edits in the *SLRECQ4 and SlZIP4* genes. Overall, eight different lines were identified that were *Slzip4 Slrecq4* double mutants with various sizes of DNA deletions. These T_0 plants were further evaluated with flow cytometer to determine the DNA content of leaf nuclei by staining with the blue-fluorescent DNA stain, DAPI. Subsequently the stained nuclei were plotted in the form of a histogram to distinguish different ploidy levels based on DNA content as the use of phytohormone Zeatin during the transformation can induce unwanted triploid or tetraploid plants. **Figure 3.0** for sample B and C indicated that the T_0 plants were diploid, like wildtype Micro-Tom (A) germinated from seed based. Plot D indicates that the plant is potentially a tetraploid plant as it has no peak for 2C. Only diploid plants were further used in this study and tetraploid-like plants were discarded. Selected lines with deletions in both *SlZIP4* and *SlRECQ4* were backcrossed with wildtype Micro-Tom and later BC_1F_1 plants were isolated that were heterozygous for mutations in *SlZIP4* and *SlRECQ4*, without transgenes and fully fertile. Two stable lines of *Slrecq4* mutant (*Slrecq4-1* & *Slrecq4-2*)

were successfully isolated in BC_1F_2 population and again confirmed to be without the unwanted transgenes. Based on Sanger sequencing analysis, two mutations (4 bp deletion in exon 1 and 88 bp deletion in exon 3) were identified in *Slrecq4-1* while for *Slrecq4-2* a 29 bp frame shift deletion was identified on exon 3 of the *SlRECQ4* gene (**Figure 3.1**). Analysis of modified sequence for both *Slrecq4-1* and *Slrecq4-2* lead to null mutation of *SlRECQ4* gene **(Appendix 5.0 & 6.0)**. Both tomato *recq4* lines were also observed to have similar characteristics in term of pollen viability and fruit setting and further experiment only utilized *Slrecq4-1* line as a sample.

3.3.2 Reduced fertility and fruit yield in *Slrecq4* mutant

Next we performed extensive phenotypic characterization of homozygous *Slrecq4-1* mutants together with heterozygous and wildtype sister plants. We found that there is a significant reduction in fertility and yield performance for the tomato *Slrecq4-1* mutant **(Figure 3.4)**. Pollen staining analysis by Alexander staining can be used to distinguish viable pollen (dark purple stained pollen) from non-viable pollen (light green coloured/colourless pollen with uneven shape) (**Figure 3.2**). Pollen viability is significantly reduced in *Slrecq4* mutant when compared to wildtype Micro-Tom. The mean for the percentage of viable pollen in *Slrecq4* mutant is 37.25% ($p>0.0001$) which is very less than wildtype Micro-Tom with an average of 97.60% viable pollen (**Figure 3.2A**). There is a significant increase in inflorescence production from *Slrecq4* mutant at 50 days (**Figure 3.3A**). The mean for inflorescence number for the homozygous *Slrecq4* mutant is 36.6 ± 4.7 inflorescences when compared to heterozygous *Slrecq4* (28.8 ± 5.5 inflorescences) and wildtype (28.9 ± 2.7 inflorescences) sister plants. Despite there being more inflorescences in *Slrecq4* mutant, less fruit set was observed on day 60 in homozygous *Slrecq4* mutant (13.3 ± 5.2 fruits) when compared to heterozygous *Slrecq4* (31.4 ± 6.4 fruits) and wildtype (35 ± 6.1 fruits) sister plants (**Figure 3.3D**).

Reduction and delay in fruit setting observed in *Slrecq4* mutants also leads to a delay in fruit ripening (mean of 81.7 ± 2.3 days for fruit to ripe) when compared to both heterozygous *Slrecq4* mutant (75.8 ± 3.3 days) & wildtype sister plants (74.8 ± 3.5 days) (**Figure 3.3B**). There is reduction of fruit size observed in *Slrecq4* mutant when compared to wildtype and heterozygous sister plant (**Figure 3.7** and **Figure 3.8**). There is significant reduction in average fruit weight for *Slrecq4* homozygous mutant (4.3 ± 1.5 g) when compared to the wildtype (5.2 ± 1.1 g) and heterozygous (5.7 ± 1.0 g) sister plants (**Figure 3.8A**). This is also reflected to the number of seed set being produced with the average seed for homozygous *Slrecq4* mutant is 9.9 ± 6.1 seeds per fruit while higher seed number for wildtype (39.1 ± 9.8 seeds) and heterozygous (41.8 ± 13.7 seeds) sister plant (**Figure 3.8B**). Nevertheless, no abnormal observation was found for other general trait such as plant height, time start to flowering, leaf shape & morphology and flower number per inflorescence.

3.3.3 RECQ4 knockout caused abnormal chromosome behaviour in tomato

We sought to understand whether the reduction in fertility of the *Slrecq4* mutant may arise due to abnormalities in chromosome behaviour during meiosis. Therefore, further investigation was carried out on male meiosis in pollen mother cells (PMCs) of *Slrecq4* and comparison was made to the wildtype sample (**Figure 3.9**). Chromosome spreads of *Slrecq4* mutant appeared normal during the first stages of meiosis I (leptotene, zygotene and pachytene stages) when compared to wildtype PMCs, indicating that *SlRECQ4* genes is not required for pairing and synapsis (**Figure 3.9**). However, abnormal behaviour in *Slrecq4* was detected during diakinesis stage where there is a univalent chromosome formation ranging from 2 to 6 univalents were observed. 56.5% out of 154 diakinesis cells in *Slrecq4* mutant with 12

bivalent chromosomes while the rest of diakinesis cells had at least 1 pair of univalent (**Figure 3.13A**). Despite the presence of univalent chromosomes in *Slrecq4,* the morphology of bivalents in *Slrecq4* during diakinesis stage is more packed and dense compared to wildtype diakinesis suggestive of a higher number of CO on those bivalents that do form (**Figure 3.10**). We also observed that *Slrecq4* chromosomes contain fragmentation tails during diakinesis suggesting a possible defect in DSB repair. This univalent and fragmentation tail was not observed in diakinesis stage for wildtype sample which has clear rod or ring bivalent formation without any fragmentation tail. Univalent formation was also observed in metaphase I confirming that there is a problem with obligate crossover formation in *Slrecq4* mutant. As there is univalent formation in both diakinesis and metaphase I, there was also unequal segregation that was observed in late anaphase I stage supporting that *SlRECQ4* gene may be necessary for assurance of one crossover event per chromosome pair. Moreover, formation of chromosome bridges with chromosome fragmentation in early anaphase I was also observed in *Slrecq4* mutant (34 out of 52 anaphase I stage cell). During meiosis II, chromosome fragmentation and delayed segregation was again observed in some metaphase II (15/37 cells) and anaphase II (21/38 cells) of *Slrecq4* mutant (**Figure 3.10**). As a result of delayed chromosome segregation and fragmentation, the formation of triads, abnormal tetrads with micronuclei or polyads was also observed unlike wildtype where only formation of tetrads was observed.

3.3.4 Partial suppression of infertility in tomato class I CO by *Slrecq4*

As described in chapter 2, the tomato class I CO pathway is required for normal fertility where in the *Slmlh1* mutant exhibits a massive reduction in fertility while the *Slzip4* mutant is classed as both male and female sterile. Both mutants have a large reduction in total CO events and abolishment of the

obligate crossover. As *RECQ4* mutation in Arabidopsis can restore the infertility of Arabidopsis class I CO mutant, we decided to produce the *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants. Crosses were successfully carried out and genotyping of F_2 population managed to isolate both double mutants *Slmlh1 Slrecq4* and *Slzip4 Slrecq4*. Pollen viability tests conducted on both double mutants showed that tomato *Slrecq4* can suppress the low percentage of viable pollen in *Slmlh1* and *Slzip4*. The mean value for the percentage of viable pollen in *Slmlh1 Slrecq4* double mutant is 35.71% while for *Slzip4 Slrecq4* double mutant is 35.50%. This represents a significant increase of viable pollen for the *Slmlh1 Slrecq4* (pollen viability is 22.4% higher than *Slmlh1* ($p<0.0001$)) and *Slzip4 Slmlh1* (pollen viability is 34% higher than *Slzip4* ($p<0.0001$) (**Figure 3.2A** and **B**). However, there is no significant differences on phenotypes behaviour between double mutants *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* when compared to the *Slrecq4* single mutant indicating that *Slmlh1* and *Slzip4* are epistatic to *Slrecq4* (**Figure 3.5** and **3.6**). It was also observed that suppression of infertility of class I CO mutants by *Slrecq4* influences the fruit set, fruit weight and seed number. As shown in *Slrecq4* mutant, reduction in fertility triggered plant to produce more inflorescence and in *Slmlh1 Slrecq4* double mutant, there is significant increase of inflorescence number at day 50 with homozygous double mutant *Slmlh1 Slrecq4* produced 33.0 ± 1.7 inflorescences when compared to wildtype sister plant with mean value of 24.6 ± 1.5 inflorescences ($p=0.0112$) (**Figure 3.3A**). However, is not significant for *Slzip4 Slrecq4* mutant as the mean number of inflorescences for homozygous mutation is 29.4 ± 2.8 inflorescences when compared to wildtype sister plant with 25.4 ± 3.4 inflorescences ($p=0.2616$). Similarly observed in *Slrecq4* single mutant, both double mutants of *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* have significant reduction of fruit setting on day 60 as a result in reduced percentage of viable pollen (**Figure 3.3D**). Reduction of fruit size and seed number per fruit in double mutant *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* was similarly observed like those in *Slrecq4* mutant (**Figure 3.7A** and **B**). The

mean number of fruit size in homozygous *Slmlh1 Slrecq4* is 17.7 ± 4.2 fruits when compared to the heterozygous (44.3 ± 5.4 fruits) and wildtype (42.6 ± 3.5 fruits) sister plants ($p<0.0001$). Same pattern for *Slzip4 Slrecq4* homozygous plants, the mean value of fruit number is 33 ± 10.3 fruits and is much lower than heterozygous (47.0 ± 7.3 fruits) and wildtype (44.6 ± 3.6 fruits) sister plants ($p=0.0099$). The weight of homozygous *Slmlh1 Slrecq4* double mutant fruit is slightly reduced with average fruit is 4.4 ± 1.4 g but not significant when compared to wildtype (5.0 ± 1.0 g) and heterozygous (5.0 ± 1.1 g) sister plants ($p=0.52$). Nevertheless, there is significant reduction in fruit weight of homozygous *Slzip4 Slrecq4* double mutant with 4.1 ± 0.9 g of average fruit weight when compared to wildtype (5.6 ± 0.7 g) and heterozygous (5.6 ± 1.2 g) sister plants ($p<0.0001$) (**Figure 3.8A**). However, fruit size and number are not the main criteria to determine the fertility rate as stimulation of various factor such as growth condition can affect the size and fruit number. Seed set produced by mutant will give more accurate description on plant performance. In term of seed number, both double mutants of *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* showed significant reduction in seed number which is reflecting to the viable pollen that being produced (**Figure 3.8B**). In homozygous *Slmlh1 Slrecq4* mutant, on average 9.9 ± 7.3 seeds per fruit were produced with wildtype and heterozygous sister produced 39.2 ± 16.4 and 35.1 ± 11.8 seeds per fruit respectively. Similar observation in homozygous *Slzip4 Slrecq4* mutant, 10.1 ± 5.7 seeds per fruit were produced which is less than wildtype (39.9 ± 12.2 seeds) and heterozygous (38.9 ± 14.4 seeds) sister plants ($p<0.0001$). Compare to single mutant of *Slmlh1* and *Slzip4* as described in chapter 2, *RECQ4* mutation introduced to both class I CO mutants has improved the seed set of *Slmlh1* mutant and abolish the sterility phenotype resulted from *SlZIP4* mutation.

3.3.5 RECQ4 knockout increase CO frequency through class II CO pathway

As described in chapter 2, mutation of class I CO genes *SlMLH1* and *SlZIP4* genes reduced or abolished class I CO in tomato, respectively. Therefore, partial restoration of fertility in double mutants of *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* indicating that crossover formation may come from different class of meiotic CO. Chromosome spreading of pollen mother cells (PMCs) for *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants showed similar behaviour as observed in *Slrecq4* mutant (**Figure 3.11** & **Figure 3.12**). The leptotene, zygotene and pachytene stages were observed normal in both double mutants. This observation was observed similar in each of single mutant of *Slmlh1*, *Slzip4* and *Slrecq4* showing these genes affect the fate of crossover without influencing the mechanism of synapsis. Again, abnormal PMCs behaviours was detected during diakinesis stage where many univalent formed as observed in *Slrecq4* single mutant ranging from 2 to 6 univalent in the large percentage of diakinesis-stage meiocyte cells (**Figure 3.13**). In *Slmlh1 Slrecq4* mutant, 63.8% of diakinesis-stage meiocyte cell were normal with twelve complete bivalents while the rest contained at least 1 pair of univalent. Diakinesis cell of *Slmlh1 Slrecq4* without normal 12 bivalent pair consist of 26.9% with 11 bivalents pair, 7.7% with 10 bivalents pair and 1.5% with 9 bivalents pair. In a similar manner 58.5% diakinesis-stage meiocyte cell of *Slzip4 Slrecq4* were normal with complete 12 bivalents pair while 28.9% has 11 bivalents pair, 11.1% has 10 bivalents pairs and 1.5% has 9 bivalents pair. As seen in *Slrecq4* single mutant, similar behaviour of chromosome morphology during diakinesis was observed in *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutant with bivalent pairs are more packed and dense and also chromosome fragmentation tail was observed suggesting that there is a delay in DSBs repair. Formation of univalent was also observed in metaphase I confirming the disruption of obligate crossover. Chromosome bridges during early anaphase I in both double mutants indicates that there is a problem in chromosome segregation.

There is also presence of - unequal segregation during late anaphase I resulting from the univalent formation as observed during diakinesis and metaphase I. Chromosome fragmentation and delayed segregation was also observed in double mutants of *Slmlh1* or *Slzip4* with *Slrecq4* during metaphase II and anaphase II stages. Triad, tetrad with micronuclei or polyad formation confirming the behaviour of class I CO mutation introgression with *RECQ4* mutation resembling the behaviour of *Slrecq4* single mutant with suppression of class I CO infertility.

3.4 Discussion

3.4.1 SlRECQ4 is important for the fertility of tomato

Mutation of *RECQ4* caused a significant reduction in fertility for inbred tomato based on the percentage of viable pollen, fruit weight, fruit number and seed number. This evidence was not previously described in *recq4* mutant in both *S. lycopersicum* and interspecific hybrid between *S. lycopersicum* and *S. pimpinellifolium* (de Maagd et al., 2020a; Mieulet et al., 2018). Based on F_2 generation hybrid tomato between Micro-Tom and M82, there is no differences in fertility rate between wildtype and *recq4* mutation although there is a slight increase in seed number (Mieulet et al., 2018). However, previous paper on *recq4* mutation in tomato do not provide any information on the percentage of pollen viability, fruit number and weight. In these findings, we provide an extra evidence on how the percentage of viable pollen is significantly reduced in BC_1F_2 generation of *Slrecq4* stable line mutant and this result is in line with number of seeds being produced and also the abnormal chromosome behaviour of *Slrecq4* mutant.

Previously, RECQ4 mutation in inbred Arabidopsis do not give any effect on fertility as *Atrecq4* mutant produced normal seeds per fruit which is similar to the wildtype Arabidopsis (Séguéla-Arnaud et al., 2015). No fertility issue was observed in *Atrecq4* probably resulted from species-specific and genomic features as the size of Arabidopsis is around 135 Mb with only 5 pairs of chromosomes. Meanwhile, plant species with larger genome such as barley, rice and pea having more complex genome structure and more chromosome pairs and therefore, mutation of RECQ4 caused instability in the DSBs repair. This can be observed as reduction in fertility for RECQ4 defect in rice, pea and barley were previously reported (Arrieta et al., 2021; Mieulet et al., 2018). In this case, tomato with 12 pairs of chromosome and larger genome size (900 Mbp) having more complex genome features with the mutation of RECQ4 function might cause disturbance in DSBs repair. Thus, this led to many intermediate fragments were leave unrepaired and resulted in aberrant chromosome and subsequently produced unviable gamete.

Based on reduced fertility in *Slrecq4* mutant, it is suggested that organisms with larger and more complex genome required NCO mechanisms for the proper DSB repair. Probably, larger genome may have more DSB and therefore cells need to be more efficient to repair a higher number of DSB. This was reported for *SlTOP3a*, a component of RTR complex where mutation lead to embryo-lethal in tomato with larger genome but still can have higher fertility rate in *A. thaliana* (Séguéla-Arnaud et al., 2017; Whitbread et al., 2021). This is also observed in another NCO mutant, *fancm* and reduced fertility was observed in plant with larger genome size such as pea (4.3 Gbp), lettuce (2.5 Gbp) and both tetraploid (10 Gbp) and hexaploidy (16 Gbp) wheat (Desjardins et al., 2022; X. Li et al., 2021; Mieulet et al., 2018).

3.4.2 DNA fragmentation and univalent formation affect fertility of *Slrecq4* mutant

As RECQ4 is required for the dissolution of intermediates for HR by unwinding the D-loops formation, therefore, mutation of *RECQ4* might cause off pathways resulted in increased number of joint molecule (Séguéla-Arnaud et al., 2015, 2017). In case of tomato, probably more number of DSB due to the bigger size of the tomato genome when compared to Arabidopsis and mutation of *SlRECQ4* will lead to higher joint molecule of HR. HR is a more complex process than NHEJ as it requires homolog template, extensive DNA processing and strand invasion making it slower and less efficient (Shrivastav et al., 2008). Therefore, loss of RECQ4 function in tomato may cause inefficient in DSB repair as the majority of DSB are repaired by NCO pathways. DNA fragmentation observed during anaphase I, diakinesis, metaphase II and anaphase II phases in *Slrecq4* mutant supporting this evidence showing that *SlRECQ4* gene responsible for the repairing of DSBs in tomato and play an important role in maintaining genome stability.

As reported in human, *RECQ4* gene was identified as essential step for homologous recombination (HR)-dependent DNA double-strand break repair (DSBR) and loss of the function of this gene resulted in genome instability (Lu et al. 2016). It is suggested in tomato, loss of RECQ4 function may cause some joint molecules remain unrepaired as likely they are too complex to resolve thereby resulting in chromosome fragmentation and genome instability. Genome instability was reported for the mutation of *BLM* gene, a homolog for *RECQ4* leading to chromosome instability, excessive homologous recombination, and a greatly increased number of sister chromatid exchanges that are pathognomonic of the syndrome (Cunniff et al., 2017). During diakinesis stage, it is also observed the chromosome pairs in *Slrecq4* mutant are more dense and compact when compared to the wildtype. This probably caused by an increased number of COs in *Slrecq4* and lead to the chromosome

pair to be interlocked with others at more positions along the chromosome. Based on *Slrecq4* mutant behaviour, it is also suggested that SlRECQ4 together with TOP3α are the main component of RTR protein complex to perform DSB repair as previously reported on RMI1 function which is not required for DNA repair or meiosis in tomato (Whitbread et al., 2021).

Another explanation is that this could also be caused by the distribution of the CO in *Slrecq4* mutant as the CO may also happen closer to pericentromeric region rather than close to the telomere region. As CO comes from class II CO pathway, there will be a greater chance of CO occurring close to each other as class II CO is interference insensitive. X. Li et al., (2021) reported that knockout of *FANCM* a NCO gene, is important for shaping the distribution of meiotic class I COs in plants based on the distribution of HEI10 signal in lettuce and Arabidopsis *fancm* mutant which is marked differently from wildtype plant. Meanwhile in barley, increase of 2-fold CO in *Hvrecql4* mutant through class II CO pathway do not give any changes in the distribution pattern of CO (Arrieta et al., 2021). It is also observed in wheat *fancm* mutant where CO are significantly increase while its genomic distribution is comparable to the wild type suggesting that chromatin may influence the recombination landscape in similar ways in both wild type and *fancm* (Desjardins et al., 2022).

Another evidence that lead to reduce fertility in *Slrecq4* mutant is the formation of univalent that might cause unequal segregation and further produce imbalance formation of gamete cell which lead to aberrant and reduced pollen being produced. Univalent formation during plant meiosis causes unequal segregation during anaphase I leading to formation of aneuploidy (De Storme & Geelen, 2020). Univalent formation was observed in *Slrecq4* mutant meiocyte indicating that RECQ4 in tomato play an important role in pro-crossover pathways although formation of univalent in *Slrecq4* is milder and not as severe as observed in tomato class I CO mutant. As applied

in most organism, obligate crossover should occur in every chromosome pair with at least one crossover per homolog pair in order to ensure normal segregation (Lloyd, 2022; Mercier et al., 2015; Wang & Copenhaver, 2018). Around 43.5% of diakinesis cell of *Slrecq4* contained at least 1 univalent pair and this suggests that RECQ4 in tomato is involved in influencing the formation of intermediate for pro-crossover. This also shows that *SlRECQ4* activity may be placed in the upstream position of tomato recombination pathway as *SlRECQ4* may regulate earlier mechanism for initiating CO and NCO fate. Similarly reported, significant number of univalent formation were previously observed for other NCO mutants, *fancm* both in lettuce and Brasicca crops indicating that possibility of sharing pathway in upstream position between CO and NCO mechanisms (Aurélien Blary et al., 2018; X. Li et al., 2021). Apart from its anti-CO function, the BTR complex possibly promotes maturation of a subset of Class I CO, as a low frequency of univalents at metaphase I were observed in certain *rmi1* and *top3a* mutants (Séguéla-Arnaud et al., 2015, 2017). Chromosome size may influence the fate of univalent and non-segregation error and in human it was reported that larger chromosomes from female meiocytes are more prone for the formation of univalent (Klaasen & Kops, 2022).

However, association of DNA fragmentation and univalent formation with reduced fertility in *recq4* mutation was not described in the previous work on *recq4* mutant of tomato and interspecific hybrid tomato. There is no work on the chromosome behaviour of *Slrecq4* mutant for the Micro-Tom and F_2 tomato hybrid as the fertility rate was similarly observed when compared to wildtype plant even though there is significant increase in CO rate (Mieulet et al., 2018). However, work conducted in interspecific hybrid acknowledged that higher percentage of chromosome bridges and DNA fragmentation during anaphase I and II which is similarly observed in our *Slrecq4* mutant suggesting that *recq4* mutation affects both sister-chromatid repair and homolog repair pathways (de Maagd et al., 2020). Moreover, work on *recq4* interspecific

hybrid do not described any percentage of malformed pollen production or production of seed per fruit in their work although they described on formation of abnormal chromosome behaviour in their interspecific hybrid tomato mutant. As we described univalent formation as a culprit for unequal segregation that also influences fertility rate in *Slrecq4*, however, previous work on tomato and interspecific hybrid tomato do not provide any evidence of the presence of univalent in their mutants (de Maagd et al., 2020; Mieulet et al., 2018).

3.4.3 *SlRECQ4* mutation induced class II CO formation

Despite having reduction in fertility, mutation of *RECQ4* gene in tomato still capable to partially restore-infertility in tomato class I mutant. As being reported in many plant species, class I CO mutant leads to many univalent chromosomes indicating that obligate crossover was disturbed resulting in unequal segregation (Chelysheva et al., 2007; Desjardins et al., 2020; Luo et al., 2013; Mao et al., 2021; Xin et al., 2021). As being described in Chapter 2, *Slmlh1* and *Slzip4* mutants have lost obligate crossover with at least 1 univalent formation in every meiocytes cell and this leads to the reduction in fertility or sterility. However, combination of either *Slmlh1* or *Slzip4* with *Slrecq4* improved fertility significantly and formation of bivalent was increased when observed in meiocytes. This indicates that in the absence of obligate crossover formation from class I CO pathway, introduction of *RECQ4* mutation in both *MLH1* and *ZIP4* mutation background increases CO suggesting that recombination event comes from class II CO pathway. RECQ4 is responsible for the unwinding of D-loop formation and therefore loss of function of RECQ4 resulted in off-pathway that increase the joint molecule between non-sister chromatid (Croteau et al., 2012). Some of this joint molecule will be further cleaved by MUS81 nuclease through class II CO pathway for the chromosome pair to be segregated equally (Geuting et al., 2009).

Manipulation of NCO associated genes to accelerate more CO is an approach which is expected to improve tomato breeding by introgressing beneficial trait from the crosses with wild relative tomato. Introgression of valuable traits from wild tomato relatives can perhaps improve robustness of domesticated tomato toward biotic and abiotic stresses (Bai & Lindhout, 2007; W. Li et al., 2023). Many valuable trait from wild relative especially within the self-incompatible species like *S. chilense* and *S. peruvianum* can be introduced in tomato breeding (Vidavski et al., 2008). Based on abnormal phenotype behaviour of *Slrecq4* mutant, unfortunately it seems that mutation of RECQ4 in tomato is not suitable to be used in tomato breeding. Although mutation of *SlRECQ4* gene can increase CO frequency in tomato, however reduction in fertility resulted from DNA fragmentation and also univalent formation limit the use of this trait for future tomato breeding.

3.5 Conclusion

Based on observations of null mutations in *Slrecq4*, it is suggested that NCO associated gene- behaviour depends on the nature of species-specific genomic properties of plant species. Unlike those described in Arabidopsis, tomato with larger genome and more chromosome pair exhibited a significant loss in fertility and produced large percentage abnormal chromosome defect which indicated that *SlRECQ4* gene contributed to large proportion of DNA repair activity in tomato. It is also noteworthy that *SlRECQ4* gene contributed to the large percentage of univalent formation in tomato which is something interesting to look more in depth as probably *SlRECQ4* may also indirectly involve in regulating pro-crossover pathways.

3.6 Materials and methods

3.6.1 Plant materials and plant growth

Solanum lycopersicum cv. Micro-Tom was used in this study and the seed is obtained from Tomato Growers Supply Company (Florida, USA). The seeds were soaked in the clean water and later was treated with saturated Na_3PO_4 and was rinsed with water a few times. Later, treated seeds were placed on wet tissue paper in petri dish to assist germination and germinated seed was transferred to germination pots containing peat moss. Tomato seedlings were transferred to potting soil after 3 weeks in growth chamber with 25°C, 16 hours light and around 60-70% of humidity. The tomato plants were fertilized weekly using 0.4%(v/v) Wuxal® Super (Precision Laboratories, Germany) and watering every 2 days interval.

3.6.2 Construction of CRISPR/Cas9 construct for the development of *Slrecq4* mutant

A single CRISPR/Cas9 transformation construct targeting both *SlZIP4 and SlRECQ4* was produced. Validated sgRNAs for *SlRECQ4* gene (de Maagd et al., 2020a) were utilized and sgRNAs targeting the *SlZIP4* gene was designed using 2 different online programs (CHOPCHOP (https://chopchop.cbu.uib.no/) and CRISPR-P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR)). sgRNAs against *SlZIP4* were selected by comparing sgRNA ranking by both programs and was further validated using Cas-Offinder (http://www.rgenome.net/cas-offinder/) in order to determine the potential off-target sites of Cas9 RNA-guided endonucleases. Primers assembly for selected sgRNAs to knock out the both *SlRECQ4* and *SlZIP4* genes were further designed based on the Golden Gate Assembly protocol as being described by Čermák et al.,(2017). The sgRNAs primers **(Table 3.0)** was then synthesized, amplified by PCR, ligated by ligase and cloned into pDIRECT22C vector

(containing 35S:Cas9) and was transformed into TOP10 *E. coli*. Transformed *E. coli* colonies were screened by using colony PCR amplification and sequencing analysis. Plasmid with the correct validated *SlZIP4* and *SlRECQ4* sgRNAs cloned into pDIRECT22c later are known as pWZ3 **(Appendix 1.2)**. After positive sequencing results from the pWZ3 plasmid, it was finally transformed into *A. tumefaciens* GV3101.

3.6.3 Transformation and transgenic line regeneration

One colony of *Agrobacterium* containing pWZ3 was inoculated in 150 mL of YEP medium containing 50 mg/L Kanamycin, 30 mg/L Gentamicin and 10 mg/L Rifampicin and was incubated at 28°C with 220 rpm shaking for 48 hours. The culture was used as an inoculum for further transformation experiment where 0.5-1 mL of culture was inoculated into new 150 mL of YEP medium and was incubated for overnight. On the same day, 3-4 weeks old of Micro-Tom seedlings were previously grown in the ½ MS was used as an explant where the leaves were cut in 6 x 6 mm size on sterile glass petri dish. The leaf explants were cut one by one and the bottom side were placed on MS media supplemented with 2 mg/L Zeatin Riboside (Duchefa Biochemie, Netherland), 0.1 mg/L Indole-3-acetic acid (IAA) (Merck, Germany) and 73 mg/L of Acetosyringone (Merck, Germany) in the sterile petri dish. The explants were placed at 25°C in the dark room or growth chamber for at least overnight and the next day were co-cultivated with *Agrobacterium*. 5 ml of overnight *Agrobacterium* culture containing pWZ3 with OD_{600} around 800 was diluted with 100 mL of LB broth to 1:20 ratio and the incubated leaf explants were infected with diluted culture for 15 minutes with mild shaking. The leaf explants were then placed on sterile dry Whatmann filter paper to remove excessive liquid and further placed in the same MS media plate for 48 hours in the dark. After that, the co-cultivated explants were transferred to new MS media containing 1.5 mg/L Zeatin Riboside, 100 mg/L Kanamycin and 500

mg/L Carbenicilin and were incubated for 1 weeks in the 25°C with 16 hours day light. The explants were then sub-cultured into new MS media and placed in the same condition and were sub-cultured every 2 weeks. After several sub-cultures, any green and resistant embryoid or shoot emerged from the explant was further transferred to the MS supplemented with 1.0 mg/L Zeatin Riboside, 100 mg/L Kanamycin and 250 mg/L Carbenicilin. Developed shoot from resistant embryoid with at least 20 mm in length was further transferred to rooting media, ½ MS supplemented with 1 mg/L IAA and cultured for at least 3 weeks until enough number of roots formed before being transferred into soil **(Appendix 2.0 & 3.0)**.

3.6.4 Tomato meiotic mutant genotyping

Genomic DNA (gDNA) from tomato meiotic mutants and wild type tomato plants were isolated using BioSprint 96 DNA Plant Kit (Qiagen, Germany) according to recommended protocol described by manufacture. Isolated gDNA was further used as a template for the amplification of DNA fragment for various genes/transgenes using primers as described in the **Table 3.1**. Amplification of DNA fragment by PCR was carried out using Mango*Taq*™ DNA Polymerase (Meridian Bioscience, United Kingdoms). The cycle of the PCR used are: Initial denaturation; 5 minutes, denaturation; 30 seconds, annealing: 30 seconds, extension; 30 seconds and final extension; 3 minutes. Amplified PCR product was observed using electrophoresis 1% (w/v) agarose gel for *ACT*, *NPT* and *AtCAS9* and 3% (w/v) agarose gel for *SlRECQ4* and *SlMLH1* genes. High Resolution Melting (HRM) using qPCR was used to genotype homozygous, heterozygous and wild-type *SlZIP4* gene for *Slzip4* mutant. HRM analysis also using the same type of polymerase that was used for PCR amplification with addition of 0.25 µL of LC Green® Plus (BioFire Defence, LLC, USA) for every 12.5 µL reaction. The thermal cycles for HRM-qPCR are: Initial denaturation; 3 minutes, denaturation; 30 seconds, extension; 30 seconds. After complete

40 cycles, HRM was performed by temperature ramping from 65°C to 95°C with increment of 0.2°C for every 5 seconds. HRM-qPCR analysis was performed using CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA). The melting curve plot for HRM to differentiate homozygous, heterozygous and wild-type for *SlZIP4* gene was performed using Bio-Rad Precision Melt Analysis Software (Bio-Rad, USA). Same primers were also used for the identification of *SlRECQ4*, *SlZIP4* and *SlMLH1* genes sequence in those mutants and also wild-type using Sanger sequencing **(Appendix 5.0 & 7.0)**.

3.6.5 Identification of ploidy level of plant regenerated in vitro

The ploidy level of tomato plant regenerated from *in vitro* culture was analysed using CytoFLEX flow cytometer (Beckman Coulter, USA). Tomato leaf with approximately 2 cm x 2 cm in diameter was finely chopped in 0.5 mL Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0). The mixture was then filtered using green filter (30 µM) and 20 µL of 100 µg/mL DAPI was then added. The mixture was incubated on ice for 15 minutes and further analysed using flow cytometer. Wild-type Micro-Tom leaf from seed-derived plant was used as a diploid control.

3.6.6 Phenotyping experiment

All mutants used in this experiment were derived from T_0 regenerated plant that was backcrossed with wildtype Micro-Tom. Cross was made between heterozygous *Slmlh1* plant and heterozygous *Slrecq4* plant in order to produce double mutant *Slmlh1 Slrecq4*. Backcrossed with wildtype Micro-Tom is to reduce any possibilities of off-target from CRISPR-Cas9 mutagenesis and to remove unwanted *AtCAS9* and *NPT* transgenes. The BC_1F_2 population without those transgenes was selected for the phenotypic experiment and the progeny will be segregated into wildtype, heterozygous and homozygous deletion. Protocol to grow and the condition of the phenotypic experiment as described

in the section (a). The growth parameters such as vegetative, inflorescence, fruit data was observed from days 22 to days 90 after seed germination and comparison was made between homozygous, heterozygous and wild-type genotypes. Phenotypic characteristics that associated with meiosis and reproduction systems was main focus such as the flowering times, number of inflorescence and flower, pollen viability, fruit set and size and also seed number.

3.6.7 Pollen viability staining

The percentage of pollen viability was carried using Alexander Staining protocol (Alexander, 1969). The secretion of pollen from full bloom flower was assisted using pollination vibrator tool and secreted pollen was collected in the glass staining cavity block. After that, 20 µL of Alexander staining solution was added, mixed evenly with the pollen, covered with glass lid and leaved for at least one minute in the fume hood. Then, 10 µL of the stained pollens were placed on the glass slide and further covered with glass lid. The stained pollen slide can be observed immediately or leaved overnight. The image of the stained pollen was captured using Axioplan 2 Imaging Microscope (Zeiss, Germany) and was further processed using Labscope software and Adobe Photoshop.

3.6.8 Cytological analysis

Classic chromosome spread protocol for observation of chromosome behaviour in tomato was carried out using SteamDrop method with modification (Kirov et al., 2014). *S. lycopersicum* cv. Micro-Tom flower bud with size approximately around 2-2.5 mm was collected fresh and immediately soaked into fresh fixative solution, Ethanol: Acetic acid (3:1). The fixative solution was refreshed 3 times in 1 hour and ideally the fixed flower bud was leaved in the fixation solution at least for 1 day before being used in cytology experiment.

The fixed flower buds can be stored in 4°C or -20°C up to 6 months. 6-8 flower buds were taken out from the fixation solution and were soaked for 10 minutes in the sterile water. The flower buds were the rinsed with the sterile water twice and further soaked in 1 mL of 10 mM citrate buffer, pH 4.5. The flower buds were dissected on glass slide with few drops of citric buffer to keep it moist. The outer layer of immature petal, calyx, stigma and ovary will be removed. The anthers were separated individually and were transfer into 1.5 mL microcentrifuge tube containing 200 µL 10 mM citrate buffer. Citrate buffer was gently discarded using micropipette and the same volume of enzyme mixture containing 0.3% (w/v) cellulase, 0.3% (w/v) pectolyase Y23, 0.3% (w/v) driselase in citrate buffer were added. The digestion of flower buds by the enzyme mixture was carried out for 3 hours in 37°C incubator. After 3 hours, the digestion mixture tube was plugged on ice and 600 µL of TE buffer, pH 8 was added. Then, the solution mixture was gently push up and down using micropipette to separate the digested cell and create the suspension cell of meiocytes. After that, the mixture was centrifuged at 10,000 rpm, 4°C for 45 second and supernatant was gently discarded using micropipette. The pellet was resuspended in 96% (v/v) ethanol and was mix thoroughly using micropipette. Again, the resuspended cells were centrifuged at 10,000 rpm, 4°C for 45 second and the pellet was finally resuspended in 400-500 µL of in 96% (v/v) ethanol and was mix thoroughly using micropipette. Obtained suspension cell can directly be used for the preparation of chromosome spread slide or it can be stored in -20°C up to 6 months. In order to prepare chromosome spread, 10 µL of well mixed suspension cell were dropped onto the slide and were leaved until the surface become granule-like as the ethanol meniscus occurred on the top of the cell within 10-15 seconds. Then, 20 µL of fixative solution (Ethanol:acetid acid=3:1) were added onto the slide and leaved until the surface become granule-like and the layer of fixation become thin within 25-35 seconds. The slide was then turn upside down toward steam for approximately 8 seconds at 10-15 cm distance from water surface of water

bath with 55°C temperature. Immediately after that, the slide was air dried toward the airflow of a table fan for 15-20 seconds. Finally, 10 µL of DAPI (2 µg/mL) were added on the meiocytes slide after it has dried completely and the meiocytes slide was covered with glass cover. The meiocytes images from the slide were acquired and processed using a ZEISS microscope (AXIO-Imager.Z2) under 100× of oil immersion objective and further analysed using ZEN software. Figures prepared from images were prepared using Adobe Photoshop. The meiocytes slide prepared as above mentioned can also be stored in 4°C or -20°C until further used.

3.6.9 Statistical analysis

Data visualisation and statistical calculations were conducted using GraphPad Prism 9.0.0 software. P value was calculated using Kruskal-Wallis one-way ANOVA, a non-parametric method for multiple comparisons. Meanwhile, T-Test was used to compare the means of two groups.

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3.8 Tables

Table 3.0: SgRNAs and primers used in Golden Gate Assembly cloning sgRNAs for editing *SlZIP4* and *SlRECQ4* genes in tomato, colony PCR screening and DNA sequencing validation.

Promoter Primer	Primer Sequence
CmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC

SgRNA	SgRNA Sequence	Primer	Primer Sequence
***SlZIP4*-1**	TCTATCAGAGACTTCCCAAGCGG	**ZIP4_1R_gRNA1**	TGGTCTCCGTCTCTGATAGACTGCCTATACGGCAGTGAACCTG
		ZIP4_2F_gRNA1	TGGTCTCAAGACTTCCCAAGGTTTTAGAGCTAGAAATAGC
***SlRECQ4*-1**	ATAAGCTTCCAAAAGCCAACTGG	**RECQ4_2R_gRNA1**	TGGTCTCCTTGGAAGCTTATCTGCCTATACGGCAGTGAAC
		RECQ4_3F_gRNA1	TGGTCTCACCAAAAGCCAACGTTTTAGAGCTAGAAATAGC
***SlZIP4*-2**	GTTAGTCTAAGAAGCAGCCACGG	**ZIP4_3R_gRNA2**	TGGTCTCCTCTTAGACTAACCTGCCTATACGGCAGTGAAC
		ZIP4_4F_gRNA2	TGGTCTCAAAGAAGCAGCCAGTTTTAGAGCTAGAAATAGC
***SlRECQ4*-2**	CCAAGAGTTACACAAGACCAGGG	**RECQ4_4R_gRNA2**	TGGTCTCCTGTAACTCTTGGCTGCCTATACGGCAGTGAAC
		RECQ4_5F_gRNA2	TGGTCTCATACACAAGACCAGTTTTAGAGCTAGAAATAGC
***SlZIP4*-3**	GGTTTTATAGAAGAAGGATGCGG	**ZIP4_5R_gRNA3**	TGGTCTCCTTCTATAAAACCCTGCCTATACGGCAGTGAAC
		ZIP4_6F_gRNA3	TGGTCTCAAGAAGAAGGATGGTTTTAGAGCTAGAAATAGC

Terminator Primer	Primer Sequence
CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Colony PCR and Sequencing Primers	Primer Sequence
TC320	CTAGAAGTAGTCAAGGCGGC
M13F	GTAAAACGACGGCCAGT

Table 3.1: Primers used for PCR amplification of *SlACT* housekeeping gene, *SlMLH1*, *SlZIP4*, *SlRECQ4*, *NPT* and *AtCAS9* transgenes and HRM analysis for *SlMLH1* and *SlZIP4* genes

Genes	Primers Name	Primers Sequence	Amplicon Size (bp)
***S. lycopersicum Actin* (*SlACT*)**	ACTF ACTR	AGCAGGAACTTGAAACCGCT ACAAAAGCTCACCTGCTGGA	404
***Neomycin phospotransferase* (*NPT*)**	NPT-35sF NPT-35sR	TTCAGTGACAACGTCGAGCA GACGTAAGGGATGACGCACA	413
***A. thaliana CRISPR associated protein 9* (*AtCAS9*)**	AtCAS9-F AtCAS9-R	ATGCCACAGGTGAACATCGT GAGAGCAAGCTCGTTTCCCT	420
***S. lycopersicum MutL homolog 1* (*SlMLH1*)**	MLH1-pWZ1-F MLH1-pWZ1-R HRM1-MLH1-F1 HRM1-MLH1-R1	AAGCCATTCCAGTGCCGATT GACAGTGACGTGACCCACAT GAAGCCATTCCAGTGCCGAT ATGAGCTCTTTCACGGCAGA	462 125
S. lycopersicum (SlZIP4/SlSPO22)	ZIP4-HRMSg1-F2 ZIP4-HRMSg1-R2	GCTTCGAGAAAGCTTCCGATCT TGTCCGCAAAAGCGAAGAGCA	186
S. lycopersicum ATP-dependent DNA helicase Q4 (SlRECQ4)	dWRe0135 dWRe0150	CTCCAACCATTTCCTTCTTGAC TAATCACACACAGTTCCCGC	364

(Appendix 5.0 & 7.0)

3.9 Figures

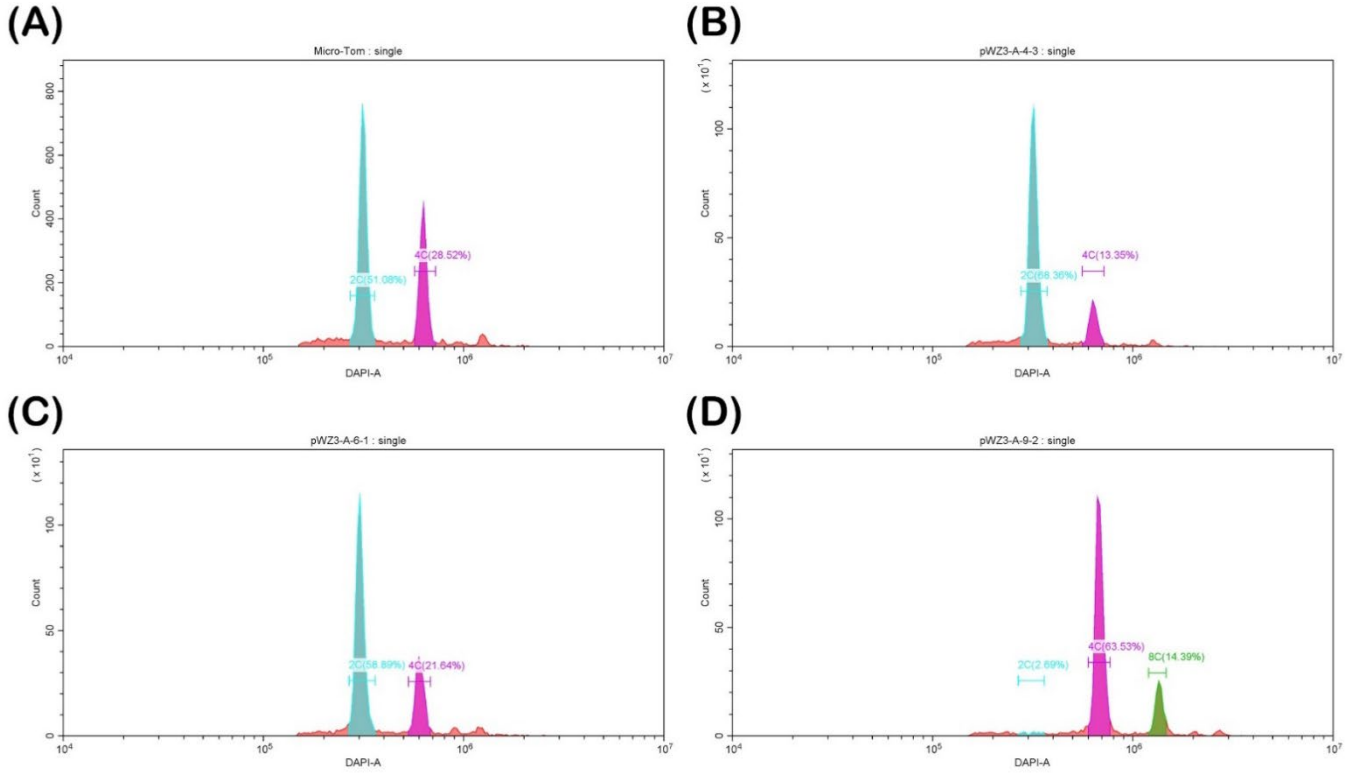


Figure 3.0: Flow cytometric histograms of diploid and tetraploid tomato (*S. lycopersicum* cv Micro-Tom). **(A):** Diploid Micro-Tom plant germinated from seed. **(B)** & **(C):** Diploid T_0 *Slrecq4* mutants regenerated from *in vitro* culture. **D:** Tetraploid T_0 *Slrecq4* mutant regenerated from *in vitro* culture

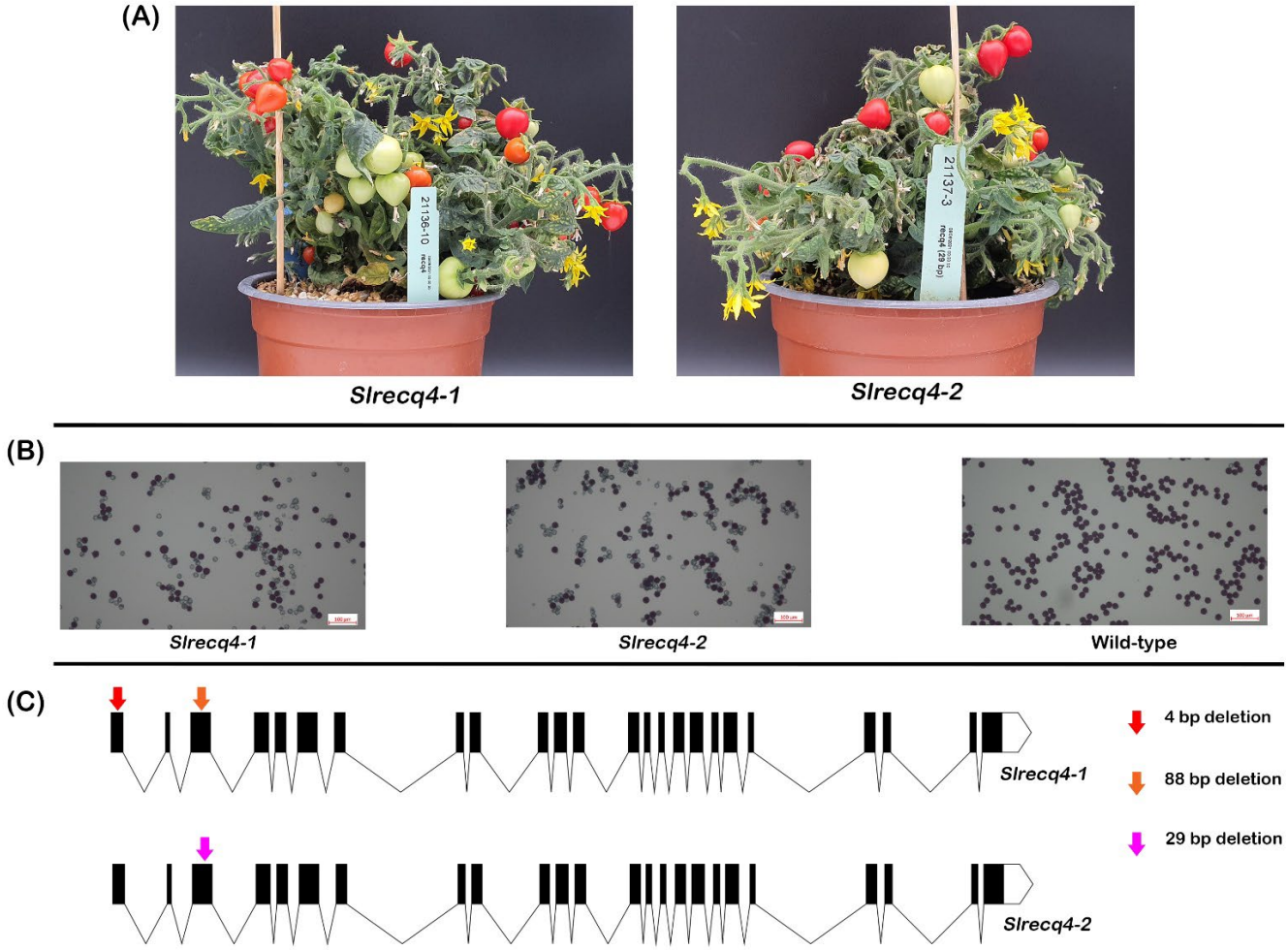


Figure 3.1: Stable lines of *Slrecq4* mutant from CRISPR/cas9 mutagenesis in Micro-Tom background. **(A):** Two different lines of *Slrecq4* mutants, *Slrecq4-1* and *Slrecq4-2*. **(B):** Pollen viability test by Alexander staining on *Slrecq4-1*, *Slrecq4-2* and wildtype Micro-Tom. **(C):** Location and size of the DNA fragment deletion on *SlRECQ4* gene for each *Slrecq4-1* and *Slrecq4-2*.

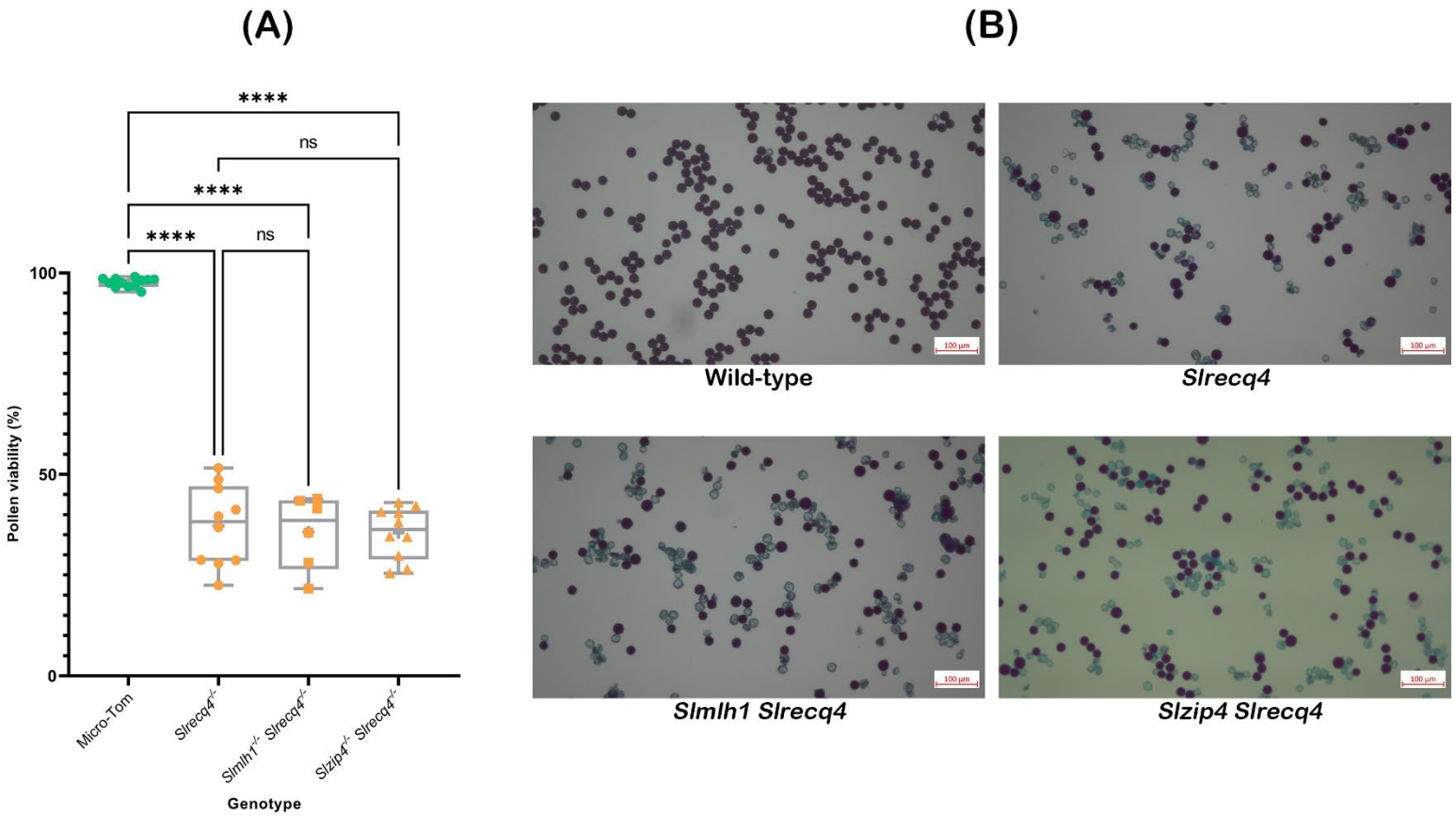


Figure 3.2: Behaviour of viable pollen for wildtype Micro-Tom, *Slrecq4*, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* mutants. **(A):** Box and whisker plot graph of viable pollen for Micro-Tom, *Slrecq4*, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* mutants. **(B):** Stained pollen image of wildtype Micro-Tom, *Slrecq4*, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* mutants at 10x magnification with dark purple indicated viable pollen and light green indicated unviable pollen.

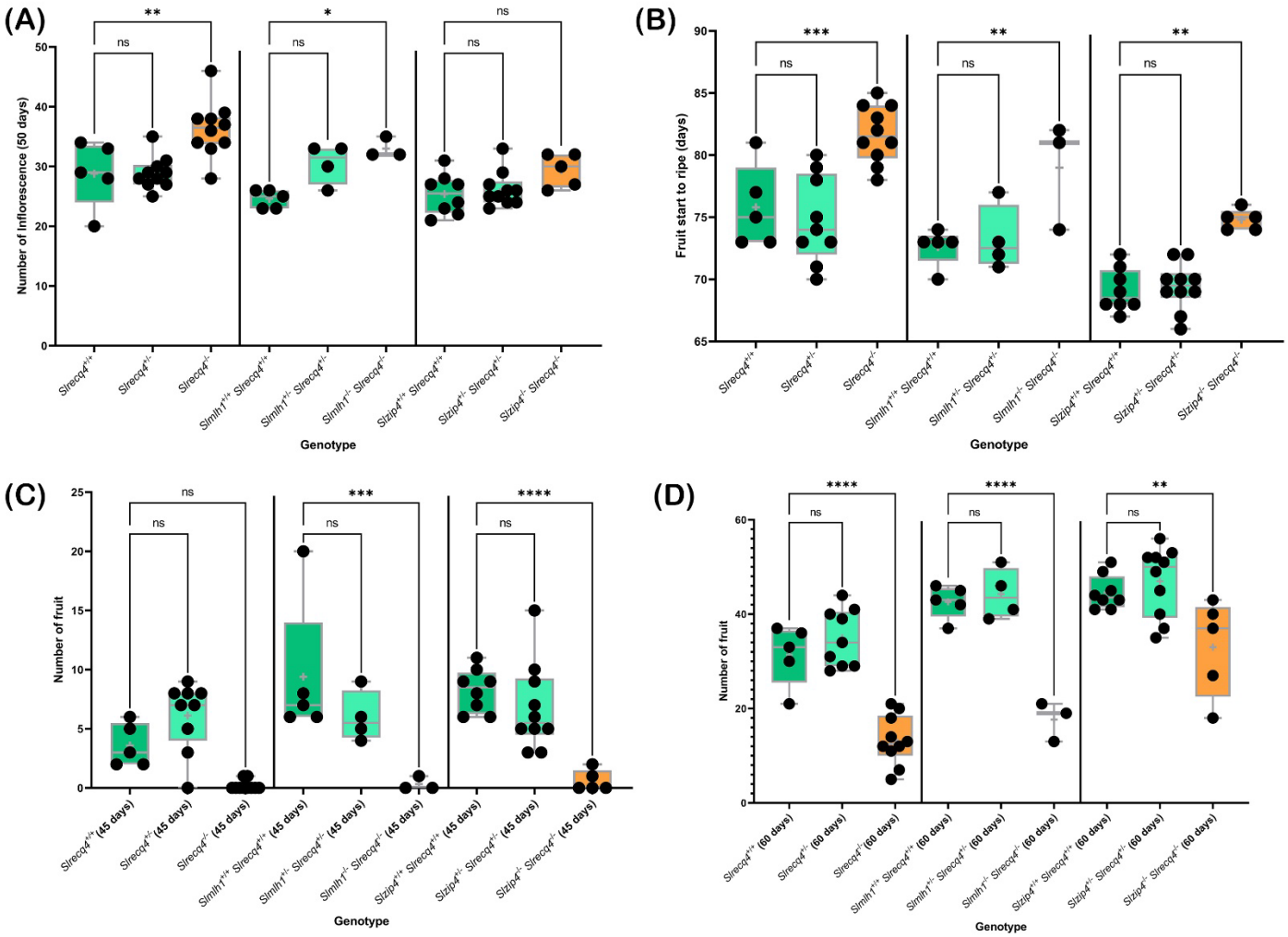


Figure 3.3: Box and whisker plot graph for the number of inflorescences, day for fruit to start ripe and the number of fruits at 45 and 60 days for wildtype, heterozygous and homozygous sister plant of *Slrecq4* single mutant, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutant. **(A):** Number of inflorescences at day 50 **(B):** Day for the plant to produce ripe fruit **(C):** The number of fruits setting at day 45 and **(D):** The number of fruits setting at day 60.

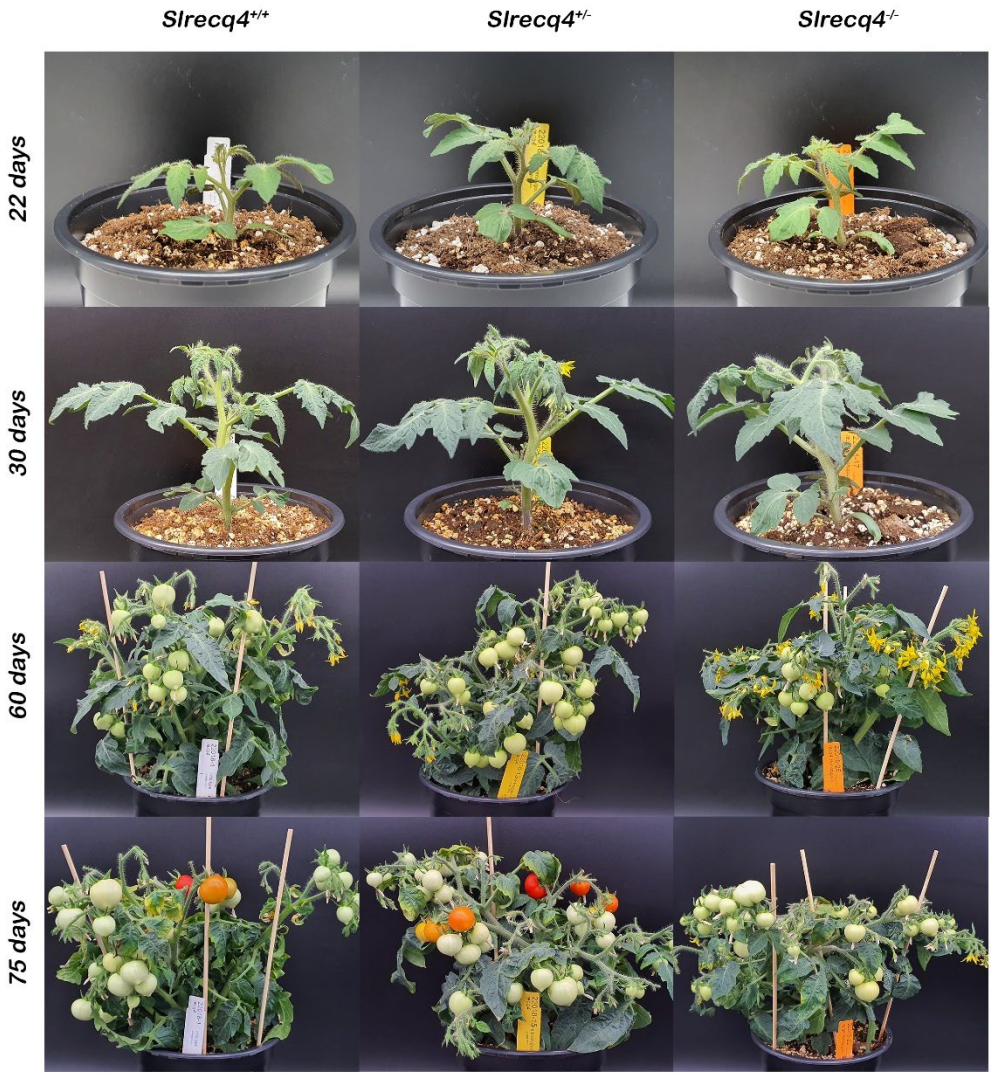


Figure 3.4: Phenotypic evaluation and comparison of wildtype, heterozygous and homozygous sister plants for *Slrecq4* single mutant at 22 days, 30 days, 60 days and 75 days after germination

Figure 3.5: Phenotypic evaluation and comparison of wildtype, heterozygous and homozygous sister plants for *Slmlh1 Slrecq4* double mutant at 22 days, 30 days, 60 days and 75 days after germination.

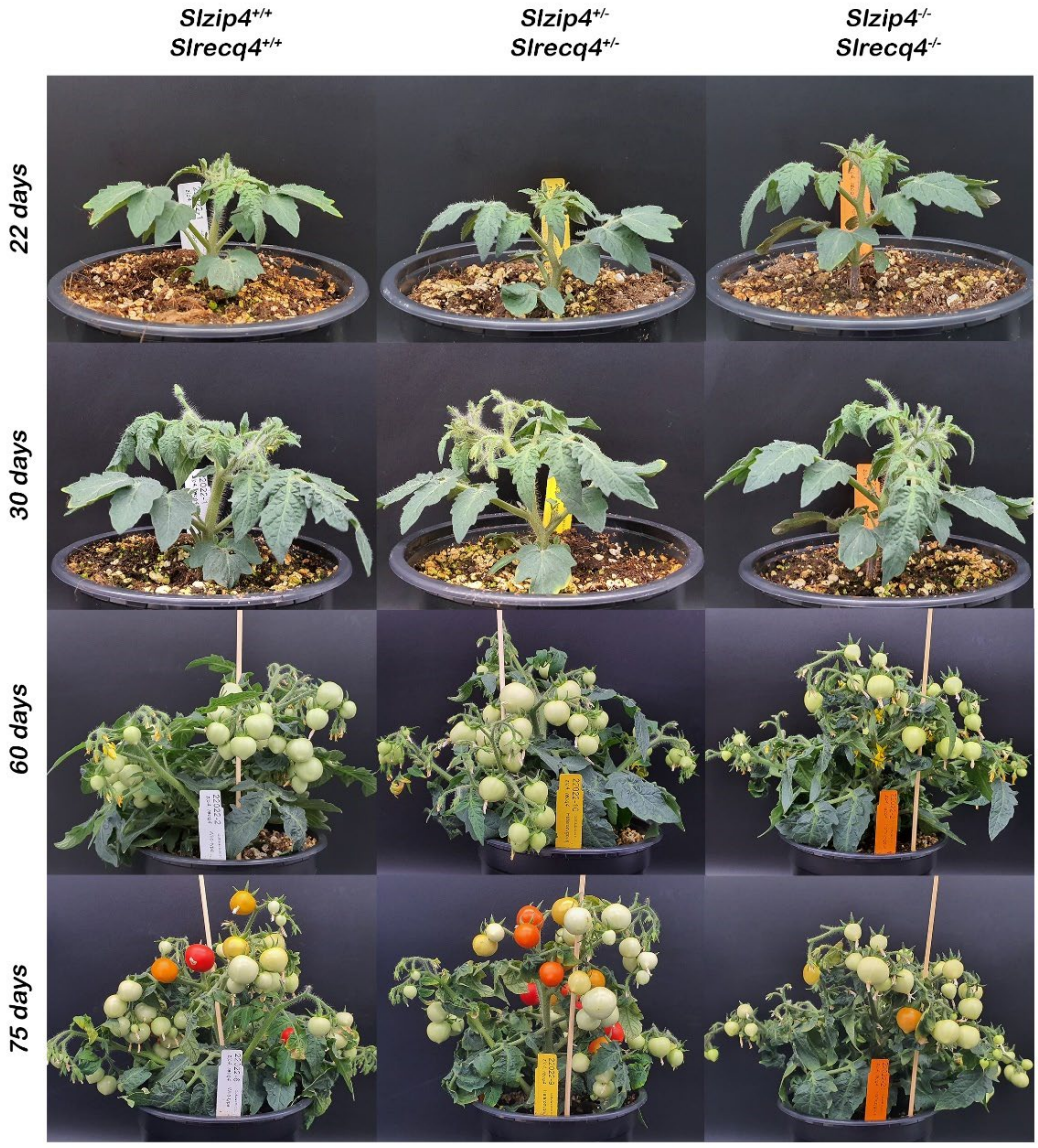


Figure 3.6: Phenotypic evaluation and comparison of wildtype, heterozygous and homozygous sister plants for *Slzip4 Slrecq4* double mutant at 22 days, 30 days, 60 days and 75 days after germination.

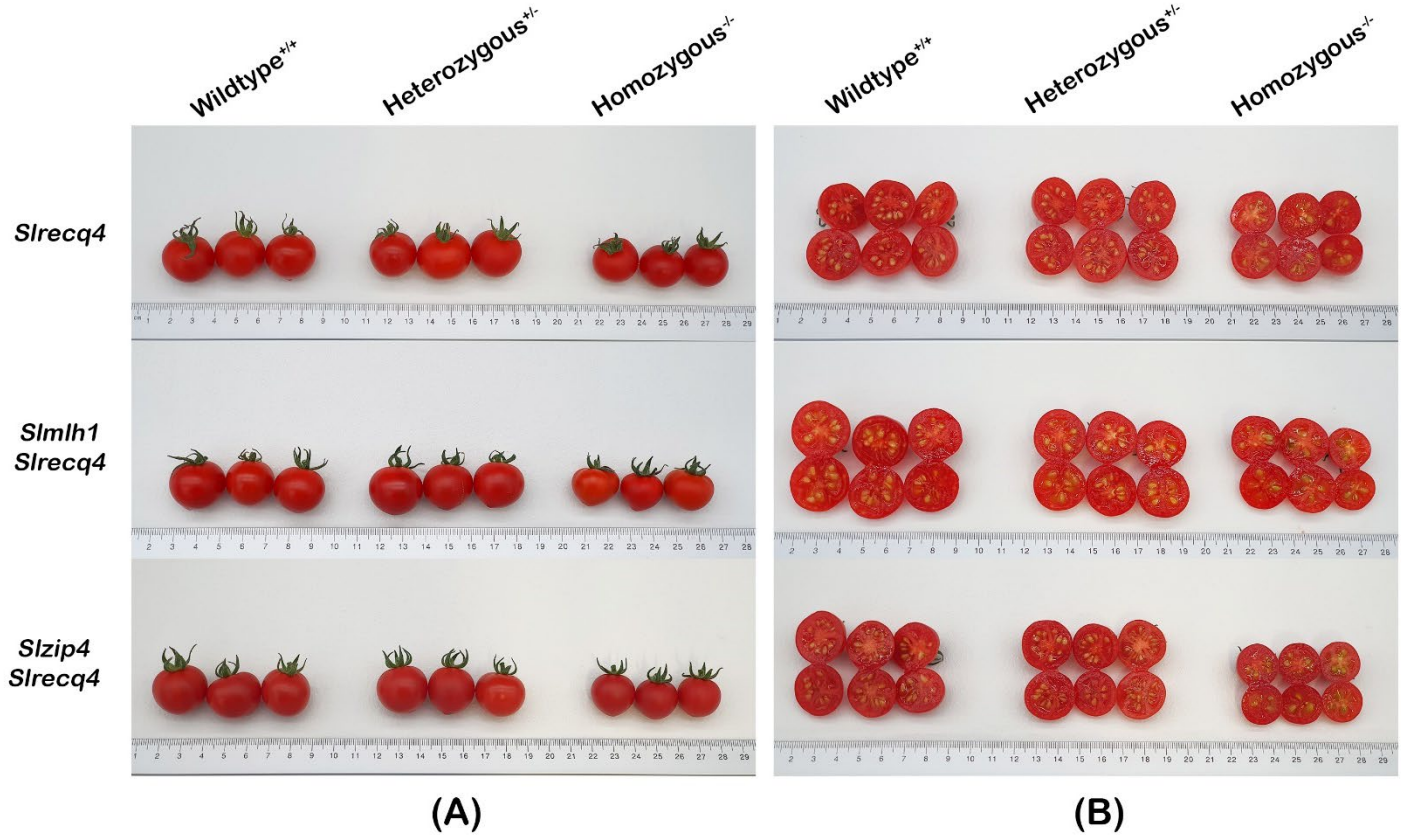


Figure 3.7: Fruit evaluation and comparison from wildtype, heterozygous and homozygous sister plant of *Slrecq4* single mutant, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutant. **(A):** Phenotypic of full ripe fruit. **(B):** Cross-section of full-ripe fruit.

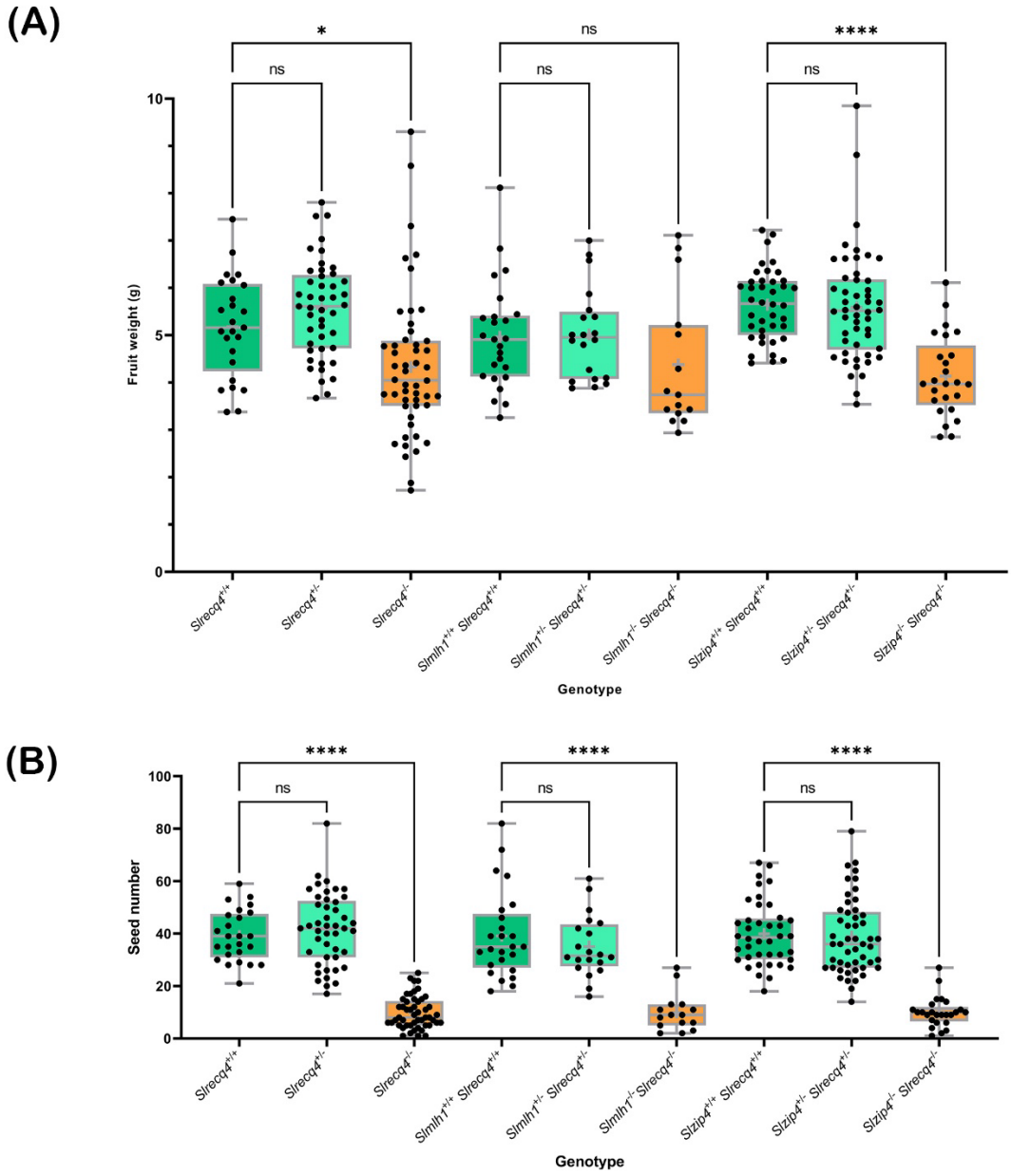


Figure 3.8: Fruit weight and seed number of *Slrecq4* single mutant and *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants in wildtype, heterozygous and homozygous sister. **(A):** Fruit weight of full ripe fruit from *Slrecq4* single mutant and *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants. **(B):** Average seed number per fruit in *Slrecq4* single mutant and *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants.

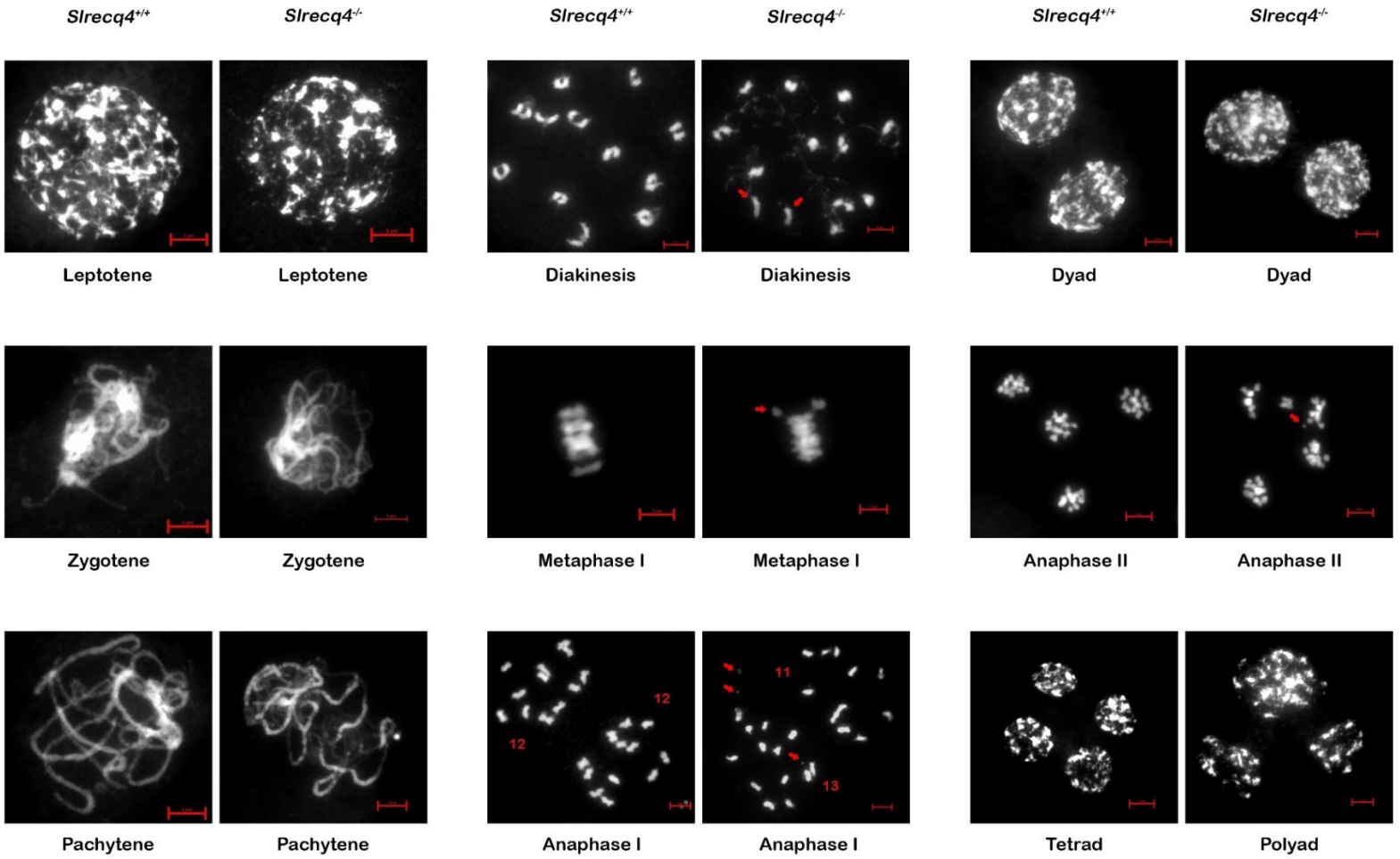


Figure 3.9: Comparison of chromosome behaviour between wildtype and *Slrecq4* mutant by chromosome spread and DAPI staining during meiosis I and II stages. **Meiosis I stages:** Leptotene, Zygotene, Pachytene, Diakinesis, Metaphase I, Anaphase I and Dyad. **Meiosis II stages:** Anaphase II & Tetrad/Polyad. Scale bar= 5 µm

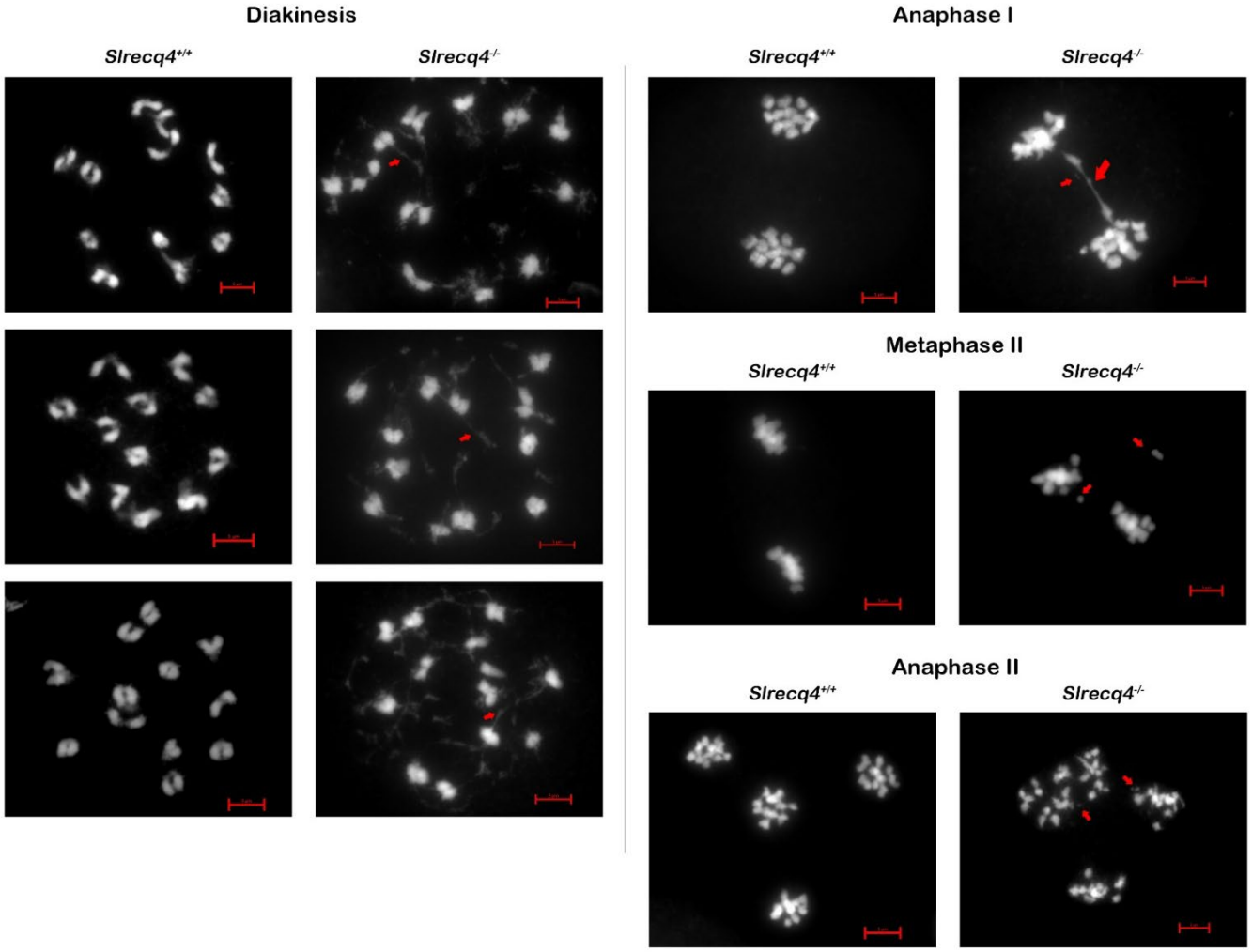


Figure 3.10: Prominent chromosome behaviour *of Slrecq4* mutant during diakinesis, anaphase I, metaphase II and anaphase II. Fragmentation tail during diakinesis. Delayed in chromosome pair segregation during anaphase I. Univalent and chromosome fragmentation during metaphase II and chromosome fragmentation during anaphase II. Scale bar= 5 µm

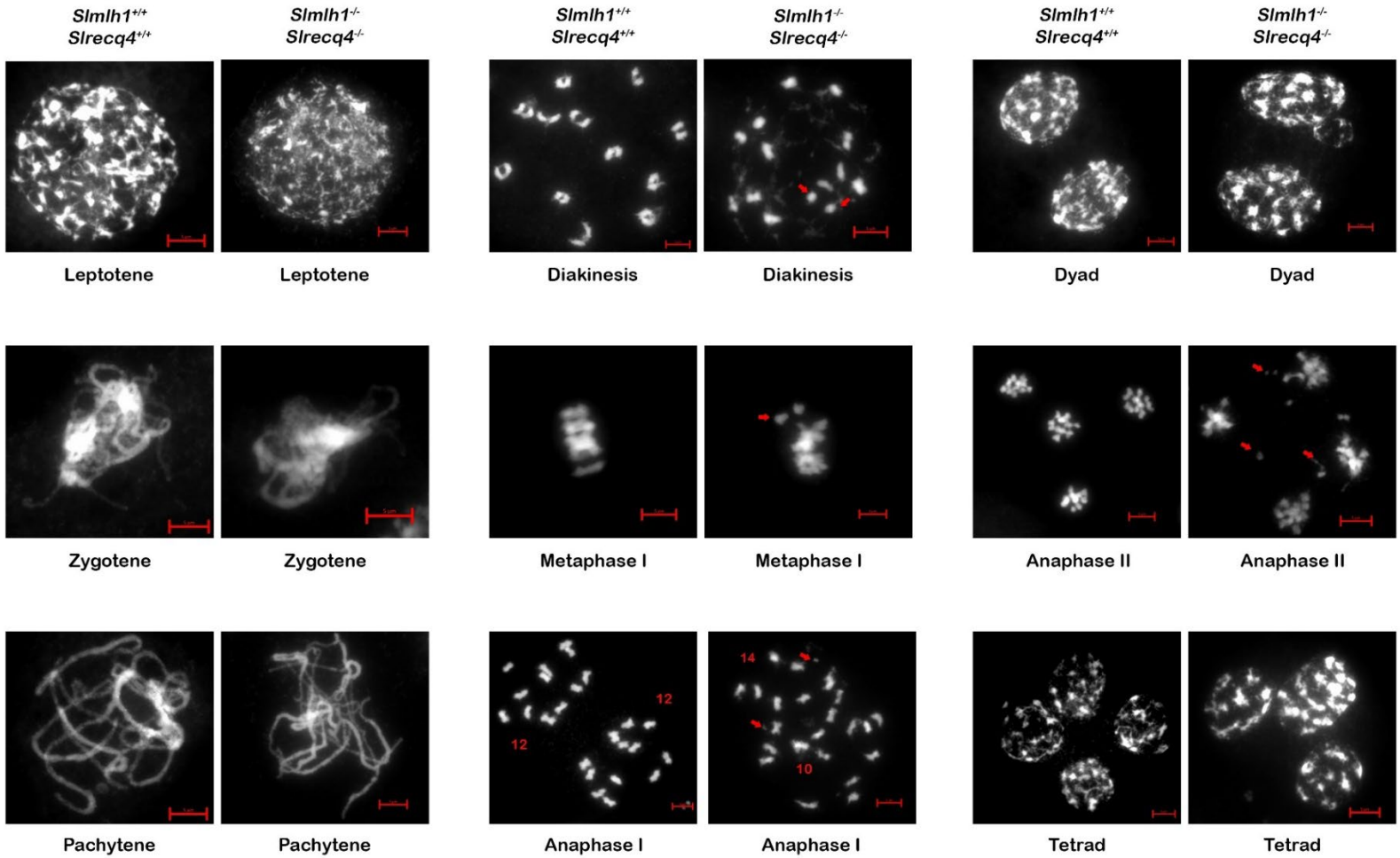


Figure 3.11: Comparison of chromosome behaviour between wildtype and *Slmlh1 Slrecq4* mutant by chromosome spread and DAPI staining during meiosis I and II stages. **Meiosis I stages:** Leptotene, Zygotene, Pachytene, Diakinesis, Metaphase I, Anaphase I and Dyad. **Meiosis II stages:** Anaphase II & Tetrad/Polyad. Scale bar= 5 µm

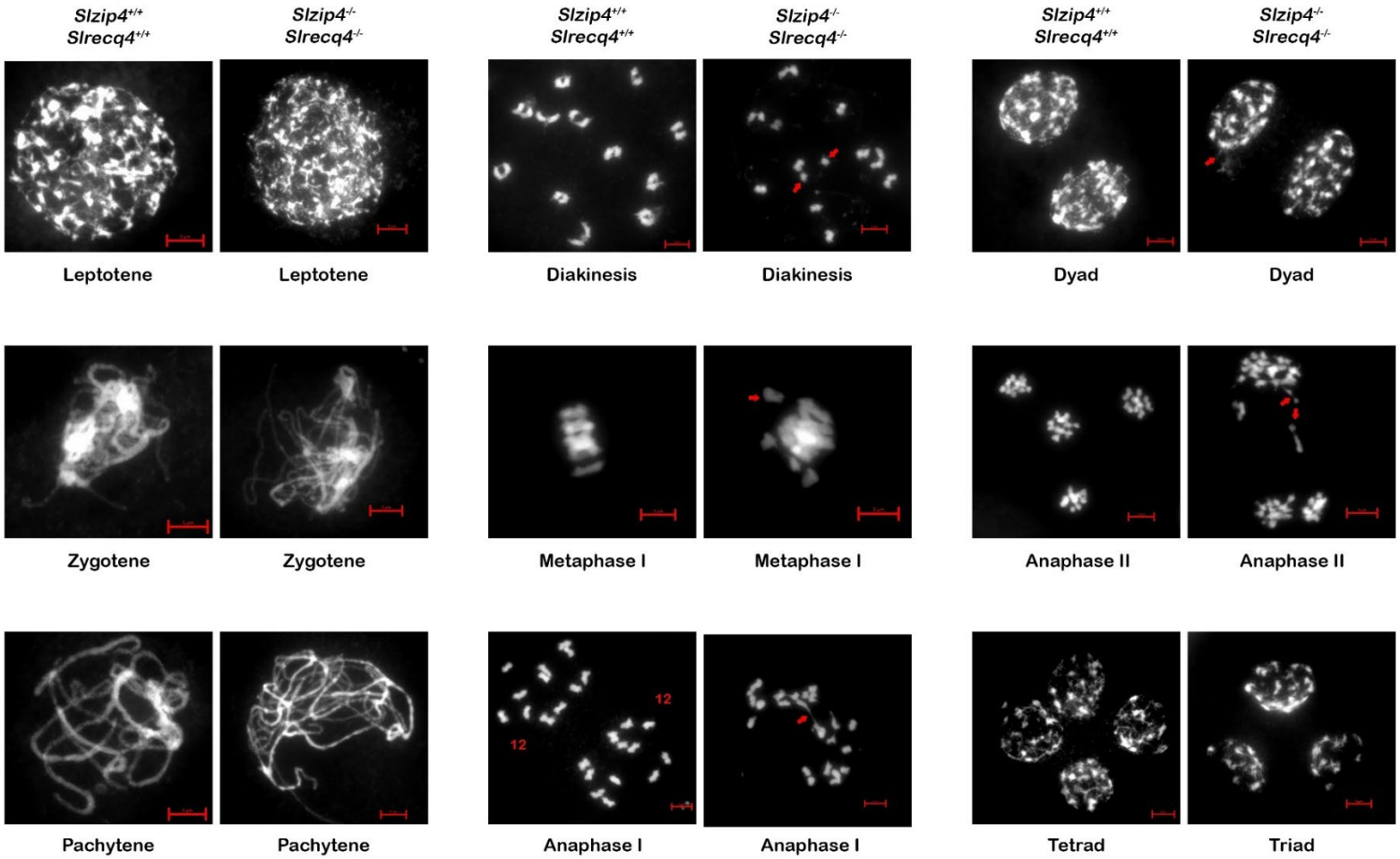


Figure 3.12: Comparison of chromosome behaviour between wildtype and *Slzip4 Slrecq4* mutant by chromosome spread and DAPI staining during meiosis I and II stages. **Meiosis I stages:** Leptotene, Zygotene, Pachytene, Diakinesis, Metaphase I, Anaphase I and Dyad. **Meiosis II stages:** Anaphase II & Tetrad/Polyad. Scale bar= 5 µm

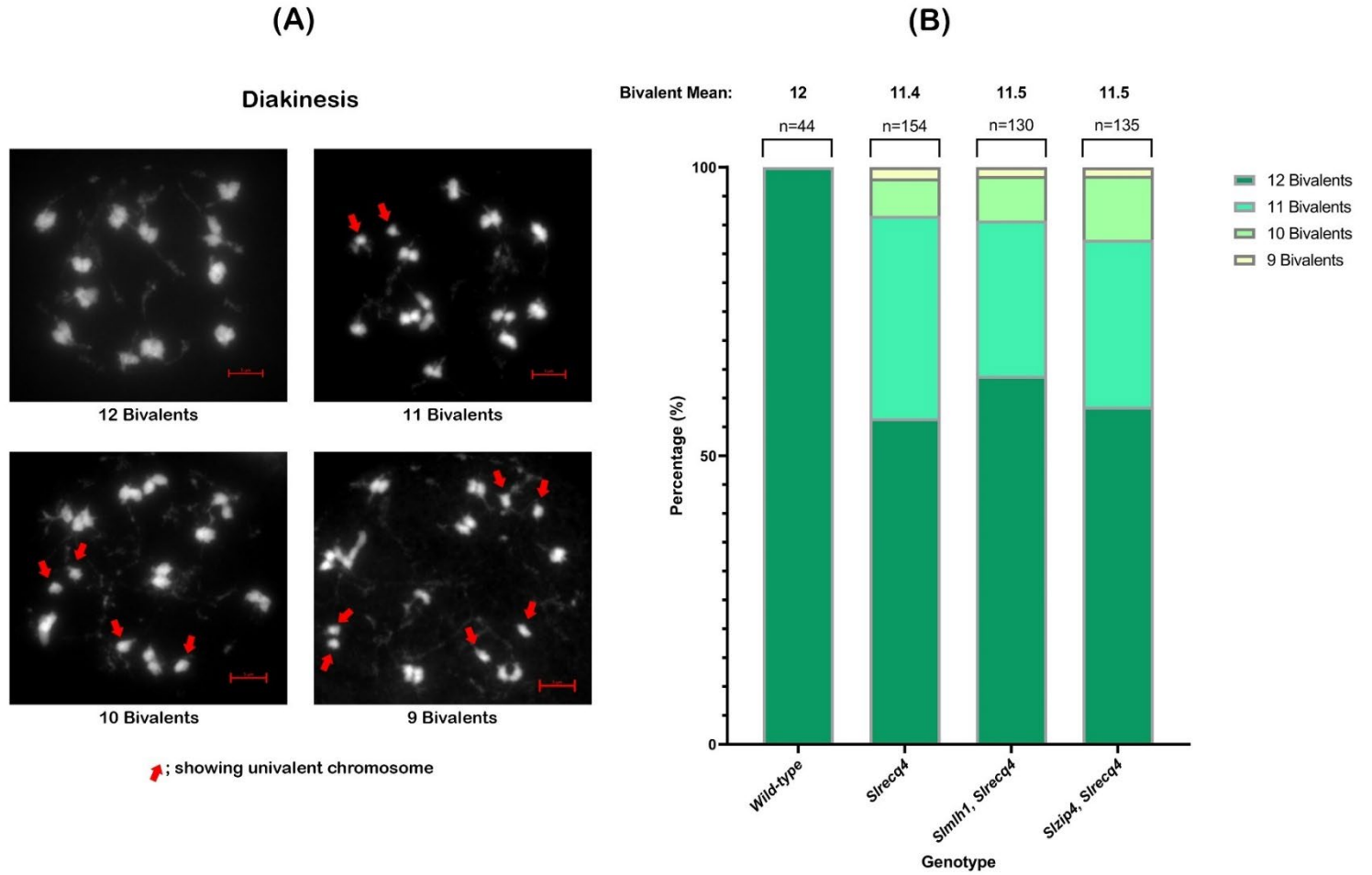


Figure 3.13: Formation and percentage of univalent during diakinesis stage in *Slrecq4* single mutant, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants. **(A):** Chromosome spread of diakinesis stage with 12, 11, 10 and 9 bivalents formation. **(B):** Histogram graph for the percentage of univalent and bivalent and total bivalent mean during diakinesis stage between *Slrecq4* single mutant, *Slmlh1 Slrecq4* and *Slzip4 Slrecq4* double mutants.

Conclusions and perspectives

In this study, investigations were conducted to unravel the function and behaviour of class I CO genes (*SlMLH1* and *SlZIP4*) and a NCO repair gene (*SlRECQ4*) in tomato. Chapter 2 describes how class I CO genes including both ZMM (*SlZIP4*) and non-ZMM (*SlMLH1*) factors are important for meiotic CO and fertility in tomato. I also identified in chapter 2 that introgression of the *mlh1* mutation into a wild tomato species can increase meiotic CO and fertility rate in interspecific tomato. Meanwhile, chapter 3 provides evidence on how the NCO gene, *SlRECQ4* in tomato is involved in pro-crossover events, is crucial in DNA repair, and promotes class II CO pathway activity leading to partial suppression of class I CO mutant defects.

In general, the function of genes related to meiotic CO is largely conserved between different species, however phenotypic differences can also be found between species. In plant meiosis studies, the majority of observations have been made on *A. thaliana* mutants which may not always fully represent the function of genes in other plant species. This was validated on tomato where the involvement of class I CO genes (*SlMLH1* and *SlZIP4*) and NCO gene (*SlRECQ4*) were found to be very essential for the fate of DSB repair and CO and eventually very essential for the fertility of tomato. It is observed that similar behaviour of *Slmlh1* and *Slzip4* phenotypes that lead to very large reduction in fertility and sterility. In comparison to *A. thaliana*, tomato with more chromosome pair and larger genome size depends on class I CO not only to ensure there is a reciprocal exchange of genetic information between non-sister chromatid but it also prevent the mis-segregation of chromosome during meiosis that will ensure intact chromosome behaviour. Adding to this idea, I also found that the wild tomato species, *S. pimpinellifolium* appears to contain a gene that boosts meiotic CO rate in the *Slmlh1* mutant. This unknown potential meiotic CO regulator in *S. pimpinellifolium* may not be present in domesticated tomato as 7,000 years of tomato domestication and selective breeding may have led to its loss.

In this research, I also showed that the behaviour of class I CO mutants depends on how essential the genes is in the formation of CO events. As ZMM protein is not only crucial in assisting class I CO, potentially SlZIP4, a ZMM protein may also be involved in synapsis. Failure to synapse may also influence the DSB repair and cause aberrant chromosome behaviour. My experiments showed that the *Slzip4* mutant has more severe defects in meiotic CO than the *Slmlh1* mutant indicating that ZIP4 is more crucial for CO formation that MLH1 in tomato. The behaviour of *Slmlh1* mutant is far severe than those *mlh1* observed in *A. thaliana* and rice but still retain a very small percentage in fertility. This suggestion that the presence of alternative heterodimer (PMS2-MLH3) and class II CO pathway may act as a minor DNA repair backup for the functional defect of *SlMLH1* gene.

As for the NCO gene, *SlRECQ4* was surprisingly found to be required for all homologous chromosomes to form crossovers whereas the *recq4a/b* mutant in *A. thaliana* has no defect in CO formation (only an increase). As similarly observed in *A. thaliana*, mutation of *Slrecq4* induced CO from class II CO pathway as it can increase the fertility of both class I mutants studied (*Slmlh1* and *Slzip4*). It is interesting that the partial suppression of class I mutants can take place despite there being a significant reduction in fertility in the tomato *recq4* mutant. The reduction in fertility of *Slrecq4* due to univalent and DNA fragmentation indicates that RECQ4 may play a major role in DNA repair and potentially stimulate intermediate precursor for the fate of both pro-CO and NCO pathways during meiosis in tomato. In this research, the class II CO pathway can be suggested to have a minor role in DNA repair and also meiotic CO in tomato as the defect of both class I CO and NCO mutants can only repaired partially by the native class II CO regulations.

In the future, the findings in this research can be further elaborated using immunocytology. The role of the SlMLH1, SlZIP4 and SlRECQ4 proteins during synapsis and the formation of CO can be further validated

using antibodies associated with either synapsis and CO foci. The number of CO for class I mutants (*Slmlh1* and *Slzip4*) and NCO (*Slrecq4*) can be validated with immunocytology using MLH1 and Hei10 antibodies. This would give a stronger evidence of the involvement of these proteins during synapsis and meiotic CO.

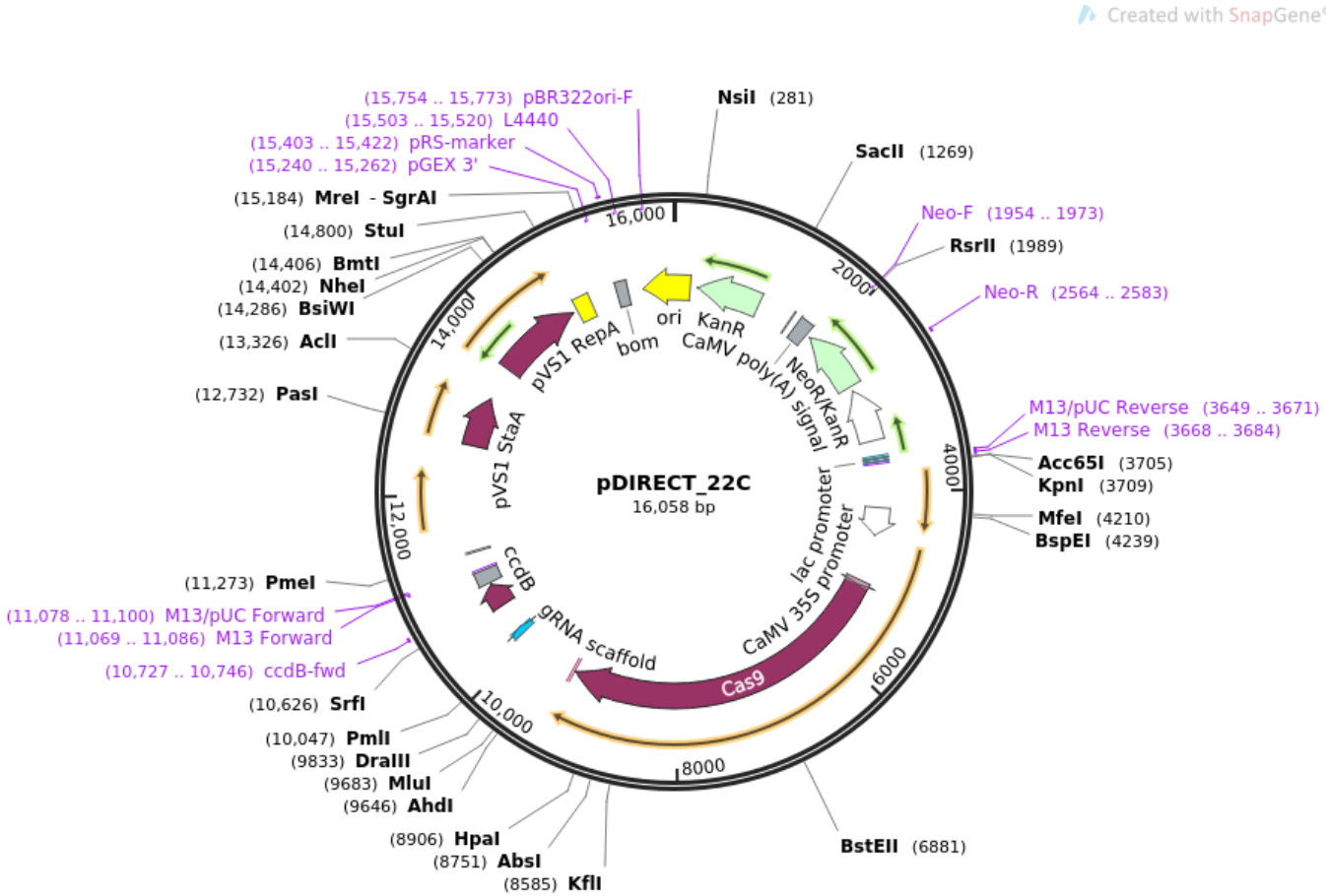
Further to this, there is also a potential to find a new meiotic CO regulator from the interspecific hybrid tomato. The increased meiotic CO in *S. lycopersicum* x *S. pimpinellifolium* hybrid *mlh1* mutant compared to the *S. lycopersicum mlh1* mutant suggests there is a possibility to find an unknown CO regulator in *S. pimpinellifolium* using fertility rate screening and genome sequencing in recombinant populations. Another potential experiment is utilizing *Slmlh1* mutant in forward genetic screening to search for a new class I CO suppressor gene. This was already begun by me when I mutagenized *Slmlh1* mutant using EMS in order to mutate genes associated with class I CO suppressor.

Another potential research can be made for NCO mutant, *Slrecq4* is to combine it with overexpression of a class I CO gene. As overexpression of class I gene for example *SlMLH1* or *SlHEI10* may help in correcting the univalent formation and DNA fragmentation observed in *Slrecq4* mutant but at the same time accelerate CO through class II CO pathway. Thus, this approach is predicted to stimulate greater CO rate that come from both class of CO and reduced the infertility stemming from univalent and fragmentation formation (due to the *slrecq4* defect). As SlRECQ4 may also be involved in pro-crossover pathways, thorough observation can be made using immunocytology during strand invasion on the D-loop formation as this will predict the actual activity of *Slrecq4* in regulating intermediates for CO or NCO events.

As a final remarks, the behaviour of CO and NCO related genes in tomato in regulating meiotic CO or NCO do not always reflect previous observations made in *A. thaliana*. There are many factors and unknown mysteries in regulating meiotic CO in plants especially those which is

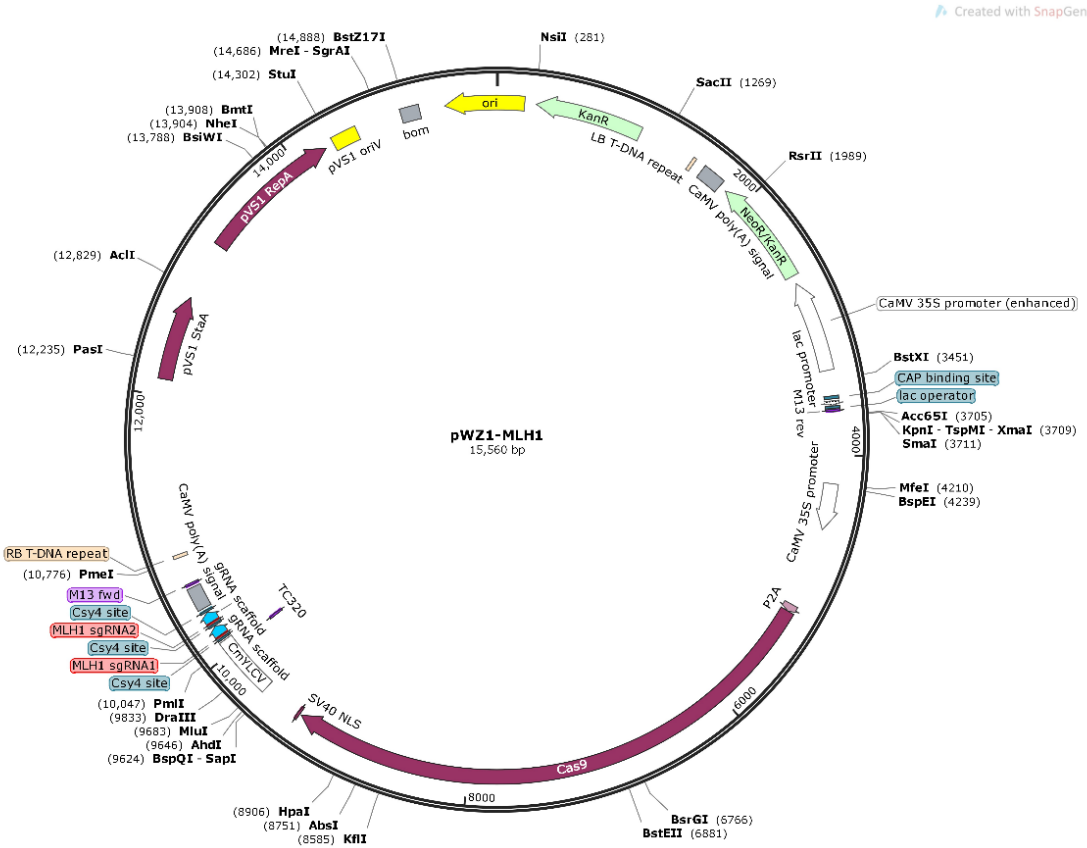
derived from different clade and species. Potentially, evolution not only preserves certain protein to perform functions in regulating meiosis, but also create other regulatory modules or dependencies that may emerge during the evolution of different organisms. For these reasons, these available class Ⅰ CO and NCO mutants in tomato can generate an opportunity to unravel in depth the mechanism of meiotic CO especially in higher dicot plant species. Hence, deeper understanding on the regulation of meiosis in tomato can be achieved and the right combination of CO and NCO manipulation can be identified in future. Therefore, this can be very useful in the breeding of highly vigorous, super tomatoes that can withstand very challenging abiotic and biotic stresses.

Appendix 1.0: pDIRECT_22C CRISPR-Cas9 and plant expression transformation vector

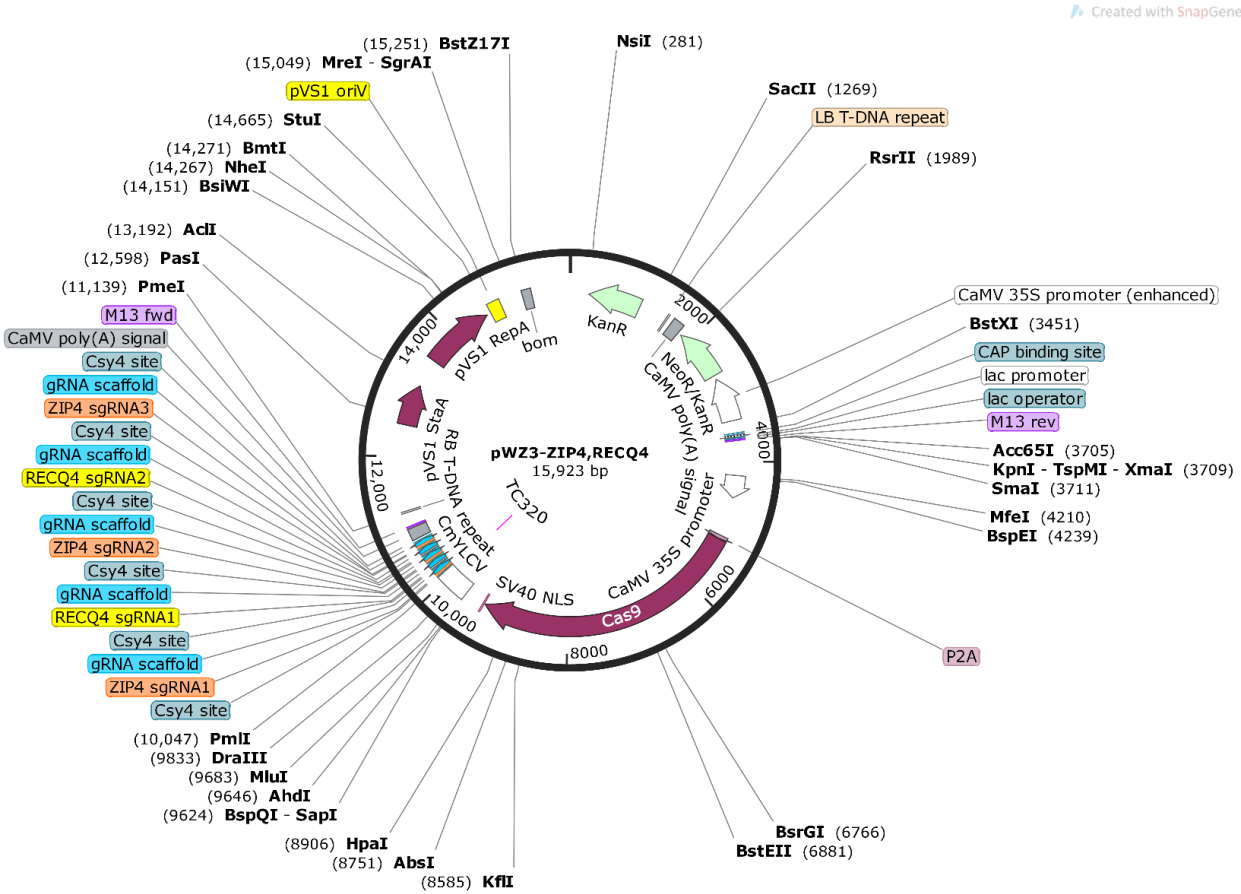


pDIRECT_22C can simultaneously express multiple sgRNAs using the Csy-type ribonuclease 4 (Csy4) enzyme. Engineering Reagent: 35S:Csy4-P2A-AtCas9 + CmYLCV:gRNAs with Csy4 spacers , Plant Selection: 2x35S:npt II (Čermák et al., 2017).

Appendix 1.1: pWZ1 is a pDIRECT_22C based vector with 2 sgRNAs of *SlMLH1* gene



Appendix 1.2: pWZ3 is a pDIRECT_22C based vector with 3 sgRNAs of *SlZIP4* gene and 2 sgRNAs of *SlRECQ4* gene



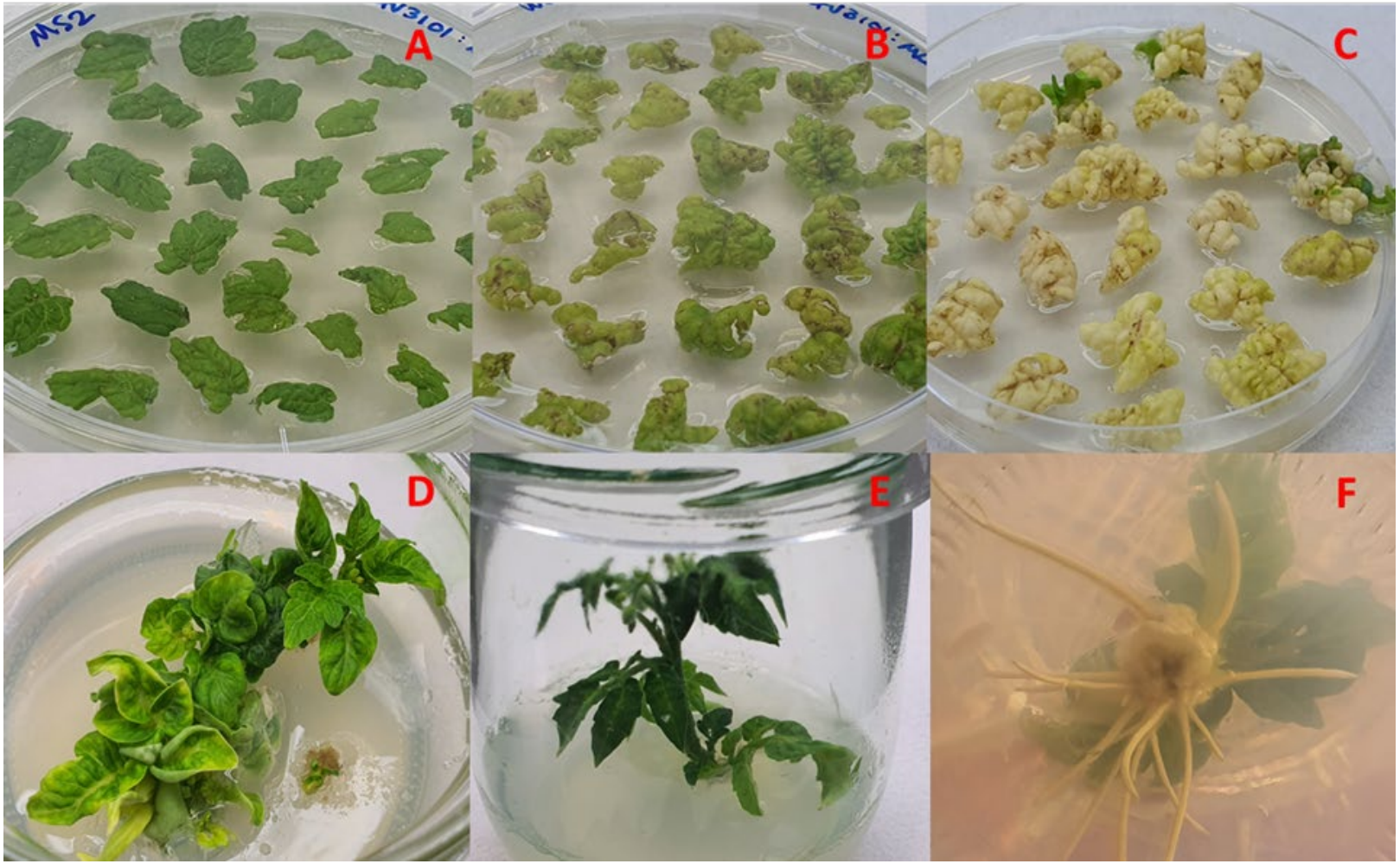
Appendix 2.0

MPIPZ Tomato transformation & selection media (With modification for Micro-Tom)

Media	Mixture
MS0 (seed germination)	• ½ MS (2.15 g/L) • ½ myo-inositol (50 mg/L) • 15 g sucrose • 125 mg/L Carbenicillin • 7 g phyto-agar • pH 5.9 • Sterile seed is germinated in 0.7% phyto-agar plate & after cotyledon produced, transfer to MS0. 1 jar consist of 4 plants
MS1 (Co-cultivation)	• Normal MS, myo-inositol, sucrose • 2 mg/L Zeatin riboside (2 ml from 1 mg/ml stock) • 5 µl of IAA (20 mg/ml IAA stock) • 73 mg/L acetosyringone (73 mg acetosyringone is dissolved in few drops of DMSO, vortex until fully dissolved & added into MS) • 7 g phyto-agar • pH 5.9
MS2 (first & second* subculture)	• Normal MS, myo-inositol, sucrose • 1.5 mg/L Zeatin riboside (1.5 ml from 1 mg/ml stock) • 500 mg/L carbenicillin (2 ml from 250 mg/ml stock) • 100 mg/L kanamycin (2 ml from 50 mg/ml stock) • 7 g phyto-agar • pH 5.9
MS2 (Third or later subculture in plate or jar for plant regeneration)	• Normal MS, myo-inositol, sucrose • 1.0 mg/L Zeatin riboside (1.0 ml from 1 mg/ml stock) • 250 mg/L carbenicillin (1 ml from 250 mg/ml stock) • 100 mg/L kanamycin (2 ml from 50 mg/ml stock) • 7 g phyto-agar • pH 5.9
MS3 (Rooting)	• ½ MS (2.15 g/L) • ½ myo-inositol (50 mg/L) • 15 g sucrose • 250 mg/L carbenicillin (1 ml from 250 mg/ml stock) • 50 mg/L kanamycin (1 ml from 50 mg/ml stock) • 1.0 mg/L IAA (50 ul from 20 mg/ml stock) • 7 g phyto-agar • pH 5.9

*Second subculture was carried out after 7 days (reduced *Agrobacterium* contamination & increase selection stringency for transformant callus/embriod). After that, subculture every 14 days.

Appendix 3.0



Appendix 3.0: *In vitro* selection, regeneration & rooting of tomato meiotic mutants. **A:** Transformed explants on kanamycin selection MS media (Day 1), **B:** Transformed explants after 2 weeks on kanamycin selection MS media, **C:** Resistant embryoids after 1 months on kanamycin selection MS media, **D:** Shoot elongation, **E & F:** Transformant plantlet in rooting media.

Appendix 4.0

A) T_0 *Slmlh1* mutant lines regenerated from CRISPR/Cas9 gene editing transformation and *in vitro* selection

Slmlh1 mutant lines	Plants transferred to soil
pWZ1-A-1	6
pWZ1-A-2	25
pWZ1-A-3	10
pWZ1-A-4	16
pWZ1-A-5	20
pWZ1-A-6	2
pWZ1-A-7	2
pWZ1-A-9	4
Total mutants	**85**

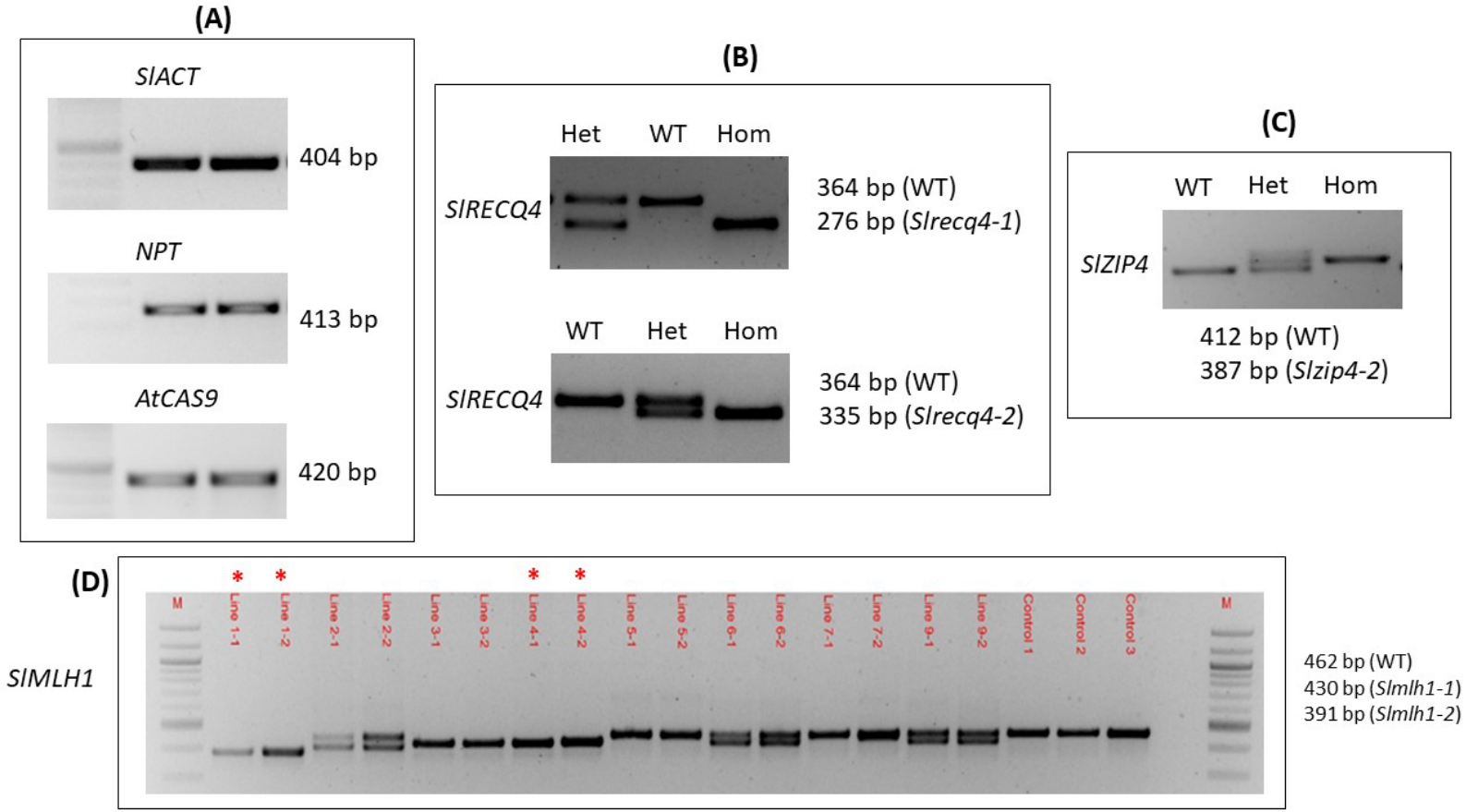
* Backcross progeny from pWZ1-A-4 line was used for phenotypic and cytology experiments with *Slmlh1-1* having 32 bp deletion on intron 1.

B) T_0 *Slzip4 Slrecq4* double mutant lines regenerated from CRISPR/Cas9 gene editing transformation and *in vitro* selection

Slzip4 Slrecq4 mutant lines	Plants transferred to soil
pWZ3-A-2	4
pWZ3-A-3	3
pWZ3-A-4	7
pWZ3-A-5	1
pWZ3-A-6	13
pWZ3-A-7	1
pWZ3-A-8	2
pWZ3-A-9	2
Total mutants	**33**

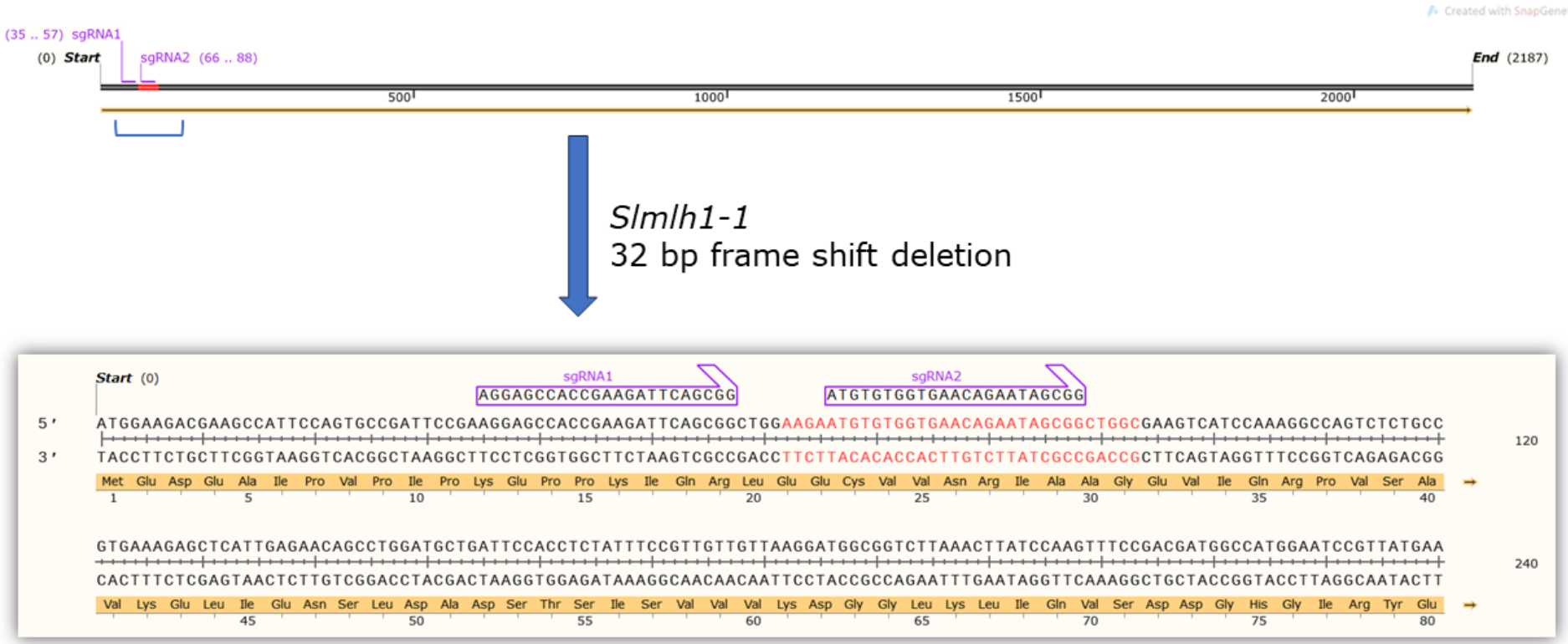
*Backcross progeny from pWZ3-A-4 line was used for phenotypic and cytology experiments. *Slzip4-1* (8 bp deletion) and *Slrecq4-1* (4 bp deletion on intron 1, 88 bp deletion on intron 3) single mutants were isolated from segregation of this line were used in phenotypic and cytology experiment.

Appendix 5.0

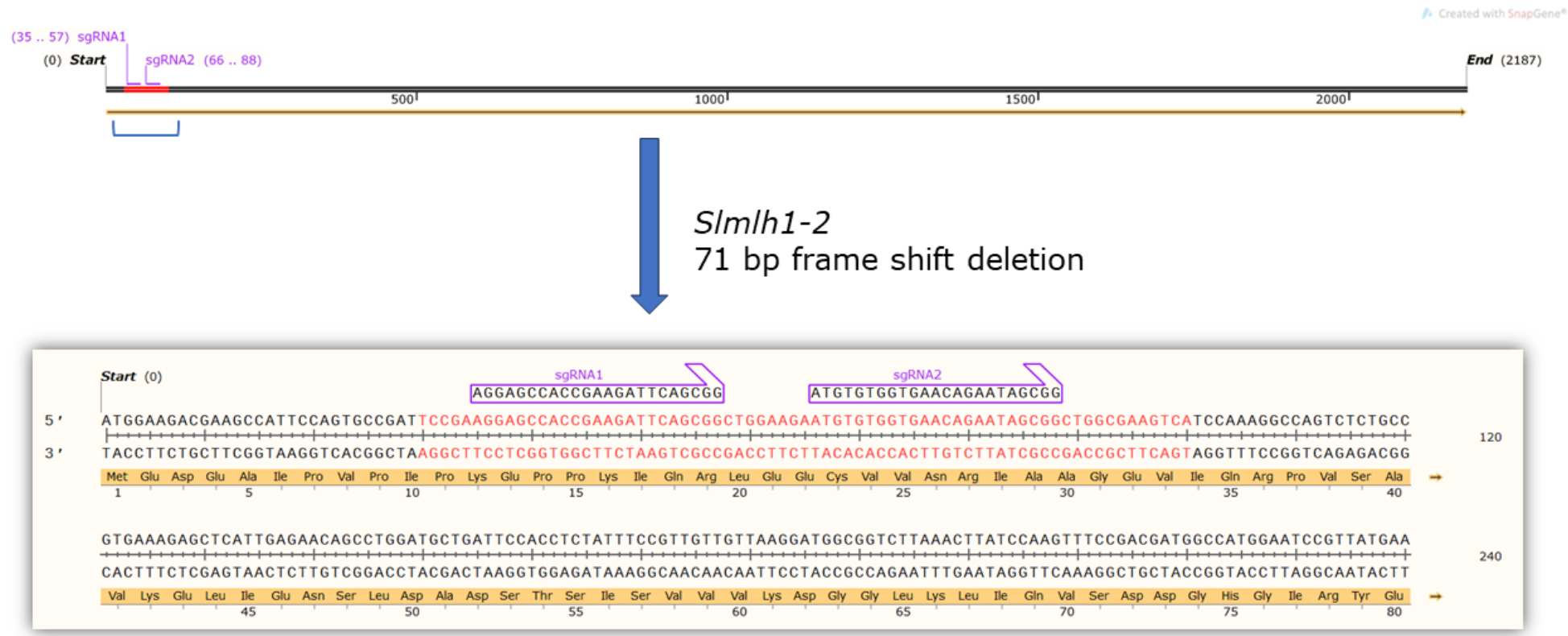


(A): PCR genotyping for tomato housekeeping gene, *Actin* (*SlACT*), kanamycin resistant gene, *Neomycin phosphotransferase* (*NPT*) and *A. thaliana CAS9* gene (*AtCAS9*). **(B):** PCR amplification of *SlRECQ4* gene for genotyping *Slrecq4* mutant (*Slrecq4-1* & *Slrecq4-2*). **(C):** PCR amplification of *SlZIP4* gene for genotyping *Slzip4-2* mutant. **(D):** PCR amplification of *SlMLH1* gene for all 8 different *Slmlh1* mutant lines. *Slmlh1-1* derived from line 4 was used in genotyping and cytology experiment. HRM genotyping was used to identified *Slzip4-1* mutant with 8 bp deletion. HRM method also developed for *Slmlh1-1* mutant (32 bp deletion).

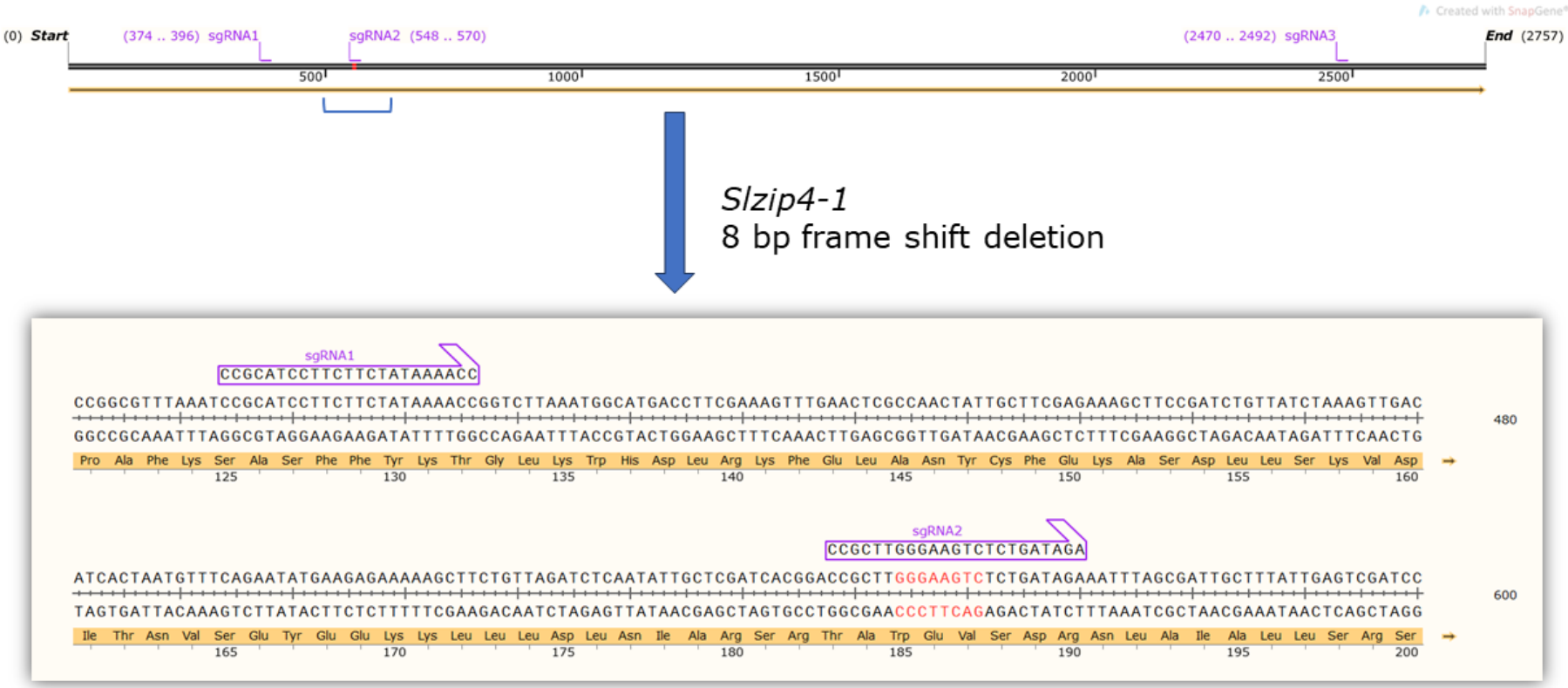
Appendix 6.0: *Slmlh1-1* mutant with 32 bp deletion located at sgRNA2 with the deletion alter the overall protein codon of SlMLH1 protein. Red colour DNA sequence is the location of the DNA deletion.



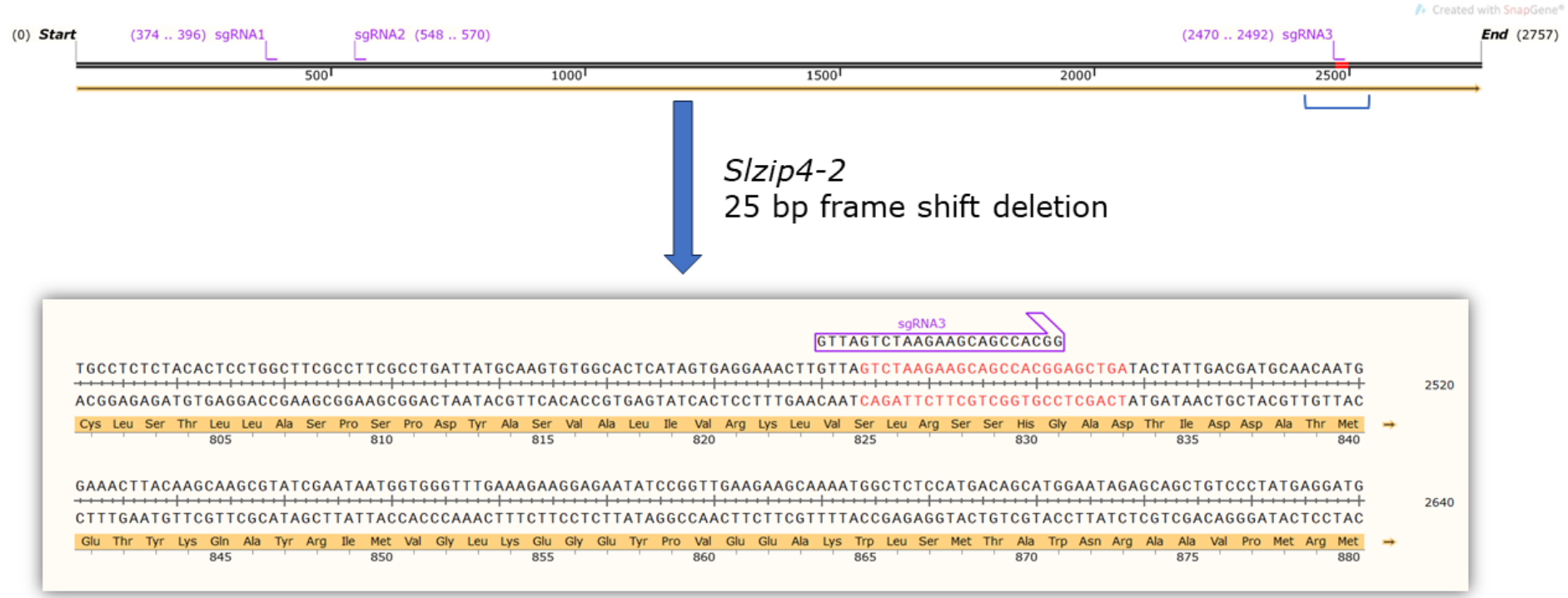
Appendix 6.1: *Slmlh1-2* mutant with 71 bp deletion located at both sgRNA1 and sgRNA2 with the deletion alter the overall protein codon of SlMLH1 protein. Red colour DNA sequence is the location of the DNA deletion.



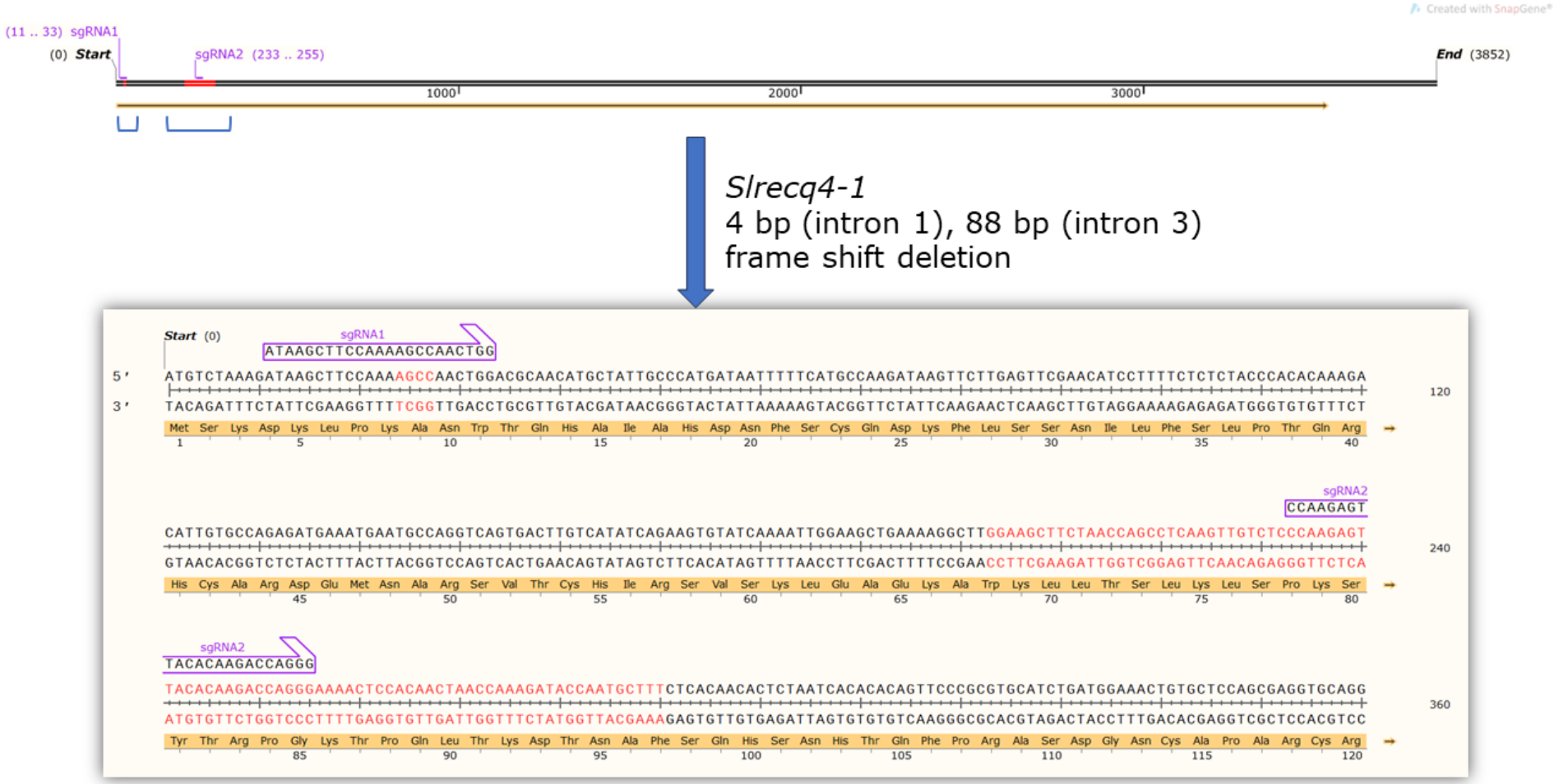
Appendix 6.2: *Slzip4-1* mutant with 8 bp deletion located at sgRNA2 with the deletion alter the overall protein codon of SlZIP4 protein. Red colour DNA sequence is the location of the DNA deletion.



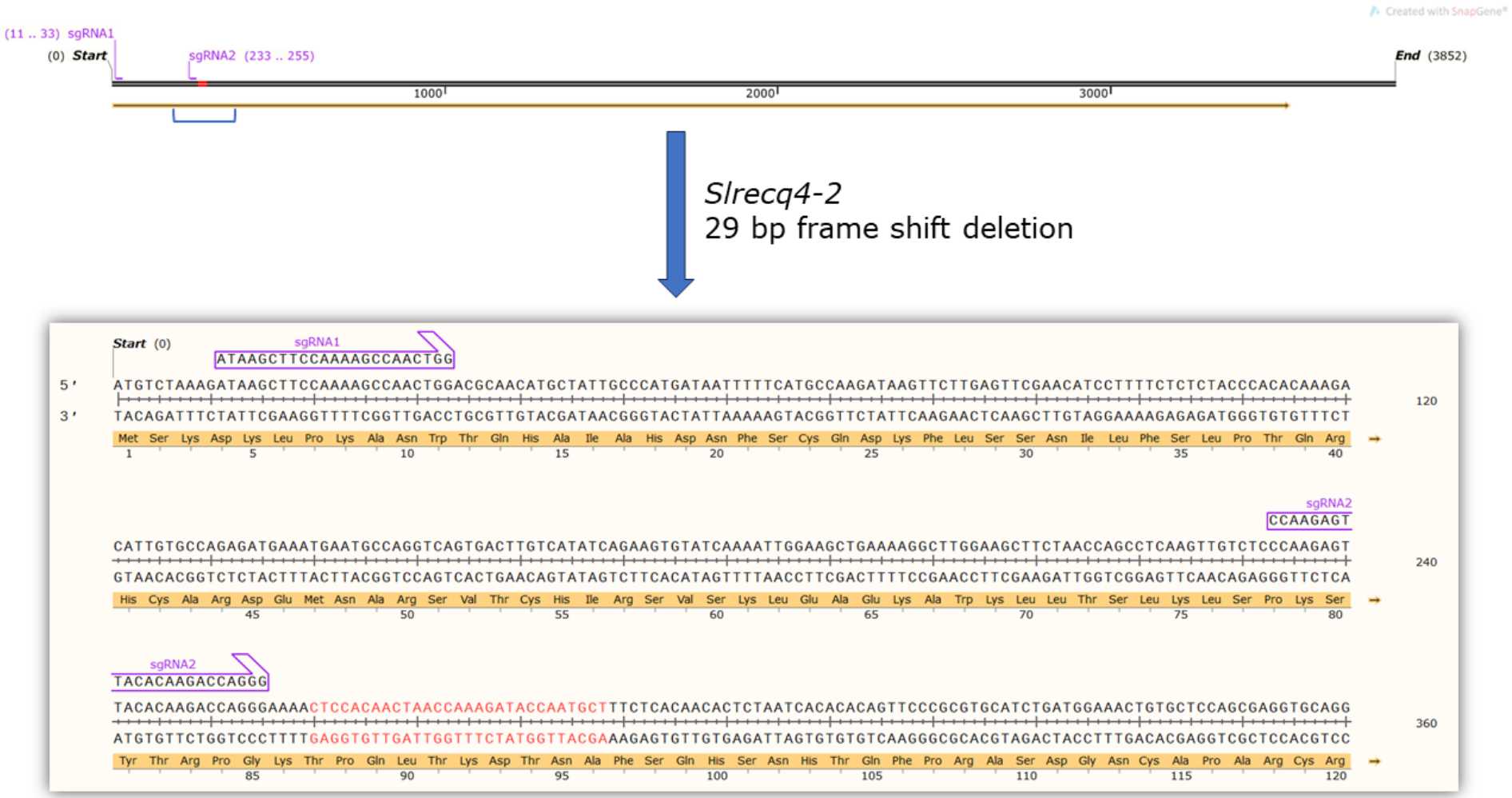
Appendix 6.3: *Slzip4-2* mutant with 25 bp deletion located at sgRNA3 with the deletion alter the overall protein codon of SlZIP4 protein. Red colour DNA sequence is the location of the DNA deletion.



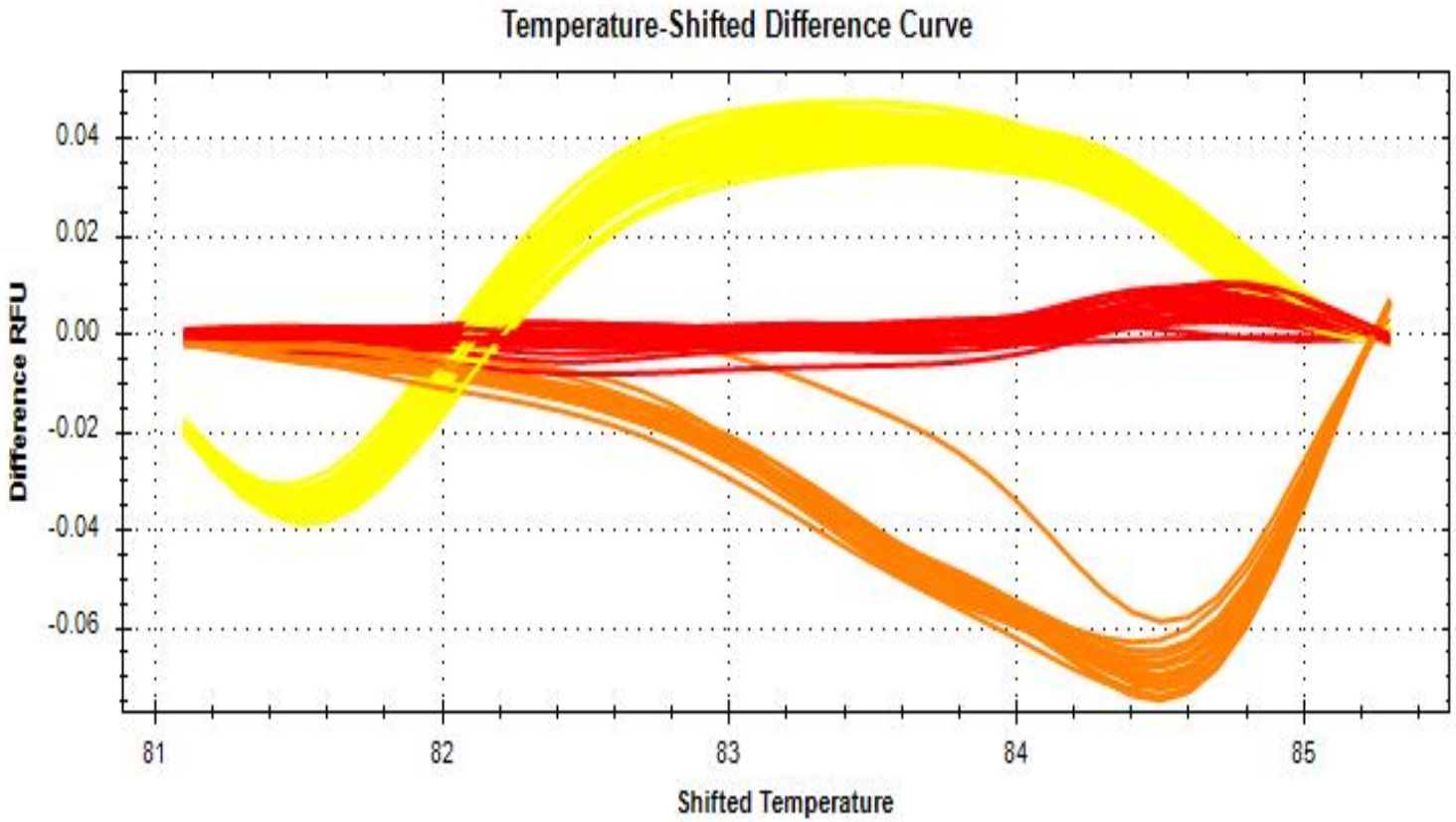
Appendix 6.4: *Slrecq4-1* mutant with 4 bp deletion located at sgRNA1 and 88 bp deletion located on sgRNA2 with the deletion alter the overall protein codon of SlRECQ4 protein. Red colour DNA sequence is the location of the DNA deletion.



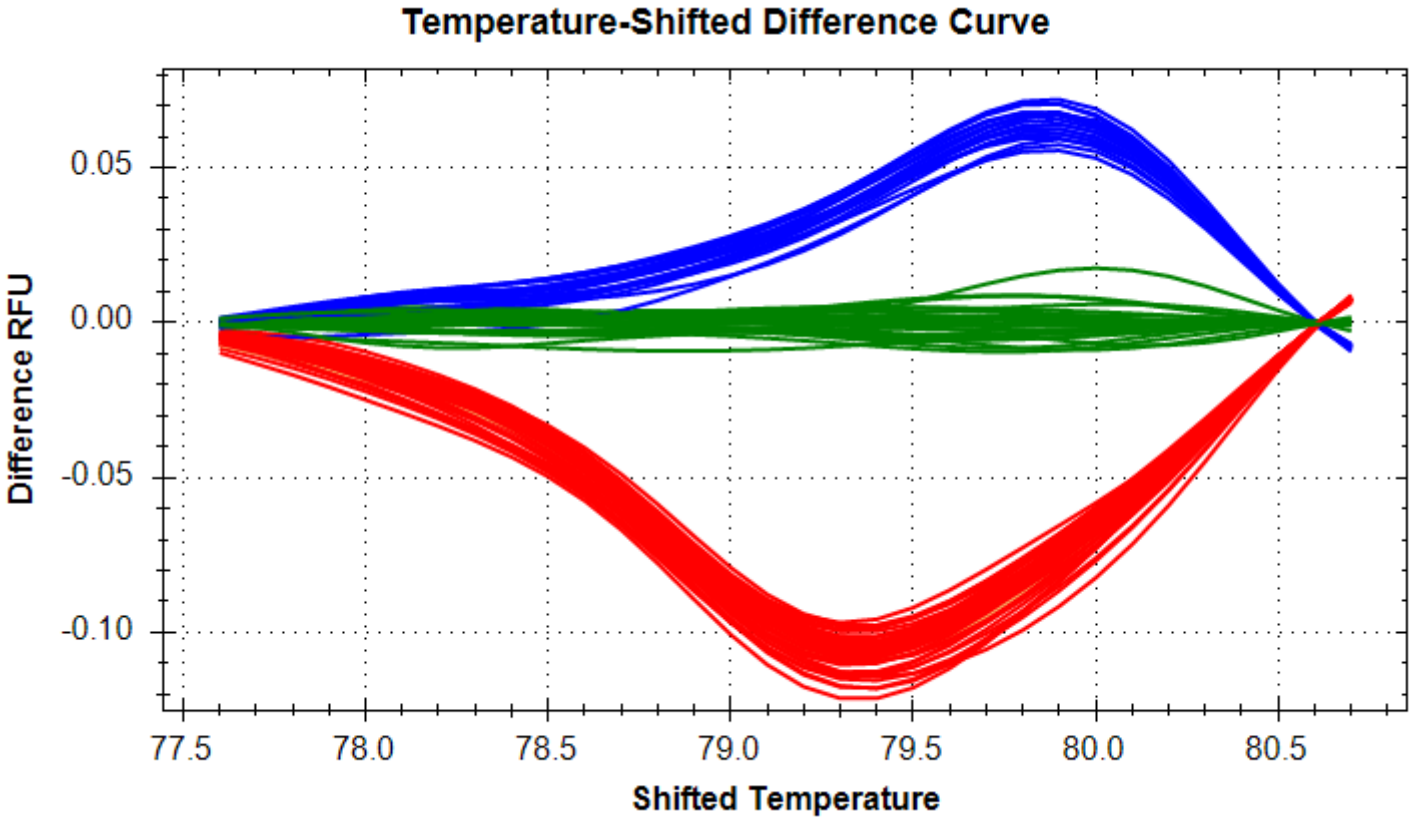
Appendix 6.5: *Slrecq4-2* mutant with 29 bp deletion located at sgRNA2 with the deletion alter the overall protein codon of SlRECQ4 protein. Red colour DNA sequence is the location of the DNA deletion.

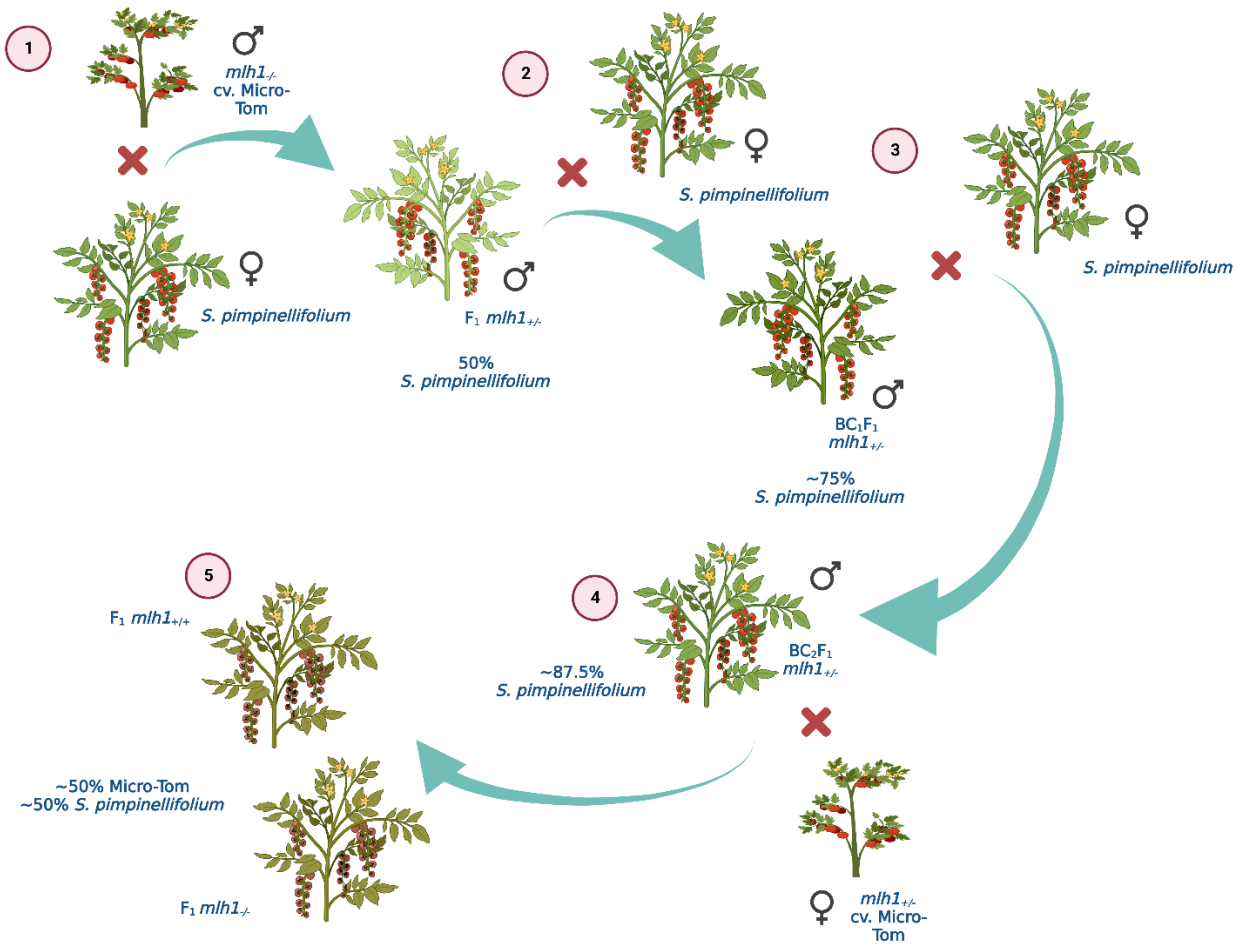


Appendix 7.0: High Resolution Melting (HRM) for quantitative analysis of the melt curves of product DNA fragments following PCR amplification of *SlMLH1* gene. HRM for wildtype (red), heterozygous 32 bp deletion (yellow) and homozygous 32 bp deletion (orange) of *SlMLH1* gene (125 bp for wildtype). Melting curve temperature for wildtype (82.6˚C), heterozygous 32 bp deletion (81˚C) and homozygous 32 bp deletion (82.4 ˚C) of *SlMLH1* gene.



Appendix 7.1: High Resolution Melting (HRM) for quantitative analysis of the melt curves of product DNA fragments following PCR amplification of *SlZIP4* gene. HRM for wildtype (green), heterozygous8 bp deletion (blue) and homozygous 8 bp deletion (red) of *SlZIP4* gene (186 bp for wildtype). Melting curve temperature for wildtype (80.6˚C), heterozygous 8 bp deletion (80˚C) and homozygous 8 bp deletion (80.4˚C) of *SlZIP4* gene.





Appendix 8.0: Genomic distribution behavior of crossover in Micro-Tom x *S. pimpinellifolium* hybrid (*mlh1*$^{+/+}$) and *Slmlh1-1* x *S. pimpinellifolium* hybrid (*mlh1*$^{-/-}$). **1-3:** Cross and backcrossed with *S. pimpinellifolium* to obtain ~87.5% *S. pimpinellifolium* and ~12.5% Micro-Tom background. **4:** BC_2F_1 crossed with heterozygous *Slmlh1-1* mutant to obtain ~50% *S. pimpinellifolium* and ~50% Micro-Tom background (F_1). **5:** F_1 progeny for both wildtype and homozygous 32 bp deletion of *SlMLH1* gene.

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gemäß der Promotionsordnung vom 12. März 2020

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(*This version must be included in the doctoral thesis*)

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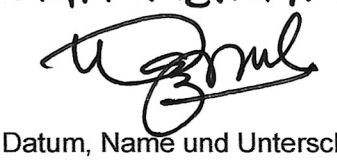

Datum, Name und Unterschrift

Erklärung zum Gesuch um Zulassung zur Promotion

gemäß der Promotionsordnung vom 12. März 2020

1. Zugänglichkeit von Daten und Materialien

Die Dissertation beinhaltet die Gewinnung von Primärdaten oder die Analyse solcher Daten oder die Reproduzierbarkeit der in der Dissertation dargestellten Ergebnisse setzt die Verfügbarkeit von Datenanalysen, Versuchsprotokollen oder Probenmaterial voraus.

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☒ Trifft zu.
In der Dissertation ist dargelegt wie diese Daten und Materialien gesichert und zugänglich sind (entsprechend den Vorgaben des Fachgebiets beziehungsweise der Betreuerin oder des Betreuers).

2. Frühere Promotionsverfahren

Ich habe bereits einen Doktortitel erworben oder ehrenhalber verliehen bekommen.

Oder: Für mich ist an einer anderen Fakultät oder Universität ein Promotionsverfahren eröffnet worden, aber noch nicht abgeschlossen.

Oder: Ich bin in einem Promotionsverfahren gescheitert.

☒ Trifft nicht zu

☐ Zutreffend
Erläuterung:

3. Straftat

Ich bin nicht zu einer vorsätzlichen Straftat verurteilt worden, bei deren Vorbereitung oder Begehung der Status einer Doktorandin oder eines Doktoranden missbraucht wurde.

Ich versichere, alle Angaben wahrheitsgemäß gemacht zu haben.

Datum	Name	Unterschrift
18.06.2023	Mohd Wazrul Adly Mohd Zaidan	