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CONTROL OF MEIOTIC RECOMBINATION IN PLANTS

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Abstract

This project aims to locate the DNA-PKcs in *Arabidopsis thaliana* and identify the role by analysing *Atdna-pkcs* mutants. The silico, genetic and cytology data for DNA-PKCs in *Arabidopsis thaliana* showed that putative *AtDNA-PKa* and putative *AtDNA-PKb* are the two genes of *AtDNA-PKcs* in *Arabidopsis thaliana*. The expression data for both genes proves that AtDNA-PKCs are expressed in different tissues, but the strongly expressed in flower buds particularly in opened buds. Also, AtDNA-PKcs were expressed in male and female organs with more expression in the female organs. The data collected of double homozygous mutants suggests that *Atdna-pkcs* is sterile null mutant, AtDNAPKcs do not play any role in meiosis, but could be important after meiosis or in mitosis to repair DSBs through NHEJ as proves by pollen grain reduction, some anaphase bridges during mitosis, and the effect of DNA damage agents on the growth of the *atdnapkcs* plants.

This project also aimed to study the outcome of residual DSB during meiosis in *Arabidopsis thaliana*. Genetic and cytology analysis was performed for *Atprd2* homozygous mutant and induced DSBs by cisplatin in the absence of AtSPO11-2. The result collected demonstrates some DSB formation is observed through the presence of γH2AX foci, These DSBs are repaired by HR and producing some crossovers, and some clear rod bivalents are showed in metaphase I. However, these data suggested that partly synapsis between homologous chromosomes can produce COs through meiotic recombination machinery. Thus, study Zinc Finger Nucleases (ZFNs) has used to induce DSBs in specific sequences in the *Arabidopsis thaliana* genome (on *TT4* and *ADH1* loci) in an *Atspo11.2* null mutant background. Inducible system has used to express the ZFNs and produce a specific targeted DSB during meiosis has allowed us to study the different outcomes of these DSBs. This data of induced DSBs by ZFN indicated that targeted DSB could be synopsised and repaired by meiotic recombination machinery and initiate

homologous pairing and recombination in that genomic region. Nevertheless, because the sequence recognised by the ZFNs is present in both homologous chromosomes and both sister chromatids, it could produce DSBs in all these regions which would not be possible to be processed by the meiotic recombination machinery and would produce some fragmentations. This thesis will produce new insights into the control of meiotic recombination in plants.

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I would like to thank my parents for everything they have provided to me until I reach this stage in my life. I am very grateful to my wife for her encouragement and keenness to complete my Ph.D. Many thanks to Taif University in Saudi Arabia for giving me this opportunity to continue my higher education. Also, I would like to thank my colleague in the department for their help and support during my research.

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CHAPTER ONE

INTRODUCTION

DNA

Deoxyribonucleic acid, DNA, is a polymeric molecule that contains the genetic information necessary for important biological functions. DNA is formed of subunits or monomers named nucleotides that are strung together to form a chainlike pattern (Bates and Maxwell, 2005). Watson and Crick (1953) characterized the structure of the DNA which is a three-dimensional double helix. Both DNA strands are held together with the help of hydrogen bonds that are present between the nucleotides. Furthermore, there is a deoxyribose sugar which is present in the nucleotides which are attached to a particular base including thymine (T), guanine (G), cytosine (C) or adenine (A) and also it is attached to a group of phosphate. The phosphates and sugars connect the nucleotides in a structure that is presented in a chain leading to a formation of a backbone which presents the alternating sugar-phosphate-sugar phosphate (Watson and Crick, 1953). T is complementary paired with A always and C is paired with G. Therefore, the backbones of the sugar-phosphate are held together along the length of the molecule of the DNA, at an equal distance (Campbell, 2005). Watson and Crick (1953) presented that to increase the base-pair packing efficiency; a double helix is formed by both the backbones of sugar-phosphates, additionally, with a single complete turn, ten base pair per turn (**Figure 1**).

DNA is functionally important, because, within its four simple nucleotide bases, it can code for all the information necessary for any cell to develop, included sexual gametes, and to the existence of multicellular organisms. DNA provides the units of biological information and the inheritance mechanism required to maintain specific traits from generation to generation (Sinden, 1999). While genes have many separate and unique functions, DNA's primary purpose is to deliver the information that makes the genes functional (Watson and Berry, 2009). The number of chromosome sets is known as ploidy.

The somatic cells of the higher eukaryotes are diploid, but the sexual gametes are haploid as they contain half of the number of the chromosomes (Gupta, 1992). Diploid cells ($2n$) contain two copies from each chromosome that are inherited from parental cells. Haploid cells (n) contain only one copy from each parental chromosome. The diploid cell undergoes meiosis to produce four haploid cells. Fertilization of haploid cells is needed to produce a diploid cell that has two copies of chromosomes (one maternal chromosome and one paternal chromosome). Then, the diploid cell undergoes mitosis to create a diploid multicellular organism (Auletta, 2011).

Diploid cells contain two copies of chromosomes. Thus it contains two copies of the gene (alleles). Alleles can be different from each other because of differences in the gene sequence provided by the maternal or paternal side. A copy of a dominant allele would produce a dominant trait or phenotype, while a recessive allele must have two copies of the recessive allele to appear that specific trait on the phenotype. If the individual contains a copy from each type of alleles (one dominant and one recessive), the dominant phenotype will prevail (Wet *et al.*, 1987).

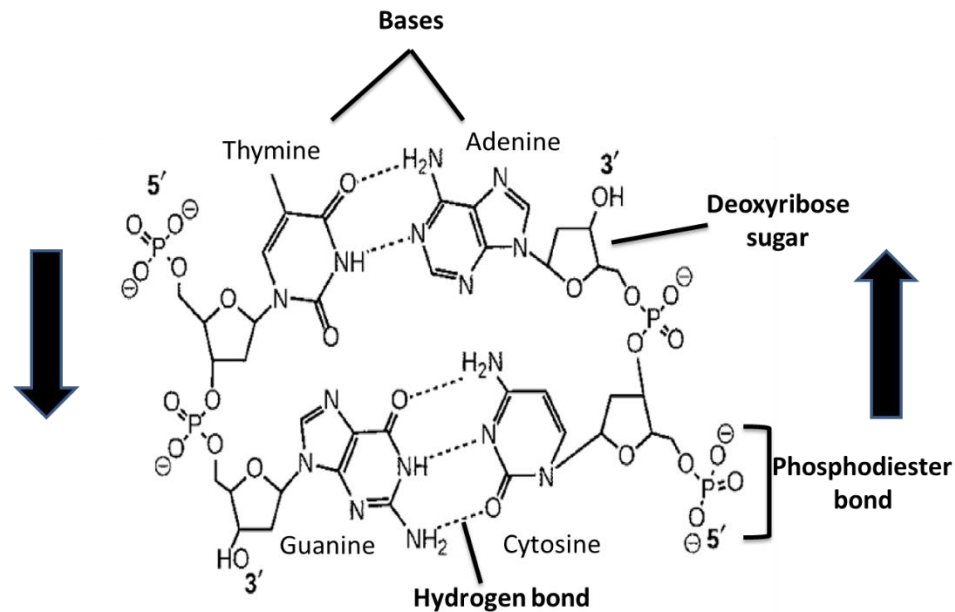


Figure 1: Complementary base pairing in the DNA double helix.

(Watson and Crick, 1953) The Image is modified from ("DNA structure and sequencing: Figure 3) <https://courses.lumenlearning.com/wm-nmbiology1/chapter/reading-structure-of-dna/>

CHROMATIN

The formation of chromatin is required to solve two main problems for DNA that are: packaging and accessibility (Maeshima *et al.*, 2010). DNA is packed by the association of proteins into **chromatin** in a way that would allow the packaging of the molecule into the small nucleus of the cell permitting continuous access to the genetic information. Histones are the main protein components in chromatin; this help to build the nucleosomes where two copies of 4 histone cores (H2A, H2B, H3, H4) are wrapped by the DNA (Cooper, 2000). Histones consist of H1 (linker protein) and H2A, H2B, H3 and H4 (core proteins). Nucleosomes contain eight histones (octamer), two copies of each of the core histones (2xH2A, 2xH2B, 2xH3 & 2xH4). Oligomerization of histones is initiated by the histones' H3/H4 tetramer formation, which interacts with DNA followed by the assembly of H2A/H2B dimmers during the formation of a nucleosome (Grunstein, 1997). The nucleosome is considered the most stable protein-DNA complex with structural and functional importance. Although it is considered to be vital for packaging, it is also highly

Important for the structural integrity and biological functions of the cell such as DNA replication, DNA repair, transcription and cell division (mitosis and meiosis). Histone modifications such as methylation, acetylation, phosphorylation, and ubiquitination are very important in regulating all these different biological functions (Li *et al.*, 2007).

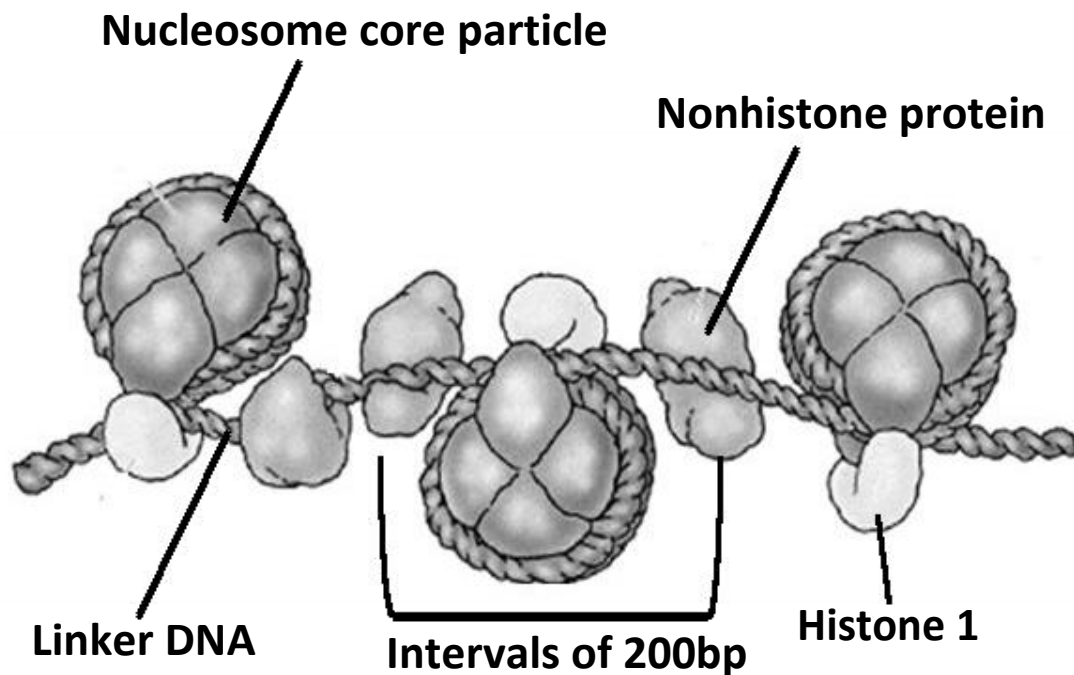


Figure 2: Organization of chromatin around the nucleosome.

According to Luger, *et al.* (1997, pp. 31). The nucleosome is formed by a histone octamer wrapped by 147 bp of DNA about 1.65 times. The points at which the DNA contact with proteins are considered to be 14 (Cooper, 2000, pp. 13).

DNA Repair

Repairing damaged DNA because of environmental and biological assault is essential.

There are different mechanisms of DNA repair in higher eukaryotes involving many Proteins (Cooper, 2000; Clancy, 2008). Many chemical reactions can take place in DNA molecules. DNA serves as a permanent cellular genetic source of basic information,

therefore any changes within its structure or sequence would carry out important consequences compared to other biomolecules like RNAs or proteins. Mutations can be due to the incorporation of inappropriate nucleotide bases during the process of DNA replication. Moreover, several chemical changes occur in DNA either spontaneously or because of chemicals or radiation exposure. Mutation in the DNA has various consequences like replication or transcription blockage, consequently producing unacceptable variations during cell reproduction. Thankfully, to maintain genomic integrity, cells have evolved various repair mechanisms to restore the damaged DNA. The DNA repair mechanisms have developed two different ways to repair:

- (1) Direct reversal of DNA damage through chemical reactions.
- (2) Removal of the damaged bases and their replacement with the correct base by synthesis of DNA.

Whenever failure to the DNA repair occurs, further mechanisms by the cells have been evolved enabling to overcome the damage (Cooper, 2000).

Type of DNA damages

DNA lesions are separated into two classes which are single-stranded breaks (SSBs) and double-stranded breaks (DSBs). The lesions that disturb only one DNA strand are called SSBs. Some SSBs damage examples include oxidized or alkylated base damage, base loss, DNA adducts, intra-strand cross-links, and DNA photoproducts. The other type of break involves the lesions that damage both DNA strands. DSBs can be produced by inter-strand cross-links during replication by different drugs or by enzymes like topoisomerase II. DSBs lesions are considered to be the most severe type of DNA damage mostly in the genomes of eukaryotes (Vasilissa & Damian, 2015).

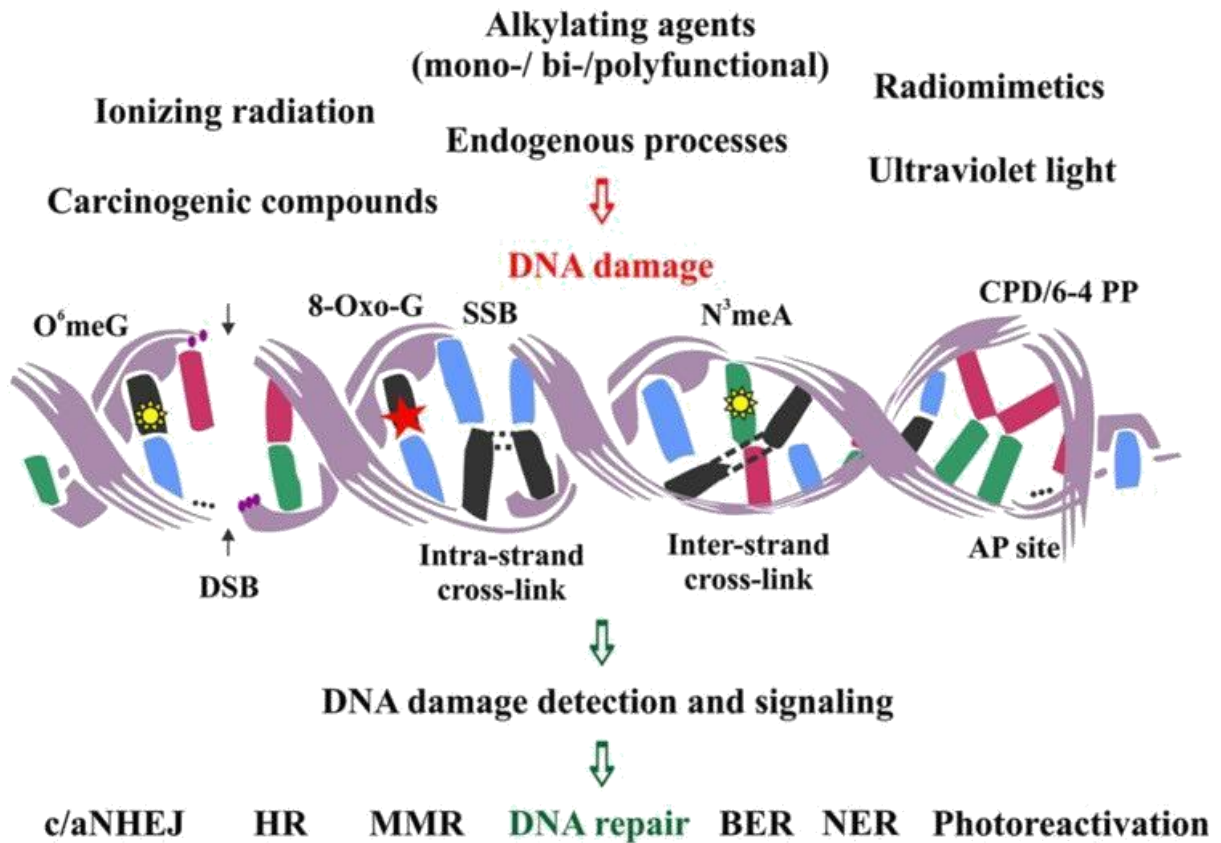


Figure 3: Schematic illustration of the most important DNA lesions induced by numerous endogenous and exogenous factors. Also, different DNA repair mechanisms employed by the eukaryotic genome repair. (From Manova and Gruszka, 2015).

Endogenous DNA damage

Deamination, replication errors, and free radical species are included in damage by endogenous agents. Free radical species such as reactive oxygen species and reactive metabolites, produced in some cases as by-products of metabolism, may have consequences towards DNA damage in a direct and indirect manner. The chemical comprises of several reactive oxygen species (ROS) and different metabolic products generation such as lipid peroxides, endogenous reactive chemicals for example aldehydes and S-adenosylmethionine similarly, chemical DNA instability (e.g., depurination) are among many that are involved in DNA damage (Friedberg *et al.*, 2005; Helleday *et al.* 2014). Spontaneous DNA damage may occur because of endogenous factors such as

oxidative DNA damage, hydrolytic DNA damage because of the reaction of DNA with water and oxygen, and enzymatic conversions. Adenine, cytosine, and guanine contain exocyclic amino groups and are subject to deamination mostly via temperature and PH dependent manner. Similarly, depurination (loss of purine bases) is also a type of DNA damage caused by the cleavage between deoxyribose and the purine bases, forming an apurinic/apyrimidinic site (AP) site) (Cooper, 2000; Helleday *et al.* 2014).

Exogenous DNA damage

Chemical mutagens and carcinogens, both ionizing and non-ionizing radiations, are an example of exogenous agents causing direct or indirect damage to the DNA (Helleday *et al.*, 2014).

i. Radiation

Ionizing radiations are energetic and a penetrating principle source of DNA damage that occurs naturally in the environment. The exposure varies among individuals, but X-ray and γ -ray photons are known to deposit energy in a highly dispersed manner within tissues. Ionizing radiation-induced bystander effects (RIBE) is the occurrence of biological effects after exposure to radiation in the cells that are un-irradiated. RIBE have been shown to produce different DNA damages including DSBs, SSBs, mutations, and chromosomal instability. Similarly, water hydrolysis because of radiation exposure creating free radicals is another major source for DNA damage in an indirect manner. Formation of hydrated electrons, hydrogen and hydroxyl, radicals as a result of water hydrolysis, of which hydroxyl radical is of prime importance in DNA damage. Covalent linkage and formation of dimers, especially cyclobutane pyrimidine dimers is the result of UV exposure. Other

products of UV exposure inducing DNA damage include purine dimers, pyrimidine hydrates, and thymine glycol (Friedberg *et al.*, 2005 and Desouky *et al.*, 2015).

ii. Chemicals

Alkylating agents are a wide variety of chemical compounds that have an affinity for nucleophilic centers for organic molecules. Based on the presence of potential reactive sites, agents can be classified as mono-functional or bi-functional. Environmental source of alkylating agents that act as mutagen and carcinogen are methyl chloride, methyl bromide, methyl iodide, and alkylate DNA (Friedberg *et al.*, 2005).

Different types of DSBs repair pathways

DSB in the DNA can be caused by both endogenous and exogenous factors such as exposure to irradiation and chemical agents or enzymes. Numerous repair pathways do exist, which include Base Excision Repair (BER), Nucleotide Excision Repair (NER), and due to the seriousness of the DSB damage there are different pathways to repair these DSBs (DSBR) (**Figure 4**). As indicated by Truong and collaborators (2014), the primary main repair pathways in DSBs are Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). More significantly, it has been asserted by Negritto (2010) that DSBs are difficult lessons for the cell as they can induce cell death if not repaired legitimately. DSBs can also prompt chromosome instability by producing deletions, insertions, and translocations.

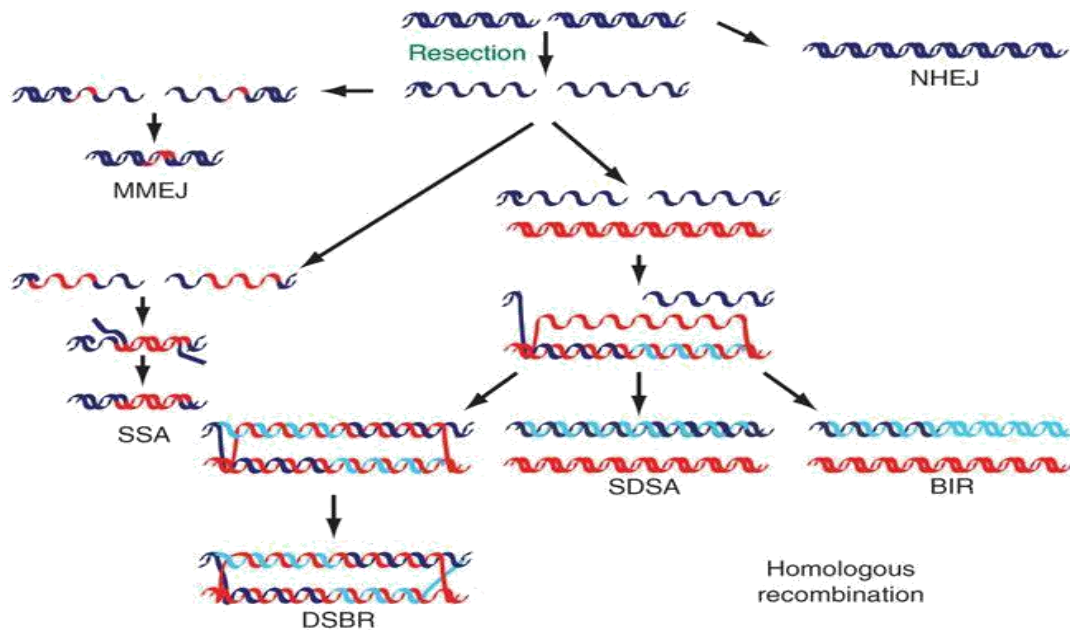


Figure 4: DNA double-strand breaks (DSBs) repair mechanisms.

DSBs repair pathways include Homologous Recombination (HR), Non-Homologous End Joining (NHEJ) and Single-Strand Annealing (SSA) (From Huertas, 2010).

Non-Homologous End Joining (NHEJ)

NHEJ is termed as "non-homologous" as breaks are ligated directly without requiring a homologous template (Zhang, 2008). NHEJ facilitates the direct re-ligation of DNA that is broken. Thus, hypothetically it is not limited to a specific cell cycle phase, while HR is thought to be able to act only during S and G2 phases of the cell cycle when a template would be available (sister chromatid). The individual steps during the mechanism of NHEJ are listed below sequentially:

- (I) DNA end recognition followed by assembly and stabilization of the NHEJ complex at the DSB.
- (II) DNA ends are bridged thereby promoting end stability.
- (III) DNA ends processing.
- (IV) Finally, broken ends are ligated followed by the dissolution of the NHEJ complex.

In the NHEJ process, proteins and enzymes are involved in each step which facilitates the repair process. Moreover, the kinase activity by DNA dependent protein kinase catalytic subunit (DNA-PKcs) in NHEJ is of prime importance (Weterines *et al.*, 2008).

Importance of NHEJ for DNA repair

Programmed DSBs are also introduced in specific cell types, such as V(D)J recombination, and are essential for the generation of meiotic crossovers and recombination events. Among different types of DNA damage introduced by numerous factors, DSBs are the most dangerous type and are detrimental to living cells, such as diseases like cancer progression. Repairing breaks are essential, and if not repaired, they may be associated with chromosomal aberrations (Burma *et al.*, 2006).

Defects in the NHEJ pathways within the cell cause sensitivity to radiation and some drugs. There is a direct link between genome instability and cancer predisposition because of the mutations in DSBs responsive genes. The breaks in DNA may generate selection pressure that can overrule the cell cycle checkpoints, thereby, bypassing the repair pathways leading to the genome instability and cancer (Burma *et al.*, 2006).

Is Non-homologous End Joining important in Meiosis?

During cellular mechanisms, cell is subjected to various stressors which affect the integrity of DNA molecule – the genetic content in the cell. Among various DNA damages encountered by the prokaryotic and eukaryotic cells, induction of double stranded breaks (DSBs) is recognised most detrimental (Tang *et al.*, 2014). There have been identified various repair mechanisms through which the induced DSBs are joined together including homologous repair (HR) and non-homologous end joining (NHEJ) (Roy 2014). NHEJ repairs DSBs in DNA in the absence of an intact template by covalently joining together the two broken ends of DNA strands (Joyce *et al.*, 2012). This manuscript will discuss the importance of NHEJ during meiosis with specific reference to plants and other eukaryotes.

Integrity of genome is threatened by DNA damage for which, repair mechanisms are required and the lack of efficiency of repair mechanism has potentially lethal consequences on cell and organism (Manova & Gruszka 2015). For the stability of plant genome, effective detection and rapid repair process of DNA damage is crucial as the plant genome is under persistent pressure from environmental as well as endogenous factors. DSBs induced in DNA are recognised as most cytotoxic forms of DNA damages which may result in fragmentation of chromosome. Thus, large amount of genetic information is lost due to failure of repair of DSB following cell division, thus compromising the integrity of daughter cells due to transfer of fragmented or lost chromosomes (Waterworth 2011). During meiotic prophase, formation of crossovers induces DSBs at several locations in each chromosome. For the recombination to proceed effectively, repair pathways are activated for resolution of DSBs. Homologous repair is usually preferred over error prone NHEJ during meiosis, however, the cascade leading to suppression or inactivation of NHEJ is still unclear (Takahashi et al., 2014). During meiotic prophase, NHEJ is blocked at two steps of DSB repair; based on the activity of intracellular signalling proteins. The activity of MCM-like protein is the initial blockade signal, which is required for crossover formation while the second blocking signal is transduced by Rad51-related proteins SPN-D (RAD51C) and SPN-B (XRCC3), which is crucial for physical interaction and promotion of HR pathway. Due to high error prone nature, evolution of multiple barriers has been reported to prevent NHEJ during meiosis in certain eukaryotes (Bertrand et al., 2019).

In plant models, the relatively small sized genome of *Arabidopsis thaliana* has been extensively studied due to availability of various transformation methods and rapid life cycle. These features have attracted this plant model for investigations on repair mechanisms in eukaryotic DNA and mutagenesis (Roy 2014). The major break repair pathways identified in plants are NHEJ and HR resulting in preservation of genetic stability. Other repair mechanisms include excision repair and recombination pathways activated for DNA lesions and photoreactivation, which are high specific for particular types of damages. Due to recent advancements in molecular biology, studies have identified the flexibility NHEJ as repair pathway occurring by the recruitment of required repair factors (Manova & Gruszka 2015). In other eukaryotes, back-up NHEJ (B-NHEJ) pathways have also been identified independent of the activity of KU including alternative NHEJ and microhomology-mediated end joining (MMEJ). However, activity of KU-independent DSB repair has been frequently

reported and plant cells have been observed to contain number of components of alternative NHEJ with comparatively slower rates of DSB repair (Takahashi et al., 2014).

These investigations have been confirmed in single cell level through single cell electrophoresis, quantifying the DNA damage by employing electrophoretic analysis. Moreover, immunochemistry based investigations have also revealed the disappearance and formation of DNA damage foci by employing the activity of phosphorylated histone γ H2AX in antisera (Da Ines et al., 2014). According to the findings of Tang et al., (2014), RAD51C is identified to play an important role in DNA repair process of DSBs and is a paralogue of RAD51 (Tang et al., 2014). The component has been recognised crucial for the occurrence of meiosis in rice. In plants, mammals and other complex organisms, DSBs induced in somatic cells are identified to be repaired by NHEJ pathway despite its error prone nature; during which broken ends, irrespective of sequence homology, are joined together (Tang et al., 2014). According to a report, rapid repairing of DSBs was observed in Arabidopsis NHEJ knockout mutants lig4 and ku80, the rates which were comparable to two other wild type species of plants, indicating the involvement of B-NHEJ, independent of classical pathway for DSB repair (Bertrand et al., 2019).

In higher plants, much of the understanding of DSB repair is observed through the studies on induction of DNA breaks and transposon excision mechanisms (Da Ines et al., 2014). These studies have clearly demonstrated collectively the importance of NHEJ and alternate NHEJ pathways for repairing of DSBs. Moreover, the rate of homologous recombination based repair is only 0.01% while much more frequent repair processes occur in intrachromosomal recombination (Roy 2014). In majority of repair processes, where a break is flanked by repeat sequence, the preferred mechanism is NHEJ which indicates the significance of this repair pathway in plants (Waterworth 2011). However, lack of substantial evidences on importance of NHEJ in repair of DSBs during meiosis in plants indicates a research gap which is required to be filled by in-vitro as well as in-vivo studies (Da Ines et al., 2014).

NHEJ plays a crucial role in repairing of DSBs induced as DNA damages. Examinations conducted in plants and higher eukaryotes reflect the significance of this repair pathway due to occurrence at rapid rate however, research is poorly conducted to reveal the importance of NHEJ during meiosis. According to investigations, certain blocking signals in multicellular eukaryotes are examined during

meiosis, inhibiting the activity of error prone NHEJ during meiosis. Moreover, evidences suggest the role of HR to be crucial for repairing of DSBs induced during crossover at meiosis, and to prevent the integrity of genome content of daughter cells. Nevertheless, significance of NHEJ as a DSB repair mechanism in recent investigation is attributed to its frequent activation despite its error-prone nature.

Proteins and function in the NHEJ pathway

1-Detection of the DSB and binding to the DNA ends

The DSB is detected by the Ku complex. The subunits of the Ku complex are composed of Ku70 and Ku80, contributing towards central DNA-binding core (Fell *et al.*, 2012). Ku complex binds to the DSB ends might contribute in securing the broken ends with each other. When Ku complex bound to the DSBs, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) can be recruited. The interaction of DNA-PKcs with the Ku complex stimulates the serine/threonine kinase activity of the DNA-PKcs, thus, promoting phosphorylation across the DSB (Mahaney *et al.*, 2009; Burma *et al.*, 2006).

2-DNA-ends processing

The enzyme involved in DNA-end processing is Artemis, which possess a 5'→3' exonuclease activity. In the presence of DNA-PKcs and ATP, Artemis gets activated its endonuclease activity towards dsDNA/ssDNA (Davis *et al.*, 2013)

3-DNA-ends ligation

After the DNA-ends processing, ligation is initiated to repair the DSB. DNA ligase IV complexed with XRCC4 to initiate the ligation of the pre-processed DNA-ends (**Figure 5**) (Mahaney *et al.*, 2009 and Burma *et al.*, 2006).

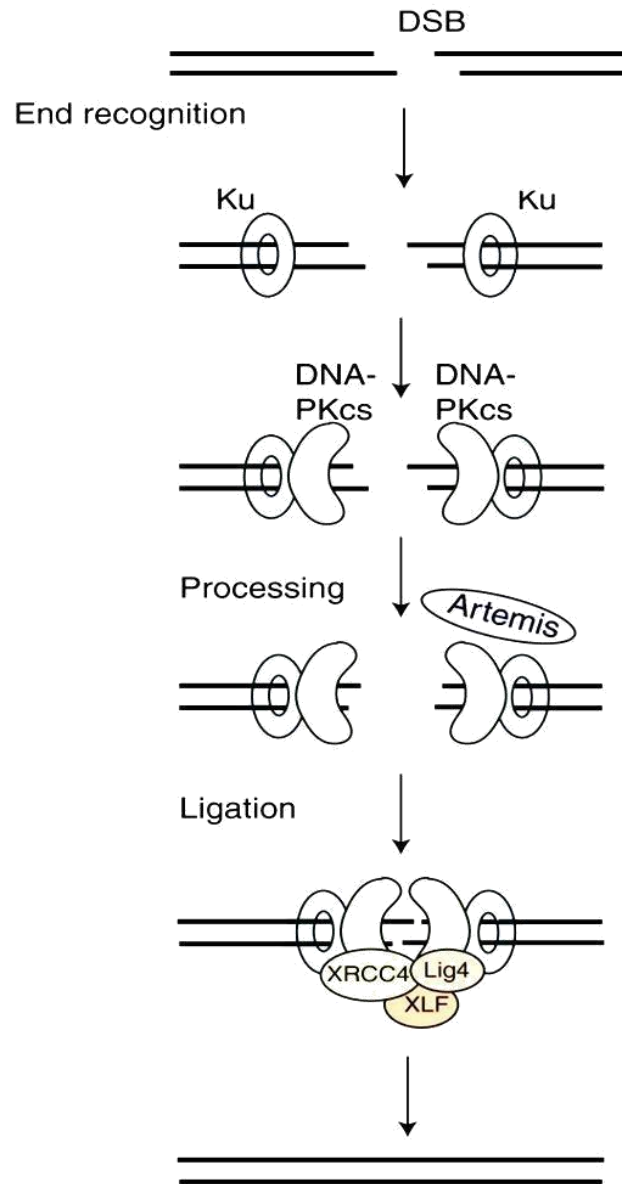


Figure 5: DSBs repair by NHEJ.

Ku70/80 heterodimer recognise the DNA ends, DNA-PKcs is recruited. If the DNA ends are not compatible, Artemis are nucleases that can cut the DNA ends. Ligation the DNA ends by XRCC4, DNA Ligase IV, and XLF. (Adapted from Brandsma & van, 2012)

Homologous recombination (HR)

Homologous recombination (HR) requires a template to repair the DSBs and is the most conservative repair pathway, typically used when cells enter S phase and G2 phase (Truong *et al.*, 2014). Homologous recombination (HR) is crucial during the DNA repair Process in mitosis as well as chromosomal pair and recombination during meiosis. Still, some HR forms can also result in unwanted DNA rearrangements. Multiple regulatory Processes have been evolved to guarantee that HR occurs correctly in terms of time, place, and method. Several processes act via control of Rad51 nucleofilaments, known to be a vital player in HR. The process occurs where some factors favor the development of these structures whereas, others caused the disassembly or usage of alternative methods for the DNA repair purpose (Krejci *et al.*, 2012).

Importance of homologous recombination in meiotic recombination

Meiotic recombination has an important role in allowing the re-assortment of genes into different combinations. This results from the exchange of homologous chromosome fragments during meiosis. This, meiotic recombination increases genetic diversity (Cooper, 2000). The connection of HR repair and meiosis was identified, and DNA DSBs were found as the initiators of meiotic recombination (Sun *et al.*, 1989). Crossing over between homologous chromosomes is known as Chiasmata which lead to the exchange of genes between the two homologous chromosomes. Generation of chiasmata is made possible after the formation of DSBs allowing genetic information exchange between paternal and maternal chromosomes that could be passed to the next generations. Chiasmata formation is an important source of genetic variation in diploid organisms (Hartl and Ruvolo, 2011).

The Single-Strand Annealing (SSA)

The single-strand annealing (SSA) pathway can normally repair DSBs that are found between direct repeats of 50bp 200bp (Paques and Haber 1999, Sugawara *et al.*, 2000). After processing the ends of the DSB an ssDNA tail is produced, if this resection is enough to detect homologous single-stranded repeat sequences, the complementary ssDNA will anneal and form heterologous flaps (always if the repeats are isolated from the break region by the required sequence) (**Figure 6**). Nucleases will then remove these flaps to fill the gap and ligate the ends (Fishman-Lobell *et al.*, 1992; Ivanov and Haber 1995). The SSA could be a source of mutation because it can produce some deletions during the process of repairing the DSB (Putnam *et al.*, 2005).

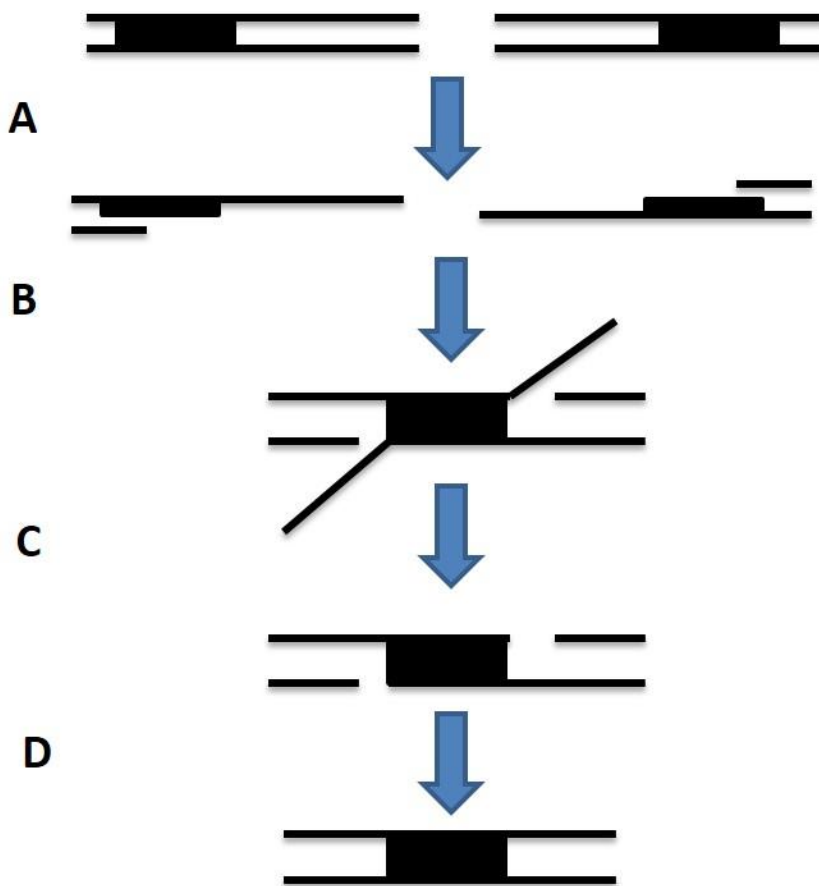


Figure 6: The Single-Strand Annealing repair pathway (SSA).

(A) 5' to 3' resection of DNA ends. (B) Annealing. (C) Flap cleavage. (D) Gap filling and ligation. (Adapted from Symington *et al.*, 2014).

Meiosis

Meiosis is a specialized cell division allowing sexual reproduction to be possible. Meiosis end products include haploid cells, containing only one member of each pair of homologous chromosomes. In all animals, specialized cells termed as germ cells, go through meiosis to produce haploid gametes, which further fuse during sexual reproduction, creating new diploid embryos. Meiosis yields genetic diversity by utilizing two mechanisms (Griffiths *et al.*, 1999):

- i. During meiotic divisions, there is an independent assortment of chromosomes
- ii. Crossing over allows some chromosomal regions to be exchanged.

By these processes a new chromosomal reorganization can be created, thereby generating genetically diverse gametes from a single individual.

Stages of meiosis

Meiosis is divided into two meiotic divisions, from an initial replication of DNA through different stages until the formation of four haploid gametes (**Figure 7**).

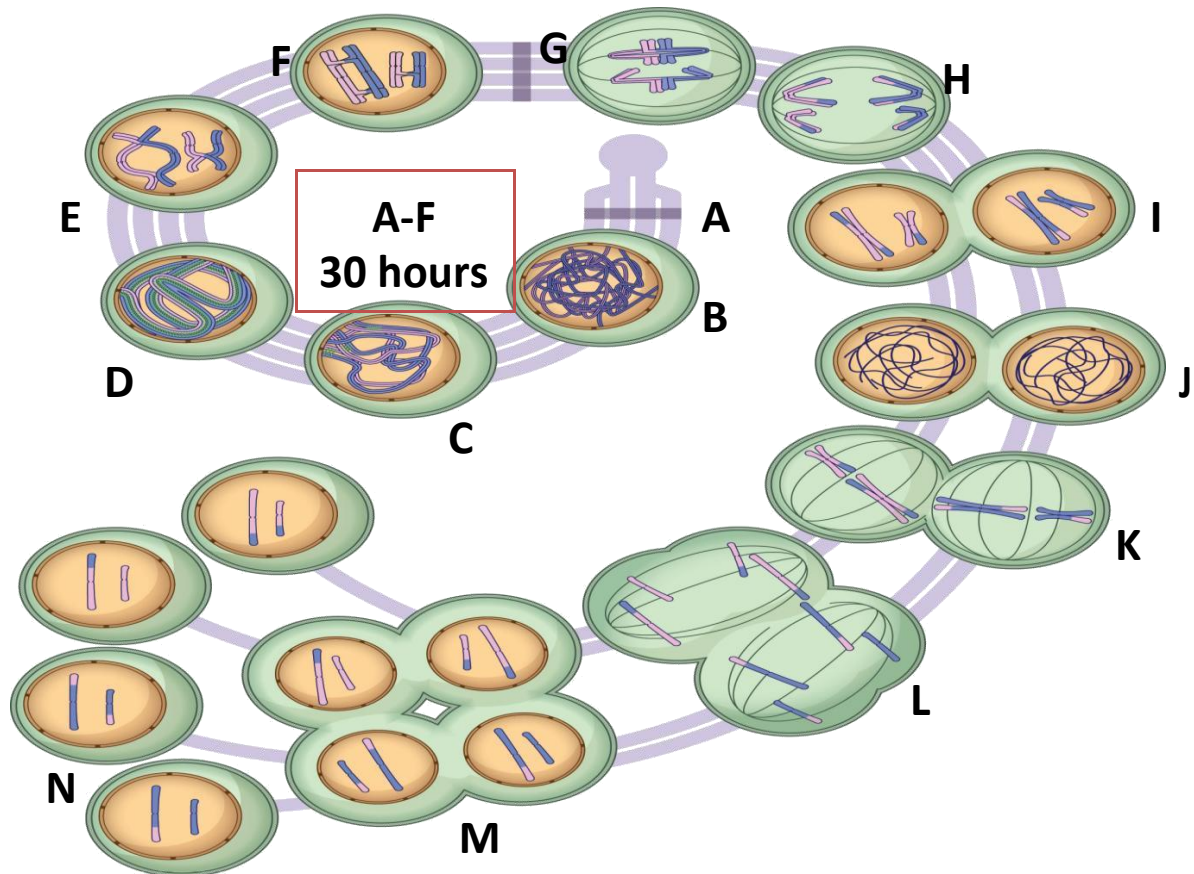


Figure 7: Stages of meiosis.

(A) S phase, in this stage the DNA is replicated to create sister chromatids for each chromosome. (B) Leptotene: the initiation of the condensation of chromosomes which appear as a thin and a long thread. (C) Zygotene: the initiation of the homologous chromosomes pairing and synapsis by the synaptonemal complex or SC. (D) Pachytene: chromosome pairing and synapsis are fully completed and maturation of crossing over (CO) that leads to the arrangement of chiasma (physical perception of COs). (E) Diplotene: The homologous chromosomes desynapse as a result of the gradual depolymerisation of the SC components, homologous chromosomes are held by the recombination regions (crossovers) that physically visualised as chiasmata. (F) Diakinesis: homologous chromosome pairs which are known as bivalents are more condensed and visible. (G) Metaphase I: The homologous chromosomes line-up in pairs on the midplane of the cell and the centromeres of the homologous chromosomes are attached to the microtubules of the meiotic spindle. (H) Anaphase I: The homologous chromosomes separate and segregate to opposite poles. (I) Telophase I: The chromosomes decondense and nuclear membrane reforms around the haploid number of chromosomes. (J) Prophase II: The chromatids of each chromosome recondense and chromosomes start to be visualised. (K) Metaphase II: The chromosomes are attached to the microtubules the center of the cell to allow the co-orientation of the sister chromatids. (L) Anaphase II: Spindle fibers attach to the sister chromatids to allow their separation to opposite poles. (M) Telophase II: The chromosomes decondense and nuclear membrane reforms around the haploid number of chromosomes and the cytokinesis occurs. (N) The result of meiosis is four daughter cells (From Mercier *et al.*, 2015).

Recombination occurs during the prolonged prophase of meiosis I

Prophase I is the longest and most vital section of meiosis, as the process of recombination occurs during this phase of meiosis. Prophase I is categorized into multiple stages based on the appearance of the meiotic chromosomes. The prophase sub-stages are leptotene, followed sequentially by the zygotene, pachytene, diplotene, and diakinesis stages. Some of the molecular events responsible for the rearrangements of chromatin occur during these sub-stages of prophase I (**Figure 8**). During leptotene, chromosomes start to be further condensed in the nucleus, and they appear thin and long (La cour and Wells, 1971). During Zygotene, homologous chromosomes start pairing, and synapsis by the polymerisation of the synaptonemal complex proteins (SC) commences. During zygotene, the SC proteins begin polymerization in a lineal manner along the homologous chromosomes, and the synapsis is fully completed at pachytene. Electron microscopy has used to study the SC as a tripartite proteinaceous structure, including two external chromosome axis or lateral elements (LEs), also known as axial elements (AEs), and a central element (CE) which are formed by transverse filaments (Roeder, 1990; Schwarzacher, 2003; Higgins *et al.*, 2005). At diplotene, the SC starts gradually depolymerising, and the homologous chromosomes begin to separate from each other holding only at the sites where homologous recombination have occurred or crossovers (COs) regions(Sanchez Moran *et al.*, 2001). During diakinesis, homologous chromosome pairs (bivalents) continuously condense, and pairs of chromosomes (bivalents) are observed.

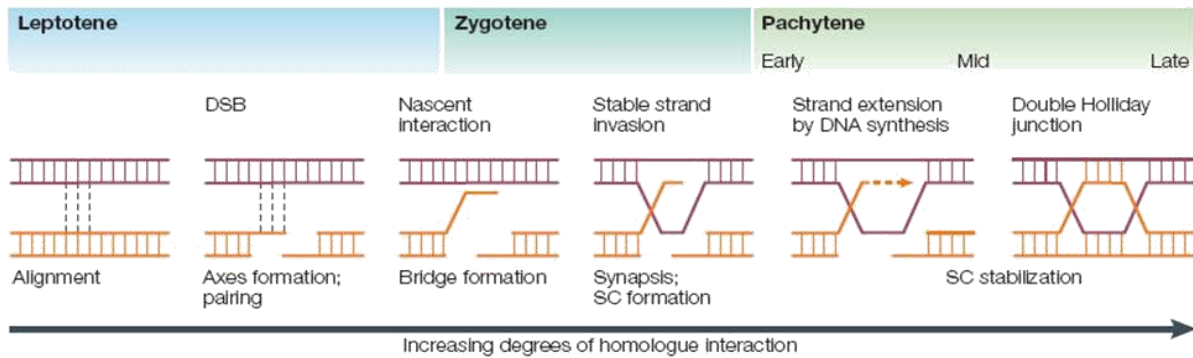


Figure 8: Homologue interactions during meiosis.

Following the generation of DSBs, one DNA strand is processed, thereby leaving a 3'-overhang, this single-stranded tail invades the homologous sequence on the homologous chromosome, this activates the development of the SC around the paired homologs and polymerization of the SC axis and central element until full synapsis is obtained at pachytene. The stability of the SC rises with the invading strand primarily extends into the homolog and is then recaptured by the damaged chromatid, establishing a double Holliday junction which could mature into a crossover site (From Gerton and Hawley, 2005).

DSBs formation during meiosis by SPO11

The beginning of meiotic recombination is favoured by the SPO11 multi protein complex, i.e., the meiotic-specific trans-esterase Spo11 along with other accessory proteins that generate DSBs within the homologous chromosomes. The ability to form DSBs at specific sites known as hotspot regions are 100-1000 times greater compared to other regions. In budding yeast, the accessory proteins include Rec114, Mer2, and Mei4 to bind to the axis, protein sites within chromosome followed by the formation of a complex with DSBs, thereby, generating a pre-DSBs recombinosome. The polymerization of SPO11 accessory proteins involve Rec102, Rec104, and Ski8, allowing DNA binding and nuclear retention (Arora *et al.*, 2004 and Panizza *et al.*, 2011). Other Spo11 accessory proteins known as RMM are the group of proteins including Rec114, Mer2, and Mei4, which form a sub-complex that partially localize on the chromatin and favor the binding of Spo11 to the DNA cleavage sites (Panizza *et al.*, 2011).

DSBs formation during meiosis by SPO11 in yeasts, *C. elegans*, and plants

In *Saccharomyces cerevisiae* (budding yeast), meiotic recombination is a consequence of mainly nine gene products, which are meiosis-specific. Among them, six are well established, i.e., *SPO11*, *MEI4*, *MER2*, *REC102*, *REC104*, and *REC114*, which code for proteins involved in the formation and following processing steps of DNA double-strand breaks (DSBs) during meiosis. Other proteins in yeast may be known to act as modulators in the DSBs formation (Keeney *et al.*, 1997). The DSBs and recombination events are thought to be conserved. Similarly, in *C. elegans*, DSB-generating enzyme SPO11 has also been found and is required for meiotic recombination and formation of DSBs. In worms, primarily SC formation is thought to be a requisite for homologous synapsis in the stabilization of pairing rather than DSBs as in yeasts. The deletion of recombination promoting regions does imply the defects in the pairing of chromosomes in *C. elegans* (Dernburg *et al.*, 1998). In *C. elegans*, meiotic recombination is eliminated in the absence of SPO11, but SC is formed between the homologs normally. The single SPO11 homolog is known to be present in yeasts and animals, but in *Arabidopsis thaliana* as well as in other higher plants two or more copies are present. SPO11-1 and SPO11-2 are known to initiate meiotic recombination and SPO11-2 are also necessary for proper chromosome segregation, whereas SPO11-3 role towards somatic endoreduplication has reported (Hartung *et al.*, 2007).

DSB Introduction by SpO11 and SPO11-1 Oligos Formation

For the meiotic recombination to occur, formation of double stranded breaks and their repair is essential. These DSBs are formed by evolutionarily conserved proteins called SPO11. The protein has also been recognised as a relative of archaeal topoisomerase. According to in vivo studies, expression of SPO11 has been examined through real time PCR (Doksani & de Lange 2016). Along with Mre11, NBS1 and Rad50, SPO11 forms a protein complex during meiotic recombination. In the structure of SPO11, it contains an

active site with a tyrosine which undergoes ligation and dissociation with DNA during the break formation process. Per strand of DNA is induced with a break by one SPO11 protein thus requiring two proteins to induce DSBs in the duplex. SPO11 has been recognised to share sequence similarity to the type-II topoisomerases' subunit A of archaea origin (Yeh *et al.*, 2017).

DSB catalysis by SPO11 is also reported to be catalysed in the same way as TOPO-IV. The dimerisation of the SPO11 is the major and significant step that brings about and facilitates the induction of DSBs. Dimerisation of SPO11 results in formation of two intermolecular DNA cleavage sites with each site containing the 5Y-CAP domain and a Mg²⁺ coordination site. The catalytic tyrosine residue resides in the 5Y-CAP domain of the protein. DSB created with a two nucleotide 5'overhang and a linked catalytic tyrosine is present within each monomer's active site attached with 5'ends. DSB induction of formation efficiency by the enzyme is affected and reduced by the mutation in of this residue (Doksani & de Lange 2016). SPO11 becomes covalently attached to the 5'ends of the breaks during induction of DSB when meiosis occurs. Single stranded endonucleolytic cleavage removes the SPO11 from the DSB thus resulting in the liberation of short lived species. This species contains a covalently linked SPO11 which is associated with a short oligonucleotide. Studies have been extensively conducted on budding yeast *Saccharomyces cerevisiae* and the SPO11 forms a DSB by generating a covalent and transient protein-DNA intermediate (Mehta & Haber 2014). Shorter form oligos observed in yeast is recognised to be 12 nucleotides long while the longer form of oligos ranges between 21 and 37 nucleotides (Carofiglio *et al.*, 2018). A positive relationship has been observed and reported between crossovers and SPO-11-1-oligos on the genome-wide scale while the correlations are observed weaker (Yamada *et al.*, 2017).

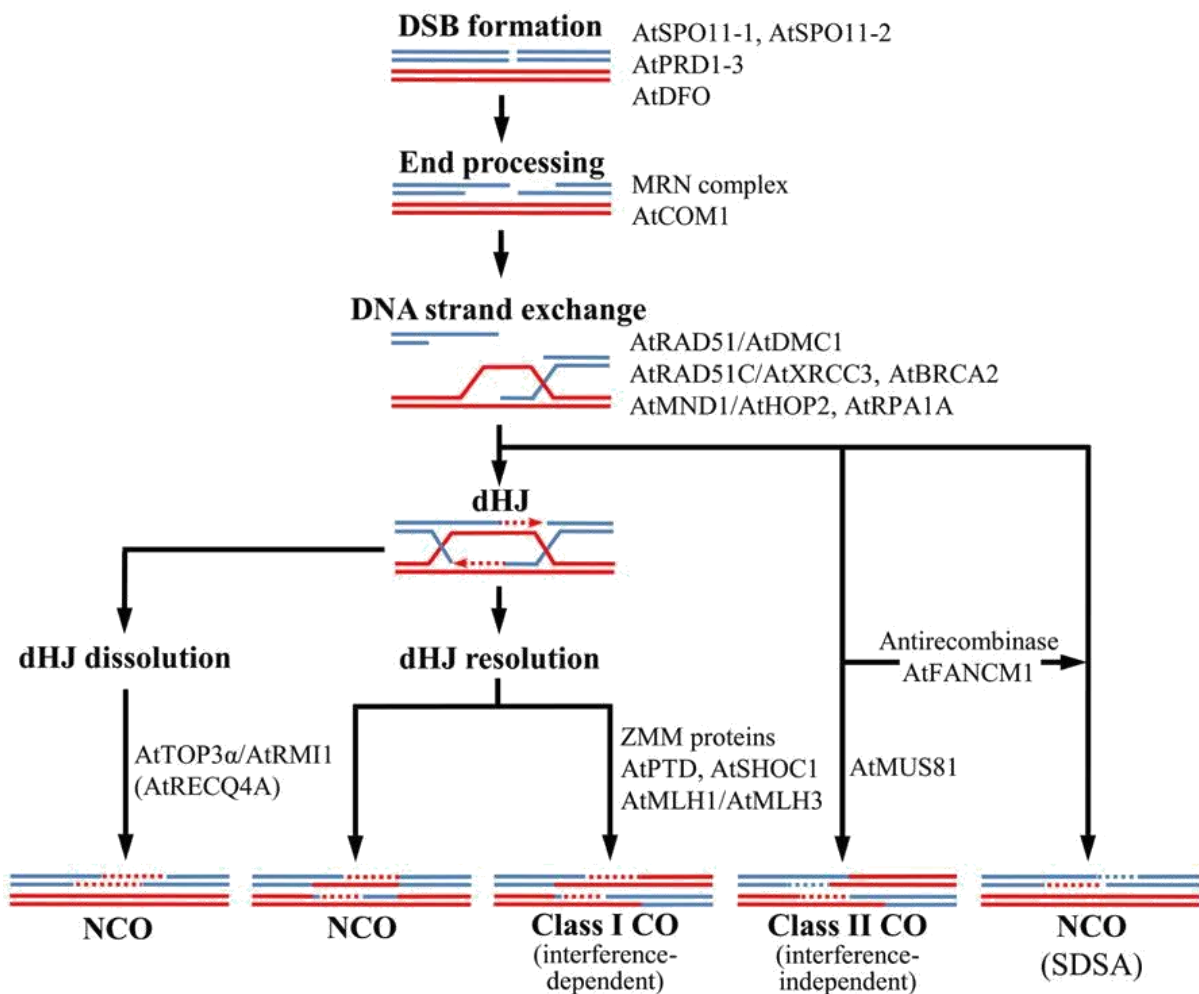


Figure 9: Model for meiotic recombination in *Arabidopsis thaliana*.

Meiotic recombination begins by the production of DSBs which get process into 3' single-stranded DNA (ssDNA). These ssDNA tails can invade the homologous chromosome, resulting in forming a D loop intermediate which can generate an interference independent COs (class II COs) which produces around 15% of total COs. Alternatively, after the formation of the D loop structure, a double Holliday junction (dHJ) can be formed, and if the second end of the DSB is captured and the strands of broken DNA are ligated could produce an interference dependent CO (class I). Most of the NCOs are likely produced by synthesis-dependent strand annealing (SDSA), when the D loop is broken before the second end capture but can also be formed by dHJ resolution (From Pradillo *et al.* 2014).

Induction of Artificial DSBs.

Different factors are present that can introduce specific and unspecific DSBs within DNA, including ionizing radiation such as γ -rays or certain chemicals like cisplatin. The purpose of introducing artificial DSBs is to study and understand the different repair pathways and the components affecting these processes (Puchta *et al.*, 1996). Meiosis is a great model to study DSB repair pathways and meiotic DSB processing (Sun *et al.*, 1989).

1. Induced DSBs by X-ray and Gamma radiation

To produce DSBs different ionizing radiation has been used (Prise *et al.*, 2001). Gamma rays (γ -rays) have a significant impact on DNA as it can produce clustered damages in the DNA. The high energy of γ -rays produces oxidized pyrimidine and DSBs in the genome. Oxidized pyrimidine clusters and oxidized purine clusters can also be induced through X-ray in human cells producing DSBs to be able to repair this damages (Gunderson & Tepper, 2015).

2- Induced DSBs by Zinc finger nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are artificial constructs carrying an endonuclease activity domain normally from the function of *FokI* and several zinc finger DNA-binding domains which together would specifically recognise a DNA sequence which could be targeted by the endonuclease action (Kim *et al.*, 2001). Dr. Daniel Voytas laboratory have constructed ZFNs in *Arabidopsis thaliana* to be able to do Gene Targeting (GT) in plants (Zhang *et al.*, 2010)

.The endonuclease activity found in ZFNs (mostly *FokI* dimerization) induce DSBs which can be subjected to DNA repair leading to both directed gene replacement and targeted mutagenesis at considerably high frequency (Mori *et al.*, 2009). ZFNs have proved to be one of the most effective and versatile classes of targeting techniques, exhibiting separate DNA-cleavage and DNA-binding domains (Urnov *et al.*, 2010).

FokI- Induced DSB

DSB formation through certain restriction enzymes has also been performed. One of the dimeric type-II restriction enzyme is FokI which has been isolated from *Falvobacterium okeanokoites*. FokI recognises a sequence to introduce double stranded cuts in the DNA. The identified recognition sequence reported in literature is 5'-GGATG-3' and the enzyme makes cut approximately 9 nucleotides away from 3' end of the recognition sequence

within the top strand of DNA. Contrarily, in complementary strand, cut is made approximately 13 nucleotides away from the 5' end. Digestion by FokI thus results in formation of DSBs in DNA collectively creating 4 nucleotide long overhangs which are also called sticky ends (Sears & Turchi 2012).

The pentanucleotide sequence specifically recognised by FokI reflects its nature of specificity. FokI is a type II restriction enzyme for which, the cleavage sequence is generally asymmetric in nature and defines the enzyme's cleavage site specificity. Part of the nucleotides of recognition sequence of FokI are shared its specificity for two other type-II restriction endonucleases (Yeh *et al.*, 2017).

Similarities and Differences of DSBs made by SPO11 and *FokI*

DSBs are induced by both proteins i.e. FokI and SPO11 and both enzymes are produced and expressed naturally. Both proteins are recognised as endogenous DSB inducers, with different DNA cleavage mechanism. Dimerisation is the common feature observed in FokI and SPO11 resulting in induction of cuts in both strands of DNA. However, DSBs are induced by SPO11 during meiosis which is an essential phenomena meiotic recombination while induction of DSBs through FokI can be performed for examinations and investigations for various purposes, apart from meiosis. In case of FokI, binding of nuclease to the DNA and sequestration of DNA binding domain is necessary which results in a conformational change upon binding. Moreover, cleavage requires a magnesium ion while no certain co-factor is required for the activity of SPO11 (Neale & Keeney 2009).

Another major difference between DSB inducers FokI and SPO11 is their source of isolation. FokI is produced by *Falvobacterium okeanoikoites* naturally for digestion of viral genomes during infection. SPO11 is expressed in testes and ovaries of mice as well as in somatic tissues of human, essentially during meiosis. SPO11 requires formation of a complex by fusing with other proteins such as Rad50, NBS1, and Mre11. Studies confirming the structure of SPO11 indicate the presence of CAP-like domain which

catalyses the DSB induction activity through ATP binding and hydrolysis. Contrarily, FokI is reported to contain two domains; a DNA binding domain and a DNA cleavage domain. The DNA binding domain acts through recognition of specific sequence by a recognition domain and a conformational change in binding domain. A dimer interface formation is essential by the DNA cleavage domain for cleavage to occur (Doksani & de Lange 2016).

Exogenous DSB Inducers versus *FokI*/SPO11

DNA damage can result as a consequence of replication stress and cellular endogenous metabolic reactions or can be induced by the exogenous sources such as chemotherapeutic agents and radiation. Damage induced by exogenous sources has been identified in several different forms such as DNA-protein cross-links, base lesions, inter and intra-strand cross-links and induction of both single and double stranded breaks (DSBs) (Woods & Turchi 2013). A class of topoisomerases inhibitors has been identified among chemotherapeutic agents such as etoposide and camptothecin; which induced DSBs and SSBs. This induction of breaks in DNA is largely dependent on trapping of covalently linked DNA cleavage complexes which appear in the form of topoisomerase-DNA cleavage complex (Sears & Turchi 2012). Among the potential exogenous DSB inducers, a potent chemotherapeutic agent has been developed called cisplatin. It is used for the treatment of various cancers such as ovarian, testicular, lung and neck cancer since decades. Contrary to the activity of FokI and SPO11 for induction and catalysis of DSB, cisplatin interacts directly with multiple components of the cell; nucleic acid, thiol-containing peptides and several proteins (Woods & Turchi 2013). Cisplatin-DNA adducts have been recognised a major cause of induction of cytotoxicity in cell due to induction of DSBs. Distortion of DNA double helix occurs primarily through covalent binding of cisplatin with purine bases. This further result in formation of intra-, inter- and mono-strand adducts. DSBs induced by cisplatin are different mechanism of induction as well as the nature of ends and can block DNA replication and transcription (Mehta & Haber 2014).

3- Induced DSBs by cisplatin

DSB repair is recognised as a critical cellular mechanism ensuring genomic stability. To cells' genetic content, DSBs are the most deleterious type of insult, which can lead to apoptosis, genomic instability and senescence (Bueno et al., 2018). Pathological consequences may also generate DSBs which are important to be recovered by cellular DNA repair pathways, and may potentially lead to cancer if poorly repaired (Woods & Turchi 2013).

DSB can be induced by exogenous agents such as chemotherapeutic agent, cisplatin. Significant tumor cytotoxicity has been reported in studies caused by cisplatin-induced DSBs and Rad-50 mutant cells of human squamous cell carcinoma cells (Cole *et al.*, 2010).

According to reports, similar DSBs have been observed to be induced by cisplatin as in case of SPO11. Examinations conducted on mice for SPO11 expression during prophase I progression report the restoration of DSB induction and partial prophase I progression by cisplatin treatment, reporting similar DNA lesions as induced by SPO11 (Doksani & de Lange 2016). In another study performed on yeast strain report mutation of *spo11* gene poorly repair a meiotic defect which had been induced through X-radiation (Cole *et al.*, 2010). Findings of the study indicate the potential of exogenous DSB induction can partially substitute for SPO11 activity for DSB introduction (Shanbhag *et al.*, 2010).

Activation of the ATM pathway for DSB induction is however distinct and different from DSB induced by exogenous agents and chemotherapeutic drugs (Yamada *et al.*, 2017). *FokI* utilises ATM pathway and generation mechanism to induce DSB which is efficiently repaired by the major two cellular DNA damage repair pathways; HR and NHEJ (Jekimovs *et al.*, 2014). Transcriptional activation by *FokI* based ATM activation during S-phase of the cell allows recognition and binding of *FokI* domains at recognition site and specific cleavage, which has been observed to distinct from DSB induced by exogenous agents (Doksani & de Lange 2016).

Aims of the project

Double strand breaks (DSBs) are serious damages in genomic DNA that could lead to cell

death and chromosome instability (Bueno *et al.*, 2018). DSBs can be occurred by endogenous and exogenous factors (Reynolds *et al.*, 2012). Homologous recombination (HR) and non-homologous end-joining (NHEJ) are the main pathways to repair DSBs (Truong *et al.*, 2014). In mammals and plants, non-homologous end joining (NHEJ) is a preferred pathway to repair DSBs. The involved proteins in NHEJ are the Ku complex (Ku70 and Ku80 proteins), the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), XRCC4 and DNA Ligase IV (Jiang *et al.*, 2015). DNA protein kinase (DNA-PKcs) work as the catalytic subunit of a nuclear DNA- dependent serine/threonine. A previous study concluded that DNA-PKcs is also required for the NHEJ pathway to binding the ends of DSBs (Goodwin *et al.*, 2015). In this project we have investigated to characterize , identify, locating, and analysing the mutants of DNA PKcs in *Arabidopsis thaliana*, which is an unexplored area in terms of the plant to date.

All higher Eukaryotes that reproduce sexually encode for a *SPO11* gene, however plants appear to have two genes *SPO11-1/SPO11-2* that catalyse meiotic DSBs to initiate meiotic homologous recombination (Hartung and Puchta, 2000). The result of homologous recombination can be either COs or NCOs. At least one formation of CO is required for holding homologous chromosomes until they are segregated to opposite poles at anaphase I (Jones & Franklin, 2006). The *Arabidopsis thaliana* DSB accessory proteins are not conserved as between other species and that are functionally diverse. In *Arabidopsis thaliana*, *SPO11-1/SPO11-2* intermediate DSB formation by interacting with different accessory proteins such as MTOPVIB, PRD1, PRD2, and PRD3(Tang *et al.*, 2017). PRD2 is known to possess several motifs as in DNA-binding proteins, so it can have a role in promoting DSB formation after binding to the chromosomes (De Muyt *et al.*, 2009).

In the present study, we have analysed a *prd2* mutant which presents a hugely reduced number of DSBs, which lead to the formation of a residual number of crossovers. We have started to analyse the different stages of meiotic recombination processing this small amount

of DSBs compared to WT and a null *spo11.2* mutant where DSBs are not catalysed. Furthermore, we have used Zinc Finger Nucleases (ZFNs) to induce DSBs in specific sequences in the *Arabidopsis thaliana* genome (on *TT4* and *ADH1* loci) in a *spo11.2* null mutant background. Using an inducible system to express the ZFNs and produce a specific targeted DSB during meiosis has allowed us to study the different outcomes of these DSBs. This study will produce new insights into the control of meiotic recombination in plants.

CHAPTER TWO

MATERIAL AND METHODS

Plant materials

Ecotype Columbia 0 (Col0) was used as *Arabidopsis thaliana* wild-type (WT) plants in this study. T-DNA insertion mutant lines (Table 1) were searched from the Salk Institute (<http://signal.salk.edu/cgi-bin/tdnaexpress>) (Alonso et al., 2003) and obtained from European *Arabidopsis thaliana* seed stock center (NASC; <http://arabidopsis.info/>). ZFNs were obtained from Dr. Daniel Voytas.

Growth conditions

Seeds were vernalized for around 48 hours at 4°C and subsequently planted on soil and kept in a glasshouse with organized temperature (18°C), humidity and light conditions (16 hours light cycle).

Name	Type	Gene Locus	Line	Phenotype
Columbia	Ecotype	-		Wilde type
<i>SPO11.2</i>	T-DNA mutant	AT1G63990	GK08409	-severe sterility -Defects in synapsis during meiosis I and reducing meiotic recombination -No chiasmata -univalent chromosomes in meiosis at metaphase I
COL-ZFN3	Transformed WT	AT1G77120	From (Feng Zhanga <i>et al</i> , 2014) http://www.pnas.org/sci-hub.club/content/107/26/12028.short	Wilde type
COL-ZFN5	Transformed WT	AT5G13930	From (Feng Zhanga <i>et al</i> , 2014) http://www.pnas.org/sci-hub.club/content/107/26/12028.short	Wilde type
<i>DNA-PKa</i>	T-DNA mutant	AT2G17930	N666820	reduce fertility
<i>DNA-PKb</i>	T-DNA mutant	AT4G36080	N655803	reduce fertility

Table 1: The details of genes that used

Data software and programs

Name	Use	Link
Basic Local Alignment Search Tool (BLAST)	For obtaining biological sequence information, such as the nucleotides of DNA sequences.	http://blast.ncbi.nlm.nih.gov/Blast.cgi
The National Center for Biotechnology Information (NCBI).	Resource for bioinformatics tools such as GenBank for DNA sequences and PubMed.	http://www.ncbi.nlm.nih.gov/
Excel	For statistical analysis and drawing graphic formats	Microsoft Excel

Table 2: The list of software and programmers

Genetics Analysis Materials and methods.

Lysogeny Broth Agar (LBA Media)

LBA media contains mineral and vitamins, casein peptones, peptides, and trace elements, that are required for plant growth. Agar is a complex of gelatinous carbohydrate. It forms a gel to make plant able to grow on the media.

B-estradiol used to induce the DSBs:

B-estradiol is a steroid and estrogen sex hormone protein with importance in control the estrous and menstrual female reproductive period, and it is activated XVE stimulated the expression of proteins under the control of the target promoter in transgenic plants (Yau & Stewart, 2013). ZFNs are induced by *B-estradiol* to induce meiotic DSB in the specific targeted genomic sequence. The method of treatment was by brushing flower buds with

The β -estradiol solution at different concentrations 50 μ M /100 μ M /200/ μ M /300 μ M /500 μ M (**Figure 10A**). The timing for the maximum expression of the ZFNs induced by *beta-estradiol* which seems to be around two days (Schlucking *et al.*,2013) and the timing that convert DSBs to COs it might take up to 1 day. Different samples at different times have been treated and found out that 2 to 3 days is the best to fix material for immunolocalisation and cytological analysis (**Figure 10B**).

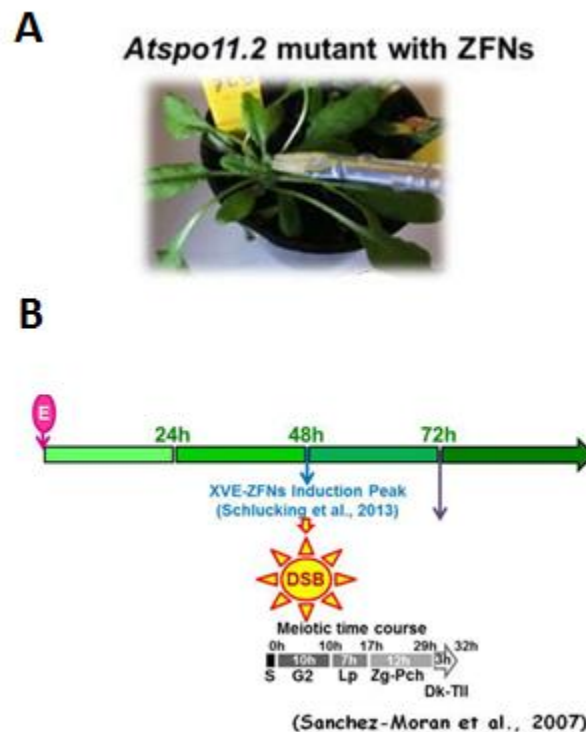


Figure 10: A-Brushing the flower buds with β -estradiol to induce the DSBs.

B-The timing of ZFNs expression and the timing for meiotic stages

Cisplatin

Cisplatin is known as cis-dichlorodiamineplatinum. It could be cross-linked with the cancer cell DNA in this manner wrecking the DNA work. It can shape intra-strand or between strand crosslink with the DNA and could make a link with DNA and protein, and can repress cell mitosis (Kartalou & Essigmann, 2001).

Bleomycin sulfate from *Streptomyces verticillus*

Bleomycin sulfate ties to DNA which is one of the reasons for ssDNA breaks at particular base successions and represses DNA amalgamation. Bleomycin requires to tie oxygen and a metal particle. Bleomycin could cleave RNA, but with a lower degree and more specifically. Bleomycin works as an inducer and controller of apoptosis and restrains tumor angiogenesis.

Bleomycin is a chemotherapeutic that induces single-strand breaks and DSBs (Burger *et al.*, 1981).

Chemical	Source
<i>B-estradiol</i>	TORIS, UK https://www.tocris.com/products/b-estradiol_2824
Bleomycin sulfate from <i>Streptomyces verticillus</i>	Sigma, UK https://www.sigmaaldrich.com/catalog/product/vetec/v900935?lang=en&region=GB
Cisplatin	Sigma, Uk http://www.chemicalbook.com/ChemicalProductProperty_EN_CB9236183.htm

Table 3: The list of drugs that used for DSBs induction

DNA extractions.

Leaf discs were taken from plants and placed inside 1.5ml sterile micro tubes. They were kept on ice throughout this procedure. Each tube was added with 40 μ L of extraction buffer. The freshly prepared extraction buffer comprised of 200mM Tris HCl Ph 7.5, 250mM NaCl, 25mM EDTA and 0.5%SDS. With the help to plastic grinders, leaves were ground directly in the microtubes. It was then vortexed for around 5 seconds by adding extra 400 μ L of extraction buffer. Samples were then centrifuged for 5 minutes at 13,000 rpm. The pellet containing the cellular remains was disconnected from the supernatant comprising the genomic DNA and pellets were rejected. 400 μ L of the supernatant from all samples was transmitted to new microtubes. After that, 400 μ L of cold isopropanol was added to each sample. This was done to allow DNA precipitation. The tubes were then mixed gently for 2 minutes at room temperature and rest on the bench. For another 10 minutes, samples were centrifuged at 13,000 rpm, and the supernatant was discarded. Freshly made 400 μ L of 70% ethanol was added to wash the pellet DNA. The samples were then centrifuged for 5 minutes at 13,000 rpm, the supernatant discarded and left for 30 minutes to dry on the bench. After that, 50 μ L of sterile deionised water (SDW) was added to each tube to wholly liquefy the DNA pellet. Then, they were incubated in the oven at 65°C for 10 minutes and then finally stored at -20°C.

mRNA extraction:

The protocol of the RNeasy Mini Kit from QIAGEN has followed to extract mRNA from both leaves and flower buds. Firstly, purify the RNA, adding 10 μ L of β - mercaptoethanol (β -ME) or 20 μ L 2 M dithiothreitol (DTT) to 1 ml RLT buffer, and adding 4 volumes ethanol to RPE buffer. After that, 1 volume of 70% ethanol to lysate is added to the sample and mixing by micropipette. Then, transfer 700 of the mixture to RNeasy tubes for centrifuging at 8000 rpm for 15 seconds. Discarding the flow-through and adding 700 μ L

RW1 buffer. Another centrifuging for 15 seconds at 8000rpm and discarding the flow-through. Adding 500 μ L RPE buffer and centrifuging for 15 seconds at 8000rpm, and the flow-through is discarding. Another, 500 μ L RPE with centrifuging at 8000 rpm for 2 minutes. Moving the RNeasy tubes are then transferred to a new 2 ml tube and adding 50 μ L sigma water and centrifuging at 8000rpm for 1 minute to collect the RNA (QIAGEN, 2017).

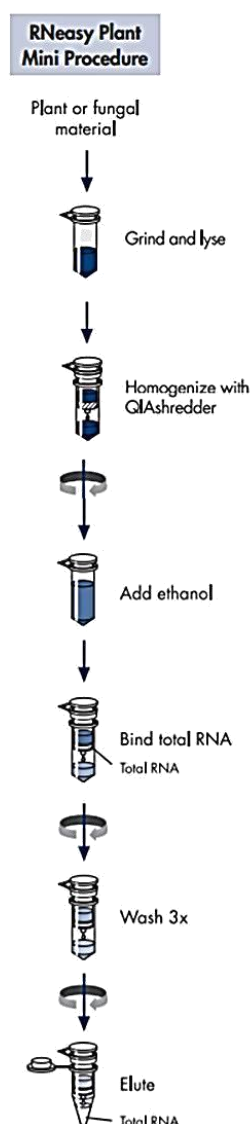


Figure 11: The steps of extraction RNA from plant materials such as flower buds and leaves. This figure is from <http://www.bea.ki.se/documents/ENRNeasy%20handbook.pdf>.

Polymerase chain reaction (PCR)

PCR is utilized in diagnostic scan and research largely. PCR method is utilised to generate greater one or more DNA templates for the duration of numerous cycles. Thousands of DNA can also be created from one DNA molecule. PCR is utilized for genotyping the distorted lines. The PCR reaction final degree was once 20 µl per tube (7µl SDW, 1µl forward primer (LP), 1µl reverse primer (RP), 1µl of DNA and 10µl of 2x Biomix. This reaction combination (20µl) was transmitted to a PCR device, the place we run for 30 cycles. The program for genotyping T-DNA intromission lines included: 1 cycle of 95°C for two minutes; 30 cycles of denaturalization at 95°C for 1 minute, annealing at 50°C for 1

A minute and then extension at 72°C for 2minutes; an ultimate extension cycle at 72°C for 4 minutes; and a limitless cycle at 4° C. The samples have been loaded in an agarose gel for electrophoresis.

Genes	Primers	Annealing Temperature
ZFN3	FP: TATAAATACCTAAAACAAACCTC	49 ° C
	RP: TAAGAAAGAAGATATGAACAAT	46 ° C
	LP-Tail4 - BP: GTTCTATAGGGTTTCGCTCATGTG	60 ° C
ZFN 5	FP : GTGATAAATTCTACATACCT	46 ° C
	RP: TTTATGGGTATTATTTGCTCACG	52 ° C
	LB-Tail4- BP: GTTCTATAGGGTTTCGCTCATGTG	60 ° C
SPOO11.2	FP: AGTGGCAAGTTTGTGTGGAG	55° C
	RP: AATCACATTCCTTGCGTGTTT	50 ° C
	GABI_039E09- BP: ATATTGACCATCATACTCATTGC	52 ° C
DNA-PKa	FP: GGTTCCTACCTCTTTGGTTGG	55° C
	RP: TCATTTTCGTGCACAGTGATTC	52 ° C
	Salk,T-DNA, BP: TGGTTCACGTAGTGGGCCATCG	61 ° C
DNA-PKb	FP : TTGAAGATGGTCTTCATGCC	52° C
	RP: GCCAACGGATACCTATCAGTG	55 ° C
	Salk,T-DNA,BP: TGGTTCACGTAGTGGGCCATCG	61 ° C
PRD2	FP :	
	AGCTCTAATTGTTGCGAGACG	57 ° C
	RP:	
	TTAAAGGAAATGCAGCTCAGG	55 ° C
	Sail,T-DNA,BP:	
	GCCTTTTCAGAAATGGATAAATAGCCT TGCTTCC	67 ° C

Table 4: The list of PCR premiers that used for genotyping the plants

RT-PCR	Premier	Annealing Temperature
<i>ACTIN2</i> transcript	FP: 5'-CGTACAACCGGTATTGTGCTG-3' RP: 5'-AGGTTTCCATCTCCTGCTCGT-3'	56° C 58° C
FOK1 transcript	FP: 5'-CTAGTCAAAAAGTGAAGTGGAG-3' RP: 5'-CTCGCCGTTATTAAATTTCCG-3'	53° C 53° C
DNA-PKa	FW ; 5'-CTCCTCCAAGACCAAAACGAA-3' RV; 5'- CACTCCTCTTTGCACTGACTG -3'	55° C
DNA-PKb	FW ; 5'-CCTCCAAGAGCAAAACGAACA-3' RV; 5'- CCACCAATTCACCTCACACCTC -3'	55° C
PRD2	FW ; 5'-GTTTATACTGATACAGCTAAT-3' RV ; 5'- CCAAAATGGCTGGCTCCACCT -3'	50° C 61° C

Table 5: RT-PCR Primers that used to check the expression of the mutants

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is used to detect the gene expression after extracting mRNA then transferring into complementary DNA (cDNA) by using the RT-PCR enzymes from different plant tissue or flower buds and after that using a specific primer to amplify targeted sequences. Promega protocol has obtained by adding 4 µL RAN and 1 µL Oligo-dT primer then incubate mixture at 70°C for 5 minutes then put it on ice for 5 minutes and then adding 5.5µL RNase free dH₂O (DEPC water) , 4µL 5X Reaction buffer , 3 µL MgCl₂, 1µL Nucleotides mix (dNTP mix) , 0.5µL RNasin (RNase inhibitor) , and 1 µL RT. Then incubate the samples at 42 °C for 60 minutes then place the samples at 70°C for 15 minutes and then 4°C to transfer mRNA into cDNA. After converting mRNA to cDNA doing a PCR by using the forward and reverse primers for the cDNA to measure the gene expression level.

Steps	Temperature	Time
1- Denature DNA	92°C	10 minutes
2- Primer Annealing	92°	15 seconds
3- Extension	60°C	45 seconds

30 Cycles

Table 6: The stages of RT-PCR

Gel electrophoresis

The gels were then organised by dissolving 0.8g of agarose (Bioline) in 100ml of 0.5% of electrophoresis buffer (TBE, 100ml 5X TBE in 900ml SDW). It was then heated with the help of a microwave. After the agarose was dissolved and cooled down, the safe red stain was added (2.5µl/100 agarose). After that, electrophoresis buffer (0.5% TBE) was added to fill the electrophoresis tank and loaded approximately 20µL of each sample per well in the gel. Gene Ruler 1Kb DNA ladder (Fermentas) was utilized to mark the different molecular weights. Electrophoresis was carried out at 90V for approximately 50 minutes. Lastly, the DNA bands were imaged using a Biorad Gel DocXR imager and software.

Materials and methods for Cytological experiments.

DAPI staining

Using 4'-6-diamidino-2-phenylindole (DAPI) diluted in a Vectashield solution (Vector) to stain the slides.

Propidium iodide:

Propidium iodide (PI) is a fluorescent molecule and an intercalating agent to stain cells. Propidium iodide is utilized as a DNA stain for the flow cytometry, to study cell viability or to analyse the DNA content during the cell cycle, and by using microscopy to observe the nucleus. PI is also utilised to recognise apoptotic, necrotic, and normal cells

(Lecoeur.,2003). In our study, we used the double staining (DAPI/PI at 10ug/ml) to check the signal from the 45S rDNA region at Diakinesis with artificial DSBs.

Fluorescence microscopy

This work was carried out using an epifluorescence microscope (Nikon ECLIPSE 90i) to check the slides equipped with filters for DAPI, FITC, and TRITC.

Imaging

Using a Hamamatsu Orca-ER digital CCD camera to acquire Images and using NIS-Elements AR software (Nikon) for analysis.

Chromosome fixation

Before studying chromosome structure and chromosomal changes, Materials fixative is required. The fixative buffer that has been prepared was around 16 ml of 3 parts of ethanol/1 part of glacial acetic acid that used to fix flower buds that were collected from both wild-type and mutant plants at room temperature. After one or two days the fixative buffer has been changed.

Chromosome spreading

The buds were then washed three times for 5 minutes each with citrate buffer consisting of consisting of 5ml of 0.1M citric acid, 5ml of 0.1M sodium citrate and 40 ml of SDW. After that, closed flower buds were transmitted into a watch glass and detached from the inflorescence; this is done by the help of forceps. These buds were then processed with a combination of enzymes (0.33% w/v pectolyase, 0.33% w/v cytohelicase and 0.33% w/v cellulose in citrate buffer). About 0.4 ml of the enzyme mixture were added to the buds and then placed into a moist chamber and transmitted into an incubator at 37°C for 1.5 hours. Water at about 4 degrees centigrade was added on the buds to stop the enzyme digestion procedure. A single bud was shifted on a slide under a microscope with a small

Amount of buffer. It was then macerated with the use of a needle. After that, a total of 10µL of 60% acetic acid was included, and for 2 minutes, the slide was placed on a 45°C hotplate. The area where the macerated material was marked with a diamond pen. Further 10µL of 60% acetic acid was added, and then, about 100µL of fixative was added around the drop of acetic acid. Extra 100µL of fixative was added. The slide was then dried with the use of a hairdryer.

Immunolocalisation

Immunolocalization techniques have been used to detect the location of meiotic proteins by using specific antibodies raised against them. The Antibodies that we used are summarised in **table 7**.

Antibody	Animal	Concentration	Described in
Anti -AtASY1	Rabbit and Rat	1:500	(Sanchez-Moran <i>et al.</i> ,2007)
Anti -γ H2AX	Rabbit	1:200	(Sanchez-Moran <i>et al.</i> ,2007)
Anti-AtSPO11	Rabbit	1:100	(Sanchez-Moran <i>et al.</i> ,2007)
Anti -AtZYP1	Rabbit and Rat	1:500	(Sanchez-Moran <i>et al.</i> ,2007)
Anti -AtDMC1	Rabbit	1:200	(Sanchez-Moran <i>et al.</i> ,2007)
Anti -AtRAD51	Rabbit	1:200	(Sanchez-Moran <i>et al.</i> ,2007)
Anti -AtMLH1	Rabbit	1:200	(Sanchez-Moran <i>et al.</i> ,2004)
Anti -AtDNA-PKcs	Rabbit	1:100-1:500	(Sigma-Aldrich) https://www.sigmaaldrich.com/catalog/product/sigma/sab4502382?lang=en&region=GB

Table 7: List of used primary antibodies

1- Slides washing

We washed carefully the glass slides by dipping them in Acetone for 10 minutes, then in SDW for 10 minutes and finally in Ethanol for another 10 minutes. After that, we left the slides on the bench to dry and keep them on a clean slide box for future use.

2- Phosphate buffer solution (PBS) and Triton X-100 detergent

Dissolving 4 tablets of PBS (Sigma-Aldrich) in 400 ml of SDW then adding 400ul Triton X-100 detergent into the PBS (PBS + 0.1% Triton X-100).

3- Block solution

Adding 0.1g of Bovine Serum Albumin (BSA) into 10 ml of PBS+0.1% Triton X-100 then mix them gently until BSA dissolved and then pass them throughout a filter of a 0.2 μ L porous by using syringe into a falcon tube and keep at 4°C until use.

Spreading technique

The smaller flower buds were selected from 5 to 10 buds for each slide under a dissecting stereoscope. Then, under the stereoscope, the anthers were dissected from the buds on a clean slide, and about 20 μ L of 0.1 % of cytohelicase were added. The slides were placed in a wet box at 37°C for 10 minutes in an incubator. After that, an extra 10 μ L of digestion enzyme was added, and the anthers were macerated using a brass rod. The preparations were placed on a hot plate at 33°C for 4 to 5 minutes. 10 μ L of 1% Lipsol were added and carefully spread by a plastic pipette. Then, 20 μ L of 4% paraformaldehyde was placed on the material and slides were left to dry under a flow hood for around 2 hours until dried. This was followed with three washes with PBS (0.1%Triton) for 5 minutes each. 50 μ L of blocking solution was added on a small parafilm piece, and this was put on top of the Material. The slides were incubated in a wet box at room temperature for 10 to 20 minutes.

Primary antibody was diluted in blocking solution and then added onto pieces of parafilm and placed on top of the material and incubated in a wet box at 4°C for 1- 2 days. Afterward, the slides were washed in PBS (0.1%Triton) three times for 5 minutes each. Secondary antibodies (WHICH ONES?) were prepared in blocking solution, then drop it on pieces of parafilm and keep them in the dark area for 45 minutes. Three washes in PBS (0.1%Triton) for 5 minutes each were carried out. Finally, the slides were stained with 7 μ L of DAPI in Vectashield (10 μ g/ml), and a cover slip was placed on top of the material.

Alexander staining:

The Alexander staining solution is made of 10ml 96% ethanol, 1ml of Malachite green in 69% ethanol, 50ml distilled water, 25ml glycerol, 5mg phenol, 5mg chloral hydrate, 5ml of acid fuchsin in 1% solution in water and Glacial Acetic acid to the final concentration of 4%. The solution must be kept in the dark place. The old flowers placed on a microscope slide and using a scalpel to cut the flowers from the bottom then removing all tissues by forceps with keeping only the anthers. Then, choosing young flowers to take the pollen from them for staining with 20 μ l of the solution. After adding the solution, putting the cover slip on the drop of the staining. Then incubating the slide at 50°C for several hours. Finally checking the slide by under a stereomicroscope (Alexander Staining, 2017).

CHAPTER THREE

IDENTIFICATION OF A PUTATIVE DNA-DEPENDENT PROTEIN KINASE (DNA-PKCS) IN *ARABIDOPSIS* *THALIANA*

Introduction

DNA-dependent protein kinase (DNA-PK) is a kinase that phosphorylates serine/threonine residues activated upon association with DNA for repair of DNA double-strand breaks (DSBs) very important for the Non-Homologous End Joining pathway (NHEJ). The integrity of the DNA is constantly experiencing different damages by endogenous and exogenous factors that have to be repaired by different processes to ensure the survival of the cell. For example, by-products generated by the oxidative metabolism can damage DNA through the release of potential reactive oxygen species (ROS), which possess the ability to introduce single-strand breaks (SSBs) and DSBs (Reynolds *et al.*, 2012). SSBs and DSBs can also be induced by the effect of exogenous agents such as certain anti-cancer drugs and ionizing radiations. These damages to DNA integrity must be repaired to reduce the chances of mutation. DSBs in DNA are also introduced during the generation of a functional immune system in vertebrates in the process called site-specific V(D)J recombination (Reynolds *et al.*, 2012). Cytotoxic consequences leading to cell death may occur if DSBs are left un-repaired or are not accurately repaired. Moreover, the chances of induced mutation are higher when DSBs are not accurately repaired, which may lead to uncontrolled proliferation and differentiation of cells and eventually cancer (Mukherjee *et al.*, 2012). To prevent these situations and combat the potential threats which might be induced by unrepaired DSBs, several repairing mechanisms have evolved in eukaryotic cells. Non-Homologous End Joining (NHEJ) has been recognized as the predominant repairing mechanism of DSBs, DNA-dependent protein kinase complex (DNA-PK) plays the role of mediator in this pathway (Bouchaert *et al.*, 2012). According to the genetic and biochemical studies performed to investigate the composition and expression of genes involved in the regulation of the DNA-PK complex, this enzyme is composed of a large catalytic subunit which has been termed DNA-PKcs. The Ku complex has been identified

as an important regulatory factor for the action of DNA-PKcs (Goodwin *et al.*, 2015). Recent studies have emphasized the significance of mammalian DNA-PK during V(D)J recombination in the immune system development and as repair machinery of DSBs induced by ionizing radiation (IR)(Mukherjee *et al.*, 2012). Moreover, recent studies analysing additional mechanisms for DNA-PK have revealed its involvement in other processes, which include telomere maintenance and the modulation of chromatin structure (Kusumoto-Matsuo *et al.*,2010). DNA-PKcs is one of the most important components for the NHEJ pathway in repairing the DSBs (Goodwin *et al.*, 2015). Revealed through structure studies, DNA-PKcs is a 460kD serine-threonine kinase. Cloning of DNA-PKcs cDNA revealed a lack of homology with other previously characterized proteins (Jiang *et al.*, 2015). However, the carboxyl terminal of DNA-PKcs which is composed of 500 amino acid residues contains the catalytic domain which possesses homology with the phosphatidylinositol 3 – kinase family (Sibanda *et al.*, 2010). Electron crystallography structure analysis has revealed that DNA-PKcs is activated by direct association with DNA (Sibanda *et al.*, 2017). Similar open channels have been reported in the structure of DNA-PKcs as in other double-stranded DNA binding proteins. DNA-PKcs contains an enclosed cavity comprising of three openings, that allow the size of single-stranded DNA to be accommodated during repair and an opening adjacent to the open channel (Crowe *et al.*, 2018). DNA strand which must be longer than or equal to the minimal length of the open channel is required for the efficient activation of DNA-PKcs, i.e. DNA strand of 12bp (Crowe *et al.*, 2018). Association patterns studied for DNA-PKcs with double and single-stranded DNA indicate separate but interacting sites in the DNA-PKcs, which allow the binding of these sites with DNA (Mukherjee *et al.*, 2012). Moreover, activation of the kinase activity has also been observed dependent on interaction with both double and single strands (Bouchaert *et al.*, 2012). These studies suggest the interaction patterns and association preferences of DNA-PKcs to repair the DNA breaks. The first regulatory factor

for DNA-PKcs identified was the Ku complex. This Ku complex was recognized as a highly specific and abundant DNA binding structure (Bartlett & Lees-Miller, 2018), during the characterization studies performed for DNA-PKcs. There are two subunits identified in the Ku complex of 70 and 83kD, associated tightly with the activity of DNA-PKcs (Radhakrishnan & Lees-Miller, 2017).

NHEJ and Alternative DNA Damage Repair Pathways

According to investigations conducted on mammalian cells, approximately ten DSBs per day per cell. DSBs present a serious threat to cell and maintenance of genomic stability and integrity of DNA is recognised crucial for different cellular activities. DSBs have also been examined to occur as programmed events such as during V(D)J recombination, meiosis and class switch recombination (CSR), which is required for diversification and functioning of immunoglobins (Chang *et al.*, 2017). Major repair pathways utilised by the DNA include homologous recombination (HR) and non-homologous end joining (NHEJ). The former repair pathway has been identified effort free while latter is considered an error prone process. However, according to some evidences, during the repair mechanism of large amount of DNA, HR is considered as a highly mutagenic mechanism. Genomic stability has also been investigated to be maintained by error free DSB repair despite their error prone molecular mechanism nature. Some of these repair processes include chromosomal translocations and complex chromosomal rearrangements (Nishizawa *et al.*, 2012).

NHEJ repair pathways operates by utilising proteins which perform the activities of recognising the DSB, binding, polymerisation and ligation of the DNA breaks in a flexible manner. Large amount of DSBs in cell rendered by various cellular metabolic activities are predominantly repaired by NHEJ which functions on a wide range of DNA-end configurations due to its flexibility. According to the observations and examinations conducted in cell culture models, two subtypes of NHEJ namely classical and alternative

NHEJ has been recognised. Alternative NHEJ processes are reported to be more error prone as they always render certain changes in nucleotide sequences of DNA (Dumont *et al.*, 2011).

One classical example of alternative NHEJ is micro-homology mediated end joining (MMEJ) which operates by unwinding of DNA and exposure of 6 to 20 nucleotide long short microhomologies. 3' overhanging flaps are cleaved after annealing of the micro-sequences and gaps are filled by ligation. MMEJ does not require ligase 4 for its activity during the ligation process as in the case of classical pathway rather ligase 1 is recruited for the gap filling activity. MMEJ results in loss of genetic material and recognised mutagenic, largely due to its non-specific nature. Recovery of genetic information through HR is based on a template and operates through strategies that are contrary to NHEJ (Donà & Scheid 2015). According to recent investigations on NHEJ, operations of third DSB resolving pathways have also been identified which function through simple end joining with a slower speed. Alternative NHEJ pathways gain functional relevance when there is local or global failure of standard repair pathways (Nishizawa *et al.*, 2012). However, alternatives NHEJ pathways are more error prone than classical repair pathways as the probability of translocation and sequence alterations at the junctions are much higher and more frequent. Due to these aspects, alternative NHEJ pathways are considered to be the major source of genomic instability. Evidences suggest that processing of dysfunctional telomeres for joining might involve both classical and alternative NHEJs while alternative pathways may process to repair naturally shortened telomere (Donà & Scheid 2015). According to recent investigations on cancer therapeutics, role of alternative NHEJs and their targeting has been evaluated as significant as their involvement in cancer development and genome instability is indisputable. However, research gaps reside in identifying the mechanism balancing the functioning and operations of classical and alternative NHEJs and require further investigations (Dueva & Iliakis 2013).

Investigations of Repair pathways Arabidopsis and the Phenotypes of Mutants

Activation and processing of repair pathways is largely dependent upon the cascade of activities promoting signal transduction. According to investigations conducted on mutants DNA-PKcs in Arabidopsis, signal transducing proteins are recognised to play an essential role in regulation of DSB repair pathways (Dumont *et al.*, 2011). Hypersensitivity has been observed by the mutants of ligase 4, ku 70 and ku80 in Arabidopsis towards the exogenous DSB inducing agents which included bleomycin, γ -irradiation, methyl methanesulfonate and ionising radiation. Evidences report the crucial role of ku 70 and 80 and the liagtion enzyme ligase 4 in regulation, processing and functioning of repair pathways fixing the DSBs in species of Arabidopsis. During the repair process, recruitment of ku70/ku80 and ligase 4 and their binding at the DSB site results in recruitment of DNA-Pkcs which function to phosphorylate the different proteins for sustained signal transduction (Chang *et al.*, 2017).

In vitro examinations have highlighted the role of backup repair pathways in mammalian cells which can repair DSBs, when chemical inhibition was performed of NHEJ factors including DNA PKcs, ku70/ku80, ligase 4 and XRCCC4. Certain other protein factors are used by these backup pathways for DSB repair which include MRN, XRCC1, DNA ligase III, (ADP-ribose) polymerase 1 and histone H (Chang *et al.*, 2017). Large amount of investigations on Arabidopsis indicate the presence of repair pathways independent of ku proteins and ligase 4 while representing distinct proteins such as AtLig1 and AtXRCC1 functioning for alternative repair pathways other than classical mechanisms. Identified in a study, *Arabidopsis thaliana* exhibits characteristic early flowering phenotype due to impairment of NHEJ pathways caused by mutation in one of the regulatory protein involved in signal transduction. The cell cycle stage, complexity of DNA and cell type plays an important role in selecting either of the homology or non-homology dependent pathways in eukaryotes (Nishizawa *et al.*, 2012). Interference or mutation in components

(proteins) of these repair pathways results in exhibition of potential phenotypes such as reduced size, reduced lifespan and less reproductive potential. Phenotypes identified in mutant *Arabidopsis* plants upon mutation of one or several NHEJ repair components included observable morphological changes such as increased pollen grain size, large flowers, size variation and the extent of phenotypic instability (Donà & Scheid 2015).

Arabidopsis mutants in Lig4 and ku70/ku 80 exhibited fertile and viable phenotype. Furthermore, no significant difference was observed in callus formation and callus regeneration from roots when wild type *Arabidopsis* was compared with ku80 mutant plants. Several evidences have confirmed more potential effects of lig4, ku70/ku80 mutations in rice than *Arabidopsis*. As a consequence of severe DNA damage; cells of *Arabidopsis* prevent cell death by entering endocycle (Dumont *et al.*, 2011). This mechanism predicts the less direct effects on viability and productivity of cells due to mutations in ku70/ku80. Several differences have been found upon investigations of effects of mutations in mammalian cells and *Arabidopsis*. According to studies on mammalian null mutants, mutation in ligase 4 lead to embryonic lethality in mice and ku mutants have been reported to exhibit accelerated senescence and are immunodeficient (Dueva & Iliakis 2013).

History of DNA-dependent Protein Kinase (DNA-PKcs)

Early studies primarily focused on the biochemical functions of the kinase activity and led the researchers to investigate and speculate the function of DNA-PK as a modulator of transcription (Anderson *et al.*, 1992; Smith and Jackson., 1999; Schild-Poulter *et al.*, 2003; Crowe, 2018). Nevertheless, following research revealed that the activity of DNA-PK was prominent in DSBs repair, reflecting the potential of DNA-PK to recover the DNA damage (Getts and Stamato, 1994, Rathmell and Chu, 1994, Smider *et al.*, 1994, Taccioli *et al.*, 1994, Finnie *et al.*, 1995, Peterson *et al.*, 1995, Deshpande, *et al.*, 2018). These studies revealed how important DNA-PK is in repair. DNA damage by ionizing radiation (Head *et*

al., 2018). Moreover, DNA-PKcs mutations were also examined showing its importance in DSB repair (Deshpande *et al.*, 2018). DNA-PKcs mutations in mouse were examined showing that they were affected with severe combined immune deficiency (Bartlett & Lees-Miller, 2018). The kinase domain in DNA-PKcs has been found to have homology with a family of protein kinases dependent of DNA which are involved in maintaining the genome stability and controlling cell cycle progression (Bartlett & Lees-Miller, 2018). At present, molecular functions of DNA-PKcs are under persistent examination through various biochemical analyses and comparative analysis with proteins containing higher homology values with the domains of DNA-PKcs (Lafont *et al.*, 2018). Activation of DNA-PKcs was reported initially in mouse spleen cell nuclear extracts by Ohtsuki and colleagues (1980). DNA-dependent protein kinases were initially discovered in extracts of HeLa cells during which other similar kinases were discovered, and their associated functions analysed (Head *et al.*, 2018). Interestingly, DNA strand binding proteins were also identified during these studies and homology measures of DNA-PK, and DNA strand binding proteins were examined. Moreover, the activity of DNA-PK was recognised to be efficient in the presence of linear DNA as compared to the activity performed in supercoiled DNA (Lafont *et al.*, 2018). Until 1990, studies confirmed the mechanism of DNA-PK as a significant enzyme in repairing DNA breaks (Zhou *et al.*, 2017). During the same time, subsequent biochemical assays indicated that the large subunit of DNA-PK was not required for DNA binding. The DNA targeting component in the DNA-PK complex was identified to be the Ku complex (Ku70 and Ku80), and its activity was observed to be non-sequence specific in binding the DNA strand (Goodwin *et al.*, 2015). Although, interaction parameters identified by certain biochemical assays for DNA-PK indicated that its activation is dependent on the association to DNA (with a maximum activation reported with linear DNA as compared to the supercoiled DNA. Greater the damage induced by the potential factors to DNA such as oxidative stress caused by the generation of reactive

oxygen species, the greater will be the expression of genes regulating DNA-PK complex. This study further confirmed the proportion of DNA-PK complex that is retained in the cells during a certain duration of time (Chirgadze *et al.*, 2017). Affinity binding capabilities of Ku subunits revealed that they could bind to dsDNA. Moreover, Ku subunits were highly associated with DSBs while they had a lower affinity for SSBs, reflecting the DSB recognition role of the Ku complex (Hammel *et al.*, 2016). Helicase and ATPase activities have also been identified in the Ku subunits, widening their functionality parameters and significance in the intracellular environment (Bartlett & Lees-Miller, 2018). Furthermore, a weak helicase activity has also been identified in the Ku subunits (Lehnert, 2000). A variety of DNA structures were exposed to the Ku subunit to detect its affinity which indicated higher affinity and association of the Ku complex with non-circular DNA while not association with circular DNA (Crowe, 2018). Structural-functional association studies have revealed that DNA-PKcs may interact with DSBs by the internalisation of these DNA breaks through these tunnels and cavities in the hollow structure of DNA-PKcs (Deshpande *et al.*, 2018). Biochemical assays and genome sequencing protocols indicated the presence of homology in the structure of DNA-PKcs and other DNA binding proteins involved in DNA replication (Sibanda *et al.*, 2017). These single-stranded DNA binding proteins provide structural support to the ssDNA by holding the strands as the DNA polymerization occurs. This mechanism prevents the annealing of DNA strands during replication, thus allowing efficient DNA replication. Structural similarity between both protein families indicates the presence of similar amino acid sequences in the domains associated directly with the holding of the DNA strand during repair or replication (Head *et al.*, 2018).

Role of DNA-PKcs in Non-Homologous End Joining (NHEJ)

Different mutations in the DNA-PK complex have been analysed in mammalian cells to analyse the role of DNA-PK in DSBs repair. NHEJ has been known to occur in the

different type of cells and integrates some important processes, which include DNA repair and telomere maintenance through the involvement of some different proteins. NHEJ pathway requires a series of proteins to be able to recognise, repair and ligate the broken ends of the DNA (Deshpande *et al.*, 2018). The most important proteins identified in NHEJ include the Ku complex (Ku70 and Ku80 proteins), the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), XRCC4 and DNA Ligase IV (Jiang *et al.*, 2015). Lack or deficiency in one or more of these proteins may result in the induction of uncontrolled induced mutations leading to cell death or cancer (Jiang *et al.*, 2015). Mutants for these proteins are normally showing hypersensitivity to DSB inducing agents like ionising radiation or some cancer drugs. The most important steps in NHEJ pathway could be summarised in the recognition and capping of DNA ends of the DSB by the Ku complex (Ku70 and Ku80) also DNA-PKcs, the activation of the proteins involved in the repair (DNA-PKcs autophosphorylation and phosphorylation of other proteins) and finally the ligation of the broken ends by the enzyme DNA ligase IV (Bartlett & Lees-Miller, 2018). The activity of DNA-PKcs in NHEJ is crucial to bring about the first two processes of the sequential NHEJ repair mechanism. Initially, Ku70 and Ku80 (Ku complex) recognize the DSB and get associated with the DNA at the recognised DSB site and then can associate with the DNA-PKcs forming the DNA-PK complex (Bartlett & Lees-Miller, 2018). The Ku complex forms the bridge between the broken ends of the DNA while supporting the DNA structurally and aligning the strand for further processing. It also protects the strand from degradation during cleavage activity and has been proposed that the phosphorylation of Ligase IV and XRCC4 might affect the interaction and association patterns of the Ku complex and other proteins involved in the repair process of DNA damages (Lafont *et al.*, 2018). The crucial step of terminal end joining in NHEJ is completed by joining of the two blunt ends of the DNA. It has been identified in some cases that before ligation can occur, terminal processing of DNA ends is significantly

required (Jiang *et al.*, 2015). Generation of blunt ends is performed by the nucleases enzymes which are successively ligated after overhanging sequences of broken DNA are joined by DNA polymerase enzyme, which synthesises the DNA strand and fills the gaps. Single-stranded DNA overhangs or sticky ends are trimmed off by the activity of nuclease enzyme. DNA-PKcs is known for the potent activity of phosphorylation which brings about either activation or inactivation of certain intracellular proteins for intracellular mechanisms (Crowe *et al.*, 2018). Upon binding with DNA-PKcs, Artemis protein, known to perform the nuclease activity, forms a complex called DNA-PKcs/Artemis complex. Phosphorylation of Artemis proteins activates its catalytic activity, and the complex is known to perform cleavage of 5' and 3' DNA overhangs to be able to join the ends (Deshpande *et al.*, 2018). Upon formation of these blunt ends, DNA ligase IV and XRCC4 are recruited to form a ligation complex to join the ends of DNA. DNA ligase has been recognised as predominant ligation enzymes but requires XRCC4 for its activity (Radhakrishnan & Lees-Miller, 2017). XRCC4 stabilizes the DNA ligase during its ligation activity and acts as a regulatory element during ligation of broken ends of the DNA (Deshpande *et al.*, 2018). It has also been identified as the major stimulator of ligase activity and possesses recognition helix that guides the DNA ligase enzyme towards the DNA break site. DNA binding capacity of XRCC4 has also been identified during the end joining mechanism of broken dsDNA (Chirgadze *et al.*, 2017).

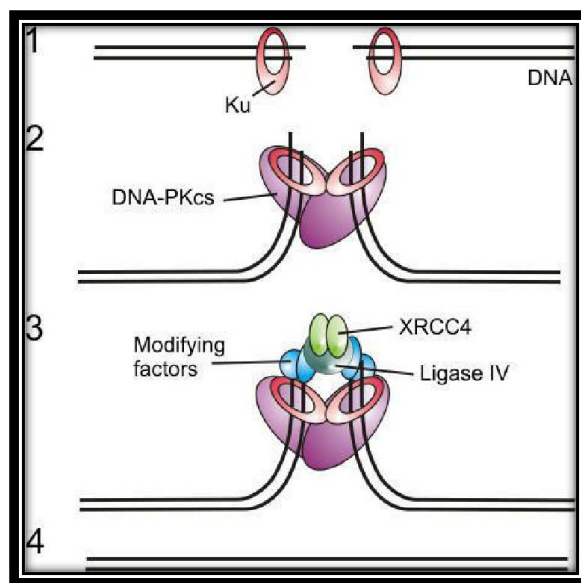


Figure 12: Schematic diagram of the NHEJ pathway

(1) Recognise DSB by Ku 70& Ku80. (2) Binding the ends of DNA by DNA-PKCS. (3) DNA ends Processing. (4) DNA ends ligation (From Weterings and Chen,2007).

Working Mechanism of DNA-PKcs

DNA-PKcs belongs to the family of proteins that brings about phosphorylation of intracellular proteins or integral membrane proteins for certain specific mechanisms. Phosphorylation has been observed as a major modulator of an active and inactive form of proteins functioning as mediators in the cells (Chirgadze *et al.*, 2017). The DNA-PKcs kinase activity has been identified as an important requirement to cleave the sticky ends of broken DNA overhangs. Ligation activity of broken DNA strands requires blunt ends, to join the base pairs (Head *et al.*, 2018). These blunt ends are generated by the catalytic complex formed through the association of DNA-PKcs and Artemis protein (Head *et al.*, 2018). However, the inactive form of DNA-PKcs is activated and directed by the Ku complex proteins that translocate along the length of DNA and recognises the broken end. Recognition is followed by an assessment of DNA-PKcs and Artemis protein complex, to perform cleavage at the recognised sticky ends of dsDNA. Alongside, the activity of DNA-PKcs is also required during the V(D)J recombination process required for the correct development of antibodies (Head *et al.*, 2018). Recruitment of DNA-PKcs catalytic activity during V(D)J recombination is crucial to generating diversity in the antibodies generated and developed

(Crowe, 2018). According to studies performed to detect the effect of deficiency of DNA-PKcs in V(D)J recombination in mice, Severe combined immunodeficiency (SCID) was reported in mice lacking the enzyme for this recombination due to lack of diversity in the antibodies (Hammel *et al.*, 2016). In the repair pathway of DNA breaks, the efficient activity of these catalytic subunits has been identified in dsDNA as compared to the activity ratio in ssDNA. Conformational change has also been reported by the activity of catalytic subunit of DNA-PK at the end of NHEJ which allows end processing enzymes to assess and reach the broken ends of the DNA (Deshpande *et al.*, 2018). DNA damage checkpoints involved in the controlled process of the cell cycle have also been studied affected by protein phosphorylation by DNA-PKcs. These and similar other studies performed to assess the functioning properties and structural features of DNA-PKcs are still an ongoing process of extensive investigation (Chirgadze *et al.*, 2017). DNA-PK was thought to be only present in vertebrates and absent from other organisms. DNA-PK was found in *Dictyostelium discoideum* which could be required for NHEJ pathway (Block, 2005). In this chapter, we have analysed the *Arabidopsis thaliana* genome to find this homolog and identified two putative genes to study their role in DNA damage.

Results

Human DNA-PKcs

The human DNA-PKcs protein has been thoroughly investigated, and its amino acid (aa) sequence is well known (NP_008835). The protein sequence has a length of 4,128 aa (See sequence in **Appendix 1**).

Conserved Domains for *Homo sapiens* (Human) DNA-PKcs

The human DNA-PKcs sequence was entered to the National Centre for Biotechnology

Information (NCBI) Conserved Domains (CD) search tool

(<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to identify similarities with other

conserved domains in the database (CDD) available (Marchler-Bauer *et al.*, 2017) Different CD was discovered: NUC194, PIKKc-DNA-PK, and FAT (**Figure 13**). NUC194 conserved domain is defined as the domain B for the catalytic subunit of the DNA-PK. The FAT (FRAP, ATM, and TRRAP) domain is a conserved domain found in PIK-related kinases. PIKKc_DNA-PK corresponds to the catalytic domain for the DNA-PKcs and is included in a subfamily similar to other kinases such as the typical serine/threonine/tyrosine protein kinases (PKs), aminoglycoside phosphotransferases, choline kinases, and RIO kinases (Marchler-Bauer *et al.*, 2017).

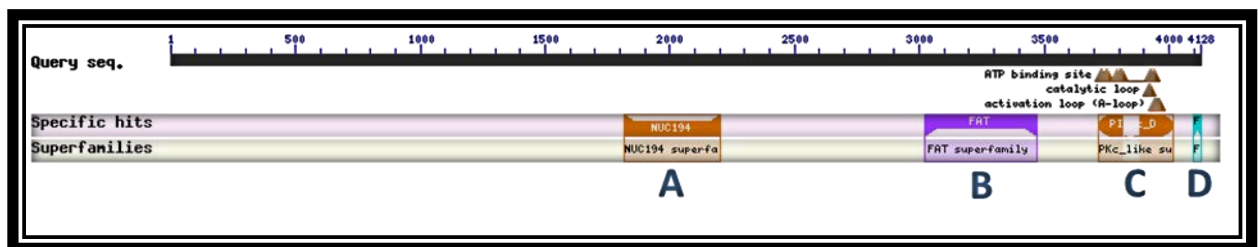


Figure 13: Conserved Domain for human DNA-PKcs.

(A) NUC194 domain B in the catalytic subunit. (B) FAT (FRAP, ATM, and TRRAP) domain. (C) PIKKc-DNA-PK domain which is catalytic. (D) FATC (FAT C-terminal domain).

DNA-PKcs in the amoeba *Dictyostelium discoideum*

DNA-PKcs homolog has been identified in the amoeba *Dictyostelium discoideum* (Hudson *et al.*, 2005). Previously, other DNA-PKcs had been identified by sequence homology in other invertebrates (Dore *et al.*, 2004). Amoebas included *Dictyostelium* are situated evolutionary before the eukaryotic division on animal and plant branches. Thus, the *Dictyostelium* protein sequences show similarities to animal and plant sequences. Using the NCBI database the *Dictyostelium* protein was identified (PRKDC_DICDI), showing a length of 4,299 aa (see **Appendix 2**).

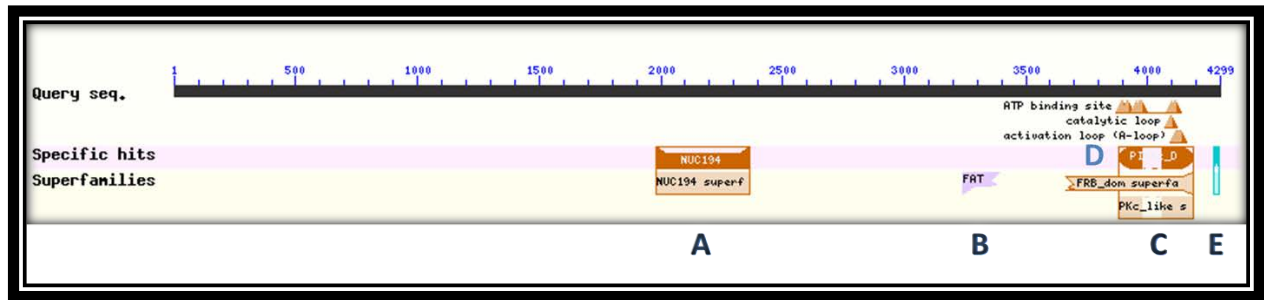


Figure 14: Conserved domains for DNA-PKcs in *Dictyostelium discoideum*.
 (A) NUC194 domain. (B) FAT domain (FRAP, ATM and TRRAP). (C) FRB-super family domain. (D) PIKKc_DNA-PK catalytic domain. (E) FATC domain (FRAP, ATM, TRRAP C-terminal domain).

Arabidopsis thaliana WU-BLAST search for a homolog of DNA-PKcs using the human sequence

The human DNA-PKcs protein sequence was used to search for homologs in *A. thaliana*. using WU-BLAST2 in the *A thaliana*. Information Resource (TAIR) free online tool

(<https://www.arabidopsis.org/wublast/index2.jsp>). **Figure 15** illustrates the summary of results for this BLAST in TAIR. The results show the homology of human DNA-PKcs with different protein kinases in the plant *Arabidopsis thaliana*. One of the homologs that appear in *Arabidopsis thaliana* is a protein related to TOR proteins (AT1g50030). The target of Rapamycin (TOR) proteins are present in yeast and mammals and are involved in the regulation of cell growth in response to nutrients. TOR proteins are phosphatidylinositol 3-kinases and are targets for rapamycin. Human DNA-PKcs shows a 25% identity to AtTOR

with 45% positives and 11% gaps in the sequence. AtTOR is involved in the control of plant growth and mediates the response to stresses (Rossenberg and Chen, 2018). Another candidate is the *Arabidopsis* ATAXIA-TELANGIECTASIA MUTATED protein or AtATM (AT3g48190), a DNA dependent protein kinase involved in Homologous Recombination pathway to repair DSBs induced by ionizing radiation and also very important during meiotic HR and the formation of crossovers (Garcia *et al.*, 2003). Human DNA-PKcs has a 28% identity to AtATM, with 44% positives and only 8% gaps. *Arabidopsis* Ataxia telangiectasia-mutated and RAD3-related (AtATR, AT5g40820)

appears to show similarities with human DNA-PKcs with 26% identities, 48% positives and 8% gaps. ATR is a cell cycle checkpoint regulator involved in DNA repair processes by HR and cell growth (Liu *et al.*, 2015). This analysis also has shown similarities with a new putative protein kinase AT2G17930 in Arabidopsis, named AtDNAPKa from now on. This protein appears to be related with a SAGA complex in the nomenclature available in TAIR. It shows identities of 23% to human DNA-PKcs, with positives of 43% and gaps of 9%. Also, another Arabidopsis protein very similar to AtDNAPKa shows similar identities to human DNA-PKcs (24%), with 43% positives and 11% gaps. This protein is AT4g36080 (AtDNAPKb from now on), and it appears in TAIR as another SAGA complex component.



Figure 15: WU-Blast in *Arabidopsis thaliana* for Human sequence (Homo Sapiens). (A) AT5g40820 ATR. (B) AT3g48190 ATM. (C) AT2g17930 New DNA dependent kinase-like (AtDNAPKa from now onwards). (D)AT4G36080 New DNA dependent kinase-like (AtDNAPKb from now onwards). (E) AT1g50030 TOR.

Arabidopsis thaliana WU-BLAST using the sequence from the DNA-PKcs from *Dictyostelium discoideum*

The *Dictyostelium discoideum* DNA-PKcs protein sequence was used to search for homologs in *A. thaliana* using WU-BLAST2 in the Arabidopsis Information Resource (TAIR) free online tool (<https://www.arabidopsis.org/wublast/index2.jsp>). **Figure 16** illustrates the summary of results for this BLAST in TAIR. The results show the homology

of *Dictyostelium discoideum* DNA-PKcs with different protein kinases in the plant *Arabidopsis thaliana* similarly to that obtained by using human DNA-PKcs.

AtTOR appeared with 29% of identities, 49% positives and 9% gaps. AtATM also appeared with 28% of identities, 44% positives and 8% gaps in the sequence. AtATR with 26% of identities, 48% positives and 8% gaps. This analysis also has shown similarities with the already shown new putative protein kinases AT2G17930 (AtDNA-PKa) and AT4g36080 (AtDNAPKb), with identities of 23/24% respectively to *Dictyostelium discoideum* DNA-PKcs, positives of 43% in both and gaps of 9/11% respectively.

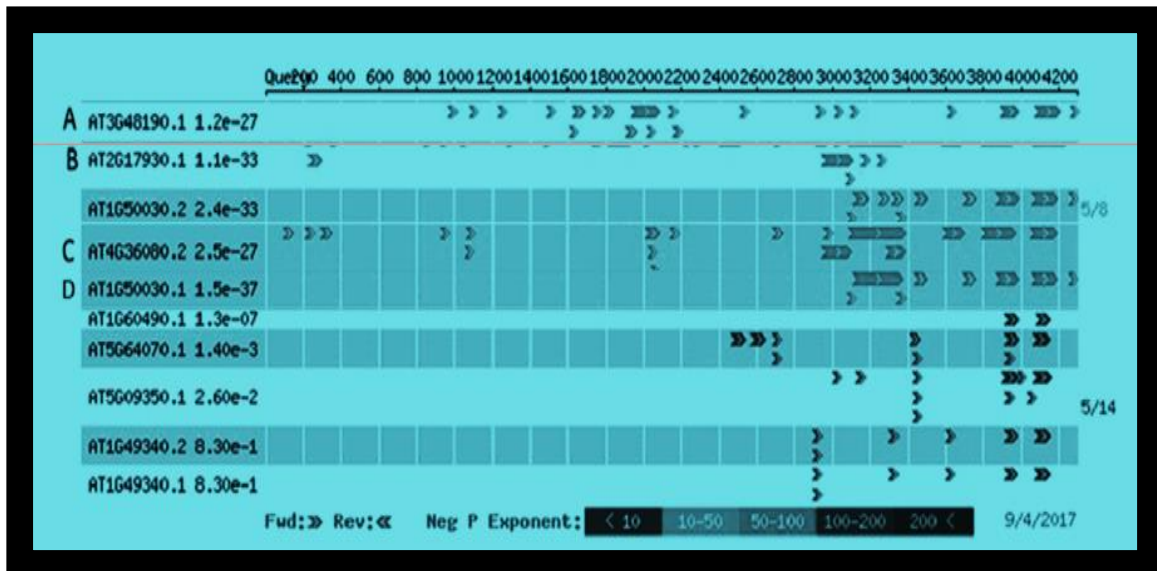


Figure 16: WU-Blast in *Arabidopsis thaliana* for *Dictyostelium discoideum*
 (A) ATM was known as (AT3G48190). (B) AtDNA-PKawas known as (AT2G17930). (C) AtDNA- PKb known as(AT4G36080). (D) TOR is known as (AT1G50030).

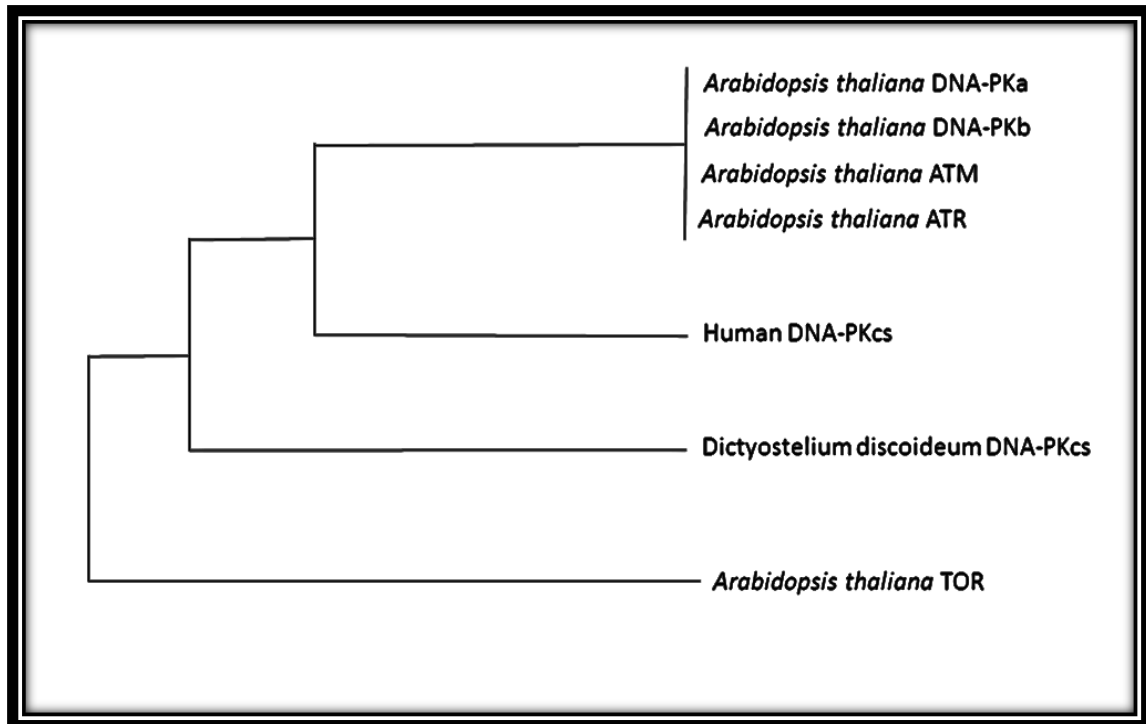


Figure 17: Summary of BLAST analyses by a phylogenetic tree of all the homologous sequences

Conserved domains for *Arabidopsis thaliana* ATR and ATM

The NCBI conserved domain search tool was employed to investigate the CD for ATR protein in *A.thaliana*. ATR is a contributor factor in both DNA damage (Lavin, 2008).

Furthermore, it provides stability to the replication fork, which facilitates the connection between the DNA polymerase and the fork (Flynn and Zou, 2011). The conserved domain analysis shows three main CDs: PIKKc_ATR, FRB domain, and UME. PIKKc_ATR is defined as a catalytic domain for Ataxia telangiectasia and RAD3 related (ATR) protein. FRB_dom super family is is an FKBP12-rapamycin binding domain. UME is a domain from UVSB PI-3 kinase which functionality remains unclear (**Figure 18A**). The conserved domains in AtATM are comprised of a FAT super family domain (FRAP, ATM, and TRRAP) (**Figure 18B**).

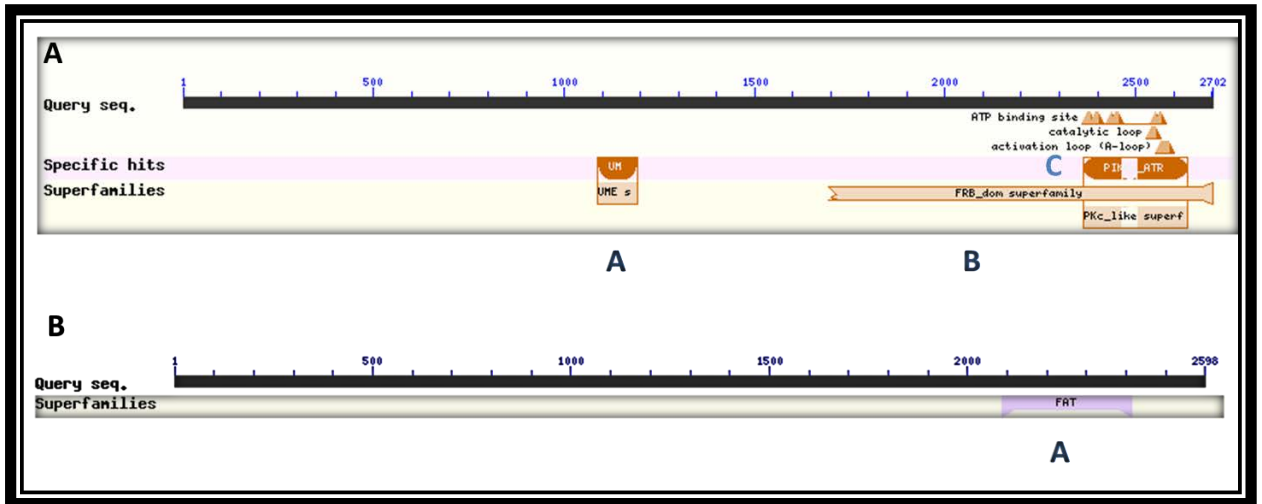


Figure 18: Conserved domains for *Arabidopsis thaliana* ATR and ATM :

Figure 18A. Conserved domain for Arabidopsis thaliana ATR. (A) PIKKc_ATR is a catalytic domain of ATR. (B) FRB_dom superfamily. (C) UME domain with an unknown function.

Figure 18B. Conserved domain for Arabidopsis thaliana ATM. (A) FAT super family domain (FRAP, ATM, and TRRAP).

Conserved Domain analysis for the putative AtDNA-PKa and AtDNAPKb

AtDNA-PKa shows two conserved domains at the C-terminal location. A PIKK_TRRAP domain which is the apparent catalytic domain for the DNA-PKcs kinases studied so far. And the FRB_dom found in the Dictyostelium discoideum DNA-PKcs which is an FKBP12-rapamycin binding domain. **(Figure 19A)**. Similarly to the CD analysis in AtDNAPKa, two conserved domains at the C-terminal location were recognized in AtDNAPKb. A PIKK_TRRAP domain (catalytic domain for the DNA-PKcs) and the FRB_dom **(Figure 19B)**.

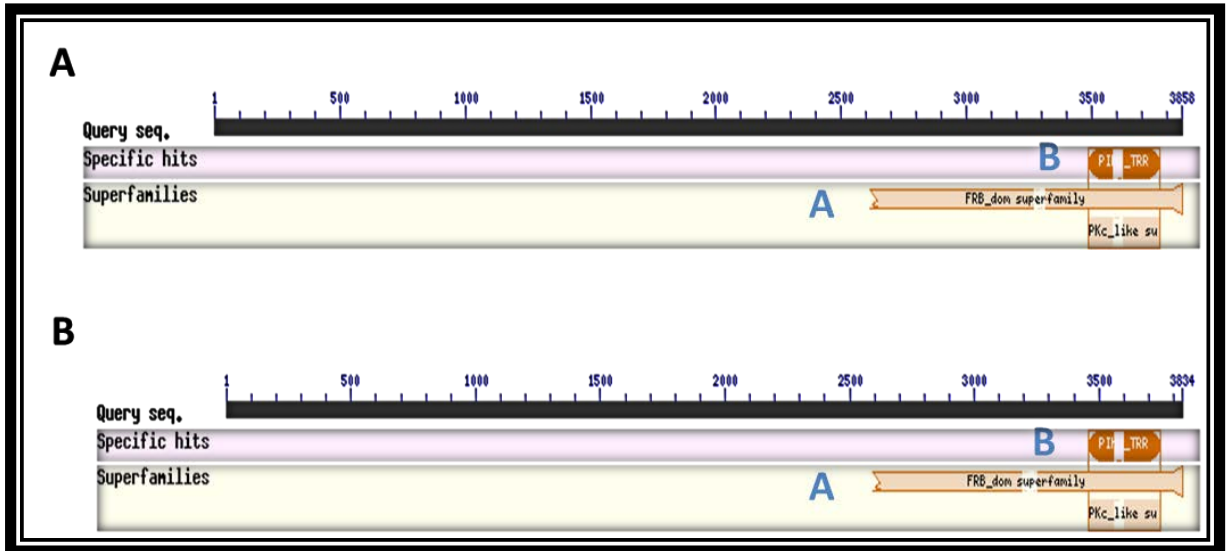


Figure 19: Conserved Domains analysis for the putative *AtDNAPKa* and *AtDNAPKb* in *Arabidopsis thaliana*.

Figure 19A. Conserved domain analysis for the putative *AtDNAPKa*. (A) FRB_dom superfamily. (B) PIKK_TRR domain.

Figure 19B. Conserved domain analysis for the putative *AtDNAPKb*. (A) FRB_dom superfamily. (B) PIKK_TRR domain.

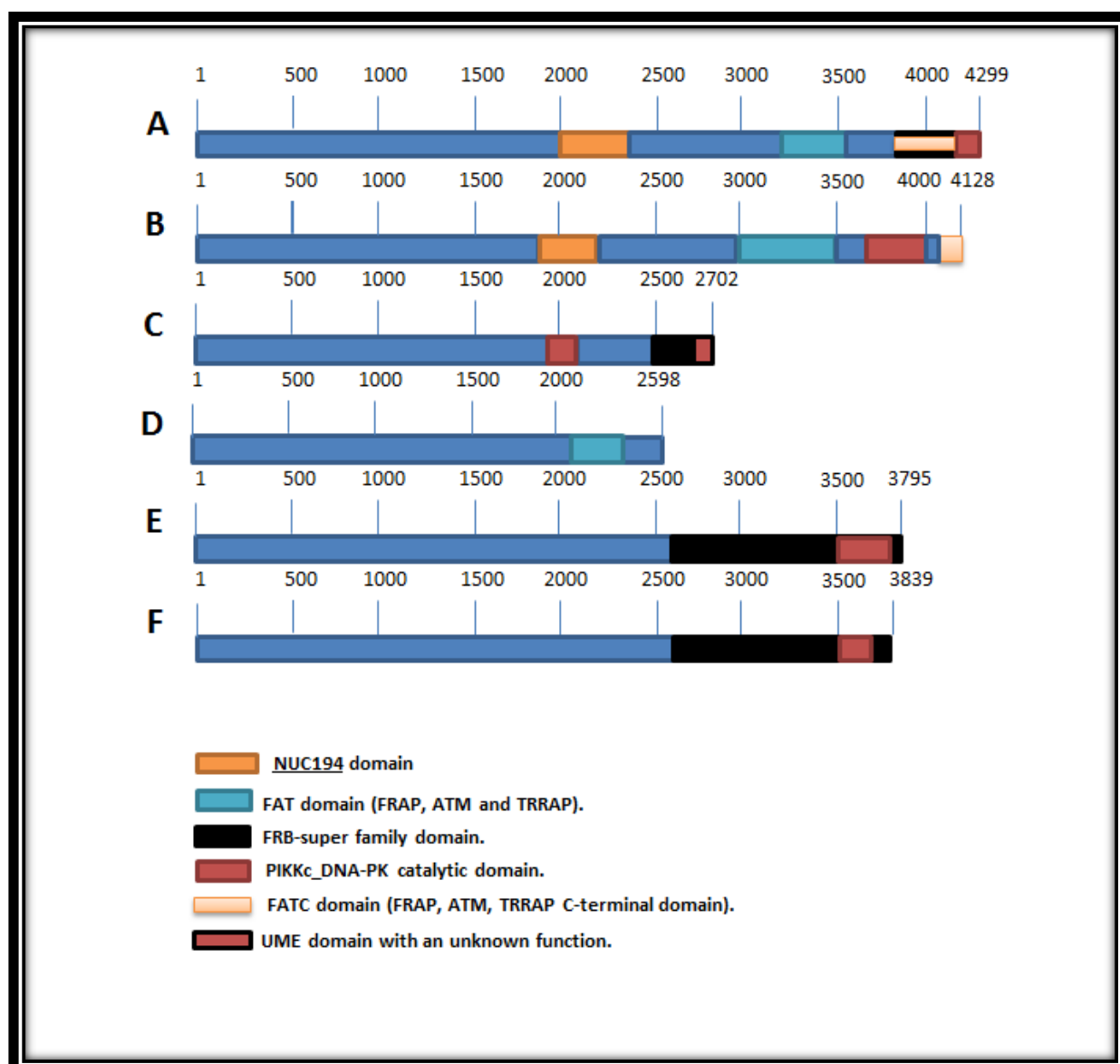


Figure 20: stick diagram shows the comparisons of the domain layout and protein sizes relative to each other. A-Conserved domains for DNAPKs in Dictyostelium discoideum(4,299 Amino Acid).B-Conserved domains for DNAPKs in Human(4,128 Amino Acid).C-Conserved domain for *A. thaliana* ATR(2,702 Amino Acid).D-Conserved domain for Arabidopsis ATM(2,598 Amino Acid).E- Conserved domain analysis for the putative DNAPKa in *A. thaliana* (3,795 Amino Acid).F-Conserved domain analysis for the putative DNAPKb in *A. thaliana* (3,839 Amino Acid).

The locus structure of the putative *AtDNA-PKa*.

The locus *AtDNA-PKa* (AT2G17930) is located at chromosome 2. The gene length is 18,317 bp. The *AtDNA-PKa* gene encodes for a 3,858 aa protein (44kDa) with domains related to phosphatidylinositol3 and has kinase family of proteins.

We obtained a T-DNA insertion mutant line (Salk_096167) from the Nottingham Arabidopsis Stock Center (NASC: <http://arabidopsis.info>). The T-DNA insertion is located in exon number 30 (**Figure 21**).

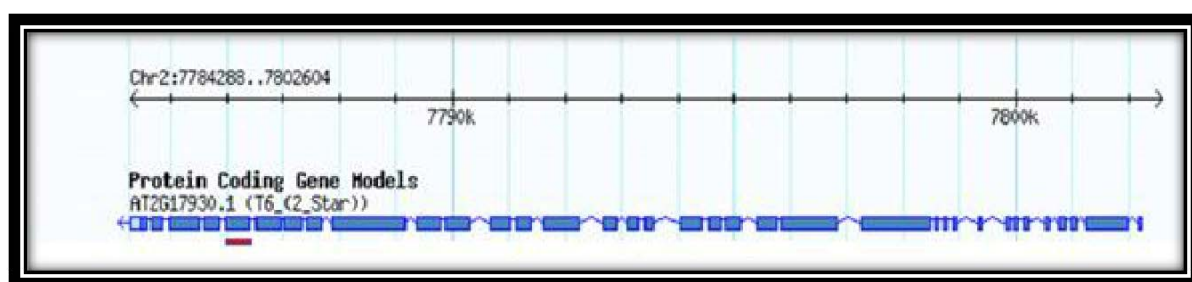


Figure 21: The Gene Model for putative *AtDNAPKa*:

The diagram shows the 34 exons (blue boxes) and 33 introns of *AtDNAPKa*. Full genomic sequence is available in **Appendix (3)**. The red box indicates the location of the T-DNA insertion in the line Salk_096167.

The locus structure for the putative *AtDNA-PKb*

AtDNA-PKb locus (AT4G36080) is located on chromosome 4. The gene *AtDNA-PKb* has a sequence length of 12,133bp. The *AtDNA-PKb* protein contains 3,834 aa with a molecular weight of 43kDa. We have analysed a T-DNA insertion line (Salk_105303) from NASC. The T-DNA is inserted in exon number 24 (**Figure 22**).

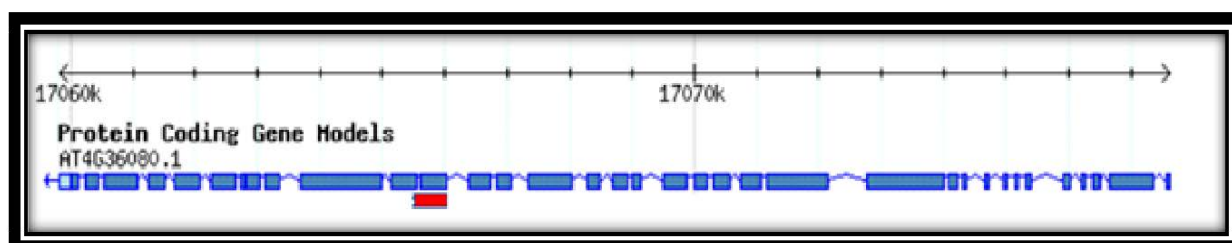


Figure 22: The Gene Model for putative *AtDNA-PKb*:

The diagram shows that *AtDNA-PKb* has 34 exons (blue boxes) and 33 introns. Also, the red box indicates the location of the T-DNA insertion for the line Salk_105303. Full genomic sequence is available in the **Appendix (4)**.

Similarities between AtDNA-PKa and AtDNA-PKb proteins

The protein sequences from AtDNAPKa and AtDNAPKb were analysed using the NCBI Constraint-based Multiple Alignment Tool (COBALT, <https://www.ncbi.nlm.nih.gov/tools/cobalt/>). COBALT is a good tool to align similar protein sequences using RPS-BLAST, BLASTP, and PHI-BLAST by matching sequence clusters (Papadopoulos and Agarwala, 2007).

The analysis shows that AtDNAPKa and AtDNAPKb are very similar with an 88% identity, 93% positives and only 0% of gaps (**Figure 23**).

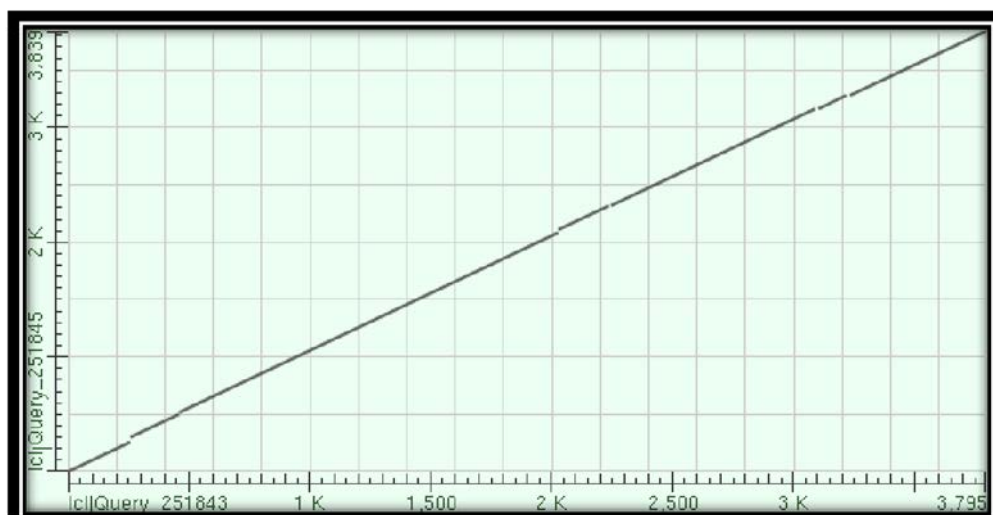


Figure 23: Comparison of AtDNA-PKa Sequence with AtDNA-PKb Protein Sequence.

Genotyping for homozygous T-DNA insertion mutant line *Atdnapka*

The results (**Figure 24**) shown very clear that Col has not T-DNA insertion therefore only the PCR reaction with LP+RP amplifies a band of about 1Kb and the reaction with primers RP+BP does not produce any amplification. Nevertheless, the homozygous mutant shows not PCR amplification using LP+RP but it produces a DNA fragment of around 750bp using RP+BP primers showing that the T-DNA insertion is in homozygosis (**Appendix 3**).

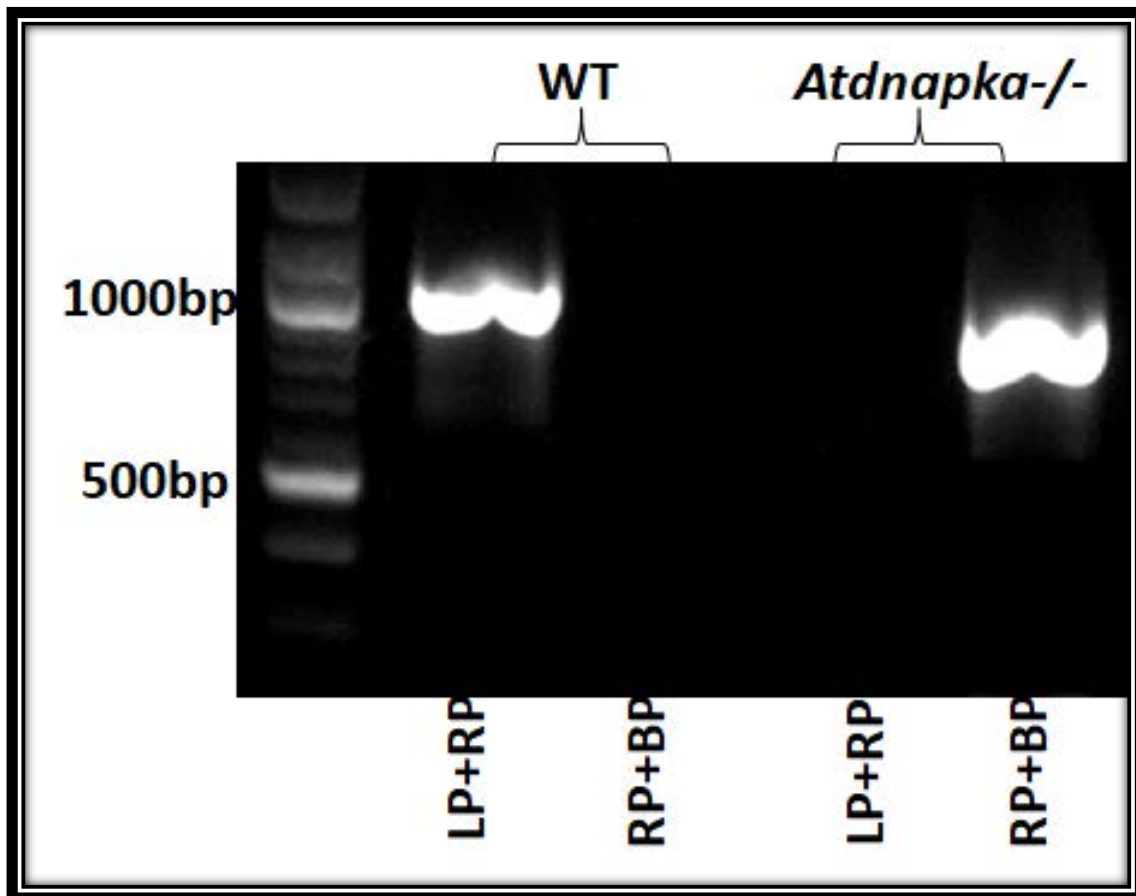


Figure 24: PCR genotyping electrophoresis for *AtDNA-PKa* in *Arabidopsis thaliana*.

Agarose gel electrophoresis was used to analyse the wild-type and mutated genotype for putative *AtDNA-PKa* in *A.thaliana* plants. Lane 1 contains the 100bp DNA ladder from *BioLabs*. Lane 2 contains *AtDNA-PKa* amplification with (LP and RP) wild-type genomic DNA showing a band of around 1031 bp as expected. Lane 3 contains the PCR product using primers RP and BP to analyse the T-DNA insertion; no band was obtained in the wild-type as predicted. Lane 4 shows no band amplification of the putative genomic *AtDNA-PKa* using LP and RP primers specific for the gene. Lane 5 contains the amplification of the T-DNA band using primers RP and BP in *Atdna-pka* homozygous T-DNA insertion line showing a band of around 750bp.

Genotyping for homozygous T-DNA insertion mutant line *Atdnapkb*

The results (**Figure 25**) shown very clear that Col has not T-DNA insertion therefore only the PCR reaction with LP+RP amplifies a band of about 1Kb and the reaction with primers RP+BP does not produce any amplification. Nevertheless, the homozygous mutant shows not PCR amplification using LP+RP but it produces a DNA fragment of around 750bp using RP+BP primers showing that the T-DNA insertion is in homozygosis (**Appendix 4**).

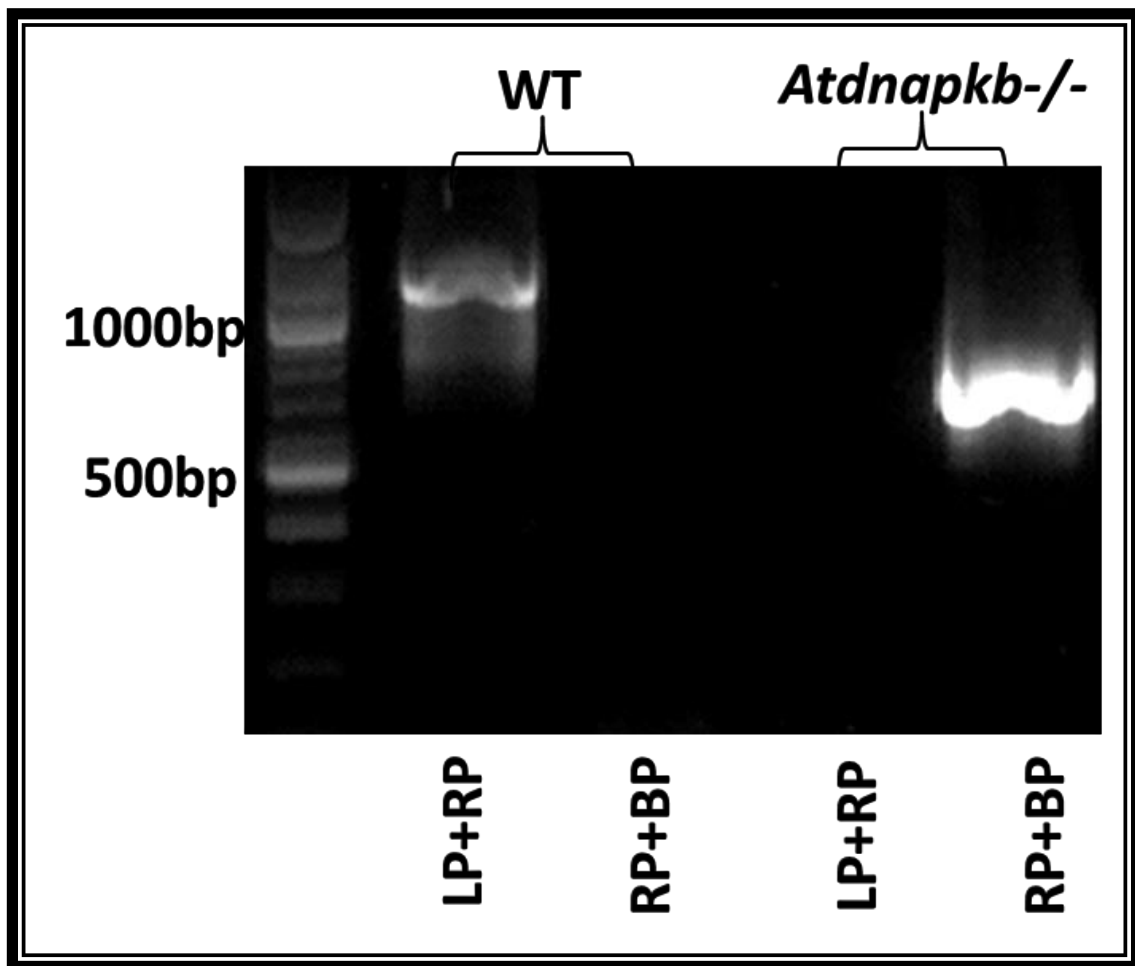


Figure 25: PCR genotyping electrophoresis for *AtDNA-PKb* in Arabidopsis.

Agarose gel electrophoresis was used to demonstrate the wild-type and mutated genotype for putative *AtDNA-PKb* in *A.thaliana* plants. Lane 1 contains the 100bp DNA ladder from *BioLabs*. Lane 2 contains *DNA-PKb* amplification with (LP and RP) wild-type genomic DNA showing a band of around 1,149 bp as expected. Lane 3 contains the PCR product using primers RP and BP to analyse the presence of the T-DNA insertion; no band was obtained as predicted in the WT. Lane 4 shows no band amplification of the putative genomic *AtDNA-PKb* using LP and RP primers in the mutant line. Lane 5 contains the amplification of the T-DNA band using primers RP and BP in *Atdna-pkb* homozygous T-DNA insertion line showing a band of around 650 bp.

Crossing both Homozygous single mutants for the putative *AtDNA-PKcs*

Cross between *Atdna-pka* homozygous mutant line and *Atdna-pkb* homozygous T-DNA insertion mutant was carried out by Dr. Sanchez-Moran and Claire Cooper in our laboratory to obtain a double mutant for both genes. A plant homozygous for *Atdna-pka* and heterozygous for *Atdna-pkb* (*Atdnapka*^{-/-} *Atdnapkb*^{+/-}) was obtained in the second generation (**Figure 26**).

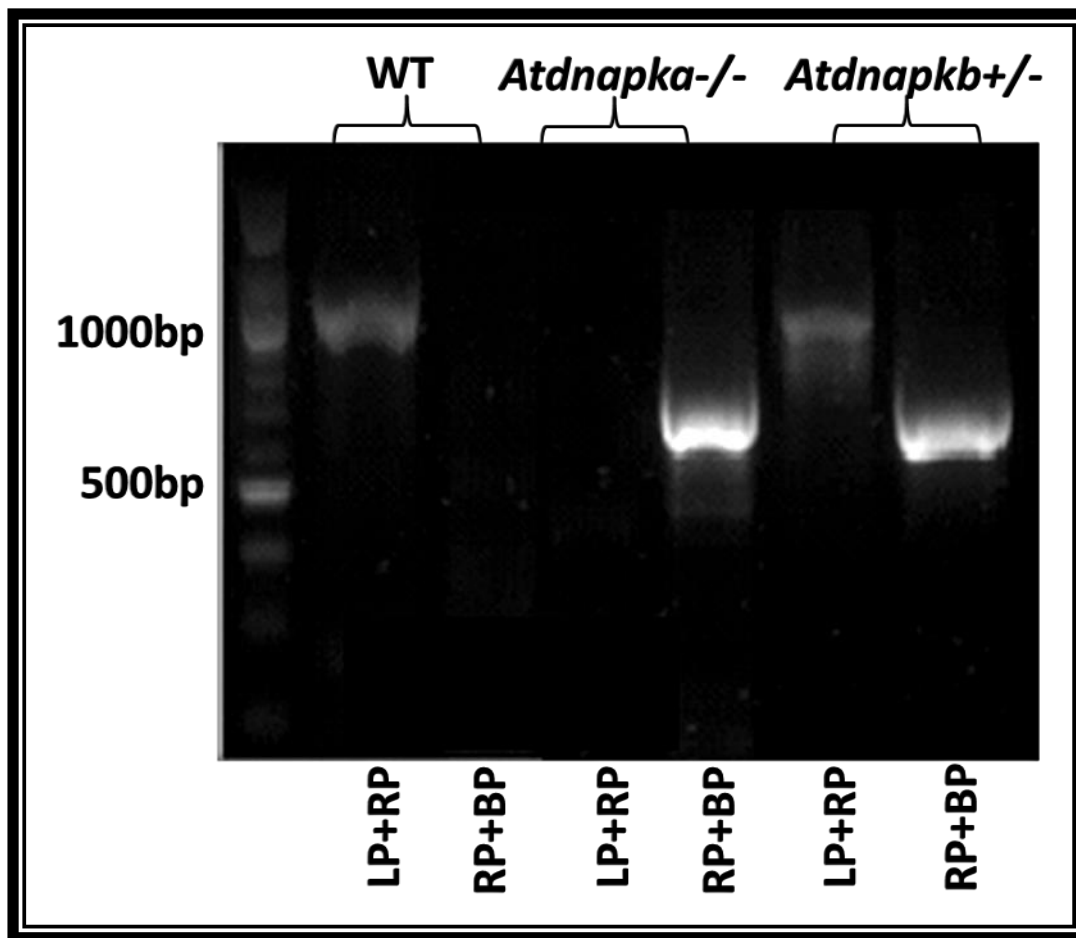


Figure 26: PCR genotyping for *Atdna-pka* and *Atdna-pkb* insertion lines

Lane 1 contains the 100bp DNA ladder from *BioLabs*. Lane 2 contains *AtDNA -PKa* amplification with (LP and RP) wild-type genomic DNA showing a band of around 1031 bp as expected. Lane 3 shows no band amplification of T-DNA insertion using primers RP and BP using WT DNA. Lane 4 shows no band amplification for the genomic *AtDNA-PKa* using LP and RP primers. Lane 5 shows the amplification of the T- DNA band using primers RP and BP in *Atdna-pka* homozygous line, showing a band of around 750 bp. Lane 6 contains *AtDNA-PKb* amplification with (LP and RP) wild-type genomic DNA showing a band of around 1,149 bp as expected. Lane 7 contains the PCR product using primers RP and BP to analyse the presence of the T-DNA insertion; a band was obtained as predicted in a heterozygous mutant for *Atdna-pkb* showing a band of around 650bp.

Over 1,000 seeds from this plant were grown in the lab over the past 5 years growing in optimal conditions at the glasshouse, during all that time only one plant was obtained to be a double homozygote for these mutations (**Figure 27**).

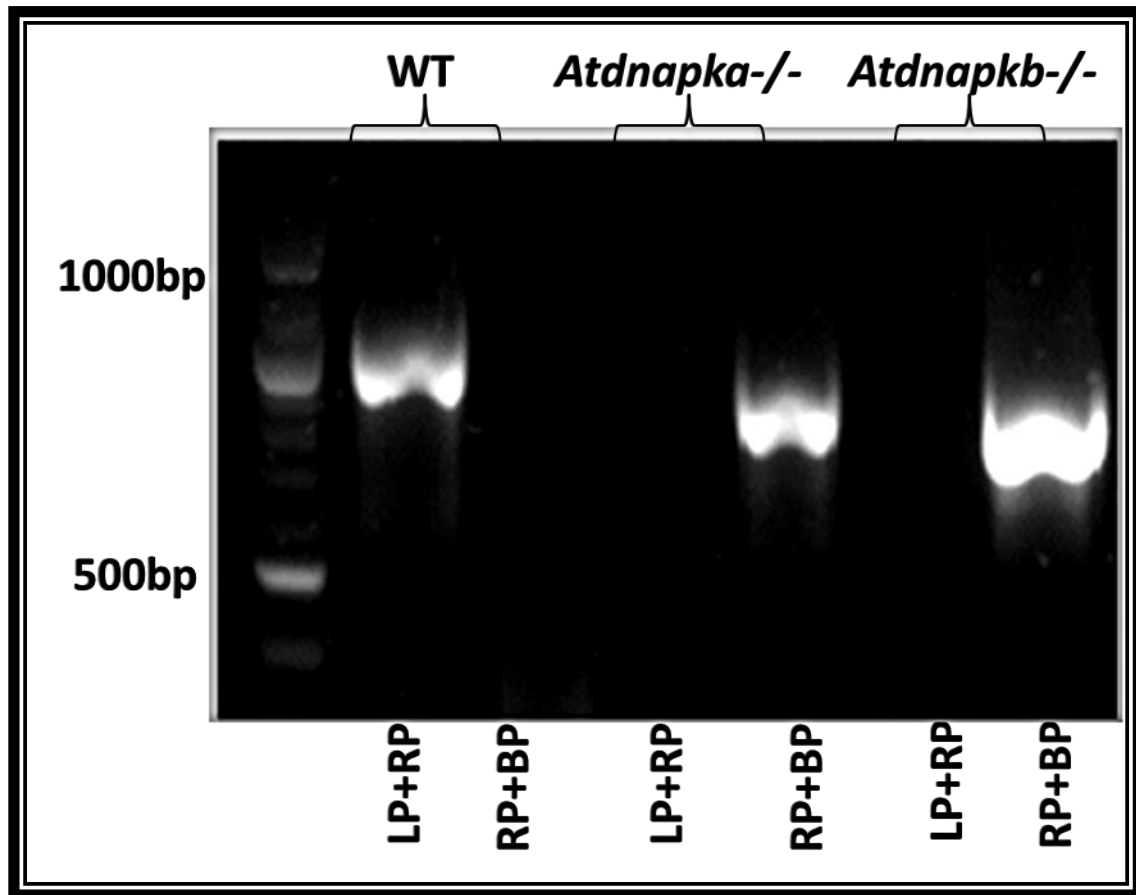


Figure 27: PCR genotyping for *Atdna-pks* T-DNA insertion lines in *Arabidopsis thaliana*.

Agarose gel electrophoresis was used to genotype a putative double homozygote mutant for *Atdna-pka* and *Atdna-pkb* obtained from. Lane 1 contains the 100bp DNA ladder from *BioLabs*. Lane 2 contains the genomic amplification for *AtDNA-PKb* with LP and RP primers and wild-type genomic DNA showing a band of around 1Kb as expected. Lane 3 shows no band amplification of T-DNA insertion using primers RP and BP in WT. Lane 4 shows no genomic band amplification *AtDNAPKa* In a homozygous mutant. Lane 5 contains the amplification of the T-DNA insertion in *Atdna-pka* homozygous T-DNA insertion line. Lane 6 shows no genomic band amplification for *AtDNAPKb*. Lane 7 contains the amplification of the T-DNA insertion band in *Atdna-pkb* homozygous T-DNA insertion line mutant. The genotyping for this plant indicates that it is a double homozygous mutant for both *Atdna-pka* and *Atdna-pkb*.

Expression of the putative *AtDNA-PKa* and *AtDNA-PKb* genes in different plant tissues

Total mRNA was extracted from different plant tissues: roots, leaves, seeds, flower buds, and siliques). Reverse Transcription-PCR (RT-PCR) was carried out by using an oligo dT to produce a library of cDNAs in these tissues. Specific primers to detect the expression of *AtDNA-PKa* and *AtDNA-PKb* were designed and used in different parts of the *Arabidopsis thaliana* wildtype cultivar Columbia (Col) and also in the respective homozygous mutants

for the T-DNA insertions. Different primers were employed in the identification of the *Actin2* housekeeping gene to ensure that the overall concentration of mRNA in each of the plant samples compared were similar. Once it was identified that the signals for *Actin2* RT-PCR were similar, it was then possible to use specific primers to analyse the expression patterns of the genes *AtDNA-PKa* and *AtDNA-PKb*. Surprisingly, the expression of both genes was highly enriched in flower buds (**Figure 28**). Nevertheless, expression was also observed in roots, leaves, seeds, and siliques but with a weaker signal.

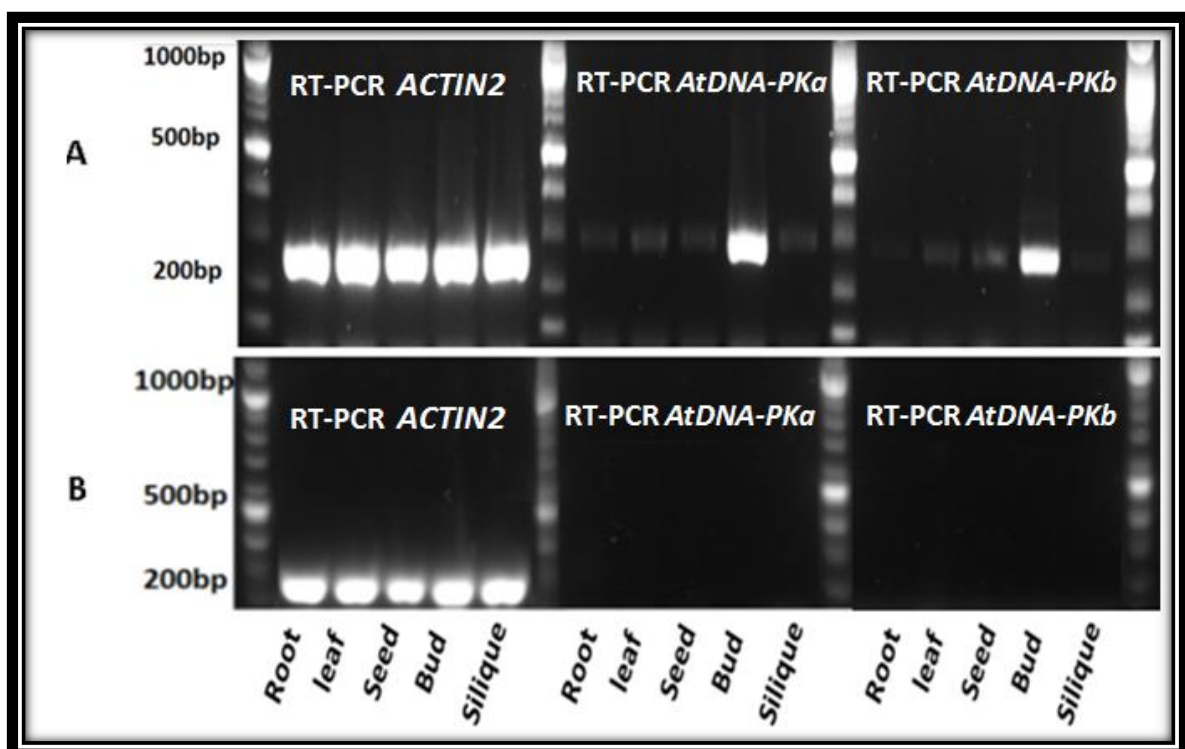


Figure 28: RT-PCR gel electrophoresis to analyse the expression patterns of *AtDNA-PKa* and *AtDNA-PKb* genes in WT and T-DNA insertion lines *Atdna-pka* and *Atdna-pkb* in different tissues.

(A): RT-PCR gel electrophoresis to analyse the expression patterns of *AtDNA-PKa* and *AtDNA-PKb* genes in WT. *Actin2* gene expression was used to ensure a similar concentration of mRNA in all the samples to compare. Col roots, leaves, seeds, flower buds, and siliques were used for this experiment. (B) : RT-PCR gel electrophoresis in T-DNA insertion lines *Atdna-pka* and *Atdna-pkb* in different tissues. RT-PCR in single mutants for *Atdnapka* and *Atdnapkb* showed that both single mutants were null mutants, as not expression could be observed in either of them.

RT-PCR analysis of the expression of putative *AtDNA-PKcs* in the mutants identified in *Arabidopsis thaliana*.

Total mRNA was extracted from flower buds and leaves, which was subsequently utilised to generate cDNA libraries from the different genetic background plants (wildtype

and mutants). Different primers were employed in the expression of the *Actin2* housekeeping gene to ensure that the overall concentration of mRNA in each of the plant backgrounds were similar (Figure 29 & Figure 30).

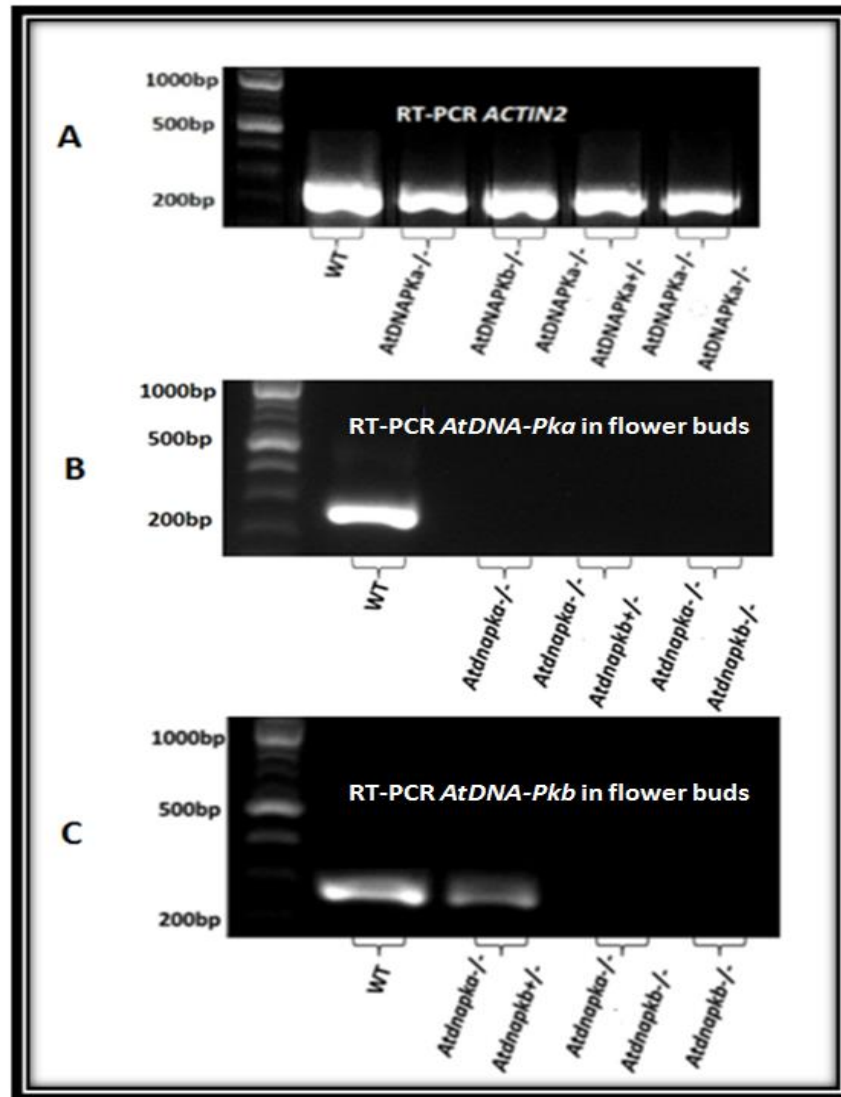


Figure 29: The expression of *AtDNAPKa* and *AtDNAPKb* genes in flower buds in the different plant backgrounds.

(A) RT-PCR gel electrophoresis showing the expression of *Actin2* gene in flower buds in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 wild-type buds. Lane 3 *Atdna-pka* homozygous mutant. Lane 4 *Atdna-pkb* homozygous. Lane 5 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 6 Double homozygous mutant. (B) RT-PCR gel electrophoresis showing the expression of *AtDNA-PKa* gene in flower buds in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 wild-type flower buds. Lane 3 *Atdna-pka* homozygous mutant. Lane 4 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 5 Double homozygous mutant. (C) RT-PCR gel electrophoresis showing the expression of *AtDNA-PKa* gene in flower buds in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 RT-PCR for wild-type leaves. Lane 3 *Atdna-pka* homozygous mutant. Lane 4 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 5 Double homozygous mutant. All the cDNA amplified bands for *AtDNA-PKa* from flower buds were around 200 bp.

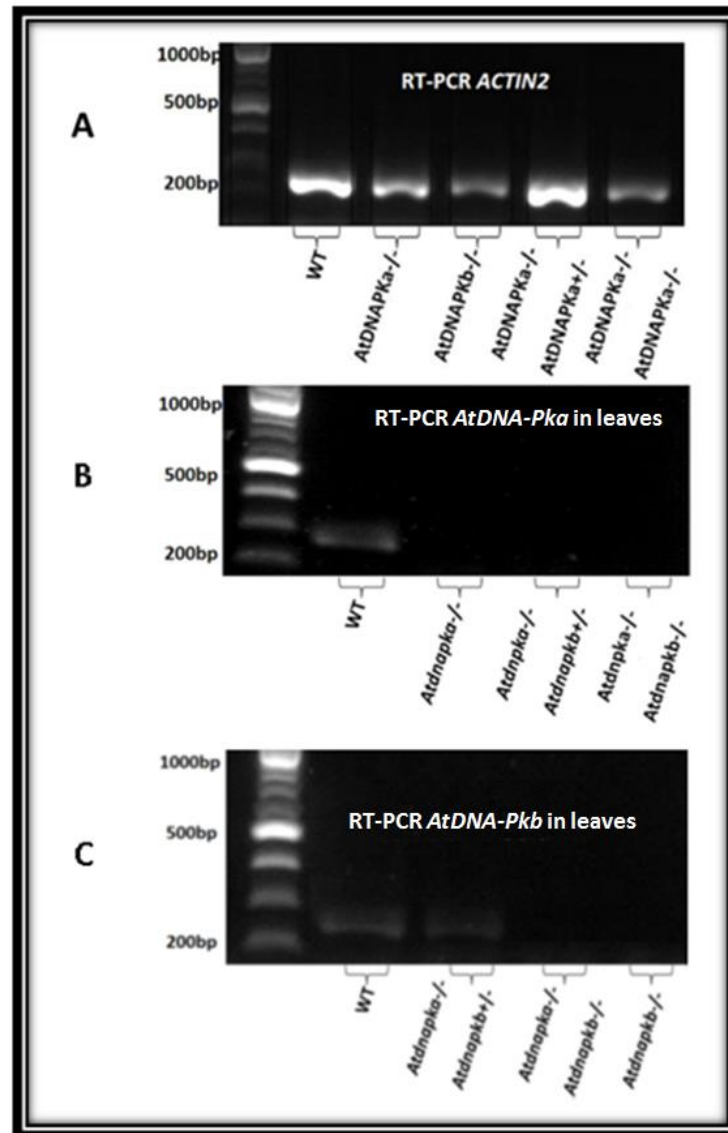


Figure 30: The expression of *AtDNAPKa* and *AtDNAPKb* genes in leaves in the different plant backgrounds.

(A) RT-PCR gel electrophoresis showing the expression of *Actin2* gene in leaves in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 RT-PCR for wild-type leaves. Lane 3 *Atdna-pka* homozygous mutant. Lane 4 *Atdna-pkb* homozygous. Lane 5 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 6 Double homozygous mutant. All the cDNA amplified bands for *Actin2* from leaves were around 200 bp. (B) RT-PCR gel electrophoresis showing the expression of *AtDNA-PKa* gene in leaves in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 RT-PCR for wild-type leaves. Lane 3 *Atdna-pka* homozygous mutant. Lane 4 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 5 Double homozygous mutant. All the cDNA amplified bands for *AtDNA-PKa* from leaves were around 200 bp. (C) RT-PCR gel electrophoresis showing the expression of *AtDNA-PKb* gene in leaves in the different plant backgrounds: Lane 1 is the marker 100 bp from *BioLabs*. Lane 2 RT-PCR for wild-type leaves. Lane 3 *Atdna-pka* homozygous and *Atdna-pkb* heterozygous mutant. Lane 4 Double homozygous mutant. Lane 5 *Atdna-pkb* homozygous mutant. All the cDNA amplified bands for *AtDNA-PKb* from leaves were around 200 bp.

Phenotype analysis of putative *AtDNA-PKCS* T-DNA insertion mutant lines

The measurement of the silique length and the quantification of the number of seeds per silique was carried out to assess the fertility for wild-type plants, *Atdna-pka* homozygous, *Atdnb-pka* homozygous, and *Atdna-pka* homozygous and *Atdna-pkb* heterozygous Mutant, and the double homozygous mutant for both. The mean length of the silique for *Atdna-pka* homozygous mutant and *Atdna-pkb* heterozygous mutant was similar to that observed at the wild-type of about 13.58 mm, while the length of the silique in the double homozygous mutant was drastically reduced (11.3mm) (**Figure 31A**). Furthermore, the plant growth and development in the double homozygous mutant plants appeared to be delayed compared to the wild-type plants. The mean number of seeds per silique in the double homozygous mutant (*Atdnapka*^{-/-} *Atdnapkb*^{-/-}) was significantly reduced to less than 5 seeds per silique compared to the 33 seeds per silique in the double homozygous mutant (*Atdnapka*^{-/-} *Atdnapkb*^{-/-}) and 55 seeds per silique in the wild-type (**Figure 31B**). The seeds of double homozygous mutant (*Atdnapka*^{-/-} *Atdnapkb*^{-/-}) were not able to grow again.

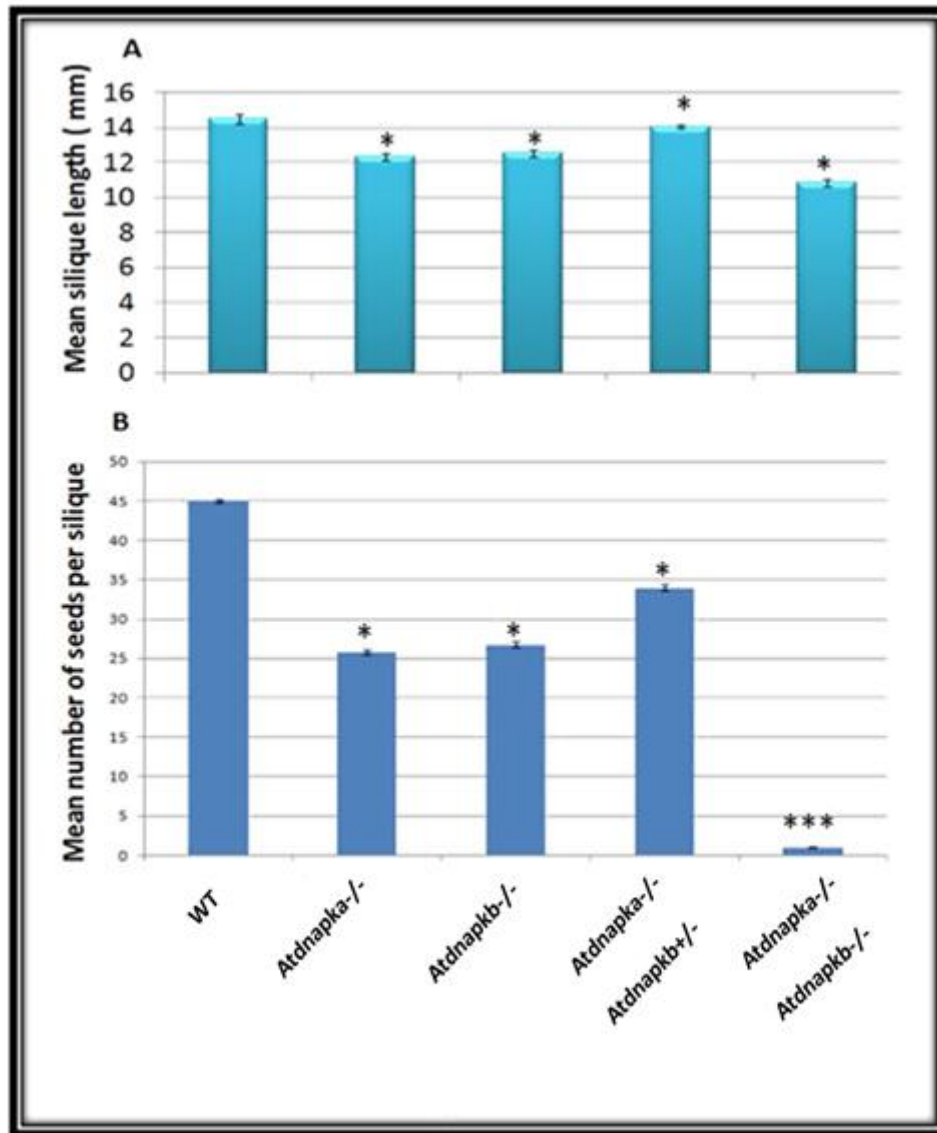


Figure 31: Plant fertility analysis, the mean number of seeds count and mean silique length for WT, and the different mutants for the putative *AtDNA-PKcs* in *Arabidopsis thaliana*.

(A) Mean silique length for 100 siliques measured from each plant. (B) Mean number of seeds per silique for 100 siliques measured from each plant. Error bar = standard error of the mean. (*) p.value < 0.05, (***) p.value < 0.001.

Fertility of *Atdnapkcs* mutant.

The viability of pollen grains was assessed using Alexander staining. The pollen from anthers was collected from three completely open flowers, partially open flowers, and completely closed flower buds. It was observed that the overall number of pollen on anthers was reduced in *Atdnapka*^{-/-} *Atdnapkb*^{+/-} mutants(**Figure 32B**) and highly reduced in *Atdnapka*^{-/-} *Atdnapkb*^{-/-} mutants (**Figure 32C**) compared to WT. Pollen staining showed that the magenta-red color cytoplasm was reduced in *Atdnapkcs* mutants. Pollen with blue-green color staining the cell wall and without viable cytoplasm was higher in number in *Atdnapkcs* mutant than in the WT.

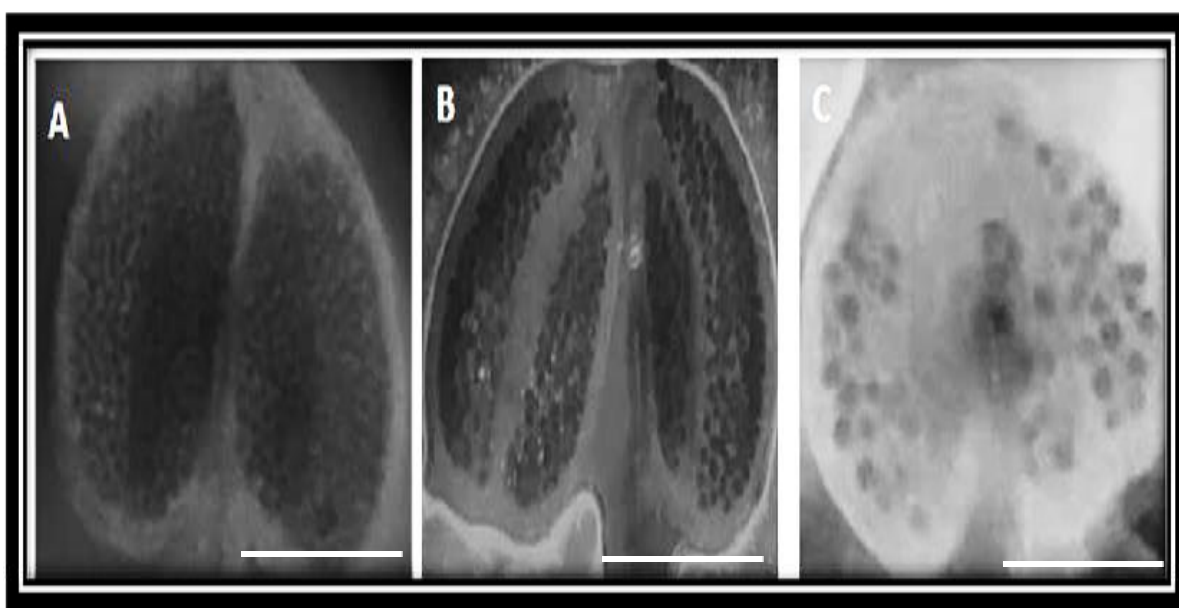


Figure 32: Photographs of Alexander staining pollen inside anthers.
(A) WT. (B) *Atdnapka*^{-/-} *Atdnapkb*^{+/-} mutants. (C) *Atdnapka*^{-/-} *Atdnapkb*^{-/-} mutants.
Scale bar =5 μ m

Plant phenotype of *Atdna-pkcs* double homozygote plants

A double homozygous mutant was obtained from the segregation of an *Atdnapka*^{-/-} x *Atdnapk*^{-/-} mutant (**Figure 33**).

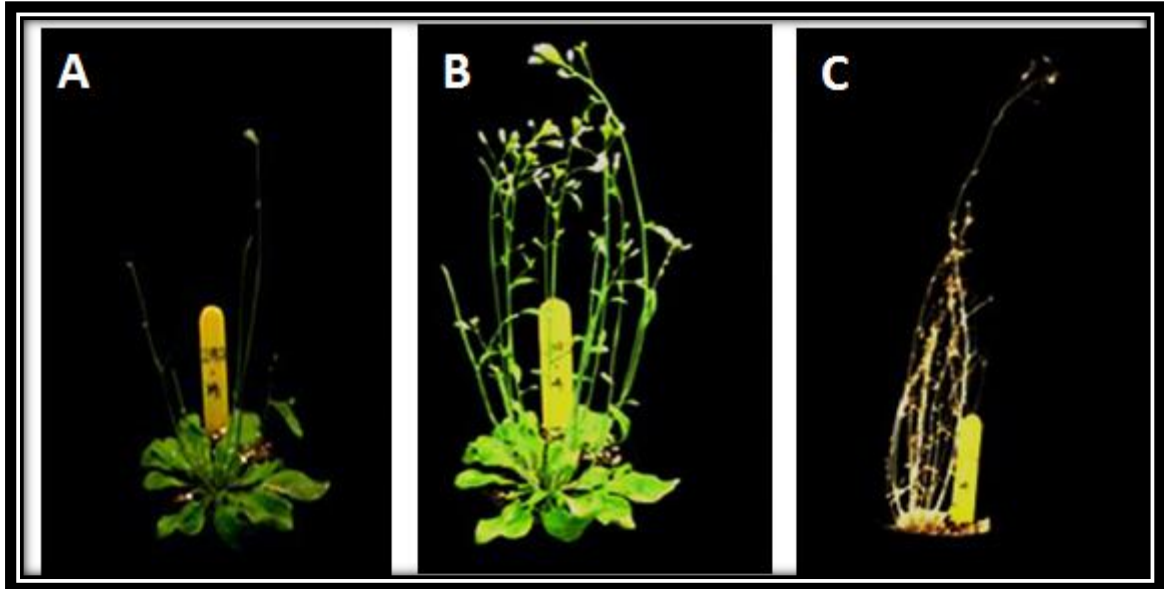


Figure 33: *Atdna-pkcs* double homozygote mutant plant photographs at different development stages.

(A) Plant at early stages of flower development. (B) A plant is producing different inflorescences. (C) Early plant death.

Meiotic analysis of *Atdnapk* mutants in *Arabidopsis thaliana*

To carry out the meiotic analysis, cytological analysis of chromosomes by DAPI staining from pollen mother cells (PMCs) was carried out, and a meticulous analysis through all stages of male meiosis was done using epifluorescence microscopy. Individual chromosomes are not yet distinguishable or paired at Leptotene in the WT PMCs. Chromosomes start pairing, and synapsis starts occurring, and become visible at some edges of the nucleus during early Zygotene. Chromosomes are synapsed and distinguishable as threads during early stages of Pachytene (**Figure 34A**). During Diplotene, homologous chromosomes start separating and unsynapsing. Five distinct bivalents are visible; homologs are held by chiasmata (the physical manifestation of crossovers (COs) during Diakinesis (**Figure 34B**). These bivalents are not aligned yet but

are partially condensed. Bivalents are further condensed and aligned on the Metaphase I plate (**Figure 34C**). During Anaphase I, homologs separate and move to the opposite poles of the cell (**Figure 34D**). Chromosomes are decondensed during Telophase I forming 2 nuclei in one meiotic product. Chromosomes recondensed and passed through the meiosis II stages, starting with Prophase II without DNA replication (**Figure 34E**). During Metaphase II, chromosomes align at the cell equator (**Figure 34F**). And sister chromatids are separated to the opposite poles during Anaphase II (**Figure 34G**). Decondensation of sister chromatids into four haploid daughter nuclei occur during Telophase II (**Figure 34H**). The four products will separate and mature into 4 individual gametes or pollen grains.

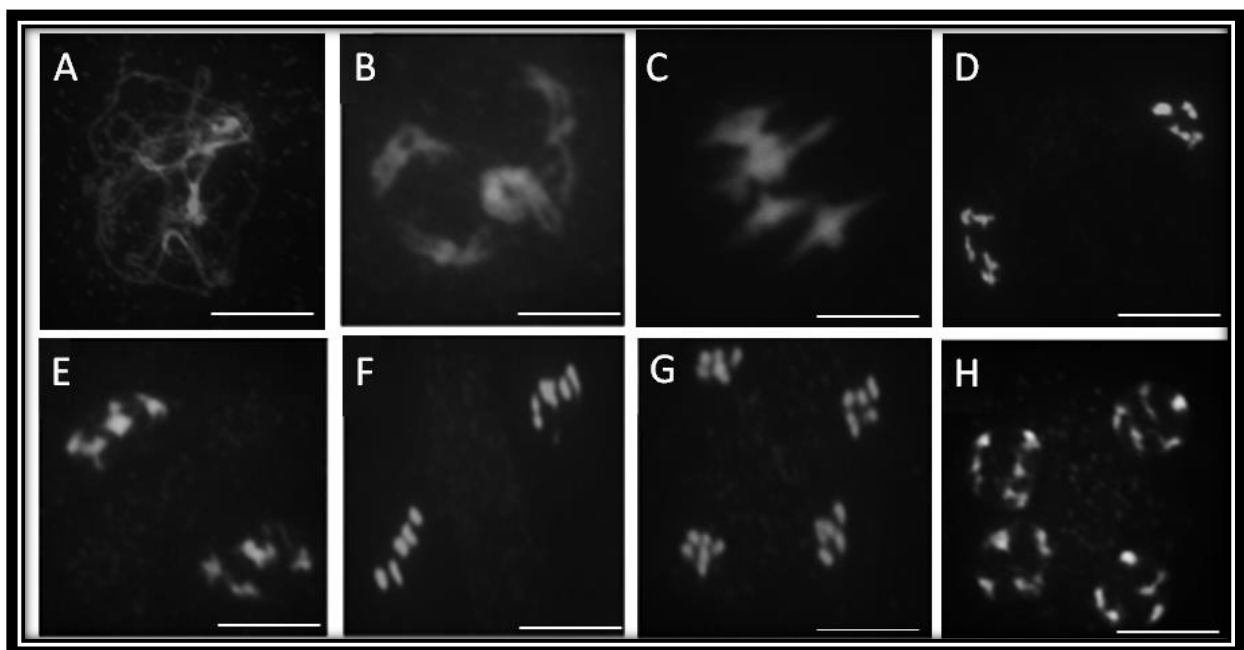


Figure 34: The meiosis stages in *Arabidopsis thaliana* Col (WT).

(A) Pachytene stage where the homologous chromosomes fully synapse at this stage. (B) Diakinesis, in which the chromosomes are shown to have more intensity, and the chiasmata are apparent between the homologous chromosomes, holding them together in bivalent configurations. (C) Metaphase I, bivalents are co-orientating at the metaphase plate. (D) Anaphase I, the homologous chromosomes are segregation to opposite poles. (E) Prophase II, at this stage, chromosomes start to condense again. (F) Metaphase II chromosome can become well arranged on the two metaphase plates. (G) Anaphase II sister chromatids separate from each chromosome. (H) Telophase II, also known as tetrad stage, decondensation of chromosomes and clear appearance of the four gametic products, the nuclei will separate and mature to form four haploid gametes or pollen grains. Scale bar = 5 μ m.

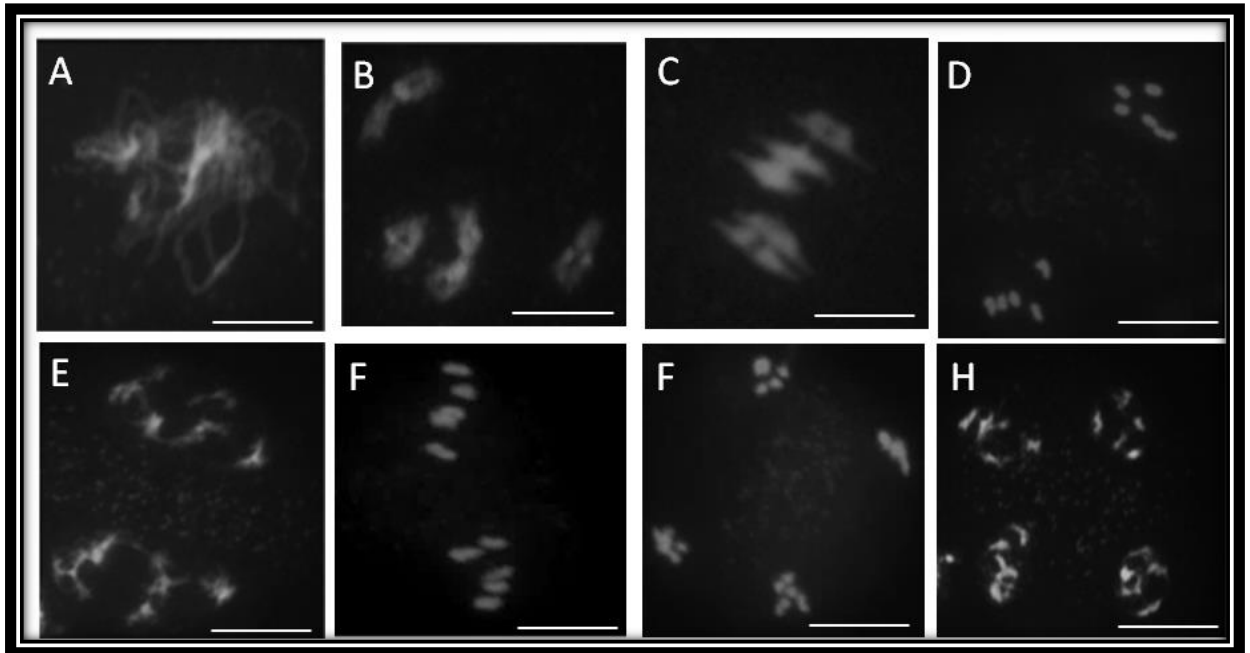


Figure 35: The meiotic stages in a double homozygous mutant for the putative *Atdnapkcs* mutant (*Atdnapka*^{-/-} *Atdnapkb*^{-/-})

(A) Pachytene, showing normal synapsis. (B) Diakinesis, showing the normal formation of bivalents. (C) Metaphase I, five bivalents are co-orientating at the plate like at WT. (D) Anaphase I, homologous chromosomes segregating to opposite poles. (E) Prophase II. (F) Metaphase II. (G) Anaphase II. (H) Telophase II or Tetrad Scale bar = 5 μm

Analysis of mitotic division in the double homozygous mutant for the putative *Atdnapkcs* mutant

Mitotic stages were analysed using cytological epifluorescence microscopy in flower bud tissue going through mitosis. In the double homozygous mutant for *Atdnapkcs*, the presence of certain anaphase bridges was very clearly observed (1/10). This shows the significance of *AtDNAPKcs* in the repair of DBSs in somatic cells for plants (**Figure 36**).

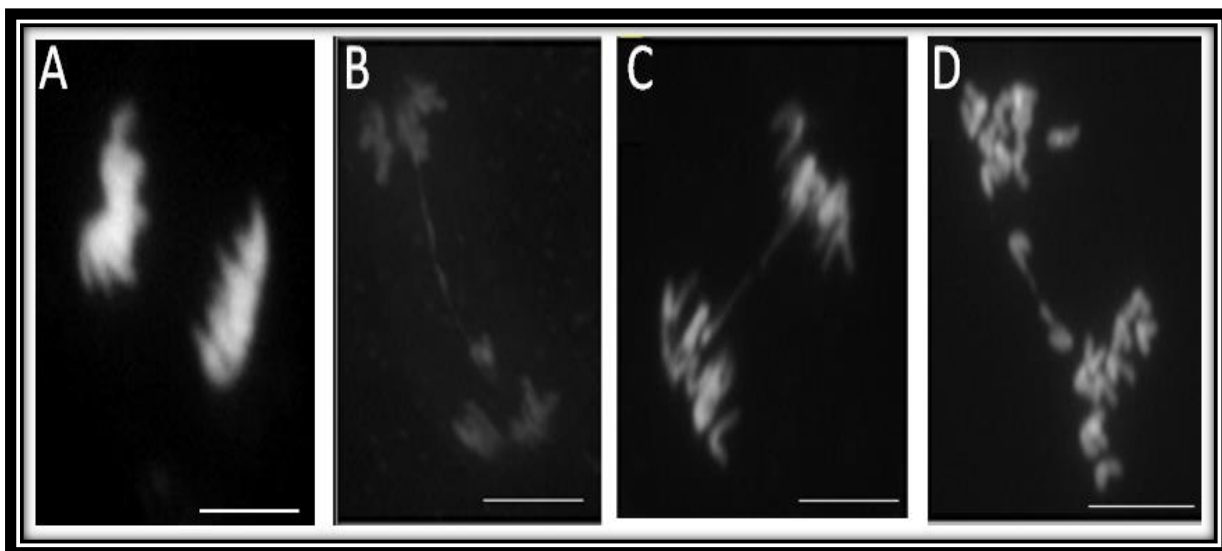


Figure 36: Fluorescence micrographs of mitotic anaphase in WT and the double homozygous of putative *AtDNA-PKCs* in *Arabidopsis thaliana*. (A) Normal chromosome segregation at mitotic anaphase in WT. (B-D) Mitotic anaphase bridges in the double homozygous for putative *AtDNA-PKCs* in *Arabidopsis thaliana*. Scale bar = 5 μ m

Expression analysis of the putative *AtDNA-PKCs* during the development of flower buds

Total mRNA was extracted from different flower bud sizes which were subsequently used to produce cDNA libraries from wildtype. Then, expression analysis of putative *AtDNA-PKCs* genes was carried out. Different primers were employed to amplify *Actin2* housekeeping gene to ensure that the overall concentration of mRNA in each sample was similar (**Figure 37A**). After checking the level of *AtDNA-PKa* and *AtDNA-PKb* expression in different sizes of flower buds (Small, medium and big open flower), it was Very clear that the strongest expression of both genes is occurring in big open flower buds which have the anthers full of fully developed pollen grains (**Figure 37B**). The expression of *AtDNA-PKa* and *AtDNA-PKb* in male reproductive organs (Anthers) and female reproductive organs (ovaries) was also investigated by RT-PCR showing that although they are expressed in both is slightly higher in female reproductive organs (**Figure 38B**).

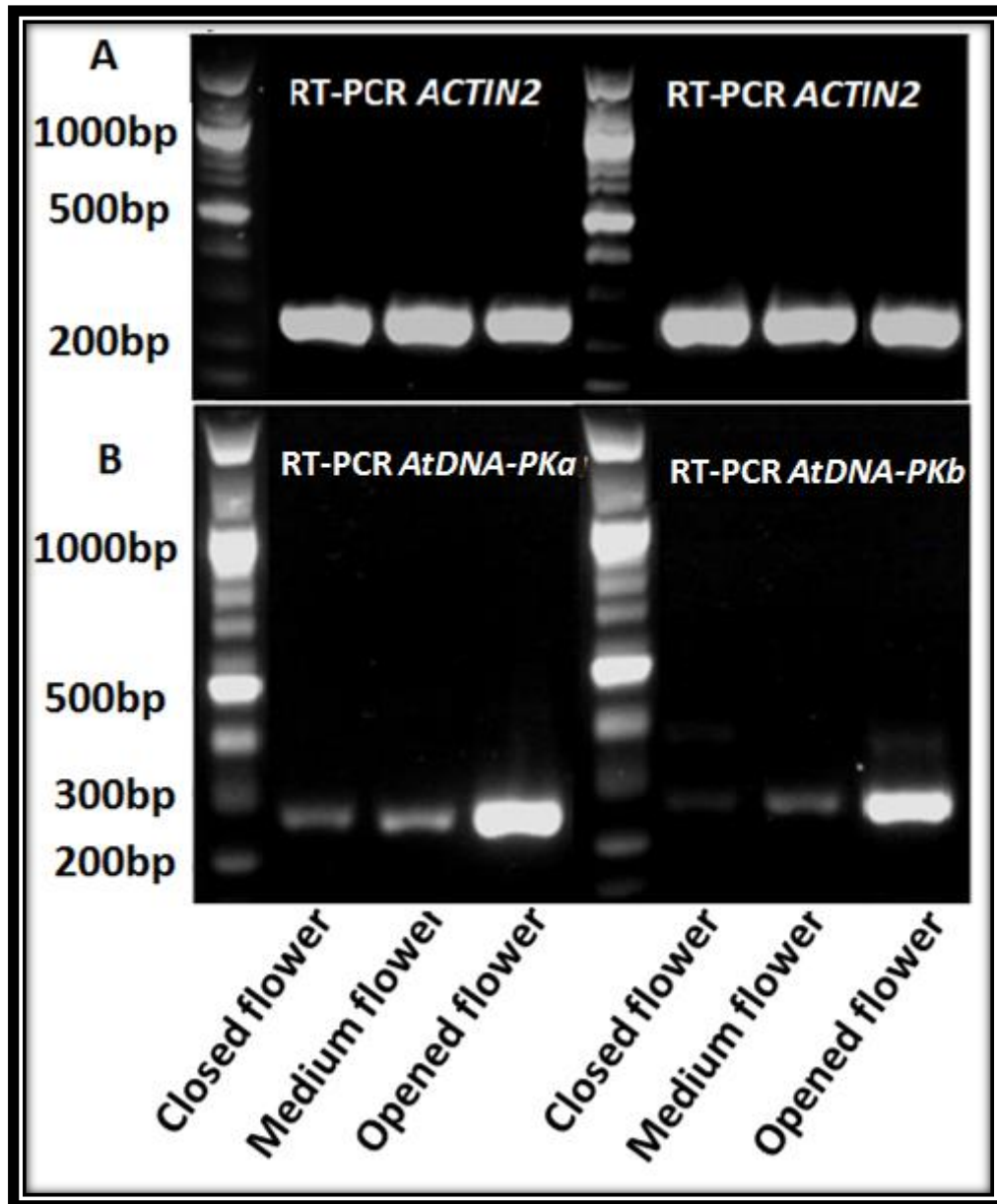


Figure 37: RT-PCR gel electrophoresis to analyse the expression of AtDNA-PKs in different size of flower buds in *Arabidopsis thaliana*.

(A) Expression of *Actin2* gene in different size of flower buds to check equal quantities in all samples. (B) Expression of *AtDNA-PKα* and *AtDNA-PKβ* genes in different size of flower buds. There is a higher expression of both *AtDNA-PKα* and *AtDNA-PKβ* genes in bigger opened flowers.

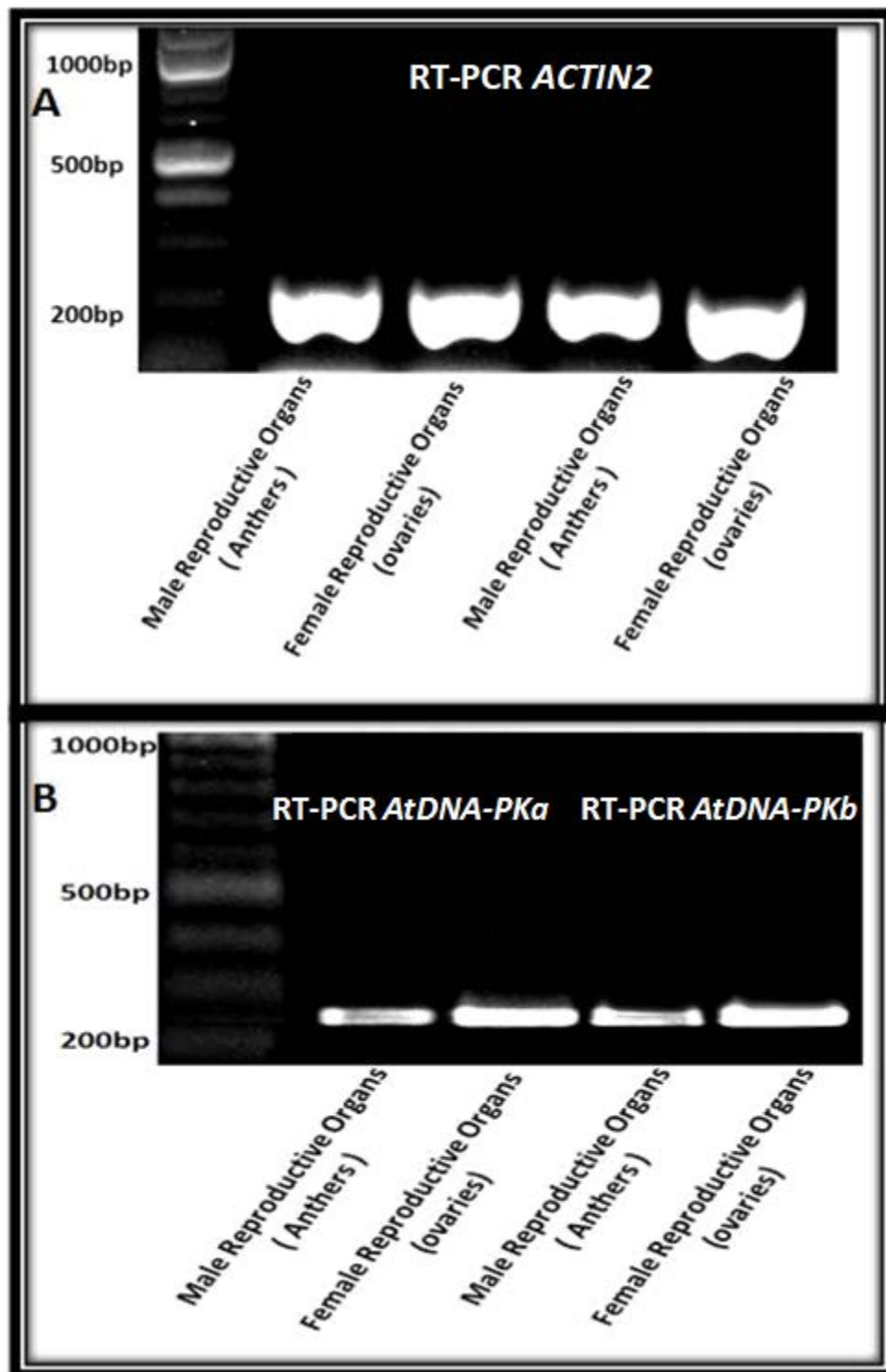


Figure 38: RT-PCR gel electrophoresis to analyse the expression of AtDNA-PKcs in male organs (Anthers) and female organs (ovaries) in *Arabidopsis thaliana*

(A) Expression of *Actin2* gene in male organs (Anthers) and Female Organs (ovaries) to analyse equal amounts in all samples. (B) Expression of *AtDNA-PK α* and *AtDNA-PK β* genes in male organs (Anthers) and female organs (ovaries). The expression of *AtDNA-PKcs* is slighter higher in female organs.

The fertility of *Atdna-pkcs* mutants crossed with wild-type in *Arabidopsis thaliana*

The measurement of the silique length and the quantification of the number of seeds per silique was carried out to assess the fertility for three types of plants. Firstly, female wild-type plant (♀) crossed with male *Atdna-pka* homozygous *Atdna-pkb* heterozygous (♂) (*Atdnapka*/- *Atdnapkb*+/-). The second type of the plant is that male wild-type plant (♂) crossed with female *Atdna-pka* homozygous *Atdna-pkb* heterozygous (♀) (*Atdnapka*/- *Atdnapkb*+/-). The third type is crossed wild-type with each other.

The mean length of the silique for the female wild-type plant crossed with male *Atdna-pka* homozygous *Atdna-pkb* heterozygous (*Atdnapka*/- *Atdnapkb*+/-) was 8.5 mm, while male wild-type plant crossed with female *Atdna-pka* homozygous *Atdna-pkb* heterozygous (*Atdnapka*/- *Atdnapkb*+/-), the mean length of the silique was higher (14.8mm) (**Figure 39A**). The mean number of seeds per silique in the female wild-type plant crossed with male *Atdna-pka* homozygous *Atdna-pkb* heterozygous (*Atdnapka*/- *Atdnapkb*+/-) was significantly reduced to 17 seeds/silique compared to the 35 seeds/silique in male wild-type plant crossed with female *Atdna-pka* homozygous *Atdna-pkb* heterozygous (*Atdnapka*/- *Atdnapkb*+/- and 45 seeds/silique in the crossed wild-type (**Figure 39B**).

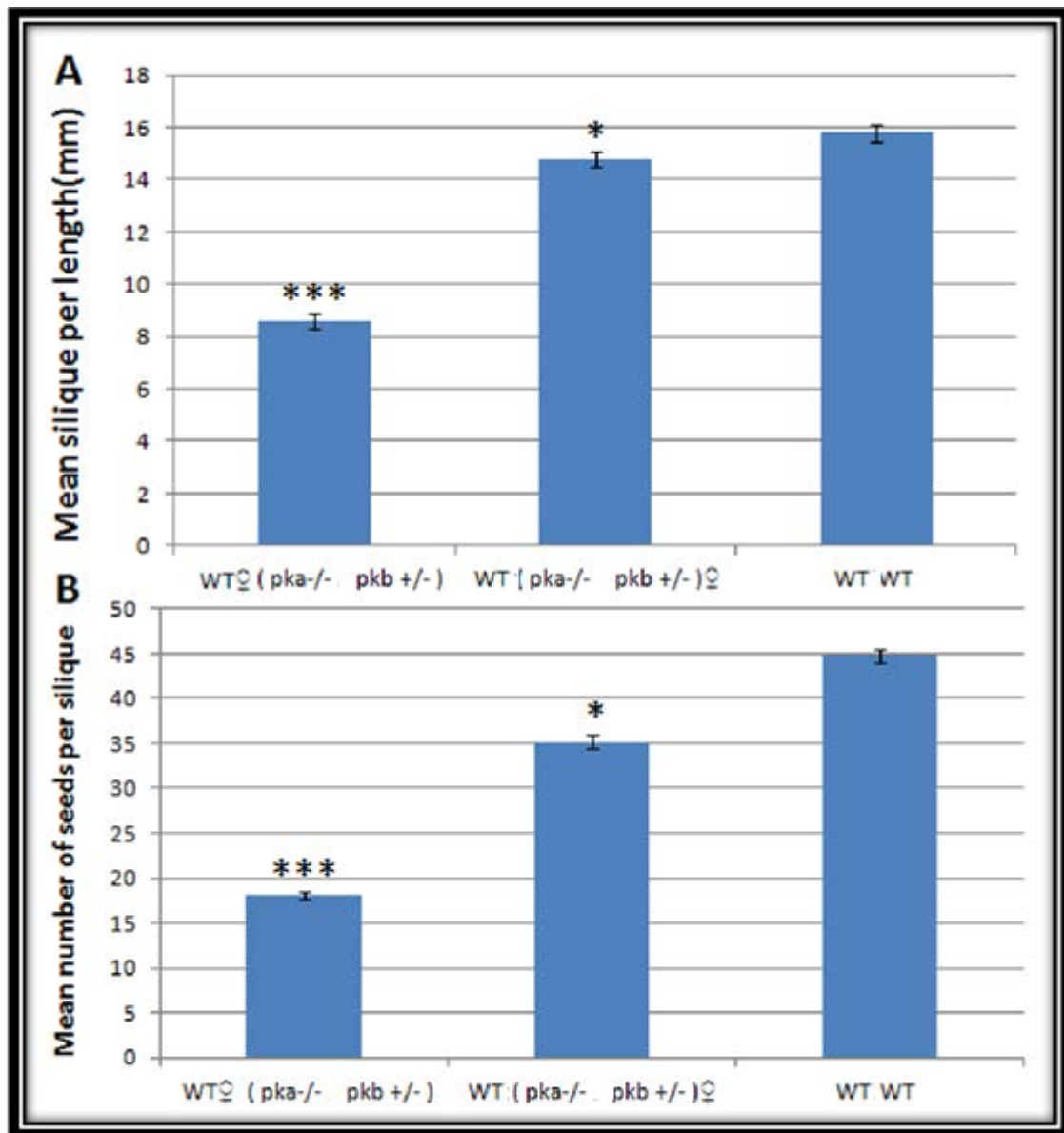


Figure 39: seed count and silique length for WT crossed with *Atdna-pkcs* mutant in *Arabidopsis thaliana*

(A) Mean silique length for 100 siliques measured from each plant. (B) Mean number of seeds per silique for 100 siliques measured from each plant. Error bar = standard error of the mean. Scale bar = 5 μ m. (*) p.value < 0.05, (***) p.value < 0.001.

The effect of different DNA damage drugs on putative *Atdna-pkcs* mutants in *Arabidopsis thaliana*

Cisplatin (Cis-platinum (II) diamine dichloride) is a chemotherapeutic reagent, which can produce inter and intra strand crosslinks with purine bases on the DNA. To be able to process these DNA crosslinks, they are excised by DSBs are produced which if they cannot be repaired will cause DNA damage and could even induce cell death (the main goal to reduce the cancer cells) (Wu and Lichten 1994; Hanneman *et al.* 1997; Kartalou and Essigmann, 2001). In this study, we have analysed the sensitivity to cisplatin of the putative *Atdna-pkcs* mutants compared to the WT (Col). Seeds of Col, *Atdna-pka*^{-/-} *Atdna-pkb*^{+/-} mutant, and single mutant for both putative *AtDNA-PKcs* genes were grown in MS agar media at different concentrations of cisplatin: 0mg/ml, 5mg/ml (**Figure 40B& Figure 40G**) and 10mg/ml (**Figure 40C& Figure 40H**). Bleomycin is one of DNA damage agents that produce different DNA lesions to induce DSBs in vitro (Hecht, 2000). Sensitivity to bleomycin was analysed in Col, and *Atdna-pkcs* mutants by growing seedlings in MS media with different concentrations of bleomycin 0 µg/ml, 2 µg/ml (**Figure 40D& Figure 40J**) and 4µg/ml (**Figure 40E & Figure 40K**).

In all cases, seeds from Col and *Atdna-pkcs* mutants were germinated on MS agar media plates and left to grow for one week at constant temperature (18°C) and light exposure. Afterward, seedlings were transferred to new MS agar plates containing the different concentrations of the drugs to analysed. Two weeks later, the plates were photographed and analysed.

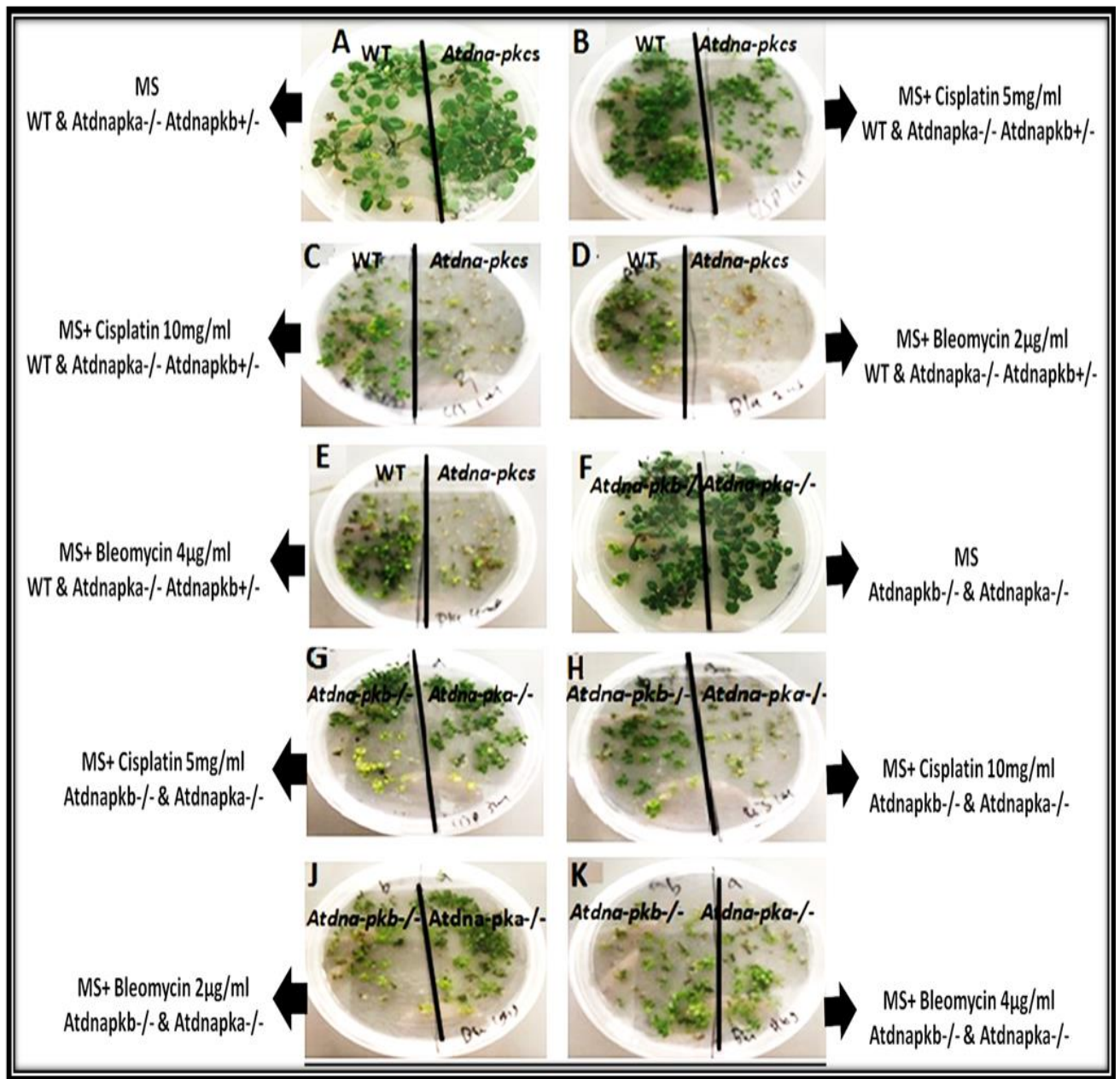


Figure 40: Sensitivity analysis to Cisplatin and Bleomycin in Wild type and *AtDNA-PKcs* mutants. (A-E) WT & *Atdna-pkcs* mutants. (F-K) Single mutant of *AtDNA-PKcs*, *Atdna-pkb/-* & *Atdna-pka/-*

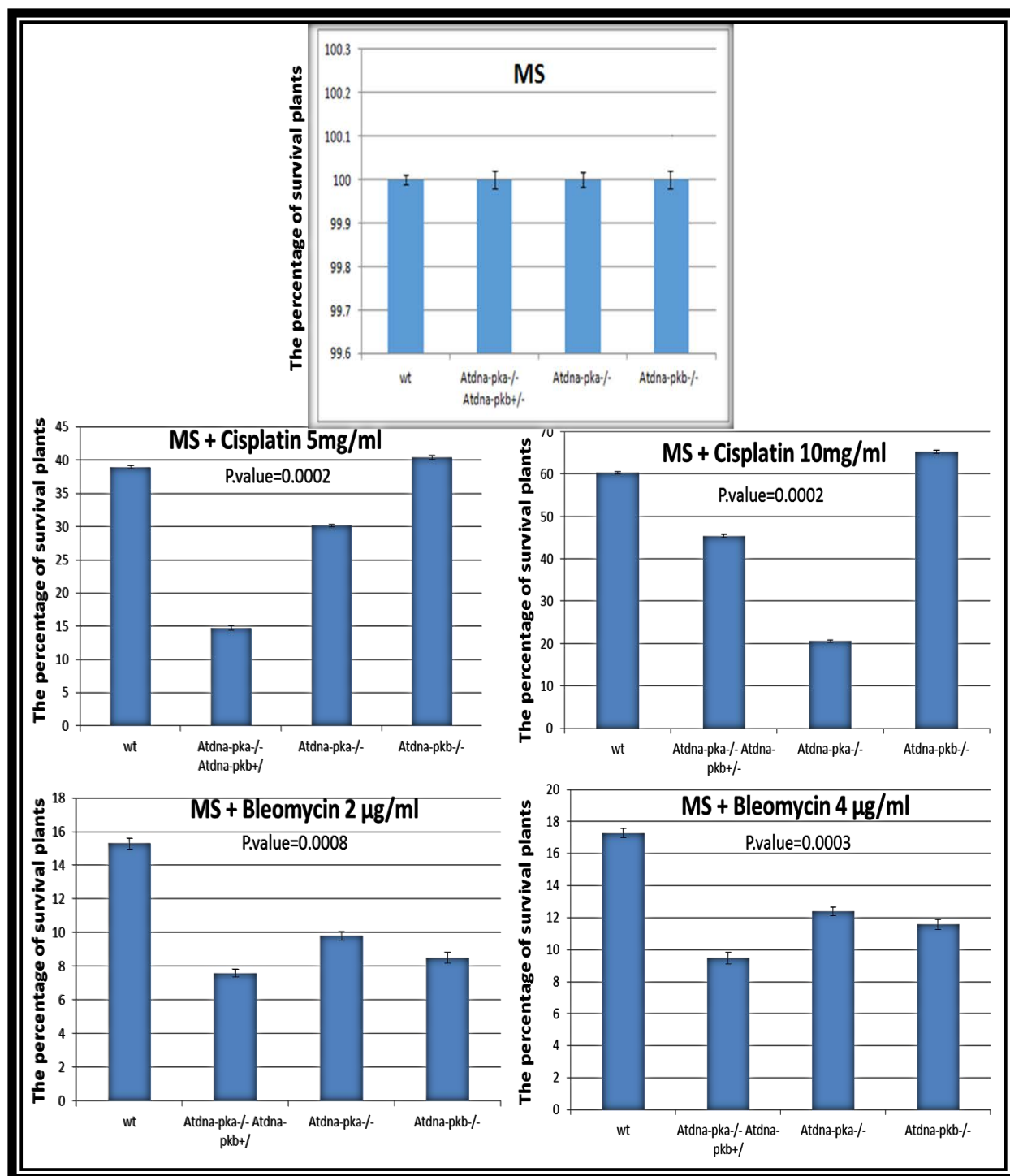


Figure 41: Effect of DSBs agents on the survival of WT and Atdnapkc mutants plants.

This figure shows the percentage of resistance WT and Atdnapkcs munts plants to cisplatin and Blumarine at different concentrations. Error bar = standard error for the mean. P.vaule was significant as shown in the graphs

Discussion

Two putative *DNAPKcs* genes have been identified in the *Arabidopsis thaliana* genome

DNA-PKcs in humans is a DNA dependent serine/threonine protein kinase highly important for the Ku-Dependent NHEJ pathway to repair DSBs (Meek et al., 2001). DNA-PKcs is also crucial in the process of V(D)J recombination to produce the variety of antibodies necessary for a correct immune system (Blunt *et al.*, 1995; Jackson and Jeggo, 1995). The DNA-PKcs kinase activity can use different proteins as substrates for Phosphorylation (Mahaney *et al.*, 2009) Furthermore, DNA-PKcs can autophosphorylate which seems to be crucial for the correct processing of DSBs during NHEJ (Smith *et al.*, 1999) Mainly, the phosphorylation cascade initiated by DNA-PKcs is necessary for the activation of specific enzymes necessary to repair the DSBs in the NHEJ pathway (Lees *et al.*, 1990; Douglas *et al.*, 2005). DNA-PKcs activity in NHEJ is similar to that one carried out by ATR and ATM in HR in the process of repairing DSBs. *DNA-PKcs* has been identified and analysed in humans and other mammals, but for a while, it was predicted that only was present on vertebrates as it was not possible to identify a homolog in non-vertebrate species. In silico analysis was improved by the availability of more and better resolved genomic sequences in other species and allowed to identify putative DNA-PKcs in non-vertebrates and to analyse for the first time one DNA-PKcs in *Dictyostelium discoideum* (Block *et al.*, 2005). Identification and characterization of NHEJ components like Ku70, Ku80, Ligase IV and XRCC4 have been done very successfully in *Arabidopsis thaliana* (Tamura *et al.*, 2002).

Nevertheless, until now, the existence of *DNA-PKcs* in plants has not been demonstrated. In this study, we have been able to use human and *Dictyostelium discoideum* DNA-PKcs protein sequences to identify putative *Arabidopsis thaliana* DNA-PKcs (AtDNA-PKcs). BLAST analysis in this study has allowed us to find several candidates among which some have been previously characterized as other proteins like ATM, ATR, and

TOR not involved in NHEJ. Nevertheless, another two candidates have appeared throughout this analysis which have not been previously characterized and that we have named putative AtDNA-PK α and AtDNA-PK β . The comparison between *DNAPKcs* in humans and *Dictyostelium discoideum* (COBALT, NCBI) has shown us that between both sequences there is only an identity of 28%, 47% positive, and gaps of the 11% on their sequences (**Figure 42**). This comparison is very informative because the two *Arabidopsis thaliana* putative DNA-PKcs show similar identities, positives and gaps values with human and *Dictyostelium discoideum* DNA-PKcs (**Figure 42**). The identity between the human DNAPKcs and the putative AtDNAPK α candidate is 24%, with 43% positives and 9% gaps (**Figure 42**).

Similarly, the identity between human DNA-PKcs and the putative AtDNAPK β is 25%, 43% positives and 11% gaps (**Figure 42**). These values, are very similar to those observed when comparing human and *Dictyostelium discoideum* DNAPKcs protein sequences. Furthermore, very similar values are observed when comparing *Dictyostelium discoideum* DNA-PKcs and the putative AtDNAPK α and AtDNAPK β candidates (**Figure 42**). This shows that these *Arabidopsis thaliana* genes could be the best candidates to identify DNA-PKcs for the first time in the Plant kingdom. When comparing the amino acid sequence of both *Arabidopsis thaliana* AtDNAPK α with AtDNAPK β , it is very clear that both are nearly identical; with a very high identity value of 88%, 93% positives and not gaps (0%) (**Figure 42**). Perhaps one of the reasons why DNA-PKcs has not been characterized in plants could have been that there is a gene duplication so mutant identification for them would have been obscured by gene redundancy.

DNA PKcs Source	DNA-PK α			DNA-PK β		
	Identities	Positive	Gap	Identities	Positive	Gap
Human	24%	43%	9%	25%	43%	11%
Dictyostelium discoideum	28%	47%	11%	24%	42%	8%

DNA PKcs Source	DNA-PK α		
	Identities	Positive	Gap
DNA-PKβ	88%	93%	0%

Figure 42: COBALT (NCBI) multisequence alignment of four DNA-PKcs protein sequences in human, *Dictyostelium discoideum*, and *Arabidopsis thaliana* (DNAPKcsA & DNAPKcsB).

Kurmas and colleagues (1999) that the size of the catalytic subunit of 470-kDa DNA-PKcs serine/threonine kinase is considerably large for humans. This catalytic subunit (DNA-PKcs) shares similarities with kinases belonging to the phosphatidylinositol (PI) 3-kinase superfamily. In this work, the conserved domains were analyzed to study the similarities of the PI-3-kinases and DNA-PKcs in different organisms to identify the correct DNA-PKcs in plants. This analysis has established that the *human* and *Dictyostelium discoideum* conserved domains are very similar, sharing NUC194, PIKKc-DNA-PK, and FAT domains. Nevertheless, an FRB-domain has been found in the *Dictyostelium discoideum* DNA-PKcs sequence which is absent in the human one (**Figure 20**). Amoebas included *Dictyostelium* are evolutionarily located before the eukaryotic division between the animal and plant kingdoms. *Dictyostelium* protein sequences share similarities to both animal and plant kingdom protein sequences. DNA-PKcs homolog has been previously identified in

Dictyostelium discoideum (Hudson *et al.*, 2005). Interestingly, both DNA-PKcs putative copies in *Arabidopsis thaliana* that we have identified contain the share CDs between human and *Dictyostelium discoideum* DNA-PKcs and the FRB domain from *Dictyostelium discoideum*. Furthermore, this FRB domain is also shared by another PI-3-kinase in *A. thaliana*, ATR. ATR and ATM are both protein kinases that facilitate the phosphorylation-mediated signal at the outset for repairing DSBs during Homologous Recombination (HR). Additionally, they enable the phosphorylation of the histone variant H2AX surrounding the region that has been damaged. Subsequently, this causes the generation of additional factors than can intervene in the damaged DNA. The mutants characterized for *Arabidopsis* ATM have shown hypersensitivity to DNA damage, especially to agents producing DSBs (Dziegielewska *et al.*, 2001). Furthermore, the plant ATR mutants can also be impacted by the stress of replication (Garcia *et al.* 2003; Culligan *et al.* 2006). Friesner and colleagues (2005) demonstrated that both ATR and ATM are important factors in signal communication as a response to DSBs, and it has also been determined that there is an important dependence on ATM in plants. The outcomes of this investigation of the conserved domains for both AtDNAPKa and AtDNAPKb indicate that both candidates have very similar sequences and CDs shared with other organisms DNA-PKcs.

The characterization of the putative *Arabidopsis thaliana* AtDNA-PKcs

The outcome of this investigation proves that there is a strong level of similarity among the Human, *Dictyostelium discoideum* and *Arabidopsis thaliana* protein sequences. AtDNA-PKa locus is located on chromosome 2 (AT2G17930) with a total of 33 introns and 34 exons. To characterize the role of this locus, an *Atdna-pka* T-DNA insertion line mutant was analysed (**Figure 21**). AtDNA-PKb locus is localised in chromosome 4 (AT4G36080) with a total of 33 introns and 34 exons (**Figure 22**). PCR genotyping allowed us to identify *Atdna-pka* homozygous mutants (**Figure 24**) and *Atdna-pkb* homozygous mutants (**Figure 25**). The phenotype of *Atdna-pka* homozygous mutants was similar to wild type with small variations

such as short the length of silique and reduction of seeds production. This lack of (or reduced) effect in these single mutants could be due to gene redundancy as both genes are very similar and expressed similarly. To analyse the role of these putative DNA-PKcs in *Arabidopsis thaliana*, the two single homozygous mutants were crossed to obtain a double homozygous mutant. Through segregation of a double heterozygous mutant an *Atdnna-pka* homozygous and *Atdna-pkb* heterozygous mutant plants (*Atdna-pka*^{-/-}*Atdna-pkb*^{+/-}) were obtained (**Figure 26**). Subsequently, after growing the offspring of these plants (about 1,645 seeds, one plant being a double homozygous for both T-DNA insertions (*Atdna-pka*^{-/-} *Atdna-pkb*^{-/-}) was obtained (**Figure 27**).

The level of AtDNA-PKcs expression has been studied in different tissues of the organisms. In human cells, the expression of DNA-PKcs and Ku80 was usually at the same level, but it was higher in neurons and glial cells of the central and autonomic nervous system, lactating breast epithelium, and spermatogenic cells, but the expression of DNA-PKcs is reduced after meiosis. The expression of DNA-PKcs and Ku80 was highly selective between the cells in the same tissue, and the majority of cancerous tissues were positive for the proteins excluding invasive carcinoma of the breast (Moll *et al.*, 1999).

In Vegetative Dictyostelium Cells, the expression of Ku80 or DNA-PKcs remains at the same level during the life of this organism and the absence of both proteins do not have any effect on the development (Hudson *et al.*, 2005). While in *Arabidopsis thaliana*, the expression of AtDNA-PKcs level was slightly similar in all tissues except the flower buds was strongly higher, but in the absence of AtDNA-PKcs, AtDNA-PKcs were not expressed in all tissues (**Figure 28**). Meiosis is a unique form of cell separation that is common to eukaryotes and is a critical component in sexual reproduction. To study the role of AtDNAPKcs in meiosis in *Arabidopsis thaliana*, the results showed that the meiotic phenotype of *Atdna-pka*^{-/-}*Atdna-pkb*^{-/-} were identical to the for WT which leads to AtDNAPKcs do not have any a role during meiosis(**Figure 35**). DNA-PKcs and Ku70 have

been reported to be not expressed in spermatocytes of the mouse at leptotene and zygotene (Goedecke *et al.*, 1999). Silique length and seeds number are generally utilized to examine the fertility in plant research (Puizina and Šamanić, 2013). Seeds production was significantly reduced to less than 5 seeds/silique in the double homozygous mutant (*Atdna-pka* ^{-/-} *Atdna-pkb* ^{-/-}) in comparison to 55 seeds/silique in the wild type. Furthermore, a reduced mean number of seeds was also observed in *Atdna-pka* ^{-/-} *Atdna-pkb* ^{+/-} plants with 33 seeds/silique(**Figure 31**). This confirms that these *AtDNA-PKcs* loci must have an important involvement in fertility, perhaps in female gametogenesis or pollen maturation. Furthermore, this phenotype is associated with the homozygous mutant of AtDNA-PKcs in mice, the fertility and viability were absent due to the deficiency of telomerase that leads to fusion of short telomeres and apoptosis(Espeje *et al.*, 2002).

DNA-PKcs of the mouse was found in early spermatocytes, and the Ku70/Ku86 were down-regulated to prevent meiotic recombination. This study indicated that the presence of DNA-PK could prevent homologous recombination pathway during meiosis, In human cervical cancer HeLa cells, the study carried out by Lee ., *et al.* (2011) reported that the association of DNA-PKcs with the spindle apparatus. In the absence of DNA-PKcs, chromosome misalignment has increased during mitosis which is strong proves for the role of DNA-PKcs in mitosis. Mitotic anaphase bridges have been observed in the double homozygous mutant in *A.thaliana* showing that these loci might be required to the repair of DNA damage in somatic cells (**Figure 36**). A study in mice by Goytisolo et al.,(2001) showed that DNA-PKcs^{-/-} cells showed a high frequency of mitotic anaphase bridges during mitosis (from 0.16 to 0.70 bridges per anaphase). Kawashima *et al.*, (2014) explained the process of sexual reproduction in *Arabidopsis thaliana* which start with generating pollen mother cells and megaspore mother cells from somatic cells where in male and female reproductive tissues. Pollen mother cells and megaspore mother cells undergo meiosis to produce microspores. The vegetative cell and the generative cell is

generated from the asymmetrical division for the microspores. The mature pollen can be generated from the divided generative cell again. The megaspore takes three rounds of nuclear to create syncytial female gametophyte with eight nuclei. Then, cytokinesis occurs to create the mature female gametophyte that has the central cell, accessory cells (antipodals and synergids), and the egg cell. In *Arabidopsis thaliana*, the pollen tube that elongates from the vegetative cell deliver the immotile sperm cells to the female gametophyte to fertilize the egg cell by one sperm cell to create zygote and endosperm. Then, the first cell division takes a place to generate proembryo cell. The developed proembryo become a globular-shaped embryo, then change to the heart-shaped embryo to develop into the mature seed (**Figure 43**).

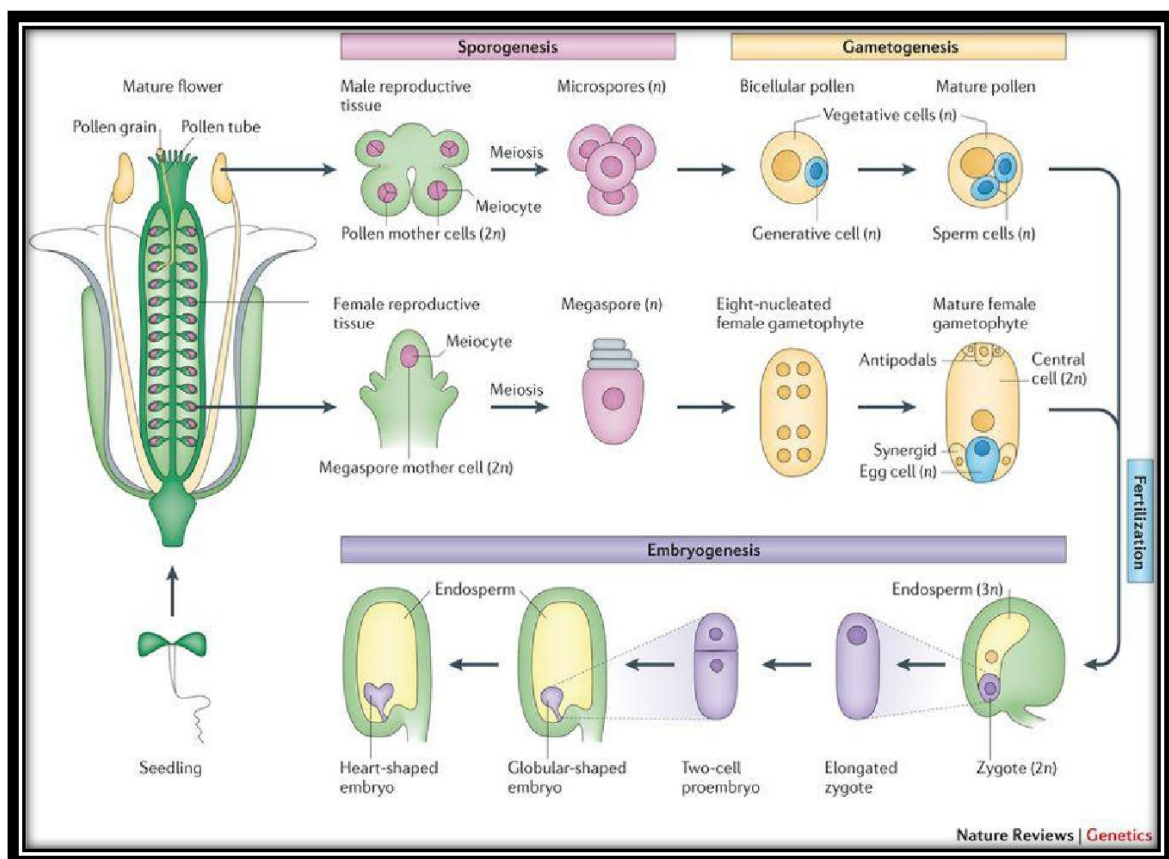


Figure 43: sexual reproduction process in flower in *Arabidopsis thaliana* (from Feng *et al.*, 2010).

The result of Alexandra staining indicated to a very strong reduction of pollen grain in the double homozygous mutant for DNA-PKcs genes which could indicate to the importance

of AtDNAPK-cs to maintain the production of the pollen grain during the process of sexual reproduction in *A.thaliana*. In Tetrahymena, DNA damage was found in all pronuclei after meiosis. Some pronucleus were selected to reair DNA damage. DNA-PKcs was expressed in that pronucleus to repair DNA by NHEJ and Rad51 was expressed in repairing DNA by HR (Akematsu *et al.*, 2017). DNA-PKcs mutation in the somatic cells of an adult might cause telomere dysfunction leading to aging and cancer (Espeje *et al.*, 2002). Another study carried by Zhang *et al.*,(2011) found that 75% of mice have died after four weeks of birth, when DNA-PKcs are absent.

Further investigation into the role of *AtDNA-PKcs* in DNA repair was carried out by analyzing the mutants sensitivity to different DNA damage drugs such as cisplatin and bleomycin. Cisplatin causes DNA breaks and intra- and interstrand DNA links that to be repaired would require to produce DSBs (Kartalou & Essigmann *et al.*, 2001). Cisplatin causes cell cycle arrest, prevent DNA replication, transcription, and Apoptosis. The previous study in human cells showed that Ku proteins are bound to repair damaged DNA by cisplatin with DNA-PK phosphorylation inactivity (Turchi *et al.*, 1996). The ligation assay with recombinant Ku, XRCC4 and DNA ligase IV has used to check the effect of cisplatin on the ligations proteins; the result indicated to cisplatin inhibits DNA ligation proteins. While cisplatin reduced the ligation in the mutant of Ku proteins (Kysela *et al.*,2003). The absence of DNA-PKcs in solid human tumors showed that the cells were high sensitivity to cisplatin after treatment. Also, silencing DNA-PKcs in cisplatin-treated cells increased necrosis and apoptosis (Sand *et al.*, 2011). Study in human gliomas by Shao *et al.*,(2008) showed that DNA-PK and Ku86 were at different of sensitive to cisplatin in primary glioma specimens, but were higher in samples that give the resistance phenotype to cisplatin in vitro. The suggestion that DNA-PKcs and ku86 play an important role in resistance cisplatin, while ku70 do not have any role in that operation. In *A.thaliana*, cisplatin was affected by the growth of WT plants, and DNA-PKcs mutant plants, but was highly affected on in the absence of DNA-PKcs.

According to Oliveira *et al.*, (2002) the involvement of DNA-PKcs in DNA repair induced by bleomycin has been reported. The Chinese hamster cells with lack of Ku 80 and

presence of DNA-PK were highly sensitive to bleomycin (Johnston *et al.*,1997; Darroudi *et al.*,1989). In the study carried by Hudson *et al.*,(2005) showed that treated cells with 100mU/ml of bleomycin and in the absence of DNA-PKcs or Ku80 in *Dictyostelium* were sensitive when HR pathway was defective, while vegetative *dnapkcs* and *ku80* strains did appear any sensitivity to bleomycin. In *Atdnapkcs* mutant the reduction of cells reduced to 11 %, while in *ku80* mutant was around 8%. A study carried by Nicolay *et al.*,(2016) in human bone marrow-derived showed that 0.4µg/mL to 1.8µg/mL of bleomycin reduced the survival cells up to 0.1%. Rosa *et al.*, (2014) used up to 2ug/ml of bleomycin to test the sensitivity of *Arabidopsis thaliana* in WT and *Atku70*. The percentage of survival plants with true leaves was around 40%, while in the absence of Ku70, the of survival plant with true leaves was less than 10 %, this result is agreed with our observation as shown in **(Figure 40 & Figure 41)**.

DNA ligase IV, XRCC4, RAD50, Ku70, and Ku80 are have identified to repair DSBs by NHEJ in the Arabidopsis (Gallego *et al.*, 2001; Tamura *et al.*, 2002; West *et al.*, 2000). According to West *et al.*,(2004) the growth of Arabidopsis was normal in mutations of some genes that are involved in repair DSBs by NHEJ which are *atku70*, *atku80*, and *atlig4*, although the presence of telomere defects in *atku70* the growth of the plants was normal. However, Osakabe *et al.*, (2006) reported that treated *Atku80* Arabidopsis plants were sensitive to cisplatin. The mutation of these genes showed that the plants were high sensitivity to bleomycin. Using two different concentrations of bleomycin (1 mg/ml & 4mg/ml) showed that the growth of wild-type Arabidopsis was slow. In *Atku80*, the plant was highly effected with lethality at both concentrations (West *et al.*,2002). However, Jia *et al.*,(2012) showed that *Atku70* plants were more sensitive to 0.2 µg/mL and 0.4 µg/mL bleomycin comper to *Atku80* and wild type plants. Another study on the NHEJ protein showed that bleomycin at 0.4ug/ml was highly affected by the growth of *Atlig4* single

mutant plants (Jia et al., 2001). We found the plants were sensitive to bleomycin, while *Atdna-pka*^{-/-}*Atdna-pkb*^{+/-} and double homozygous mutant plants were highly sensitive to the higher concentration used (**Figure 40 & Figure 41**).

Issues with Single T-DNA Mutant

Examinations based on insertional mutagenesis of T-DNA have played a vital role for plant biology researches for decades, particularly in *Arabidopsis thaliana*. In order to conduct in vivo characterisation of gene function, identified practices provide large source of mutant alleles. At present, functional genomics has been recognised to be more focused than structural genomics and more simplified after identification of complete genomic sequences of *Arabidopsis* and rice (Christian *et al.*, 2013). Insertion of T-DNA elements in a gene allows investigations on loss or gain of function mutations and facilitates the use of various vectors for in-depth study of and characterisation of regulatory elements of species of *Arabidopsis*. There have been recognised various advantages of utilisation of T-DNA as an insertional mutagen. T-DNA elements are more stable chemically and physically through multiple generations as compared to transposons as they do not transpose subsequent to insertion. Mutagenesis through T-DNA is site specific and it acts as a marker for mutation (Zhong *et al.*, 2012).

Despite these advantages, recent investigations have identified certain limitations of insertional mutagenesis. One critical issue is the risk of disruption in gene of function upon insertion of T-DNA up to 25 kb length as *Arabidopsis* genome contains very little intergenic material and has smaller introns (Thole *et al.*, 2010). By using single T-DNA mutants, the phenotypic effects cannot be identified clearly as the number of mutations rendered by insertional mutagenesis is fewer in number (Wang *et al.*, 2014). Moreover, only few numbers of plants become transgenic when large populations are transformed through insertional mutagenesis by T-DNA elements. During analysis of tagged cell lines with T-DNA mutagens, certain limitations have also been observed associated with T-

DNA inserts (Wang *et al.*, 2014). Major events include complex arrangement of T-DNA, chromosomal duplications, vector backbone sequences insertion, multiple insertions, chromosomal rearrangements and modification of DNA content per nucleus. Thus, potential issues are presented when single T-DNA mutants are used such as its complex integration patterns which are further complicated by vector sequences transfer adjacent to T-DNA borders (Ren *et al.*, 2012). Furthermore, during conduction of reverse genetics studies, concatemeric T-DNA inserted in large frequencies further complicates the PCR analysis. Small and major chromosomal rearrangements present greater disadvantages associated with the use of single mutants of T-DNA. Thus undesired mutant phenotypes can be exhibited by the model with further complications in genetic analysis of the insertions caused by chromosomal rearrangements (Wang *et al.*, 2014).

Efficient transformation systems have accelerated the integration pathways of T-DNA as an insertional mutagen in Arabidopsis, still critical analysis and evaluation of this transformation system is required to minimise the effects of associated drawbacks. Identified through recent studies, simplified insertional mutagen utilisation in the form of T-DNA has least surpassed other distinct approaches based on chemical and physical mutagenesis (Duangpan *et al.*, 2013).

The impact of or proportion of T-DNA integration in host plant genome can be analysed through truncation of functional protein products or transgene truncation analysis. This analytical procedure allows representation of effect of T-DNA integration on the basis of truncation of T-DNA, integration of plasmid backbone DNA or its rearrangement (Christian *et al.*, 2013). Reports presented through truncation analysis exhibit detailed understanding and detailed information of insertion of transgene and the protein product which has been translated from the mutagenic insert of T-DNA. This data is recognised more feasible and useful in safety assessment of genetically modified crops and tracking the genetically modified events in transgenic crops (Wang *et al.*, 2014). By analysis of

independent alleles, stronger evidence can be provided or through the strategies based on production of complementation lines of plant cells. These complemented plants cells lines provide efficient medium which are transformed by integration of T-DNA through insertional mutagenesis of functional parts of protein (Christian *et al.*, 2013). Plants' phenotypes analysed after insertional mutagenesis of independent alleles provides elaborated information of each trait in an independent manner. It also elaborates the impacts of loss or gain of function caused by the mutation, exhibiting a particular phenotype. For predicting the likelihood of impact of single nucleotide polymorphisms, mutagenesis of independent alleles is reported an effective strategy, evidenced by the studies on Arabidopsis (Ren *et al.*, 2012).

Establishment of complementation cell lines has also been reported after evaluations to analyse the effect of T-DNA mutagenesis. For development of complementation cell lines in genetic experimentations, a cross is made between two strains of an organism with distinct homozygous recessive mutations, which produce offspring with wild type phenotype and mutant genotype. For this purpose, identification of gene that has been disrupted is essential for detection of impact of mutagenesis through tests for complementation (O'Malley *et al.*, 2015). Co-segregation testing has been a preferred tool for identification of the disrupted gene as the identification of flanking sequences at insertion sites is not difficult. Insertion mutation has been recognised to cause distinct phenotypic traits evidenced by the reports based on data of analytical results of complementation lines testing. Further confirmation can be made through characterising an independent mutation within the same gene initially which was exhibiting identical phenotype (Yi & An 2013).

Allelism test can also be employed for this purpose through any recessive mutants which were observed to display same phenotype. As in case of T-DNA site directed mutagenesis, positional cloning strategies can provide access to mutations (Yi & An 2013). This strategy

has been recently identified and widely employed for analysing the dense genetic maps in *Arabidopsis* with visualisation of various molecular markers. A complete physical map is therefore analysed for this purpose which represents overlapping cloned DNA fragments and the collection of total genome sequences (Zhai *et al.*, 2013).

Conclusion

The silicon, genetic and cytological analysis carried out in *Arabidopsis thaliana* has identified two putative candidates for *AtDNAPKcs*. This could be the first time that DNA-PKcs has been discovered in the plant kingdom. *AtDNA-PKcs* is strongly expressed in opened flowers. *Atdna-pkcs* double homozygous mutants are sterile but male meiotic stages occurred without any error. *AtDNA-PKcs* genes seemed to be required for DNA repair, as anaphase bridges have been found in the double mutant. Also, the *atdnapkcs* mutant plants have shown sensitivity to some DNA damage agents, pointing to a specific role in DNA damage repair and perhaps in NHEJ as it should be expected.

CHAPTER FOUR

ANALYSIS OF RESIDUAL AND ARTIFICIAL DNA DOUBLE STRAND BREAKS (DSBs) DURING MEIOTIC RECOMBINATION IN *ARABIDOPSIS THALIANA*

INTRODUCTION

Meiotic recombination introduction

The meiotic process implies a cell division that reduces to half the genome complement for the production of sexual gametes. During the leptotene stage of meiotic prophase I, homologous recombination is triggered by programmed DNA double-strand breaks (DSBs) catalysed by a Spo11 complex. Processing of these DSBs results in either crossover (CO) or non-crossover (NCO) events during meiosis. Exchange of genetic material is the result of reciprocal CO between non-sister chromatids of homologous chromosomes (Henderson, 2012). Cytologically, the chiasmata are the physical manifestation of the recombination or CO sites (Tease and Jones, 1978).

DNA Double Strand Breaks Initiate meiotic recombination

A topoisomerase VI like activity catalysed by the Sporulation Protein 11 (Spo11) and associated proteins introduce DSBs early on at meiotic prophase I. Different studies have been done to identify the proteins involved in this process (De Massy *et al.*, 1995; Keeney and Kleckner, 1995). Keeney and Kleckner (1995) showed that many proteins including Rad50, Mre11, and Sae2 bound covalently to the 5' DNA breaks. These proteins remain bound on both sides of the DSB before their removal by resection proteins. DNA homology sequencing techniques have revealed that archaeal TopoVI subunit shares sequence homology with yeast protein Spo11. Genetic analysis in *S. cerevisiae* confirmed that Spo11 biological function is relatively different from that of the archaeal TopoVI subunit (Bergerat *et al.*, 1997). Keeney and collaborators (1997) suggested an important role for the Rad50 complex in the production of Spo11 meiotic DSBs. Spo11 is highly conserved among different species like *Drosophila*, fungi, and mouse. This protein is essential in the initiation of meiotic recombination (Keeney, 2011; Celerin *et al.*, 2000; Bowring *et al.*, 2006). *Arabidopsis* possessed three homologs for Spo11 (Hartung and

Puchta, 2000; Hartung and Puchta, 2001). Different studies confirmed that Spo11 plays a specific role in meiotic cells in fungi and *C. elegans* by generating DSBs to initiate meiotic recombination (Keeney, 2011; Ramesh *et al.*, 2005; Storlazzi *et al.*, 2003).

The mechanisms and protein involved in DSBs initiation are conserved among different species (Camerini-Otero, 2000; Grelon *et al.*, 2001). Spo11 has been extensively studied in budding yeast. Zicker and Kleckner (1999) suggested that the occurrence of DSBs is in a random pattern throughout the length of the chromosomes and that happen at chromatin loops to physically move towards the chromosome axis for further processing through meiotic HR. *A. thaliana* has three paralogues of Spo11 that are encoded by three different genes: *AtSPO11-1*, *AtSPO11-2*, and *AtSPO11-3* (Hartung and Puchta, 2001; Ronceret *et al.*, 2009). Two of these proteins, *AtSPO11-1* and *AtSPO11-2* are responsible for the initiation of meiotic recombination. These proteins contain various tyrosine residues which are responsible for the catalytic DSB induction (Ronceret *et al.*, 2009). Phenotypic analysis of *atspo11-1* and *atspo11-2* mutants in Arabidopsis have proof their key role in DSBs formation ((Stacey *et al.*, 2006)). Severe reduction of fertility was observed in *Atspo11-1* mutants as they showed only univalents during metaphase I (Grelon *et al.*, 2001). Furthermore, extreme reduction of fertility just like *atspo11-1* has also observed in *atspo11-2*, and during metaphase, I bivalents was completely absent (Grelon *et al.*, 2001). These abovementioned facts reveal that both proteins *AtSPO11-1* and *AtSPO11-2* play a key role in DSBs formation (Stacey *et al.*, 2006; Ronceret *et al.*, 2009). However, the third paralogue *AtSpo11-3* protein plays a key role in other cell processes such as DNA replication and cell growth (Hartung *et al.*, 2007). Moreover, *AtPRD1* which is a homolog of MEI1 in mammals has also been associated with *AtSPO11-1* (De Muyt *et al.*, 2007). *AtPRD2* which is an orthologue of MEI4 in mouse and *AtPRD3* which is a homolog of rice protein PAIR1, have also been reported to play an essential role in DSBs formation in *A. thaliana* (**Figure 44A**). Mutations in any of these proteins lead to the same outcome as a mutation in *Atspo11-2* where no chiasmata formation is observed and therefore resulting in

only univalents formation during metaphase 1 (De Storme *et al.*, 2011). Other proteins such as Mei4, Mre11, Rad50, Xrs2, Ski8, Mer2, Rec104, Rec114, and Rec102 have been reported in budding yeast to work together with Spo11 in DSBs formation (**Figure 44B**). Jolivet and collaborators (2006) reported the different functions of some of these orthologues in cell metabolism in Arabidopsis such as Ski8 which is not related to the meiotic process. Other proteins like AtMRE11 and AtRAD50 have different roles in meiosis not related to DSBs formation (Many *et al.*, 2009).

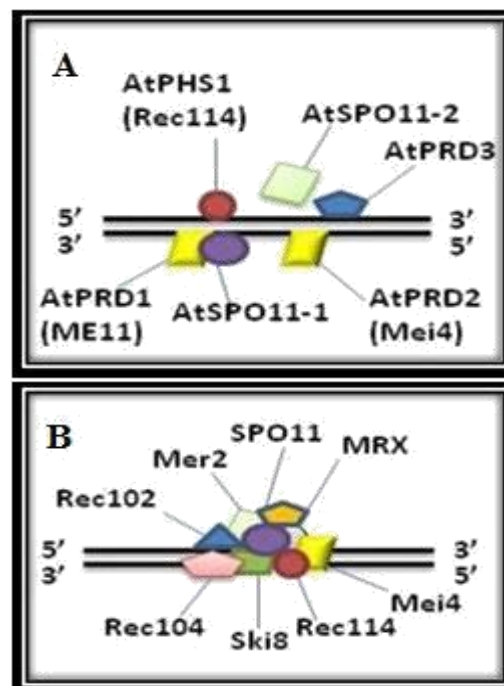


Figure 44: Representation of protein complexes that are involved in DSBs formation in meiosis.

Diagrammatic representation of multiprotein complexes that play an important role in DSBs formation in *Arabidopsis thaliana* (A) and budding yeast (B) and Adapted from Edlinger and Schlogelhofer, 2011.

Meiotic DNA DSBs processing

Neale and collaborators (2005) suggested that SPO11 remains bound to the cleavage site at the 5' end of both strands after DSB formation. It is attached to an oligonucleotide of DNA due to the endonucleolytic cleavage after its removal. MRE11, RAD50, and XRS2 proteins form a complex which covers the DNA ends and results in the excision of DNA strands in 5'-3' direction. In higher eukaryotes two recombinases RAD51 and DMC1

(homologs to bacterial Rec A protein) bind to the short piece of ssDNA up to 100bp, resulting in the formation of a nucleoprotein filament at the end of the DSB (Brown *et al.*, 2015). Both recombinases have been suggested to be bound to the opposite ends of the DSB (Neale *et al.*, 2005). Six paralogs of AtRAD51 are present in Arabidopsis, only three paralogues AtRAD51, AtRAD51C and AtXRRC3 are involved in meiosis (Pradillo *et al.*, 2014). An intermediate D-loop structure is formed by the involvement of these recombinases when an ssDNA combine with a homolog double-stranded DNA from the homologous chromosome. If this ssDNA combines with 3'-end of another DNA, a double Holliday junction (dHJ) can be formed (Neale and Keeney, 2006).

CO and NCO formation pathways

Processing of DSBs results in the formation of CO and NCO pathways. Synthesis-dependent strand annealing (SDSA) leads to NCO pathways, which do not involves the formation of dHJs. Resolution of this dHJ by a complex of proteins including AtRECQ4A leads to the generation of NCOs. ZMM (Zip1, Zip2, Zip3, Zip4, Msh4, Msh5and Mer3) proteins resolve the dHJ in interference sensitive Class I COs. The class II CO pathway is formed by the resolution of a single dHJ by AtMUS81 (Berchowitz *et al.*, 2007). This pathway generates interference independent CO (Pradillo *et al.*, 2014).

Result :

AtSPO11.2 gene structure analysis

Arabidopsis SPORULATION 11-2 (*AtSpo11-2*) gene is located at chromosome 1 (AT1G63990), and it is comprised of 2,544 bps. Severe sterility phenotypes were observed in homozygous plants for *Atspo11-2*. Furthermore, *Atspo11-2* mutant showed disruption in meiosis, which may lead to severe defects in synapsis during meiosis one leading to prevention of meiotic recombination. Both *AtSPO11-1* and *AtSPO11-2* are required for meiotic recombination initiation and have identical overlapping functions. *AtSpo11-2* gene

comprises of 10 introns and 11 exons (**figure 45**). The AtSPO11.2 protein contains 83 amino acids. GABI_039E09 T-DNA insertion line is located on exon 3.

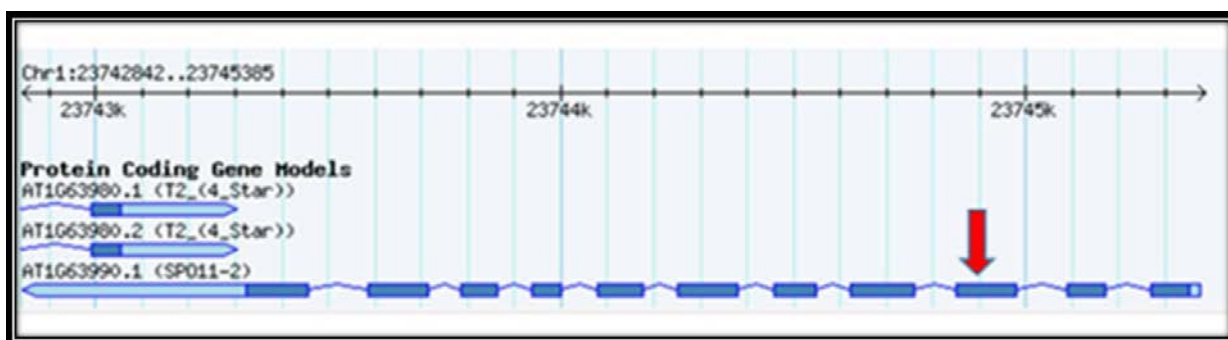


Figure 45: Gene structure for *AtSPO11.2* gene.

The gene consists of 11 exons (blue boxes) and ten introns. GABI_039E09 T-DNA insertion location in the gene is indicated by a red arrow. The gene is located at chromosome 5 and comprises of 3,286 bps (At5g57880 locus).

AtPRD2 gene and protein structure analysis

Arabidopsis Putative Recombination initiation Defects 2 (*AtPRD2*) gene is composed of 10 exons and nine introns (**Figure 46**), which code for a 38 amino acids protein of 44kDa molecular weight. Sail 94 D08 T-DNA insertion line was used to analyse the mutant phenotype, and the T-DNA insertion site is in intron 5. TAIR database was used to obtain the amino acid sequence of *AtPRD2*, and TAIR WU-BLAST tool was utilised to identify similar sequences in *A.thaliana*. Nevertheless, no other homolog for *AtPRD2* was identified by the BLAST search.

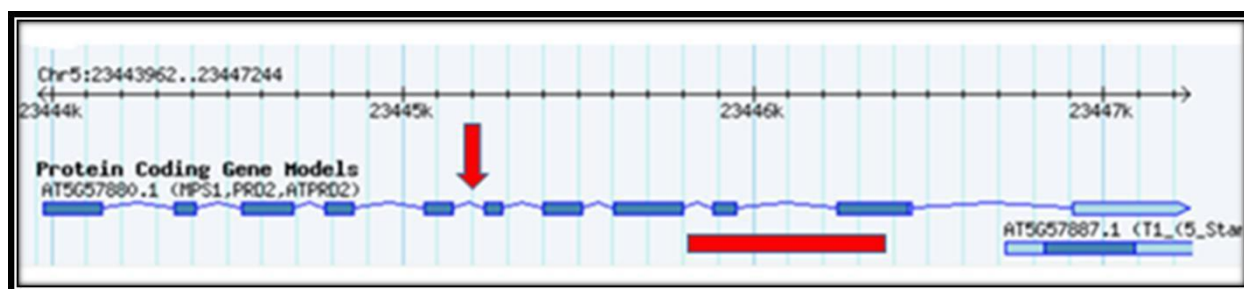


Figure 46: Representation of *AtPRD2* gene structure.

The gene presents ten exons (blue boxes) and nine introns. Sail 94 D08 T-DNA insertion is indicated by a red arrow. RT- PCR amplification region amplified to test the expression of the gene in WT and mutant is indicated with an orange box.

To find out if any conserved domains (CDs) are present in the AtPRD2 protein sequence, the amino acid sequence was analysed using the NCBI CDs tool. This search did not prove fruitful as not CD was found. To find out if AtPRD2 has similar sequences with other proteins in other organisms, BLAST P tool (NCBI) was used, but it was unable to find any putative homologs in other species but plants. Thus, it seems that AtPRD2 is a plant-specific protein (**Figure 47**).

Genotyping of *AtSPO11.2*

PCR amplification was used to genotype *AtSPO11.2* WT and mutant gene and for this purpose specific LP, and RP primers were used. Similarly, GABI_039E09 T-DNA insert was able to be amplified using BP primer. Gel electrophoresis was used to check if the T-DNA insert was or not in *AtSPO11.2* gene. During the genotyping several heterozygous and homozygous mutants were found. Gel electrophoresis indicated that the WT amplification produced 1kb single band in WT plants and a 500bp band in the mutants with the T-DNA insertion. Amplification of both bands was observed in heterozygous mutant plants representing the T-DNA insertion in one allele and the WT band for the other allele (Figure 48).

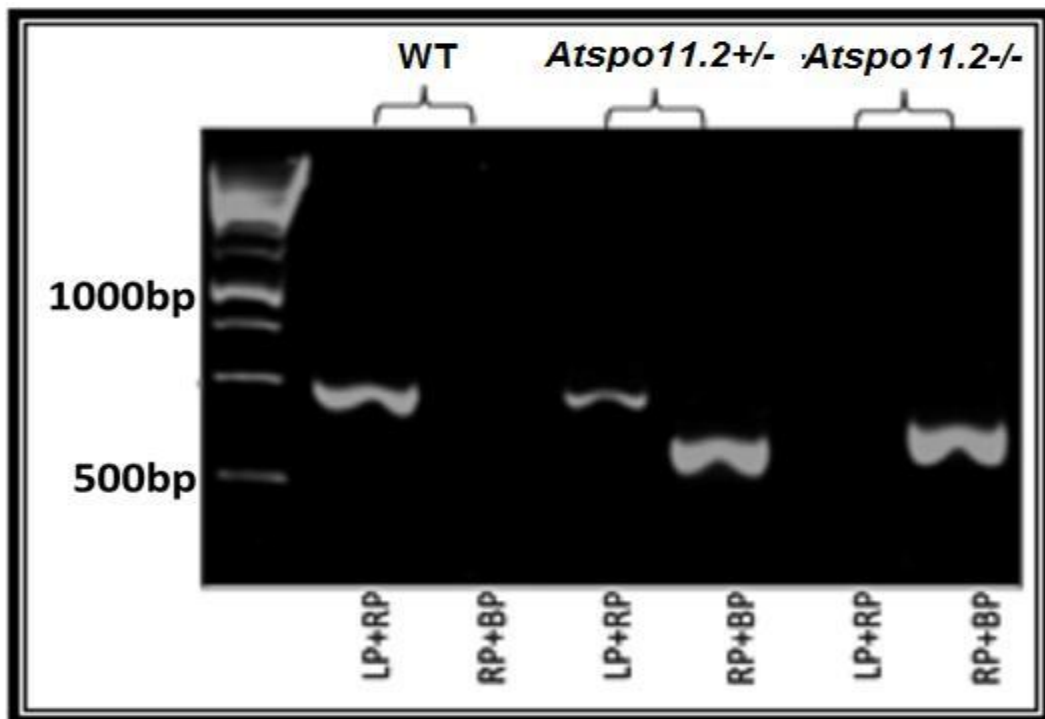


Figure 48: DNA gel electrophoresis is showing the genotyping of WT and *Atspo11.2* T-DNA insertion mutant.

Amplification by PCR of DNA extracted from plant leaves using LP, RP and BP primers specific for *AtSPO11-2* and followed by agarose gel electrophoresis. 1000bp single band is amplified in the WT allele using LP and RP, while the T-DNA insertion allele (mutant) showed a 500bp band. Both bands were only visible in plants that were heterozygous for the T-DNA insertion.

Genotyping and expression of AtPRD2

The Sail 94 D08 T-DNA insertion line was used in this study. Genotyping of plants was done by PCR amplification using BP T-DNA primer and *AtPRD2* gene specific LP and RP primers (Figure 49).

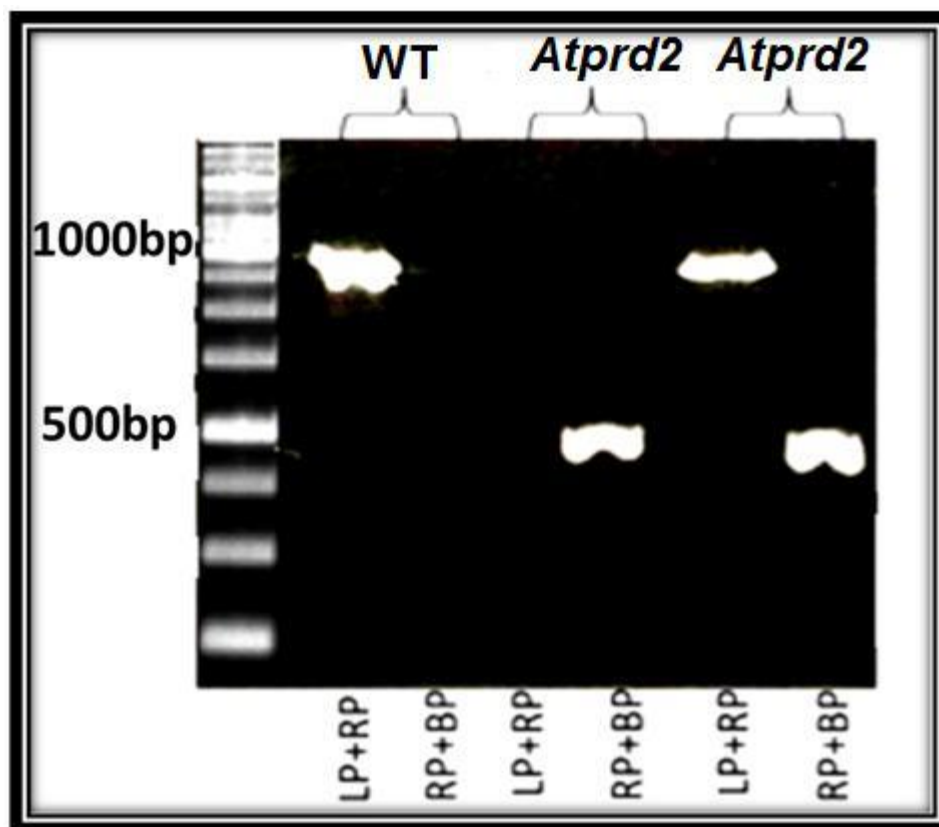


Figure 49: DNA gel electrophoresis is showing the genotyping of WT and *Atprd2* T-DNA insertion mutant.

Amplification by PCR of DNA extracted from plant leaves using LP, RP and BP primers specific for *Atprd2* and followed by agarose gel electrophoresis. 1000bp single band is amplified in the WT allele using LP and RP, while the T-DNA insertion allele (mutant) showed a 500bp band. Both bands were only visible in plants that were heterozygous for the T-DNA insertion.

To determine the expression of *AtPRD2* gene in flower buds of WT and *Atprd2* homozygous mutants, RT-PCR was carried out to produce a cDNA library of mRNAs and then a PCR with specific primers. For control, primers for *AtACTIN2* housekeeping gene were used. For the *AtACTIN2* gene, a band of 200-300bps was observed in the WT and *Atprd2* mutant for flower buds and leaf equally. This was used to check that equal amounts of cDNA were used for all the samples. A band is seen just above 200 bps for *AtPRD2* gene in WT leaf and bud. For *Atprd2* homozygous mutant in leaf and flower bud, no equivalent band can be observed (**Figure 50**).

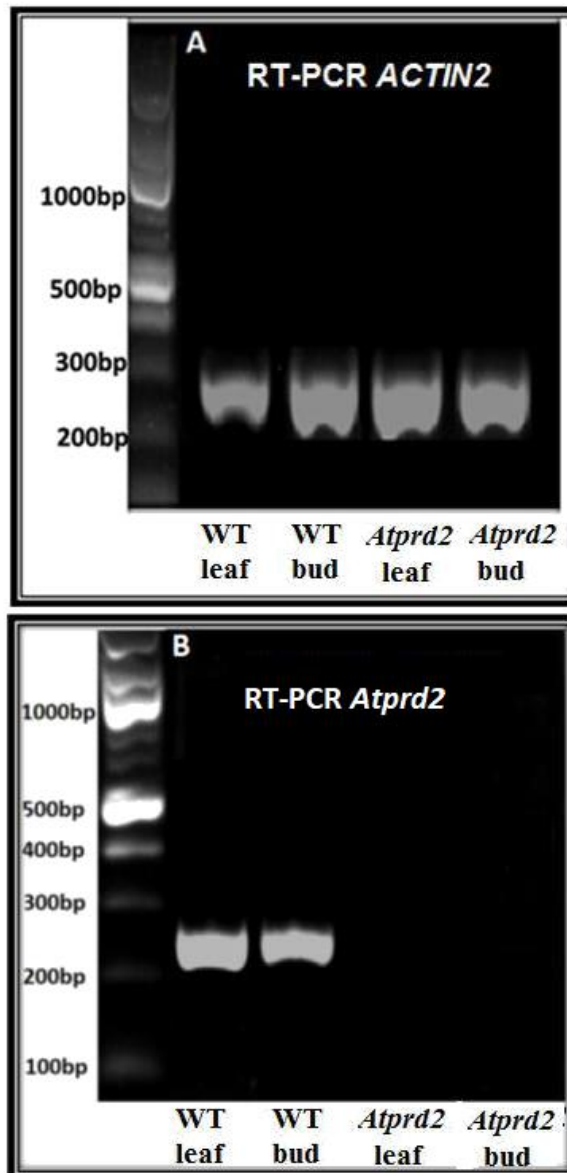


Figure 50: Gel electrophoresis photographs are representing RT-PCR analysis for *AtPRD2* gene in different plant samples.

(A) Control for an equal amount of cDNA among samples using a 200bp amplification of *Atctin2* gene. (B) RT-PCR analysis of the expression of *AtPRD2* gene in WT and mutant samples. A cDNA band is observed above 250bps in WT leaves, and WT buds, but for *Atprd2* homozygous mutant no bands could be observed.

The phenotype of *Atprd2* mutant

The expression analysis has shown us that the T-DNA insertion mutants used are null mutants as not the expression of the gene is observed. The phenotype of heterozygous and homozygous mutants was compared to WT plants (**Figure 51**). The mean silique length on heterozygous mutants was similar to the mean silique length on WT plants. Nevertheless, the mean silique length on *Atprd2* homozygous mutants was visibly shorter than the WT. It is observed that mutants were appeared to grow slower than WT (Data was not quantified on growth rates of the plant, but it was very visible). On the other hand, the growth rates of heterozygous mutants were similar to WT. Morphology of leaves and rosettes were similar to the homozygous and heterozygous *Atprd2* mutants (**Figure 51**).

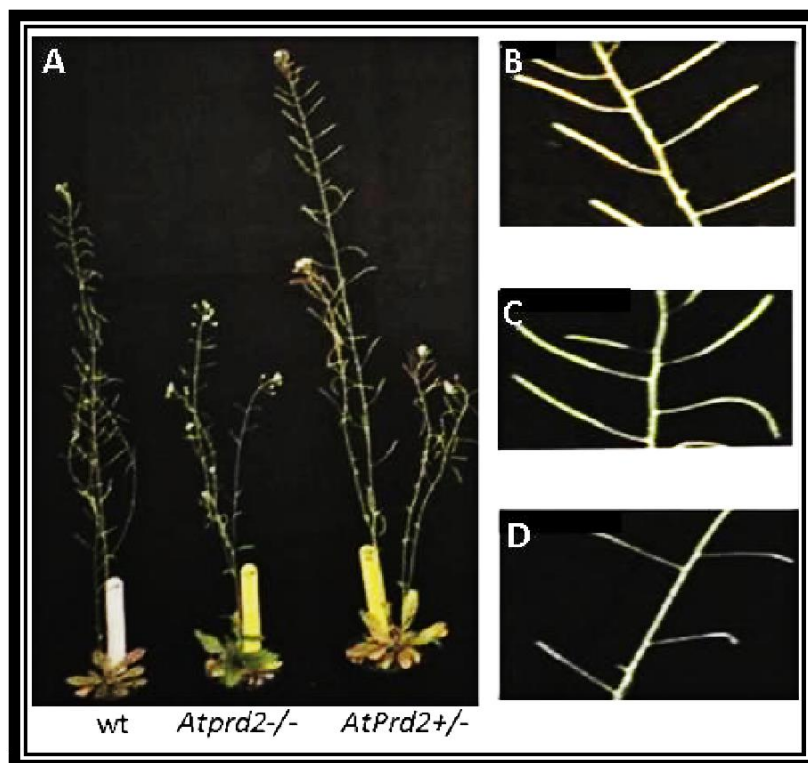


Figure 51: *Atprd2* mutant phenotype.

(A) Photographs of homozygous and heterozygous mutant plants. (B) Details of siliques in WT plants. (C) Siliques of *Atprd2* heterozygous mutant. (D) Siliques of *Atprd2* homozygous mutant showing a reduced length than WT.

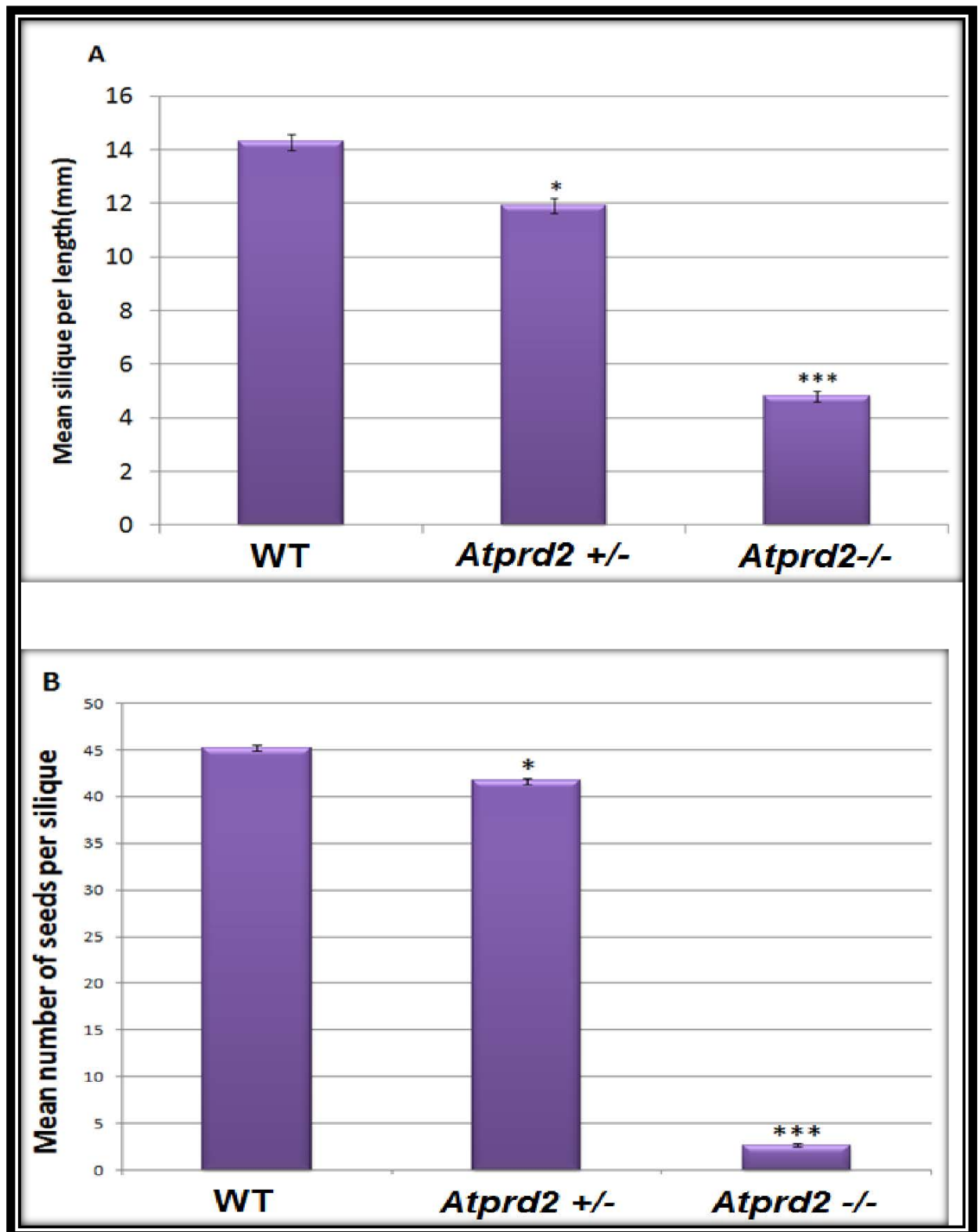


Figure 52: Seeds count and silique length data for WT and *Atprd2* mutants.

(A) Mean silique length per silique (B) Mean number of seeds per siliques (100 siliques per plant type) . Error bar = stander error of the mean. * means p.value <0.05 and *** means p.value <0.001.

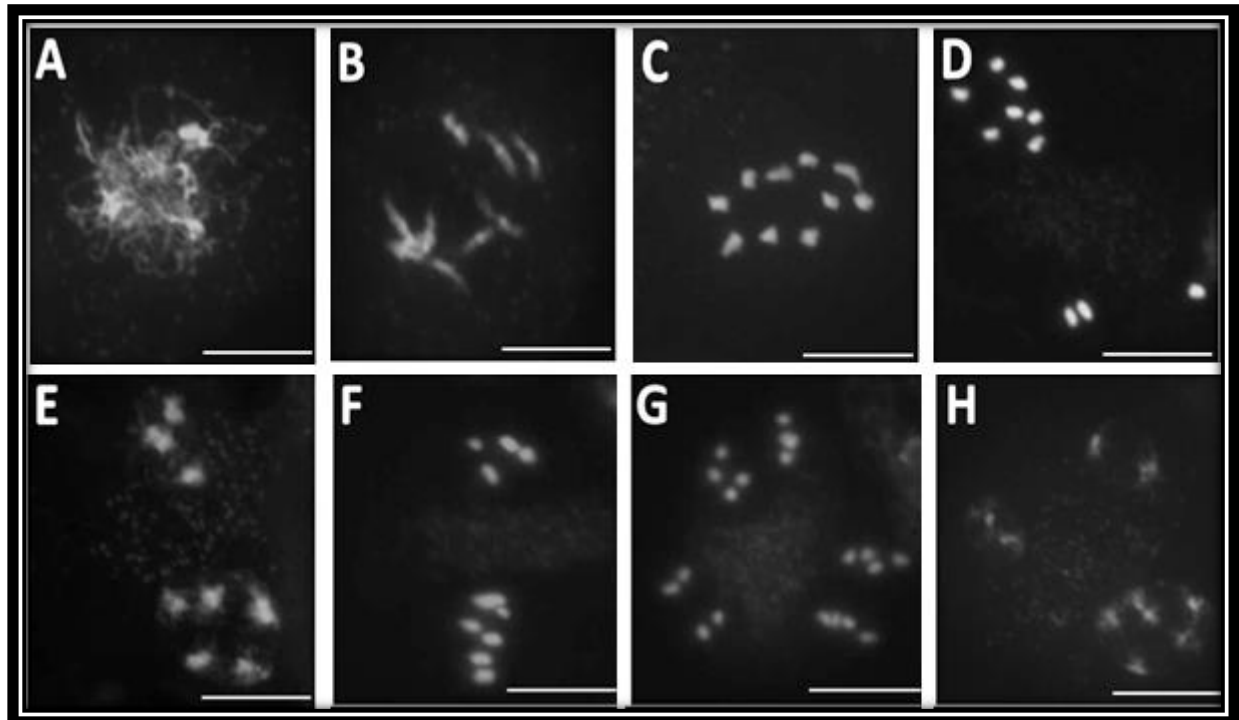


Figure 53: Dapi stained chromosome microphotographs of *Atspo11.2* meiosis. (A) Zygotene-Pachytene. (B) Diakinesis. (C) Metaphase I. (D) Anaphase I. (E) Prophase II. (F) Metaphase II. (G) Anaphase II. (L) Telophase II. Scale bar 5µm.

Meiotic stages of *Atprd2* mutant was also carried out and showed some differences from that of previous publication ([De Muyt et al. 2009](#)). *Atprd2* show chromosomes not pairing during zygotene but also some chromosome fragments could be observed at this stage (**Figure 54A**). In most of the cells, chromosomes appeared as univalents during diakinesis and metaphase I stages (**Figures 54B & Figures 54C**). Nevertheless, some cells also showed a single bivalent during diakinesis and metaphase I (**Figures 54D– Figures 54H**). Chromosome missegregation was observed at anaphase I and meiotic second division (**Figures 54J- Figures 54M**). Some cells in second division showed the presence of DNA fragments (**Figure 54 M**).

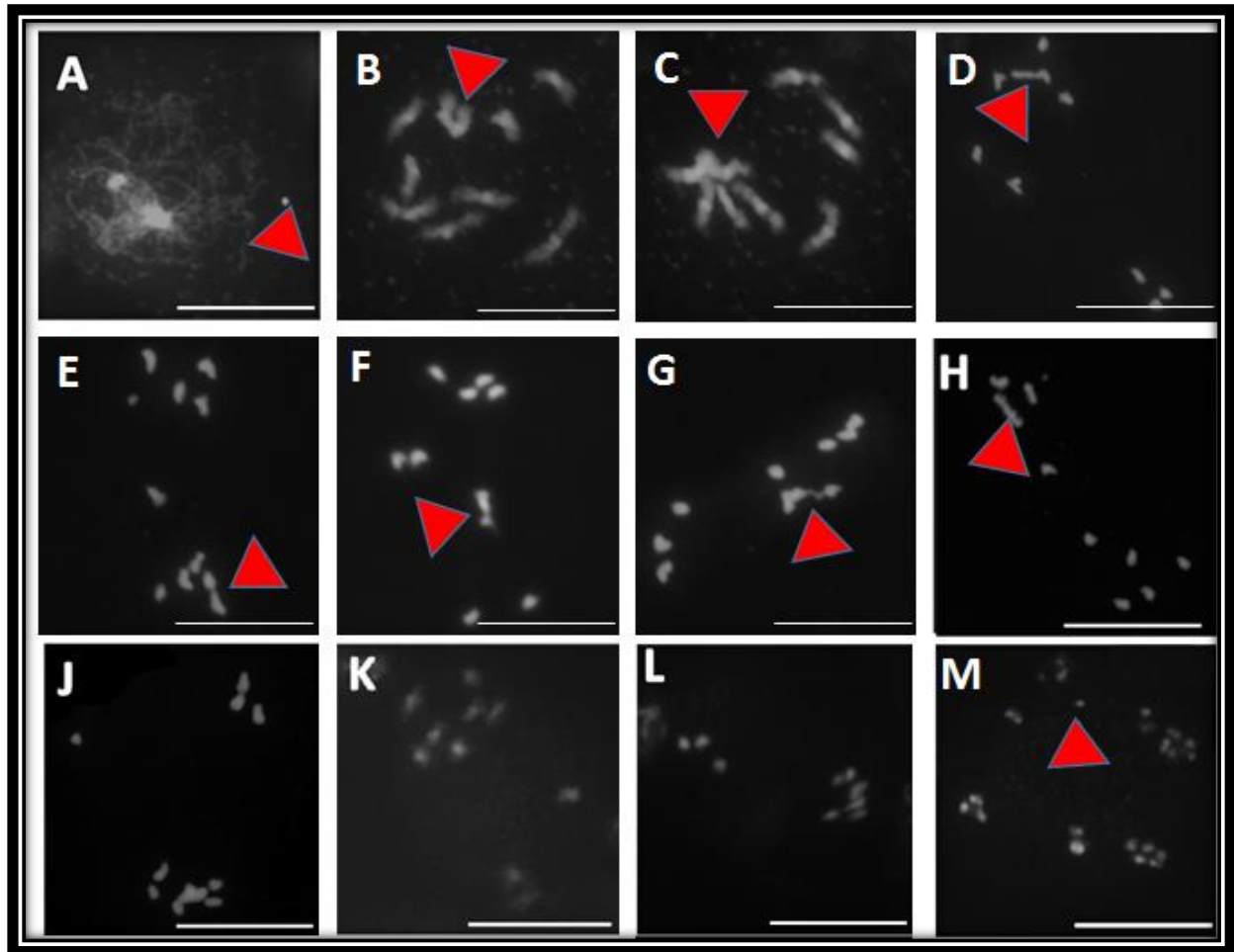


Figure 54: Different stages of meiosis in the *Atprd2* homozygous mutant.

(A) Zygotene-Pachytene. (B & C) Diakinesis. (D-H) Metaphase I. (J) Anaphase I. (K) Prophase II. (L) Metaphase II. (H) Anaphase II. Scale bar 5µm..

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Immunolocalisation of meiotic proteins in WT, *Atspo11.2* mutant, and *Atprd2* mutant

Polymerization or spatial distribution of meiotic proteins was studied in early prophase using immunolocalization in WT, *Atspo11.2* mutant, and *Atprd2* mutant. The polymerization of chromosome axis protein (AtASY1) and the distribution of the DSB formation catalysing protein (AtSP011-1) was determined by using specific antibodies against AtASY1 (green) and AtSP011-1 (red). There are co-localization and even distribution of signal for AtASY1 with AtSP011-1 signals along the chromosomal axis in the WT (**Figure 55A**). H2AX exists as an isoform of H2AX protein, which is chromatin-associated histone protein. This protein undergoes phosphorylation to form γ -H2AX. In meiosis when DSB formation started, γ -

H2AX localized near the DSB site and played important role in DSB repair by recruiting the repair protein (Paull *et al.*, 2000). Shroff *et al.*, 2004 showed that γ -H2AX is involved in making structural changes in chromosomes position at break site for assurance of efficient repair. γ -H2AX acts as an efficient marker for the site of DSBs in yeast and other organisms including Arabidopsis (Redon *et al.*, 2002; Sanchez Moran *et al.*, 2007). γ -H2AX signals were localized around the nucleus evenly in WT type with AtASY1 (**Figure 55A**). In *Atspo11.2* mutant, there was not a single signal for γ -H2AX (**Figure 55B**), but in *Atprd2* mutant, some residual γ -H2AX signal was observed in a limited number of cells. This residual γ -H2AX signal was localised in a small region of the nucleus (**Figure 55C**).

Disrupted Meiotic cDNA1 (Dmc1) is known as Meiotic recombination protein involved in the strand invasion (Neale and Keeney, 2006). It has a central role in homologous recombination in meiosis at DSBs sites and searching for homologous sequences (Habu *et al.*, 1996). RADiation sensitive51 (RAD51) mediates the homologous recombination to repair DSBs in mitosis and meiosis. In meiosis, it interacts with DMC1 for inhibition the activity of strand exchange (Cloud *et al.*, 2012). Immunolocalization of DMC1 and RAD51 was examined to follow the recombination dynamics at prophase I. DMC1, and RAD51 proteins appeared in WT meiocytes at zygotene (**Figure 55A**), while in *Atspo11.2* mutant; there was not signal for any of these proteins (**Figure 55B**). AtAZYP1 is a protein, which is present as transverse filaments in the central element of SC this protein was immunolocalised with an anti-AtZYP1 antibody (green) at the same time that AtASY1 (red) antibody. AtZYP1 synapse the homologous chromosomes together by polymerizing in a zipper-like fashion. Localization of AtASY1 signals is evenly distributed and polymerised along with the chromosomes. Localization of AtZYP1 signal is between the homologous chromosomes and its polymerization is full at pachytene (**Figure 55A**). In *Atspo11.2* mutant, the ZYP1 polymerization was completely absent (**Figure 55B**). In *Atprd2* mutant, AtZYP1 polymerization was as small individual traces (**Figure 55C**).

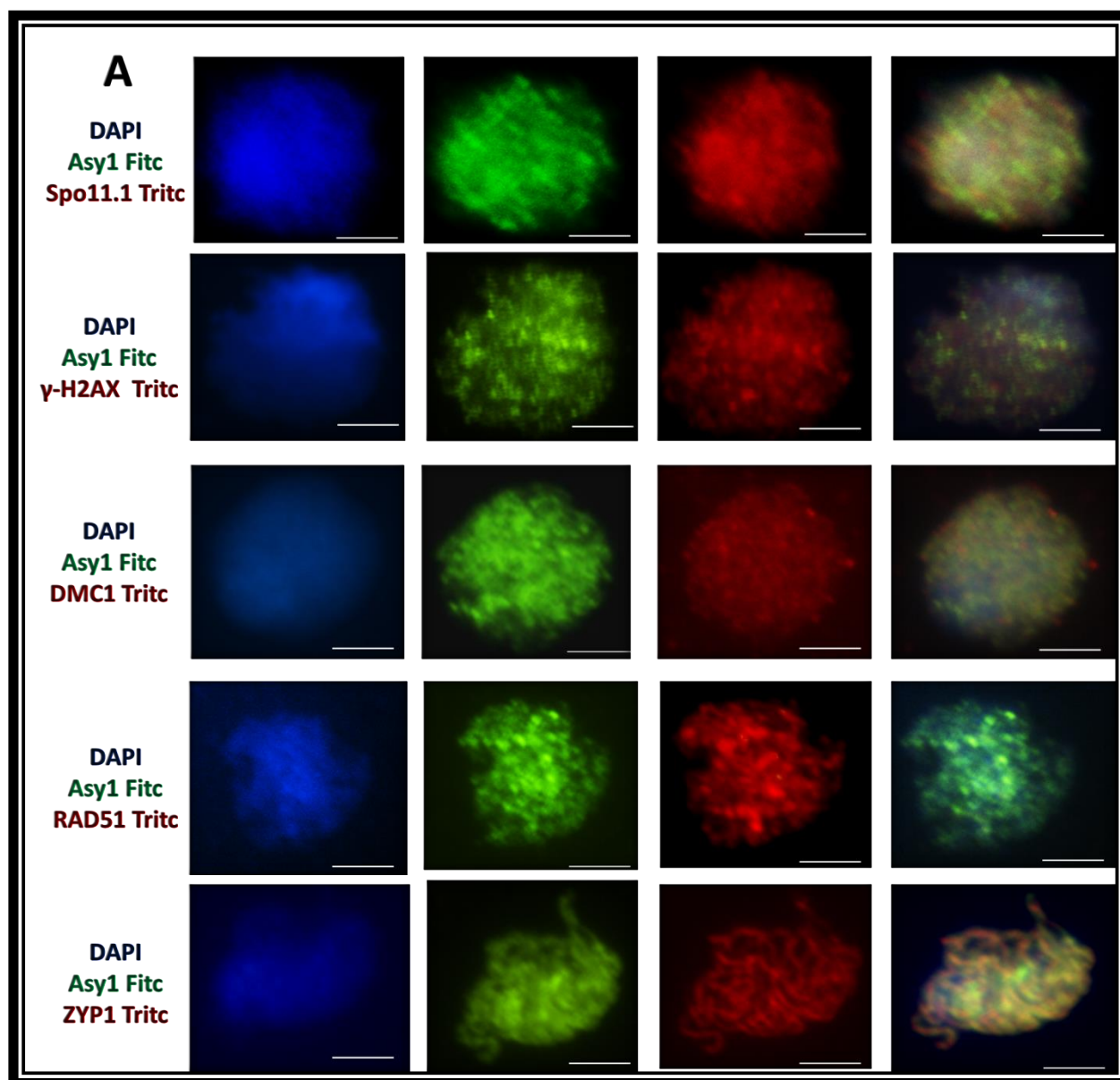


Figure 55A: Representation of dual immunolocalization of meiotic proteins in WT.
 Dual immunolocalization of AtASY1 (green) and AtSP011-1 (red), AtASY1 (green) γ -H2AX (red), AtASY1 (green) and AtDMC1 (red), AtASY1 (green) and AtRAD51 (red), and AtZYP1 (green) and AtASY1 (red)

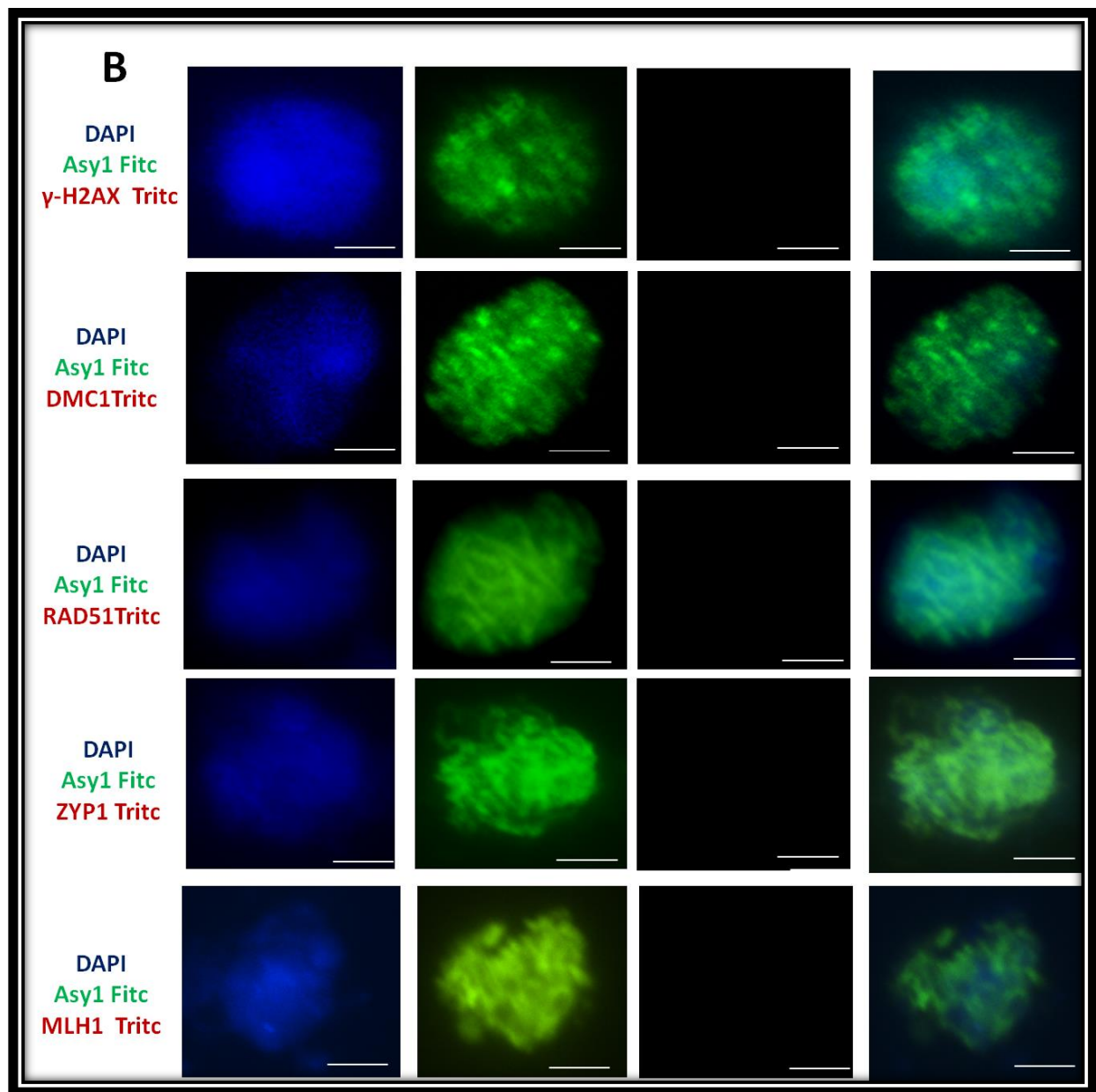


Figure 55B. Representation of dual immunolocalization of meiotic proteins in *Atspo11-2* mutant. Dual immunolocalization of AtASY1 (green) γ -H2AX (red), AtASY1 (green) and AtDMC1 (red), AtASY1 (green) and AtRAD51 (red), AtASY1 (green) AtZYP1(red) and AtASY1 (green) AtMLH1(red) .

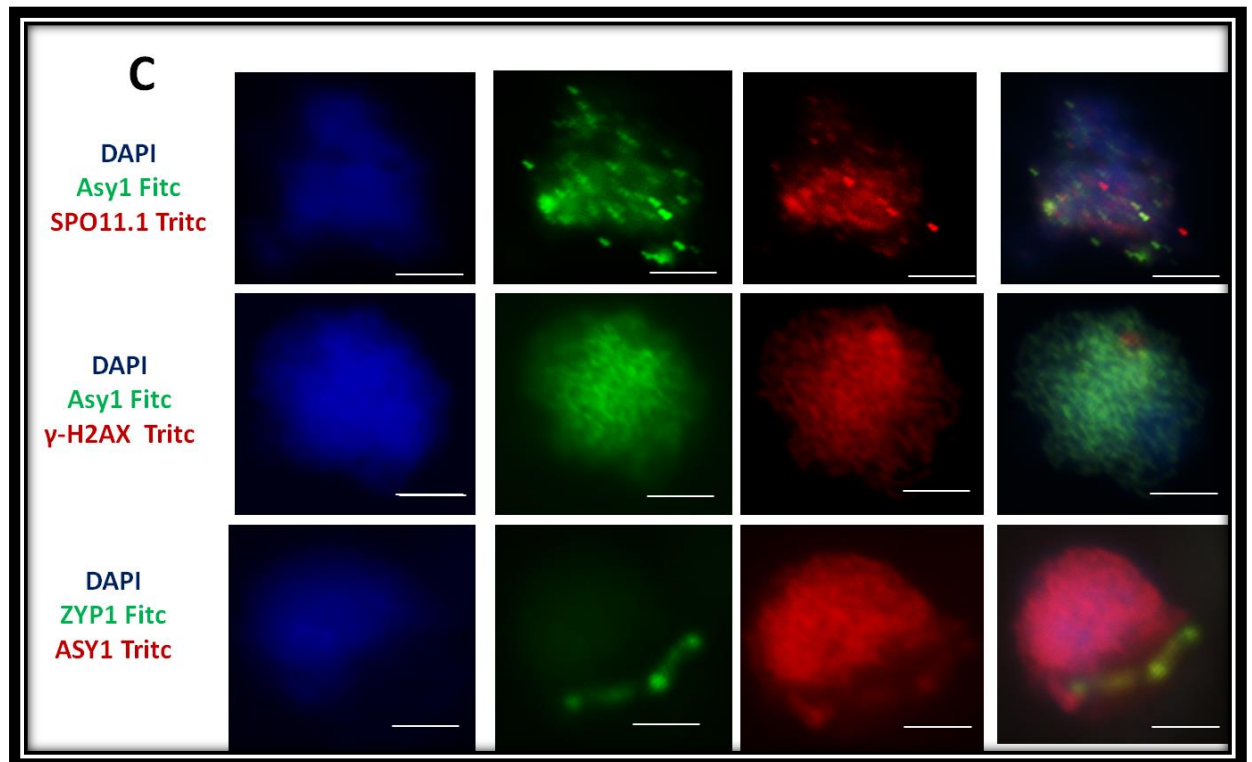


Figure 55C. Representation of dual immunolocalization of meiotic proteins in *Atprd2* homozygous mutant plants. Dual immunolocalization of AtASY1 (green) and AtSPO11-1 (red), AtASY1 (green) γ -H2AX (red), and AtZYP1 (green) and AtASY1 (red). Scale bar is (5 μ m).

Meiotic analysis for artificial DSBs by cisplatin in the absence of *AtSPO11.2*

Cisplatin (Cis-platinum (II) diamine dichloride) is a chemotherapeutic reagent, which is used in the formation of monoadducts ((Kartalou and Essigmann 2001). Cisplatin reacts with DNA to produce inter and intra stand to crosslink, DSBs are formed due to excision of DNA crosslinks to be able to repair these structures. This DSB formation after treatment with cisplatin can start the recombinational repair pathway and even meiotic recombination (Wu and Lichten 1994; Hanneman *et al.*, 1997). Meiocytes isolated from *Atspo11.2* flower buds at 42 hours after treatment with cisplatin (5mg/ml and 10mg/ml) were used to prepare chromosome spread preparations. Cytological analysis was done to study changes during meiosis (**Figure 56**). When DAPI stained prophase I chromosomes were analysed, some bivalent formation was restored in *Atspo11-2* mutant which was indicating that some DSBs induced by cisplatin (5mg/ml) could be used to produce COs

Chromosomes did not appear fully synapsed in zygotene-pachytene stage and some small fragments could be visualised (**Figure 56F& Figure 56G**). During metaphase I only a single rod bivalent was observed in some cells (1/38) (**Figure 56H – Figure 56J**). Chromosome missegregation and some fragments could be observed in some cells at anaphase I (**Figure 56K- Figure 56M**). Some prophase II cells also showed some DNA fragments (**Figure 56N**). Some metaphase II cells also showed some DNA fragments (**Figure 56O**). At higher concentration of cisplatin (10mg/ml), most of the meiocytes at zygotene showed chromosomes not fully synapsed and some meiocytes with large DNA fragments (**Figure 56P & Figure 56Q**). Some cells showed bivalents and DNA fragmentation at metaphase I (**Figure 56R - Figure 56U**). Some cells showed bivalents (up to 3 in some cells) (**Figure 56V**). Extensive DNA fragmentation was also observed at second division stages (**Figure 56W– Figure 56Y**).

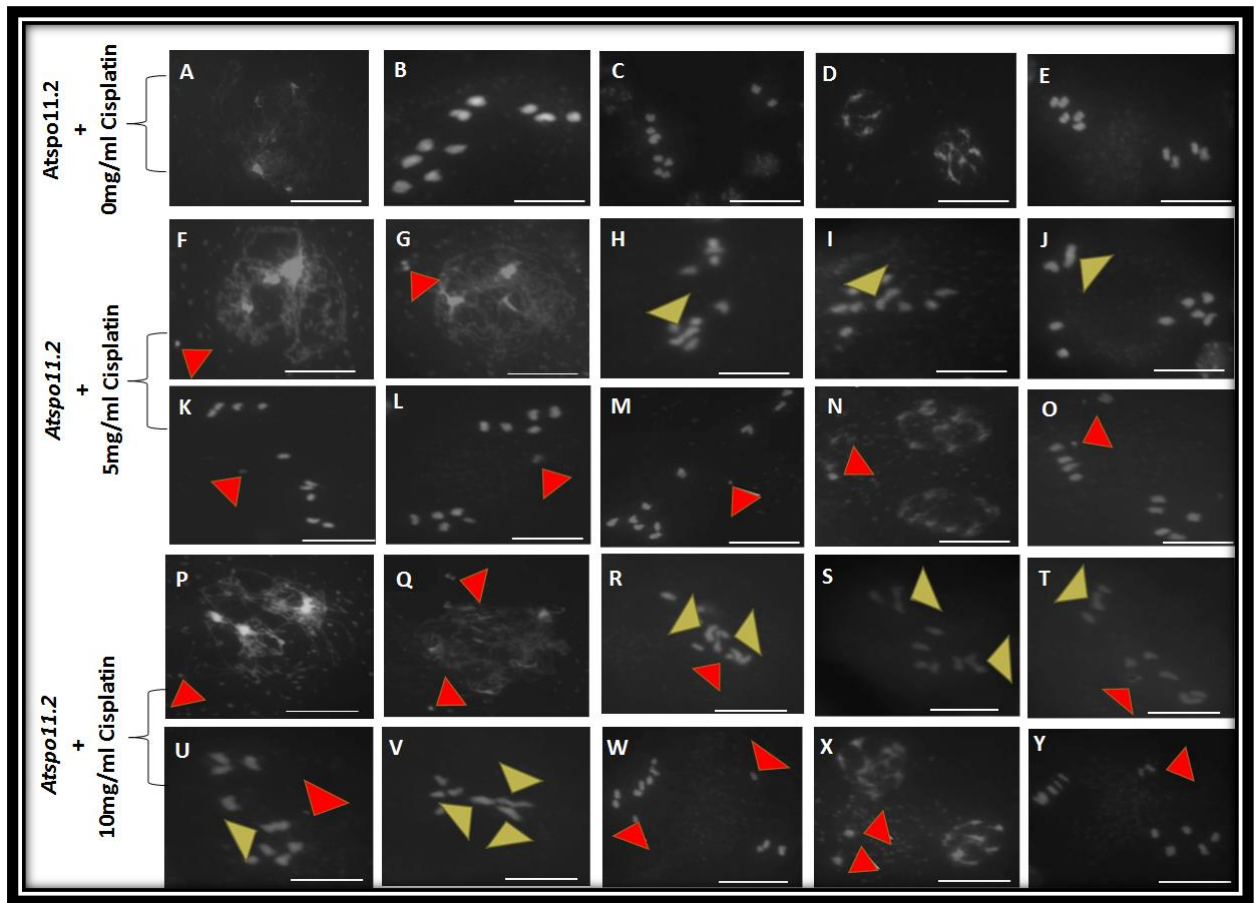


Figure 56: Different stages of meiosis in the *Atspo11-2* homozygous mutant treated with cisplatin.

(A-E) untreated *Atspo11.2*. (A) zygotene- pachytene, stages. (B) Ten univalents at metaphase I. (C) Chromosome missegregation at anaphase I. (D) prophase with chromosome missegregation. (E) Chromosome missegregation at metaphase II (F–O) *Atspo11.2* treated with 5mg/ml cisplatin. (F&G) zygotene- pachytene, stages with small fragments (red mark). (H–J) One bivalent at metaphase I (yellow mark). (K–M) metaphase I-anaphase I with small chromosome fragmentation (red mark). (N) prophase II with some fragmentations (red mark). (O) Metaphase II with small fragmentations (red mark). (P–Y) *Atspo11.2* treated with 10mg/ml cisplatin. (P & Q) zygotene- pachytene, stages with large and two small fragments (red mark). (R) Two bivalents at metaphase I (yellow mark) and small chromosome fragment (red mark). (S) Two bivalents at metaphase I (yellow marks). (T & U) One bivalent at metaphase I (yellow marks) and small fragment (red mark). (V) three bivalents at metaphase I (yellow mark). (W) Chromosome missegregation at Metaphase I with two small fragmentations (red mark) (X) prophase II with two small fragmentations (red mark) (U) Metaphase II with two small fragmentations (red mark). Scale bar = 5 μ m

Cisplatin-treated meiocytes were used to immunolocalize some meiotic proteins. In *Atspo11-2* mutant cells treated with cisplatin (10mg/ml), γ -H2AX signals were limited compared with WT (**Figure 57**) but present, indicating that some DSBs were artificially formed in *Atspo11-2* mutant by cisplatin. DMC1 and RAD51 signals indicated that artificial DSBs could be activating meiotic HR machinery (**Figures 57**). Synaptonemal complex (SC) transverse filament protein ZYP1 was immunolocalized and showed that partial chromosome synapsis was partially restored in some chromosome regions in *Atspo11-2* zygotenes treated with cisplatin (**Figure 57**).

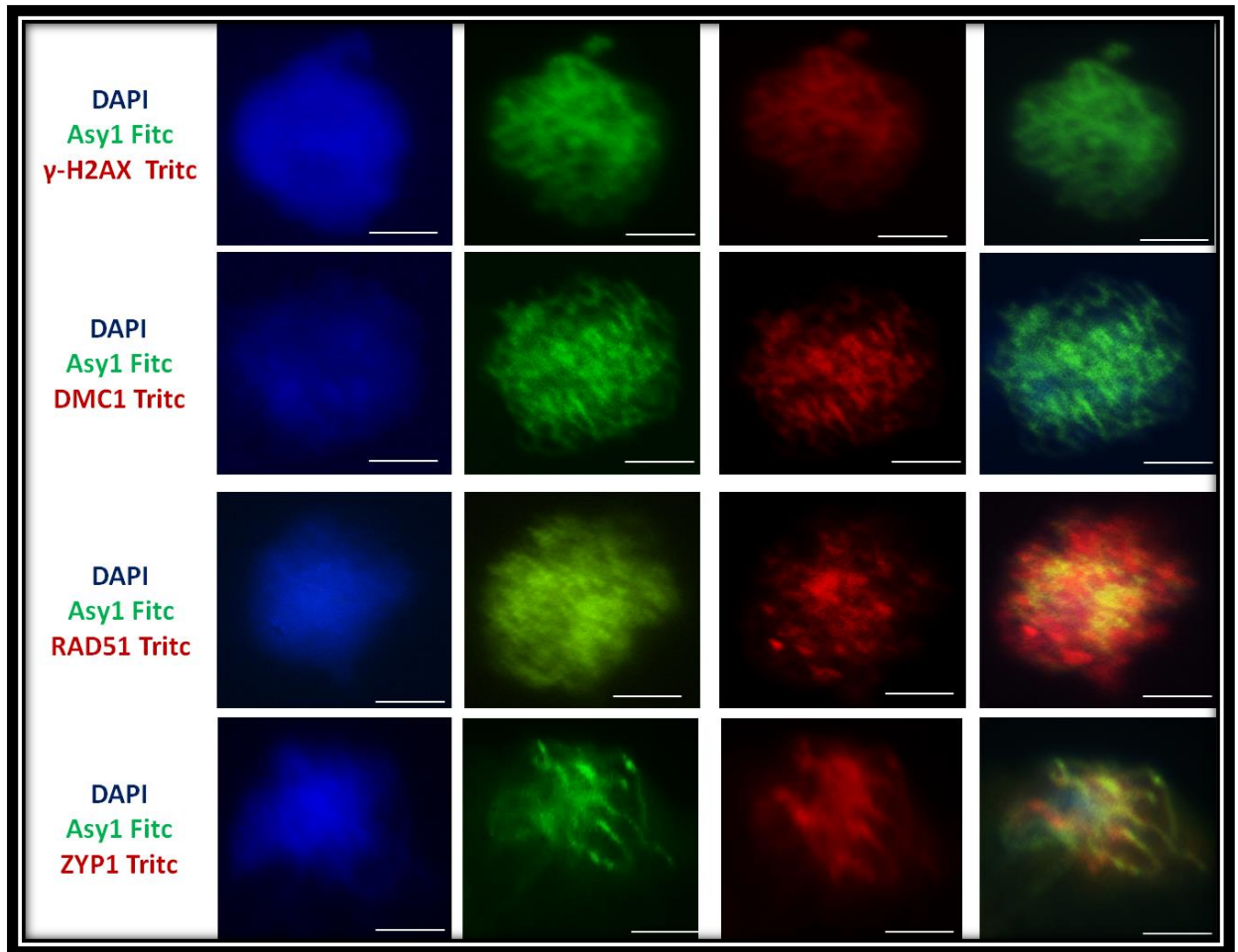


Figure 57: immunolocalization of meiotic proteins in *Atspo11.2* homozygous mutant plants treated with Cisplatin (10mg/ml).

Dual immunolocalization of AtASY1 (green) γ -H2AX (red), AtASY1 (green) and AtDMC1 (red), AtASY1 (green) and AtRAD51 (red), AtASY1 (green) AtZYP1(red) (red). Scale bar =5 μ m.

Discussion

SPO11-1 AND SPO11-2 have similar functions during the process of meiosis

Various similarities between AtSPO11-1 and AtSPO11-2 are a strong indication that both have similar functions during the process of meiosis. The phenotype of heterozygous plants *Atspo11.1* and *Atspo11.2* exhibit a moderate to strong sterile. This form of genetic interaction has additionally been found among different systems and is defined as non-allelic and non-complementation; to explain this situation, two different models have been proposed (Hubert *et al.*, 2000). Cytological research has demonstrated that SPO11 generates discrete staining formation –foci” on chromatin and at the leptoneuma substage among mice, *S. cerevisiae*, and *Sordaria macrospora* (Romanienko & Camerini-Otero 2000). This represents the stage during which DSBs are created, which means SPO11 exist at the appropriate time so that they can participate in the formation of DSBs.

Nevertheless, it is perhaps surprising that the SPO11 gene continues to act on the chromosomes through the pachytene phase in all of these organisms, which exceeds the period of DSB formation (Storlazzi *et al.* 2003; Prieler *et al.* 2005). Cytological investigation *Atspo11-1* and *Atspo11-2* mutants phenotype in *A.thaliana* showed the significant importance of both proteins (*Atspo11-1* and *Atspo11-2*) in the creation of DSBs. It has been demonstrated that *Atspo11-1* mutants lead to a considerable decrease in fertility with either a complete absence of or minimal amount of bivalents formation at metaphase I (Grelon *et al.*, 2001). In our Study for controlling meiotic recombination in *A.thaliana*, the gene of SPO11-2 at the AT1G63990 locus has been selected, and the mutant line that is used in our study is a SAIL GABI_039E09 T-DNA insertion as shown in **(Figure 45)**. Our results for *Atspo11-2* mutants have shown a similar result into *Atspo11-1*, a lack of bivalents during metaphase I. Such findings indicate that the

proteins above (*Atspo11-1* and *Atspo11-2*) are key components in the creation of DSBs (Ronceret *et al.*, 2009). In Arabidopsis, *Atspo11-2* has indicated that out of the three Spo11 homologs one is an important factor in meiosis. Also, *Atspo11-2* is an additional functional orthologue of Spo11. Disturbance of the *Atspo11-2* function in two separate insertion alleles leads to serious imperfections in the development of gametophytic development in both females and males, which consequentially causes a robust sterility phenotype. The results of the fluorescence microscopy conducted in the present study suggest that among *Atspo11-2* mutants, synapsis and recombination are significantly disrupted, and no bivalents are created at MI as we observed in our result (**Figure 53**), which frequently indicates that there is a necessity for *Atspo11-2* to create DSBs for starting pairing and recombination.

SPO11-2 is required to start the process of Meiotic Recombination

There are two distinct ways in which meiotic recombination impacts the separation of chromosomes. Firstly, research into various species, such as plants, mammals and fungi, has revealed that the process of homologous chromosome pairing is highly associated with meiotic recombination. Secondly, meiotic recombination generates actual links called chiasmata among homologous chromosomes in each bivalent. The purpose of the chiasmata is to hold the bivalents together to guarantee that they are appropriately oriented and the separation of chromosomes during the stage of the initial meiotic division (Pawlowski & Cande, 2005). SPO11 proteins are required for DSBs formation to start the process of meiotic recombination as our result of *Atspo11-2* mutant showed that in the absence of SPO11, DSBs are not formed. Thus meiotic recombination cannot occur as shown no bivalent at MI in *Atspo11-2* mutant. In *A.thaliana* (wild type),

Recombination during meiosis is started via the forming DSBs by SPO11. The entire procedure is supported by two recombination proteins, namely Rad51 and Dmc1 that are absent in *Atspo11-2* mutant as shown in our immunocloastion result because of no DSBs formation by SPO11 (**Figure 55B**).

PRD2 is involved in DSBs formation in *Arabidopsis thaliana*

The process and proteins incorporated in the creation of DSBs are conserved among the majority of species. Researchers have established that SPO11 is a key component in the process of inducing DSBs. DSBs materialise randomly along the chromatin loops and transition physically towards the chromosome axis for additional processing (Grelon et al., 2001; Zicker & Kleckner, 1999). Through the utilisation of the protein sequence homology from the yeast protein SPO11, research has discovered that there are three paralogues for the protein in *Arabidopsis* which has three genes, namely *Atspo11-1*, *Atspo11-2*, and *Atspo11-3*. It is possible that *Atspo11-1* and *Atspo11-2* work as heterodimers and are necessary factors in the commencement of meiotic recombination. All of these proteins incorporate diverse tyrosine residues, which are primary constituents in the process of inducing DSBs (Hartung & Puchta, 2001; Ronceret *et al.*, 2009). The third paralogue protein, *Atspo11-3*, has been determined to be an important factor in additional cell processes, including cell development and the replication of DNA in mitotic cells (Hartung *et al.*, 2000). In the context of (*Arabidopsis thaliana*), the A subunit is comprised of SPO11-1 and SPO11-2 as well as the new B subunit (MTOPIVIB), which are believed to generate a heteromeric complex (Vrielynck *et al.*, 2016). Contrastingly, it is necessary for various additional proteins to work in harmony with SPO11 in budding yeast for the induction of DSBs like Mei4, Mre11, Rad50, Xrs2, Ski8, Mer2, Rec104, Rec114, and Rec102 to be possible (Jolivet *et al.*, 2006). Additionally, it has been reported in the literature that other periods are important the formation of DSBs in *A. thaliana*, namely

AtPRD1 which is homolog function of MEI1 protein in mammals and is considered to have an association with AtSPO11-1. AtPRD2 is an orthologue of ME14 in mice, and AtPRD3 is homolog function of the rice

protein PAIR1. A mutation phenotype in any of the proteins above is a contributing factor to the same phenotype when *Atspo11-2* mutates, where no DBS formed. Thus chiasmata are not created at metaphase I (Osman *et al.*, 2011). Jiang and colleagues (2009) studied the gene of PRD2 at the At5g57880 locus for the first time. In our study, the same mutant line that is used by Jiang and colleagues which is a SAIL _94_D08 T-DNA insertion as shown in **(Figure 46)**. *Atprd2* homologs were identified within the plant kingdom, while homologs were not identified in humans and mice during in silico analysis. Most significant alignment of some of these homologs was with many species such as Brassica oleracea, Brassica rapa, Brassica napu, and Camelina Sativa. The function of these homolog proteins is not characterized, and their role in DSB formation is not clear yet **(Figure 47A & Figure 47B)**. The previous study by De Muyt (2009) considered *Atprd2* mutant as a knockout mutant, but the absence of a band in *Atprd2* homozygous mutant confirmed that it is a null mutant **(Figure 50B)**.

Jiang and colleagues (2009) observed that cytological analysis of these mutants showed that miss- segregation of chromosomes during meiotic process results in the sterility of male and females. Meiotic spindles showed aberrant organization when these were studied with immunolocalization work. Researchers concluded that this all is due to missegregation of chromosomes during meiosis. De Muyt and colleagues (2009) indicated the important features of *Atprd2* mutant which are a complete absence of chiasmata formation, defective synapsis, and absence of bivalents. Our result to study the role of PRD2 in meiosis indicates that DSBs formation is occurring in *Atprd2* homozygous mutant, very clear bivalent formation or some appeared as bivalent in metaphase1 was observed. This is observed in approximately 8 cells from total of 100 cells. Although in

some cells chromosomes are connected showing the bivalent formation (**Figure 54**). Research carried out utilizing the *Atspo11-1* mutant; bivalent formation appeared to be a rare, very limited number of bivalents observed. This was similar that DSB formation is effected abnormally to *Atprd2* mutants. Structures were chromosomes joined are termed as chromosome bridges and give rise to the chiasmata formation. In this study, dual immunolocalisation is performed between AtASY1 and At γ -H2AX as well as AtASY1 and AtSP011-1. In *A.thaliana* marker for DSB formation is γ -H2AX, so the presence of γ -H2AX in mutant confirmed the presence of a small number of DSB formation in the localised region of the nucleus (Sanchez-Moran *et al.*, 2007). Presence of AtSP011-1 localised as foci across the chromosome, supported the fact that DSBs machinery which is required for this catalysis is present in the nucleus (Grelon *et al.*, 2001). However, it is suggested that when AtSP011-1 and At γ -H2AX localization is compared in mutants and WT, localization of both is reduced in mutants. This indicates that DSBs producing capacity is greatly reduced in mutants (**Figure 55C**). Preparation of chromosomes spreads stained with DAPI indicate the presence of DNA satellite fragments. This result indicates the formation of some DSBs. Fragments are formed as these DSBs do not undergo repair process by CO or NCO pathway (Caryl *et al.*, 2003).

Residual DSBs can be resolved as a crossover

In wild type of Arabidopsis, after the process of strand invasion has been completed, the 3' end-infiltrating strand represents a starting point for DNA synthesis, which ultimately leads to the double-stranded homologous DNA being unwound, thus creating a displacement loop structure (D-loop) This intermediate that occurs at an early stage can potentially instigate numerous kinds of combined molecules, while the differential stabilisation and resolution of combined molecule intermediates appear to be a key determining factor in recombination processes (Petukhova *et al.*, 1998: Allers & Lichten, 2001). If the D-loops are lengthened, which can subsequently provide improved exposure

of the dislodged strand, another end of the DSB recombines with the dislodged strand of the D-loops through a procedure defined as second end capture annealing. Following the processes of ligation, a distinctive heteroduplex DNA configuration labeled the double Holliday junction (dHJ) is created which can be resolved as CO or an NCO (Schwacha & Kleckner, 1995). COs that is resolved from dHJ is defined as Class I COs, can experience interference; thus, the distance between them along the chromosome is greater than one would expect if they were randomly distributed. Börner and colleagues (2004) proved that Class I COs is based on a group of proteins that were first determined in *S. cerevisiae* and fall under the umbrella term of ZMM proteins, which covers Zip1, Zip2, Zip3, Zip4, Spo16, Msh4, Msh5, and Mer3. Hence, although it is therefore apparent that most COs are accounted for by the ZMM pathway, there are other pathways that can be considered. Cytological evidence has shown that many early recombination intermediates including ZIP4 and MER3 are defined as ZMM proteins, although only a limited number sequentially develop into COs that can be marked with MLH1 or HEI10 at pachytene phase (Lhuissier *et al.*, 2007; Wang *et al.*, 2009; Shen *et al.*, 2012). However, Class II COs is interference – independent and it is produced around 10% to 20% of COs (Berchowitz *et al.*, 2007). Genetic investigations have revealed that MUS81 is a prerequisite for the generation of Class II COs and if it is mutated, the number of COs created will be diminished by approximately 10%. In *Arabidopsis*, Higgins and colleagues (2008) found that 0.8 chiasmata per cell were observed for double mutant (*msh4/mus81*), thus implying that Class II COs can be achieved by other pathways that remain unidentified. Nevertheless, it remains undetermined regarding whether the same principle applies to plants. Research into budding yeast has indicated that the CO/NCO. The choice is made early in the process, which is generally around or immediately before the creation of the robust single-end invasion intermediate. Such an assertion is founded on an examination of *zmm* mutants, that have been determined to generate NCOs and DSBs at standard levels

at a temperature of 33 °C, but were imperfect in terms of the generation of the single-ended invasion intermediates COs and dHJs (Borner *et al.*, 2004). It is not likely that the amount of COs in each chromosome will be greater than three per bivalent, regardless of the actual magnitude of the chromosome. In terms of the plant *A.thaliana*, we observed that COs were not produced as shown in the result of *Atspo11.2* mutant. AtZYP1 was not polymerized in the mutant of *Atspo11.2* (**Figure 55B**). In wild type, Sanchez-Moran and colleagues (2007) found that meiotic recombination is instigated by 150 DSBs, 10 DSBs resolved as COs and 140 resolved as NCOs. To guarantee that a CO pair exists on every chromosome pair, it is necessary for the DSB locations to be chosen in a non-random manner so that they develop into COs, and the rest of the locations are directed towards the NCO pathway. In the case of yeast, a large number (50%) of DSBs develop into COs. Fung and colleagues (2004) believed that the CO pathway that contains ZMMs are the locations where the SC polymerisation is initiated. In the majority of different species including *Arabidopsis thaliana*, Cos number is significantly reduced (5–10%).

Nevertheless, in Arabidopsis, AtMSH4 foci that is connected with parts of SC is three times greater than the Cos number, a situation that likely exists to guarantee that chromosome synapsis occurs at the right time. It is possible that AtRMI1/BLAP75/TOP3 α Is an essential pathway for the elimination of DHJs that do not resolve as COs (Osman *et al.*, 2011). Although it has not yet been determined how and when the decision is made between COs and NCOs, research has suggested that the process is managed at numerous different level. Firstly, the creation of a minimum of one CO per homolog pair is necessary if the precise separation of homologous is to occur, defined as the obligate CO. Secondly, the existence of a CO diminishes the likelihood of there being another CO in the immediate vicinity, also known as CO interference (Jones & Franklin, 2006). Lastly, the process of CO homeostasis, which has been observed in both *S. cerevisiae* and mice, can sustain a constant number of COs, even in the situation when the amount of DSBs changes (Martini *et al.*, 2006). From our result for *Atprd2* mutant which produces small number of DSBs, dual immunolocalisation between AtASY1 and

AtZYP1 indicated some limited polymerization (**Figure 55C**). It is also observed that in mutants full polymerization and formation of SC is defective. Previous research utilizing *Atzyp1* mutants (defective polymerization & SC formation. It is evidenced that in *Atprd2* mutant some DSBs leads to form a crossover.

Full synapsis/pairing is not necessary to produce a crossover

Meiotic recombination occurs at the same time as synapsis, a procedure in which homologous chromosomes are closely linked to each other as a result of SC formation (Page & Hawley, 2004). Homolog pairing/juxtaposition, synapsis, and recombination foster the appropriate acknowledgment and connection of homologs, which enables them to subsequently separate in the correct manner. Our result showed that in the absence of SPO11-2 no synapsis or pairing occurs between homologous chromosomes (**Figure 55B**). Resultantly, it is necessary to have DSBs for the processes of pairing and synapsis homologous chromosomes in the context of Arabidopsis. However, it appears that SC is necessary to provide stability to pairing and the crossover among homologous chromosomes (Vazquez *et al.*, 2002). This result is similar to the result of *Atasy1* mutant that works published by Sanchez-Moran and colleagues (2007). *Atasy1* mutant showed highly defective synapsis, but some bivalent formation is observed. It is observed that this bivalent formation was observed due to chromosome 2. This chromosome is paired and synapsed because it is associated with nucleolus through its NORs. So it is evidenced that in *Atprd2* mutant the limited synapsis is taking place in chromosome 2 which give rise to the bivalent formation (**Figure 55C**). The data from studying *Atprd2* mutant conclude the fact that a limited number of DSB formation can be synapsed and undergo meiotic recombination to produce COs. Nevertheless, it is possible for synapsis and pairing of homologous chromosomes to be realised without the production of DSBs in different organisms. Conversely, it has been observed that pairing occurs in *Drosophila melanogaster* male meiosis despite the lack of DSBs in addition to recombination and the creation of SC (Gerton & Hawlep, 2005).

Meiotic recombination can be occurred by artificial DSBs

DNA-damaging agents are responsible for making DNA vulnerable inside cells. These agents fall in many categories such as ultraviolet rays, and mutagenic and reactive chemicals. Agents that damage DNA have successfully been applied to instigate meiotic recombination in DSB mutants as well as various wild-style plants (Sanchez-Moran *et al.*, 2007). For instance, different kinds of DNA damage are caused by cisplatin and UV exposure can be repaired by the pathway of homologous recombination, thus generating COs in meiosis. The effect of ultraviolet radiation (UV) on yeast cells in the process of meiosis has been investigated in previous studies. For example, it was determined by Simchen, Salts, and Pinon (1973) that there was a significant impact on cells during the process of premeiotic DNA synthesis as a result of intense irradiation, and they experienced meiotic arrest as well as a diminishment in meiotic recombination (Resnick, 1983). When applying irradiation Machida and Nakai (1980) found that although there was no significant impact on intragenic recombination, intergenic recombination was strengthened. The impact of X-irradiation on meiotic yeast has also been investigated, albeit to a lesser extent. Kelly, Merrill, and Parry (1983) distinctly showed reciprocal recombination. Further research into the outcomes of applying X-radiation to meiosis that is under way could be of significant interest as the well-distinguished lesions that created – both single and double-strand breaks. This increases the likelihood that double-strand breaks will in fact be received by only a small proportion of the bivalents (Orr-Weaver & Szostak, 1985). Researchers have demonstrated that X-irradiation can impact meiotic recombination in fly *Drosophila*. The result has revealed that X-irradiation causes the onset of recombination, translocations, and mutations in meiotic cells in females as well as males, who under normal circumstances do not exhibit recombination during the process of meiosis (Sankaranarayanan & Sobels, 1976; Hannah-Alava, 1964). In the absence of an efficient means of staging the point of treatment regarding the complication biology of *Drosophila*, it has been challenging to determine whether it was, in fact, the meiosis that

was being impacted. Improved timing has been accomplished in relation to the irradiation of meiocytes in *Lilium* and *Tradescantia*, whereby elevated levels of chiasmata were discovered at metaphase I. It was also recorded that there was a considerable increase in meiotic recombination as a response to X-irradiation in *Chlamydomonas reinhardtii* as well as *Caenorhabditis elegans* ((Lawrence & Holt, 1970; Kim & Rose, 1987).

Furthermore, it has been shown by physical assays that meiosis-specific double-strand breaks are not only hotspots for meiotic recombination but also in a wider sense for chromosomes (Cao, Alani, & Kleckner, 1990; Sun *et al.*, 1989).

DSBs by Cisplatin produce crossover in the absence of SPO11-2

DNA damage caused by radiation can partially remedy the viability flaw in *Atspo11* mutants, implying that certain kinds of DNA damage in meiosis can act as a substrate for meiotic recombination(Thorne & Byers, 1993; Dernburg *et al.*, 1998). In *S. cerevisiae* indicating that lesions induced by X-rays can instigate recombination provided that the treatment is applied after the commencement of premeiotic DNA synthesis; however, if the X-ray damage is induced before this stage, it can lead to arrest in the GI meiosis phase. Furthermore, the evidence is given by assays for spore viability in SPO11- 1 meiosis indicating that X-ray induced lesions that occur at later phases could be particular replacements for this mutational flaw in meiotic recombination and could cause enhancements in the meiotic disjunction. In *C. elegans*, irradiation led to the generation of crossovers in the *Atspo11* mutant. Furthermore, a highly elevated frequency of chiasmata was detected; the majority of oocytes were found to have six bivalents (i.e., 51 of the 56 oocytes scored for six animals had no univalent), which suggests that crossovers were induced on every homolog pair. This finding supports the hypothesis that the radiation-mediated rescue of SPO11 worms is caused by the functional substitution of SPO11 by DNA breaks, while the standard function of this protein involves the generation of meiotic DSBs. As there is such an increased number of recombinant progeny, this is an indication

that crossovers caused by ionising radiation can facilitate correct chromosome separation (Dernburg *et al.*, 1998). In *C. elegans*, γ -irradiation cause DNA breaks that remove the necessity for SPO-11 during meiosis and forming chiasmata (Dernburg *et al.*, 1998; Kelly *et al.*, 2000). HIM-17 is a protein associated with chromatin that acts as a connection between the chromatin state and the formation of DSBs in the process of *C. elegans* meiosis. As the *him-17(ok424)* mutant exhibits identical behaviour to the *Atspo-11* mutant, it is possible that γ -irradiation could have a similar effect by bypassing the formation of chiasma. Irradiation was particularly effective at inducing chiasmata: for example, a total of 96% of the chromosome pairs were found to be bivalents, which is the same response observed in the *Atspo11* mutant. Furthermore, chiasmata induced by radiation seem to be practical, as an elevation in the progeny viability was observed, which is likely an indication of the more efficient segregation of chromosomes. In *C. elegans*, the absence of *him-17* and *spo11*, chiasma can be created from DSBs induced by radiation (Reddy *et al.*, 2004).

Cisplatin known as cis-diaminedichloridoplatinum II creates platinum chemical complexes that interact with DNA with the consequence that intrastrand and interstrand crosslinks are formed (Olive & Banath, 2009). In mice there was a confirmation for the significant growth in recombination induced by cisplatin. The recombination rate (34%) of the mice group was almost the same as the original control group (38%) and the cisplatin group were observed to mate after 4 weeks, with a frequency of 79%, which is more than two times more than the control group. It is interesting that this is a considerably higher rate than the original group (60%) (Hanneman *et al.*, 1997). In the genus *Tetrahymena*, pairing occurs in the highly extended micronucleus (MICs). The process involved harvesting cells treated with cisplatin 4 to 5 hours after the mixing was complete. The treatment with cisplatin reinstated the crescent shape, and the cells that had been treated with cisplatin showed a large γ -H2A.X patch formation in MICs. Hence, the existence of DSBs is enough to

instigate the extension of MICs (Kazufumi et al., 2008). Lemmens et al. (2013) have used cisplatin to induce meiotic DSBs in the *Atspo11-1* mutant in *Arabidopsis*. Their result showed that cisplatin has the capability of partly restoring the number of bivalent chromosomes. The disadvantage of employing such an approach is that the DNA break ends are not covalently restricted by SPO11, to constrain the other end-connecting pathway that is prone to error, thus allowing the DNA to be repaired. In our experiments, we tried to analyse the outcome of artificial meiotic DSBs by Cisplatin in the SPO11-2 mutant in *Arabidopsis thaliana*; we found that Cisplatin could produce some COs from artificial DSBs. One rod bivalent has observed at MI in addition to some small chromosome fragmentations during meiotic stages with a concentration of Cisplatin (5mg/ml). The number of bivalents increased with 10mg/ml Cisplatin to two or three bivalents in some myocytes. Also, the chromosome fragments increased with this concentration of cisplatin as shown in **(Figure 56)**. Treated meiocytes were immunolocalized at cisplatin 10mg/ml, At γ -H2AX signals were appeared and limited after 24 hours from cisplatin treatment. Some prove indicate that artificial DSBs by cisplatin can be repaired by homologous recombination pathway, because at 72 hours post-cisplatin treatment DMC1 and RAD51 proteins were observed. At the same time point, synaptonemal complex (SC) transverse filament protein(AtZYP1)was immunolocalized as well. Partial chromosome synapsis is restored. AtZYP1 were polymerized, continuous threads are absent but small sections were appeared **(Figure 57)**. What we got from results agree with this theory " for *Arabidopsis thaliana*, the creation of an adequate volume of cisplatin-derived DSBs that could facilitate standard pairing and COs without the generation of recombination that is prone to error leading to instability in the genome is impossible (Sanchez-Moran et al., 2007). Contrastingly, in various cases, including in *Lilium* spp., that there is an increased volume of chiasma in wild-type plants after they are irradiated (Lawrence, 1961).

In conclusion, meiotic recombination partially rescued in Atspo11-2 mutant by cisplatin. We were able to detect the presence of γ -H2AX, Rad51, Dmc1, and ZYP1 which are good prove for the formation of cisplatin-induced DSBs, meiotic recombination, and synapsis, indicating that some DSBs could be effected for SC formation. Thus, meiotic DSBs and recombination by cisplatin are required and enough for stabilizing homologous synapsis.

CHAPTER FIVE

**ANALYSING THE MEIOTIC
PROCESSING OF INDUCED
ARTIFICIAL DSBS IN *ARABIDOPSIS
THALIANA* USING ZFNS**

Introduction.

Plant breeding has been exponentially innovated since the first time that plant T-DNA transformation by using *Agrobacterium tumefaciens* (Herrera-Estrella *et al.*, 1983; Paszkowski *et al.*, 1984). From that point forward, a variety of transgenic plants from different species have been used for fundamental research purposes alongside apply improvements that have been used for agricultural and market innovations. In the last decade, another innovation has been developing that could help us to control better plant transformation: Genome Targeting (also known as Genome Editing). These advancements are allowing us to target specific changes in the DNA sequence of some species, including some crops. They are based in the construction of artificial nucleases with DNA binding motives which can recognized a specific target in the DNA sequence of the individual genome to produce a DNA Double-Strand Break (DSB) in that location (Urnov *et al.*, 2010). These designed DNA binding nucleases are very productive and precise. The techniques are based on the DSBs repair pathway choice; they could be repaired by Non-Homologous End Joining (NEHJ) that could produce some mistakes (error-prone) or by Homologues Recombination (HR) that if a specific template is introduced to the cell with some sequence homology to the DSB ends, an insertion or correction could be introduced in that site (Wyman and Kanaar, 2006). These days, there are three main sources of designed nucleases for Genome Targeting: Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effectors Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated protein 9 (CRISPR/Cas9). Genome Targeting has two notable goals: to understand the role of a specific gene by introducing, identifying and analyzing mutants in specific DNA sequences and to restore a gene sequence to a functional one by introducing a template sequence to a specific site (Han *et al.*, 2015).

Introduction to Genome Targeting Techniques

The discovery of these Genome Targeting techniques occurred while researching natural biological processes. The first eukaryotic DNA sequences discovered to contain a specific DNA binding domain were transcription factors which contain zinc-finger (ZF) binding domain repeats that when characterised showed that contain a high degree of sequence specificity (Bibikova *et al.*,2001). Each ZF domain can recognize three bp of the DNA sequence to increase specificity; ZFNs normally use an array of at least 3 ZF domains to recognize a minimum of 9 bp of the site that wants to be targeted. The use of different of these ZF domains in tandem can increase the length of the DNA sequence recognised and therefore the specificity of the binding. Some plant pathogenic bacteria (i.e., [*Xanthomonas*](#)) can secrete proteins into host cells when they infect them known as Transcription activator-like effectors ([TALEs](#)) which induce infection within the hosts by binding specific genes in the host genome and regulating their activity. The DNA binding domains of these TALEs contain a repeated highly conserved 33-34 aa sequence with two variable aa (12-13) known as the Repeat Variable Diresidue (RVD). This RVD is highly divergent and can recognize a specific nucleotide. Thus, TALENs used a tandem array of these RVDs to increase the length of the targeted sequence. This recognition pattern is simple and robust. The discovery of the CRISPR-Cas system was initiated when a cluster of odd and short repeats was reported within the bacterial genome that were identified as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). These repeats were short sequences showing sequence resemblance with viral genomes. After a thorough investigation, scientist discovered CRISPR-associated (Cas) proteins that are encoded by the nucleotide sequences adjacent to these repeat clusters which some seem to facilitate the incorporation of these repeats from viral sequences into the bacterial genome whereas others seem to play a role in the cleavage and inactivation of intruding viral genomes after the original infection. These Cas proteins that cleave viral genomes use two short RNAs:

CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). crRNAs are transcribed from the CRISPR arrays in the bacterial genome. tracrRNAs have been identified to contribute towards the crRNAs processing as well as the cleavage of the invading viral DNA (Tang *et al.*, 2017). Cas9 endonuclease has been modified to have a more simple system by fusing both crRNA and tracrRNA molecules in one known as single-guide RNA (sgRNA) (Jinek *et al.*, 2012). Since then, the CRISPR/Cas9 system has been developed as one of the primary biotechnological techniques used for genome editing (Carroll, 2017).

Creating DSBs by gene editing techniques

The creation of induced DSBs by using ZFNs is obtained by its nuclease domain. Normally, *FokI* is the chosen nuclease for ZFNs. *FokI* needs to dimerize to produce a DSB. Thus, the construction of two ZFNs with sequence specificity to both sides of the target are needed to dimerize and produce a DSB in that region (**Figure 58A**). The first evidence of FokI dimerization by use of its cleavage domain originated from the work on the FokI endonuclease crystal structure. Subsequently, kinetic studies supported the view of dimerization by the nuclease domain of *FokI* enzyme and regarded as essential for the production of DSBs (Bitinaite *et al.*, 1998). In order to generate DSBs, TALENs use a C-terminal fusion with the same FokI nuclease. Similarly to ZFNs, TALENs are designed in pairs targeting DNA specificity for both sides of the region where the DSB will be produced by the *FokI* dimer (**Figure 58B**). The introduction of these ZFNs and TALENs heterodimers for FokI nuclease activity results in the minimization of off-target effects. TALENs have been successfully used to generate knock down/ knock out mutations in numerous organisms which include bacteria, yeast, plants, nematodes, fish, *Xenopus* embryos, human cell lines, rodents, and rat embryonic stem cells. In *E. coli* longer targets of almost 26- 32 bp recombined 100-fold more proficiently compared to the 14 and 20 base pairs targets which are comparatively shorter. Similarly, when compared to mammalian

cells ~20 fold efficiency is obtained with a 44bp target and ~six fold efficiency was observed with a 32 bp target (Moore *et al.*, 2014; Joung and Sander, 2013). In the CRISPR/Cas9 system, the specificity for the endonuclease activity resides in a single array of 20 nucleotides inside the sgRNA (Wang *et al.*, 2011; Gong *et al.*, 2014) (**Figure 58C**). Cas9 nuclease activity has been widely used in the field of genome-editing of different species. Cas9 can site-specifically cleave double-stranded DNA, thereby generating the DSBs and ultimately activating the DSB repair machinery. Cas9D10A is one of the different Cas9 mutants generated to increase the specificity of targets and repair pathway choice, and have a nickase activity with the ability to cleave just one strand of the DNA. Two constructs for Cas9D10A targeting nearby DNA nicks in a specific locus would produce a DSB with single strand tails which could be preferentially processed by HR. This Cas9D10A system is normally used in combination of a template with homologous sequences around the breaks to Knock-in or insert sequence in a targeted site of the genome (Chiang *et al.*, 2016). CRISPR/Cas9 system has been extensively and successfully used for introducing mutations by genome targeting in various organisms, e.g. human, bacteria, zebrafish, *C. elegans* , different plant species, *Xenopus tropicalis*, yeast, *Drosophila*, monkeys, rats, and mice (Gilles *et al.*, 2014).

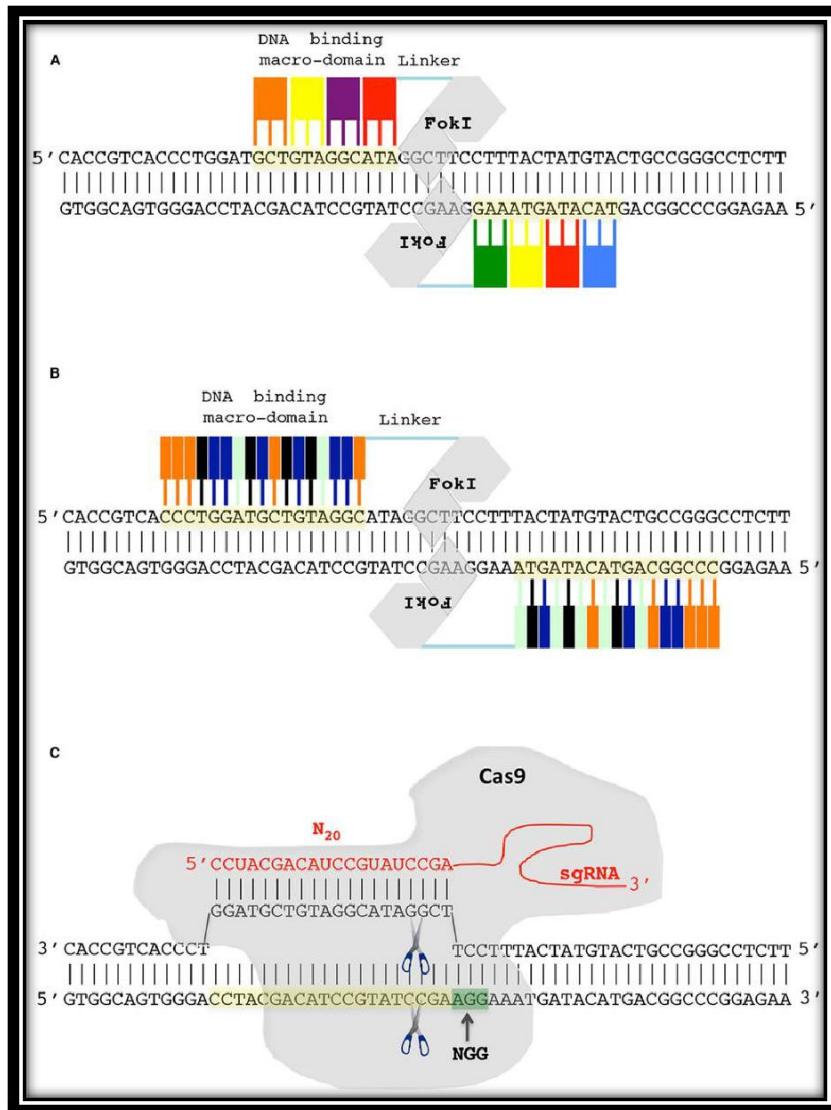


Figure 58: Genome editing technologies

(A) Zinc-Finger Nucleases (ZFNs), two ZFNs have to recognise both sides of the target sequence to FokI to dimerise and be able to produce a DSB. (B) Transcription Activator-Like Effector Nucleases (TALENs), two TALENs recognise both sides of the DNA target to allow dimerization by FokI and thus produce a DSB. (C) CRISPR/Cas9 system, 20 nucleotides inside the single-guide RNA (sgRNA) direct the nuclease Cas9 activity into the DNA target (yellow box). This figure is adapted from Ilardi and colleagues (2015).

Targeted DSBs processing

After these Genome Targeting techniques have induced a sequence-specific DSB, this will be processed by DSB repair pathways: mostly NHEJ and HR. If repaired by NHEJ, this could lead to the mutagenesis of the targeted region as NHEJ is error-prone (Gyngell *et al.*, 2017). If the DSB is repaired by HR it will be error-free, thus if a template sequence is included with homology to the DSB region then gene correction or insertion could be done

(Voytas & Baltes, 2013). Targeted mutagenesis and gene replacement have been achieved in *Drosophila* by using ZFNs (Beumer *et al.*, 2006). ZFNs have been successfully used in the model plant *Arabidopsis thaliana* as well (Zhang *et al.*, 2010). The efficacy of ZFNs has also been demonstrated in tobacco cells which have allowed to regenerate whole plants (Townsend *et al.*, 2009). The nematode *C. Elegans* has use ZFNs very successfully (Wood *Et al.*, 2011). The advent of TALENs shown to have generally better specificity than ZFNs, due to having less off targets (Mussolino *et al.*, 2011). The CRISPR-Cas9 system has shown to have higher specificity but due to the simple generation of constructs (only one sgRNA for one specific target) have put this technique ahead of any other Genome Targeting technique (Jinek *et al.*, 2012).

Results:

Using XVE fusion inducible promoter for ZFNs expression in *Arabidopsis thaliana*

The XVE process reacts to inducer concentrations ranging from 8 nM to 5 mM estradiol. Furthermore, the strength of the target promoter can attain a similar level to that of a 35S promoter at a comparatively low concentration at 0.2 mM. Additionally, estradiol swiftly activates the transcription of target genes, which can attain an increase eight times as high as a 35S promoter with 24 hours of induction. Such attributes enable transgenes to be outlined at the wanted period and level by modifying the incubation time and estradiol concentrations correctly (Zuo *et al.*, 2001). In this study, an inducible promoter has used to activate ZFNs to produce one specific DSB during meiosis in the absence of SPO11-2 in *A thaliana*. We utilize synthetic inducible, site-specific DSBs by ZFNs. The ZFNs expression is controlled by the XVE system (**Figure 59**). ZFNs controlled, and DSBs can be instigated at high effectiveness. This framework is helpful for a wide range of explants recovery either by organogenesis or substantial embryogenesis (Zuo at el, 2000).

DSB has induced by ZFN in two specific regions of the genome which are Meiotic Artificial DSB Line 1 (*MADI*) where DSB occurs in a distal position in chromosome 1 (*ADHI* gene) and Meiotic Artificial DSB Line 2 (*MAD 2*) in a more interstitial position in chromosome 5 (*TT4* gene) as shown in **(Figure 61)**. The *MADI/2* could be used by the meiotic recombination machinery and thus produce one crossover (CO) in these two specific locations. Also, to allow crossover formation which could break the genetic linkage drag present in plants and studying the different outcomes of the DSBs artificially induced by ZFNs during meiosis. The flower buds have been treated at different concentrations of *β-estradiol* (50 μ M, 100 μ M, 200 μ M, 300 μ M, and 500 μ M) to activate

The promoter of ZFNs (**Figure 59**). To monitor the progression of induced DSBs, we examined the expression by RT-PCR after *β-estradiol* treatment. The XVE fusion gene has expressed in recombinant plants. The XVE, ZFNs expression level was as expected with using different concentration *β-estradiol* treatment at different timings. The XVE, ZFNs expression gradually increased with time. From our result, the RT-PCR to measure the level of expression for ZFN genes from the cDNA at different concentration of *β-estradiol* from zero time up to 72 hours of application. At 200 μ M of *B-estradiol*. The ZFNs started to express from 12 hours, and it keeps increasing up to the third day from the application, but with 500 μ M the expression started earlier. ZFN genes started expression at 6 hours from treating the flower buds, and then highly increased up to three days **(Figure 60)**. Our outcome demonstrates that the inductions are continuously increased upon *β-estradiol* treatment.

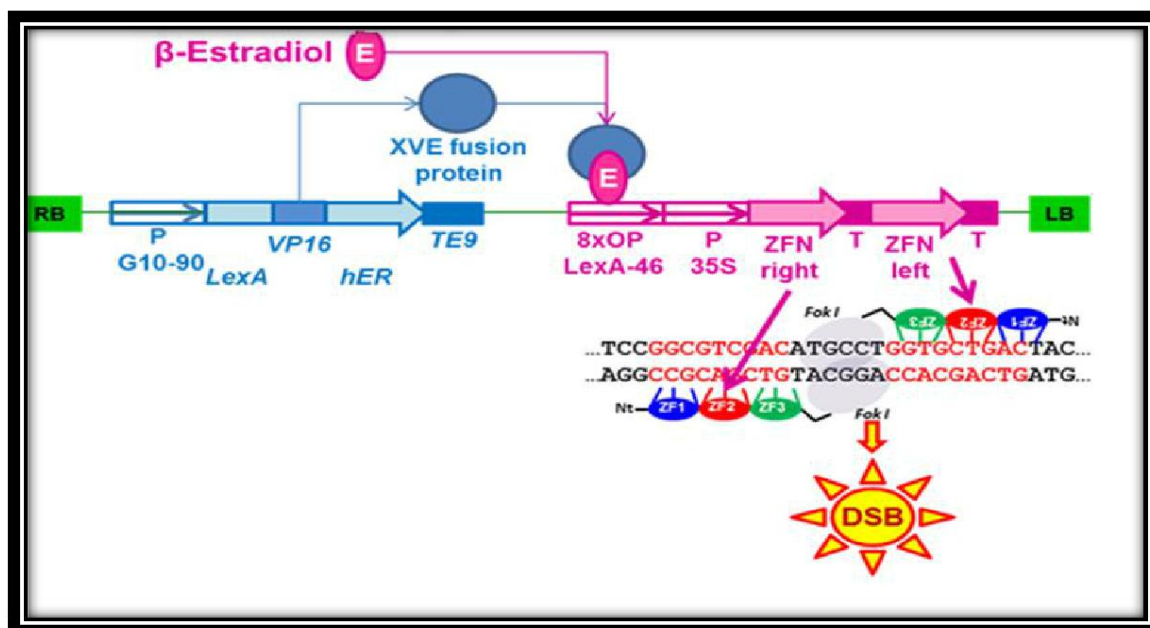


Figure 59: A schematic diagram of the inducible promoter in the ZFN lines studied in this work

Promoter G10–90 is a constitutive promoter to direct the expression of XVE fusion protein. XVE contains different parts; the DNA binding motif to bind the LexA operon, the VP16 activation transcription domain, the regulatory region of an estrogen receptor in human estrogen receptor and the terminator TE9. XVE fusion protein can bind estradiol and relocate into the nucleus to activate the expression of ZFNs through the binding to the LexA operator. (Based on a model by Zuo *et al.*, 2000)

Dr. Daniel Voytas' Lab kindly provided the seeds from *Arabidopsis thaliana* transformed with the ZFNs constructs targeting the genes for *Alcohol Dehydrogenase I (ADH1)* and *Testa 4 (TT4)* (Zhang *et al.*, 2010). *adh1* mutation allows the seedlings to grow in the presence of alcohol and *tt4* mutation shows a distinct yellow color of the seeds. These clear phenotypes were the reason why Voytas Lab chose these genes to carry out research into the use of inducible ZFNs to induce mutants (Zhang *et al.*, 2010).

Past work has already demonstrated that ZFNs could produce mutations in *Arabidopsis* using the NHEJ repair pathway (Lloyd *et al.*, 2005). In *Arabidopsis*, it is possible to use ZFNs to create a mutation in a specific sequence by NHEJ or using HR for integrating a DNA in a specific sequence on the genome (Britt and May 2003). There are some challenges for the efficiency of ZFN-induced mutation in *Arabidopsis*: (i) targeting a specific sequence without off-targets due to sequence homology and (ii) utilizing protocols that could permit recovery of genetically HR and NHEJ induced mutants at frequencies

surpassing 10%. The ZFNs analysed in this work are targeting coding sequences in the *Alcohol dehydrogenase I* gene (AT1G77120) located in a distal position in chromosome 1 and the *Testa 4* gene (AT5G13930) located in a more interstitial position in chromosome 5 (**Figure 61**). The ZFN constructs were transformed into *Arabidopsis thaliana* by T-DNA transformation and their insertion sites identified by Dr. Voytas lab. Meiotic Artificial DSB Line 1 (*MAD1*) The ZFNs T-DNA to target *ADH1* gene is inserted in chromosome 2 to target and Meiotic Artificial DSB Line 2 (*MAD2*) the ZFNs T-DNA is inserted in chromosome 1 to target the *TT4* gene.

Plants homozygous for each of the T-DNA insertions (targeting *ADH1* and *TT4*) were crossed with an *Atspo11-2* heterozygous mutant. Two generations after the cross we managed to obtain plants homozygous for each ZFNs T-DNA insertions and heterozygous for the *Atspo11-2* T-DNA mutation. The seeds from these plants were grown to analyse plants with ZFNs T-DNA insertions and *Atspo11-2* mutation in homozygosis. From now on we will refer to the lines homozygous for the ZFNs insertion targeting *ADH1* gene and homozygous for *Atspo11-2* mutant as *Meiotic Artificial DSB line 1 (MAD1)* and the lines homozygous for the ZFNs insertion targeting *TT4* gene and homozygous for *Atspo11-2* mutant as *Meiotic Artificial DSB line 2 (MAD2)*. This was done to have plants with the *spo11-2* background (without meiotic DSBs) which we could use to induce the two different ZFNs targets (*MAD1* and *MAD2*).

The first important point was to establish the timing required for the maximum expression of these ZFNs induced by β -estradiol. Previously, it was published that this inducible system has a maximum expression around two days after exposure to estradiol (Schlucking *et al.*, 2013). So experiments were carried out to study the expression timing for this ZFNs in our *MAD1* and *MAD2* lines. Another important point to bear in mind is that after the expression of these ZFNs and their attachment and cleavage to their targets (DSBs production at *ADH1* and *TT4*) we will need to allow some time for these DSBs to be

processed through the meiotic recombination machinery. Previously, the timing of meiosis in *A.thaliana*. has been carried out by BrdU labeling and a clear picture of the timing for each stage of meiosis has been established (Armstrong *et al.*, 2; Sanchez-Moran *et al.*, 2007). From DSB formation catalyzed by AtSpo11 complex to the formation of chiasmata (COs), a minimum of 24-30 hours is necessary for *A.thaliana* (Sanchez-Moran *et al.*, 2007). Thus, we started treating *MAD1* and *MAD2* lines at a different concentration of β -estradiol and fixing material to cytogenetically analysed at different times.

Three different methods have used to treat the flower buds with *B-estradiol* solution. The first way of treatment was by spraying, but it was not highly effective. This may be due to the difficulty of the *B-estradiol* solution to flower buds. The second method of treating flowers buds was cutting around 1cm to 1.5cm of plant part containing flower buds and dipping in *B-estradiol* solution at different conventions , but the solution of *B-estradiol* was toxic to the plants. Finally, we found brushing the flower buds with *B-estradiol* solution was the most effective method that could be used to treat most of the flower buds and disrupt the solution as shown in **Figure 60**



Figure 60: Treating the flower buds with different concentration of *B-estradiol*

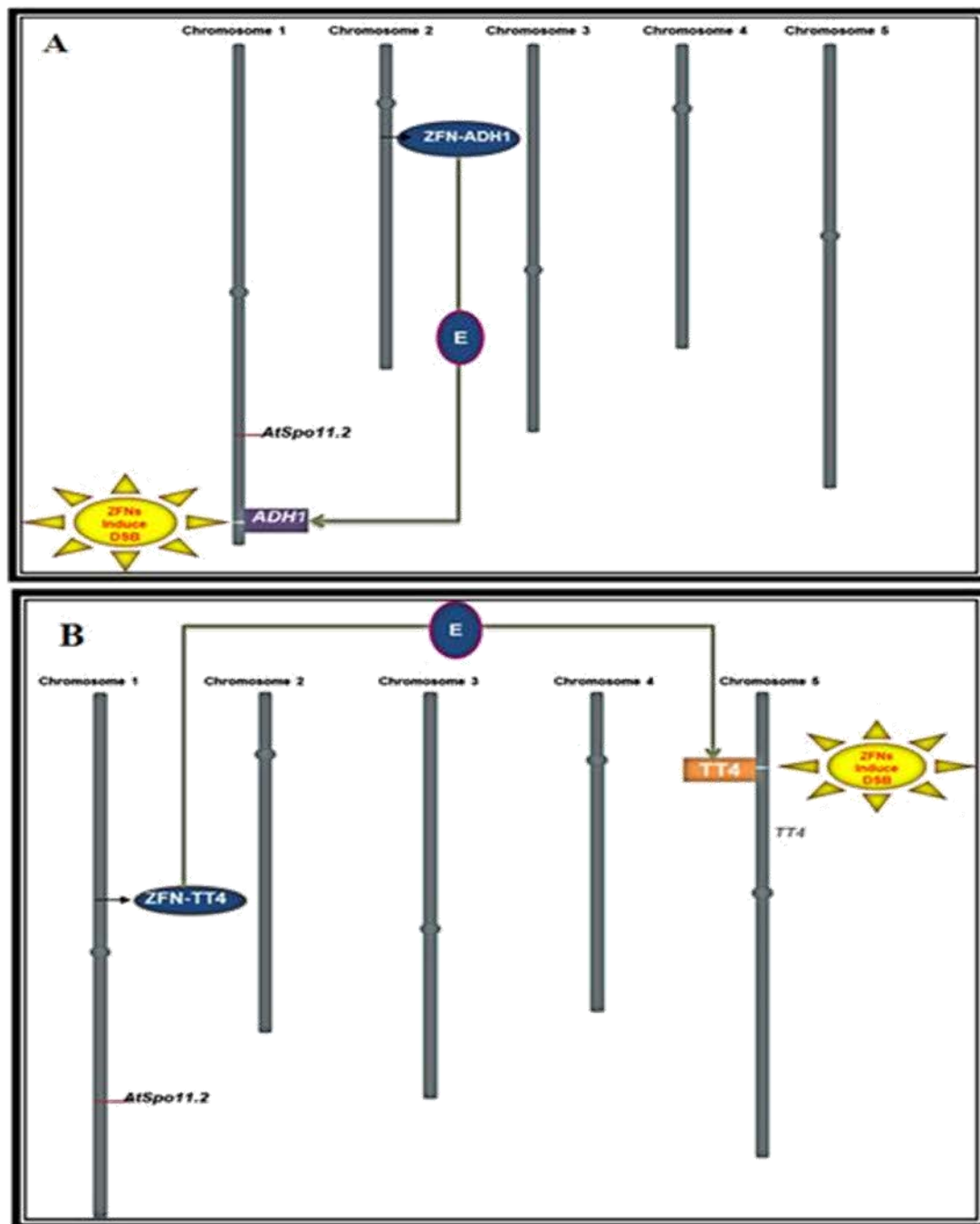


Figure 61: The position of locus *ADH1* and *TT4*

This figure explains the rejoins of Meiotic Artificial DSB Line 1 (*MAD1*), Meiotic Artificial DSB Line 2 (*MAD2*) and At on the chromosomes in *A.thaliana*. (A) It shows the rejoin of *MAD1* which is in *ADH1* (AT1G77120) in a distal position on chromosome 1 and ZFN-*ADH1* T-DNA insertion is located in chromosome 2. (B) It shows the rejoin of *MAD2* which is in *TT4* (AT5G13930) in a more interstitial position on the chromosome 5 and the ZFN T-DNA insertion is also shown on the chromosome 2.

Material	Time	Concentration
<i>MAD1/2 X ZFN</i>	48 hours	50μM
<i>MAD1/2 X ZFN</i>	72hours	50μM
<i>MAD1/2 X ZFN</i>	48 hours	100uM
<i>MAD1/2 X ZFN</i>	72 hours	100uM
<i>MAD1/2 X ZFN</i>	48 hours	200uM
<i>MAD1/2 X ZFN</i>	72 hours	200uM
<i>MAD1/2 X ZFN</i>	48 hours	300uM
<i>MAD1/2 X ZFN</i>	72 hours	300uM
<i>MAD1/2 X ZFN</i>	48 hours	500uM
<i>MAD1/2 X ZFN</i>	72 hours	500uM

Figure 62: The different concentrations of β -estradiol and time from exposure that were used to fix material to analyse meiotic stages in *MAD1 X ZFN* and *MAD2X ZFN*.

ZFNs expression by β -estradiol induction in *MAD1* and *MAD2*

To characterise the expression of the ZFNs in *MAD1* and *MAD2* a Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis was carried out. RNA was extracted from Flower buds the wild-type (Col), *MAD1/2 X ZFN* plants at different concentrations of β -estradiol (50μM, 100μM, 200μM . 300μM, and 500μM) and for different timings. Two sequence-specific primers (forwarded and reverse primers) (**Table 4**) were designed to amplify approximately 542bp of the ZFNs transcript which is not present in the WT plant genome. RT-PCR using specific primers for *AtACTIN2* housekeeping gene was carried out as a control to check equal amounts of cDNA in all samples. The *AtACTIN2* RT-PCR control showed amplified a 255bp band and showed equal intensities of cDNA from RNA extracted from flower buds of WT, *MAD1/2 X ZFN* (**Figures 63A**). To analyse if there was any genomic DNA contamination in the RNA extractions the same *AtACTIN2* premiers were used as the product of genomic DNA amplify with these primers is nearly double size than the size from the transcript (without introns) of about 550bp (**Figures 63B**). In all samples of RT-PCR from mRNAs not bands at 550bp were found showing not traces of

genomic contamination on the samples. To check the expression of ZFNs, specific primers against a region of the *FokI* gene were designed and used. The RT-PCR analysis of *MAD1/2 X ZFN* lines showed the increase level of ZFNs expression through time (0, 6, 12, 24, 48 and 72 hours) after exposure with different concentrations of β -estradiol (100 μ M, 200 μ M and 500 μ M). The ZFNs expression started after 24 hours at 100 μ M of β -estradiol showing an expected 542bp band (**Figures 63C**). At 200 μ M β -estradiol the expression started to be observed earlier after 12 hours (**Figures 63D**). The expression of ZFNs at 500 μ M β -estradiol started even earlier after 6 hours (**Figures 63E**).

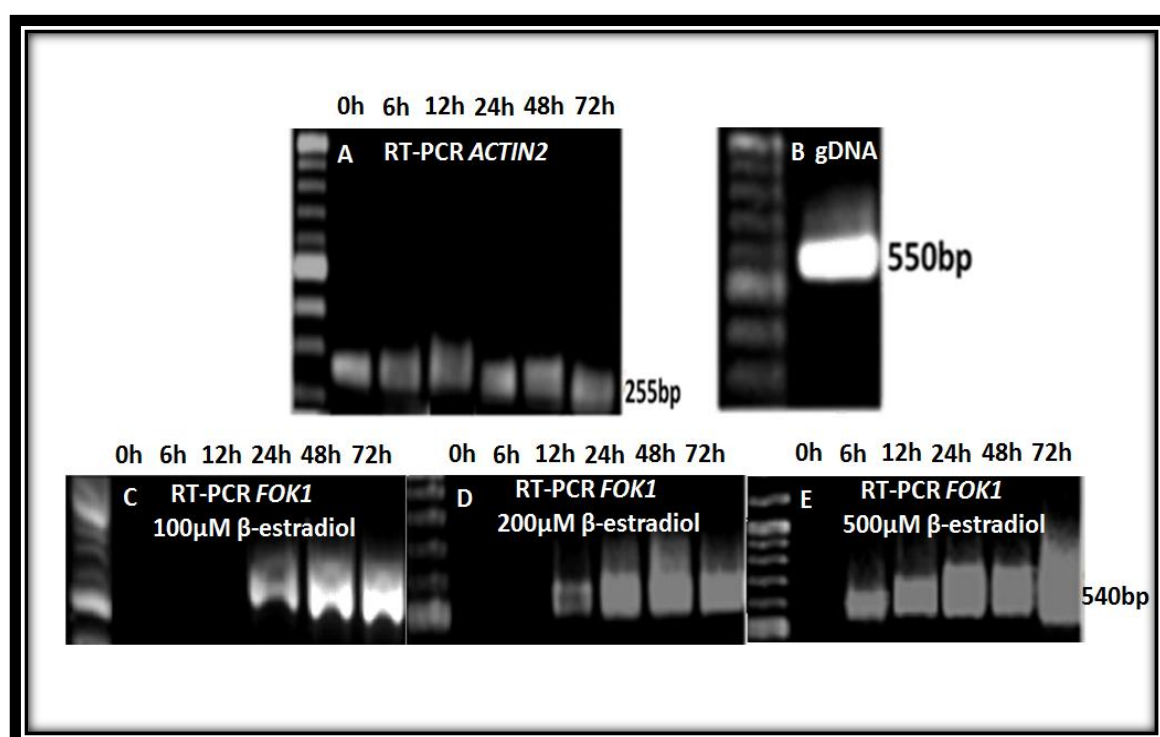


Figure 63: ZFNs expression at different concentrations of β -estradiol and at different time points after exposure.

(A) RT-PCR to check the *AtActine2* gene expression to check the equal amount of cDNA in all samples. (B) PCR gel electrophoresis of RT-PCR primers for *AtActin2* gene using full genomic DNA to confirm that there is not genomic DNA contamination on the RNA samples (Genomic band is bigger than the cDNA for the same primers due to the introns present in the genomic sequence). (C) RT-PCR gel electrophoresis to analyse ZFNs expression on *MAD1/2* lines after 100 μ M β -estradiol treatment. It is important to note that at 0 times (not exposure to β -estradiol) not expression of ZFNs is observed. (D) RT-PCR gel electrophoresis to analyse expression levels for *MAD1/2* ZFNs after 200 μ M β -estradiol treatment. (E) RT-PCR gels electrophoresis is showing the expression level for *MAD1/2* after 500 μ M β -estradiol treatment.

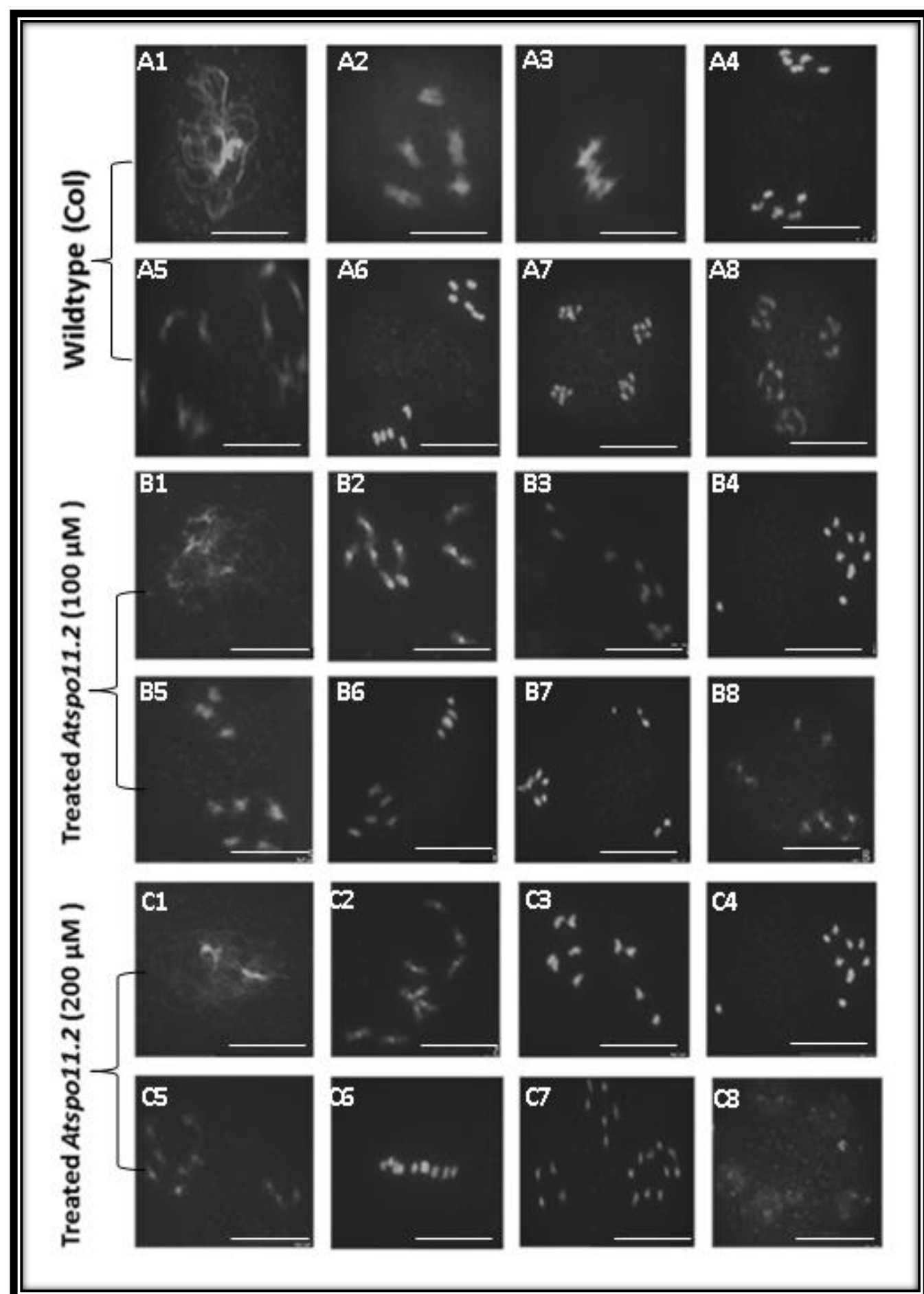
Cytogenetic analysis of meiosis in *MAD1* and *MAD2*

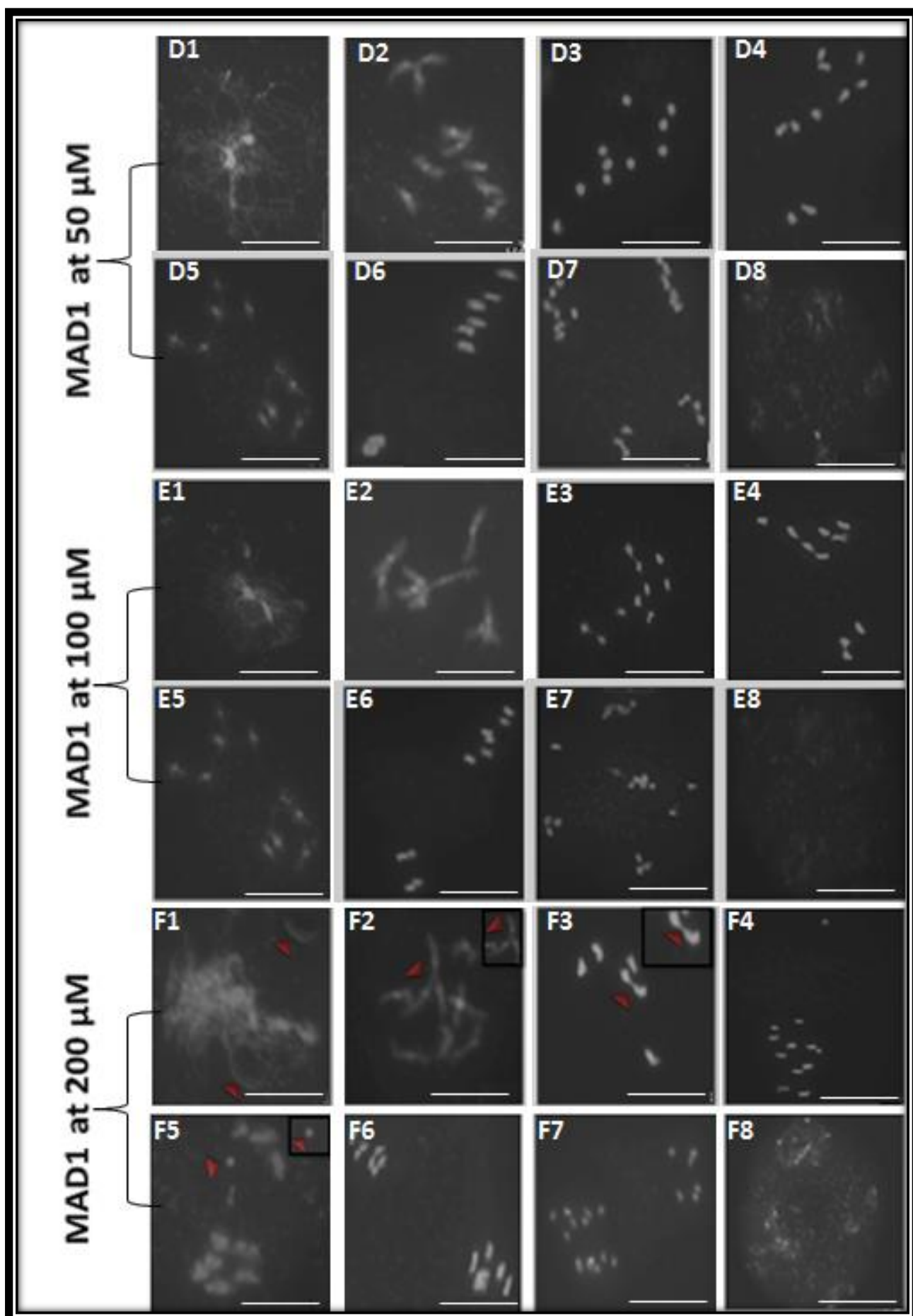
The cytological analysis in pollen mother cells to study the meiotic stages for wild type, *Atspo11.2* null mutant, and *MAD1/2 X ZFN* lines were carried out. Meiotic stages were analysed using DAPI-stained spreading chromosome preparations. WT(**Figure 64A**) and treated and untreated *Atspo11.2* plants were analysed as a control. *Atspo11.2* mutant without ZFNs insertions treated with 100 μM (**Figure 64B1- Figure 64B8**) and 200 μM β -estradiol (**Figure 64C1- Figure 64C8**) showed abnormalities in meiotic chromosome segregation as expected for an *Atspo11.2* mutant. During the early stages of meiosis I, no pairing between homologous chromosomes was observed. Ten univalents were observed at Metaphase I. At Anaphase I and second division stages missegregation of these univalents were observed. *MAD1/2 X ZFN* lines at 50 μM (**Figure 64 D1- Figure 64D8**) and 100 μM β -estradiol (**Figure 64 E1- Figure 64E8**) after 72 hours from application showed identical phenotype to that showed in *Atspo11.2* mutant where in the absence of DSBs not pairing, synapsis or chiasma formation among homologous chromosomes is observed.

Nevertheless, in *MAD1/2 X ZFN* lines treated with 200 μM β -estradiol was possible to visualise some partial pairing during zygotene (**Figure 64F1**), at diakinesis one chiasmata could be observed in some cells (**Figure 64F2**) and only one rod bivalent could be observed in one metaphase I cell from a total of 1,126 cells analysed (**Figure 64F3**).

Furthermore, some DNA fragmentation could be observed in some cells(1/628 cells, N=1,126 prophase II cells) which could come from DSBs induced in both homologous chromosomes and in both sister chromatids (**Figure 64F5**). At 300 μM β -estradiol induction one bivalent could be observed in one metaphase I cell from a total of 1,136 metaphases I cells analysed (**Figure 64G3**). After 500 μM β -estradiol treatment, there was some partial pairing during zygotene (**Figure 64H1**), one chiasmata were observed at diakinesis (**Figure 64H2**). *MAD1 X ZFN* line treated at 500 μM β -estradiol presented 8 cells with 1 bivalent of a total of 1,214 metaphases I (1/209). Also, some DNA

fragmentation was observed in this line at prophase II cells, with one DNA fragment every 300 prophase II cell and 2 DNA fragments every 600 cells (N= 1,178 prophase II cells) **(Figure 65H3)**. When we try to increase the concentration of *β -estradiol* to 1000 μ M, we found that the tissues exposed in the plant start to die to the point that not viable material could be used to analyse cytologically **(Figure 67)**.





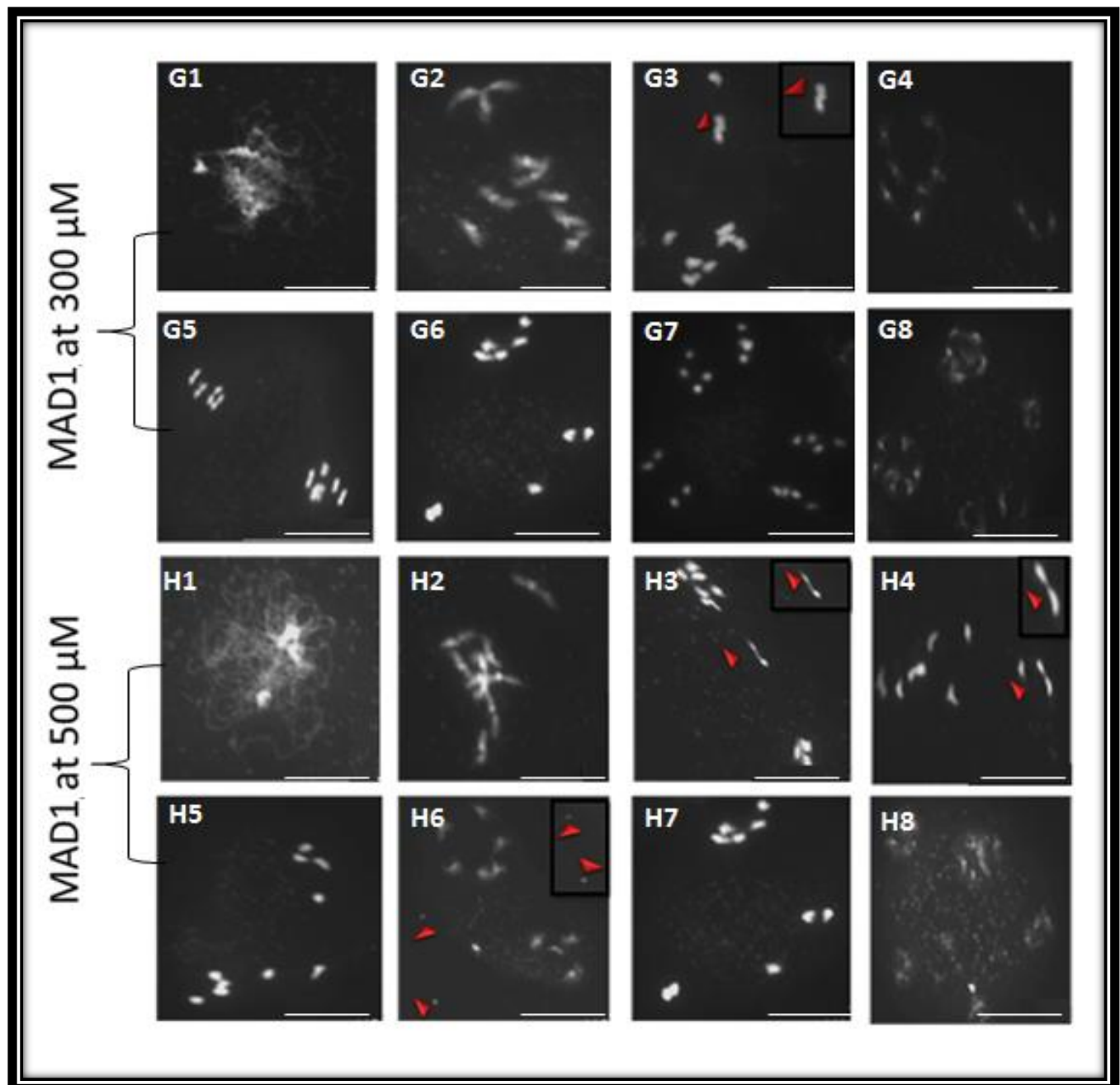


Figure 64: Meiotic analysis in WT, treated *Atspo11.2* mutant and *MAD1* treated plants.

This figure shows the stages of meiosis in the different plant backgrounds that were analysed.

(A1–A8) meiosis in wt. (A1) Pachytene (A2) Diakinesis (A3)Metaphase I (A3)Anaphase I (A4)Telophase (A5)Prophase II (A6) Metaphase II (A7)Anaphase II (A8)Telophase II.

(B1 – B8) meiosis in *Atspo11.2* mutant treated with 100 μM β -estradiol.(B1) Pachytene (B2) Diakinesis (B3)Metaphase I (B4)Anaphase I (B5)Prophase II (B6) Metaphase II (B7)Anaphase II (B8)Telophase II.

(C1–C8) Meiotic stages in induced *Atspo11.2* at 200 μM . (C1) Pachytene (C2) Diakinesis (C3)Metaphase I (C4)Anaphase I (C5)Prophase II (C6) Metaphase II (C7)Anaphase II (C8)Telophase II.

(D1- D8) *MAD1 X ZFN* at 50 μM . (D1) Pachytene (D2) Diakinesis (D3)Metaphase I (D4)Anaphase I (D5)Prophase II . (D6) Metaphase II (D7)Anaphase II (D8)Telophase II.

(E1-E8) *MAD1 X ZFN* induced at 100 μ M. (E1) Pachytene (E2) Diakinesis (E3)Metaphase I (E4)Anaphase I (E5)Prophase II (E6) Metaphase II (E7)Anaphase II (E8)Telophase II.
(F1- F8) *MAD1 X ZFN* induced at 200 μ M. (F1) Pachytene (F2) Diakinesis (F3)Metaphase I with one rod bivalent (F4)Anaphase I (F5)Prophase II with one fragmentation in some cells (F6) Metaphase II (F7)Anaphase II (F8)Telophase II.
(G1-G8) *MAD1 X ZFN* induced with 300 μ M. (G1) Pachytene (G2) Diakinesis (G3)Metaphase I with one rod bivalent (G4)Anaphase I (G5)Prophase II (G6) Metaphase II (G7)Anaphase II (G8)Telophase II.
(H1-H8) *MAD1 X ZFN* at 500 μ M. (H1) Pachytene (H2) Diakinesis (H3)Metaphase I with one-rod bivalent (H4)Anaphase I (H5)Prophase II with two fragmentations in some cells (H6) Metaphase II (H7)Anaphase II (H8)Telophase II.

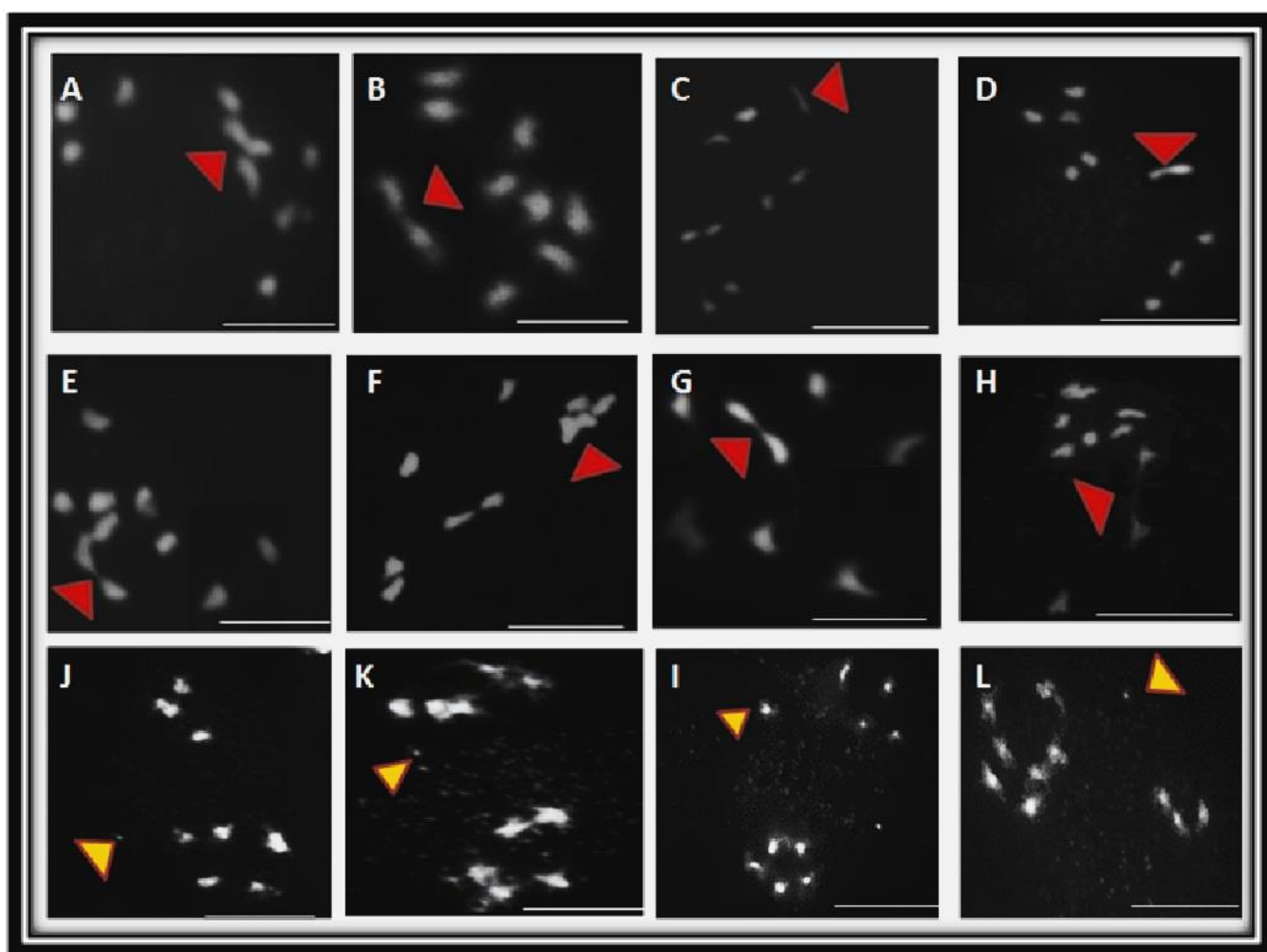


Figure 65: Bivalents formation and chromosome fragmentations in *MAD1 X ZFN* plants at 500 μ M.

(A – H) Red arrows indicate to one bivalent is formed in some M I cells, also, (J – L) Yellow arrows indicate to chromosome fragmentations presented in some prophase II.

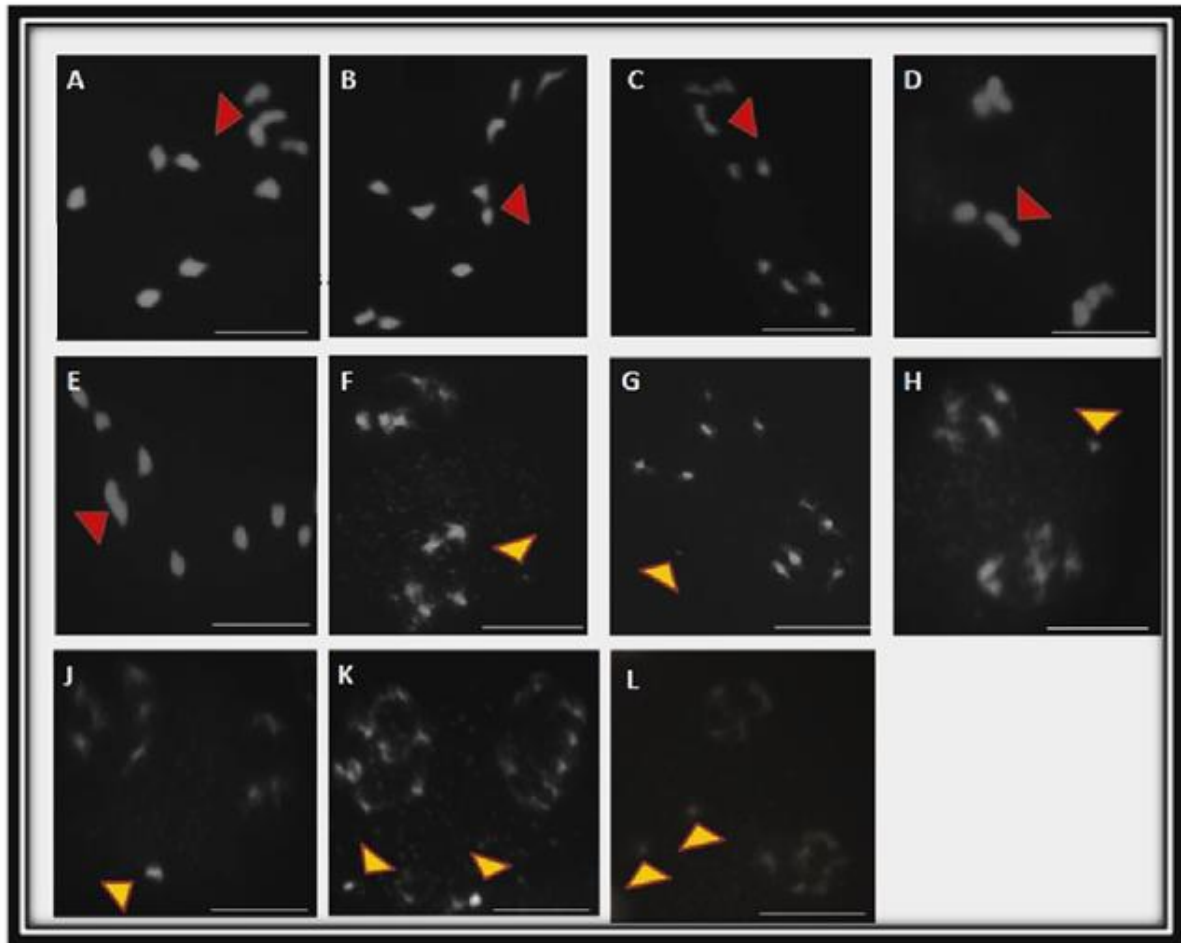


Figure 66: Bivalents formation and chromosome fragmentations in *MAD2* X ZFN plants at 500 μ M.

(A-E) Red arrows indicate to one bivalent is formed in some M I cells (F – G) Yellow arrows indicate to chromosome fragmentations presented in some prophase II.

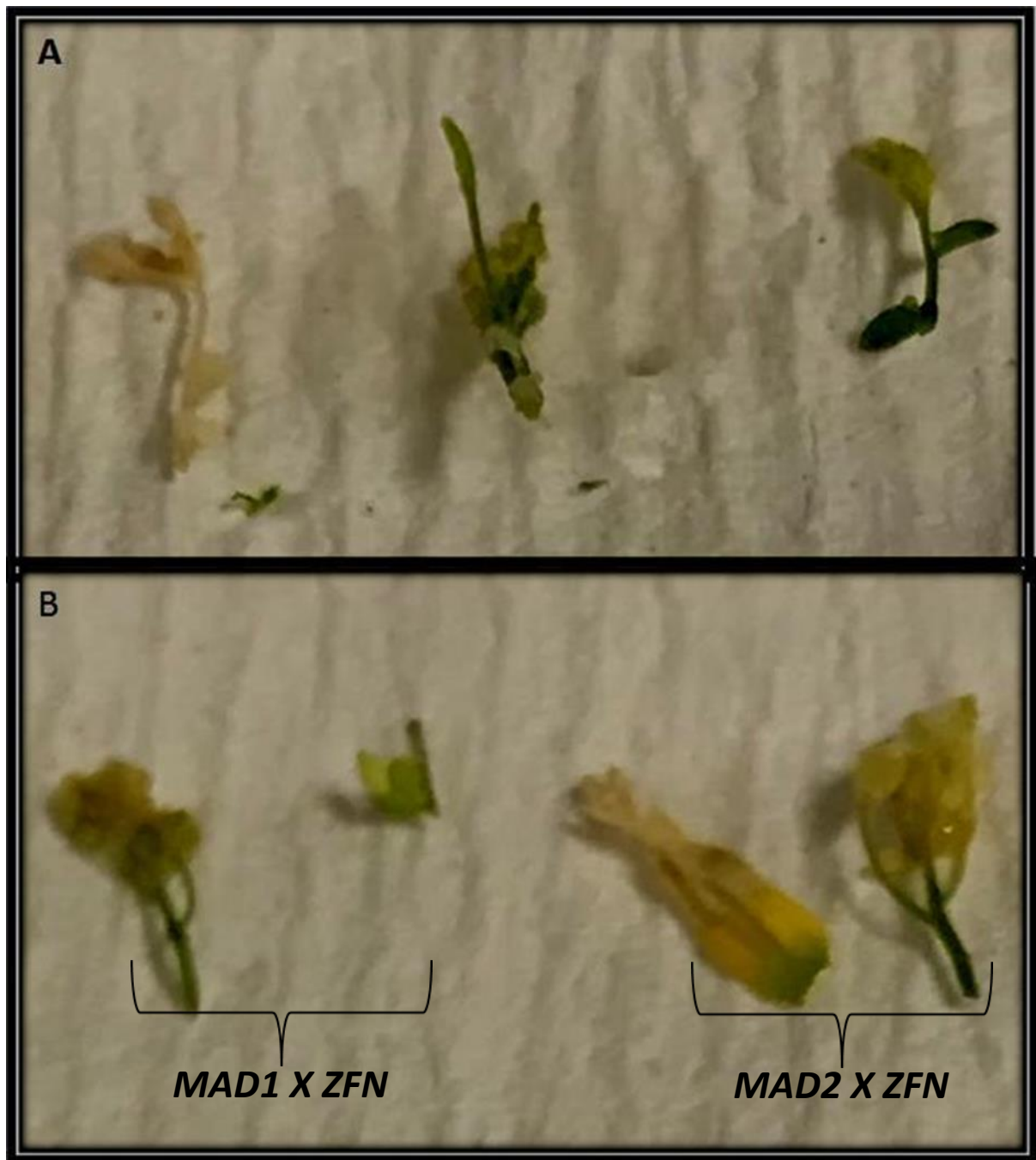


Figure 67: *MAD1/2 X ZFN* lines treated with 1000 μM β -estradiol.
(A) WT. (B) *MAD1 X ZFN* & *MAD2 X ZFN*

Immunolocalisation of meiotic recombination components

The numbers of meiotic DSBs in *Arabidopsis thaliana* have been estimated based on the number of DSBs marker proteins such as γ -H2AX. The γ -H2AX foci appear at early stages of meiotic prophase I in WT just after DSBs have been formed (Sanchez-Moran *et al.*, 2007). ASY1 gene is essential for homologous chromosome synapsis in *Arabidopsis thaliana* and is part of the meiotic chromosome axis (Sanchez-Moran *et al.*, 2007). Doubleimmunolocalization against AtASY1 and γ -H2AX has been conducted for wild-type, *MAD1/2 X ZFN* lines untreated and treated with estradiol 500 μ M (**Figure 68**). In the WT, a high number of foci for γ -H2AX were homogenously distributed on the chromosomes. In *MAD1/2 X ZFN* untreated lines no signal of γ -H2AX was observed as expected for an *Atspo11.2* mutant. Interestingly, γ -H2AX residual signals were observed in some cells in *MAD1/2 X ZFN* treated with 500 μ M *B-estradiol* (N=1,174 zygotene cells:30 cells with one single signal of γ -H2AX and 4 cells with 2 signals of γ -H2AX). Cells with 2 signals of γ -H2AX could indicate that the ZFNs could be targeting a DSB in both sister chromatids or both homologous chromosomes (**Figure 68**). Immunolocalization of meiotic recombinases, RAD51 and DMC1 was examined to follow the strand invasion dynamics at prophase I. DMC1 and RAD51 foci appeared homogeneously distributed in the WT, no signal of these proteins was observed in *MAD1/2 X ZFN* untreated material and in *MAD1/2 X ZFN* treated buds we could observed cells with none, one and two signals (N= 12 cells with 1 signal of RAD51, N = 12 cells with 1 signal of DMC1; N = 6 cells with 2 signals of RAD51; N= 4 cells with 2 signals of DMC1) for both proteins which again could indicate that the ZFNs could be targeting a DSB in both sister chromatids or both homologous chromosomes (**Figure 68**). ZYP1 is a synaptonemal complex protein which is localised on the central element., In the WT, Zyp1 polymerise between each pair of homologues and at pachytene is fully polymerised or synapsed (**Figure 68**). In the absence of SPO11.2

(*MAD1/2 X ZFN* untreated), there was not a signal of ZYP1. Nevertheless, in *MAD1/2 X ZFN* treated plants was possible to see some cells with one signal with some partial polymerisation (16 cells with 1 signal of ZYP1 of 100 cells)(**Figure 68**). MLH1 foci mark the class I COs at pachytene in the WT (Sanchez-Moran *et al.*, 2007). In *MAD1/2* untreated cells no signal of MLH1 could be observed as expected in CO absence. In *MAD1/2 X ZFN* treated material some meiocytes showed one single focus of MLH1 (**Figure 68**).

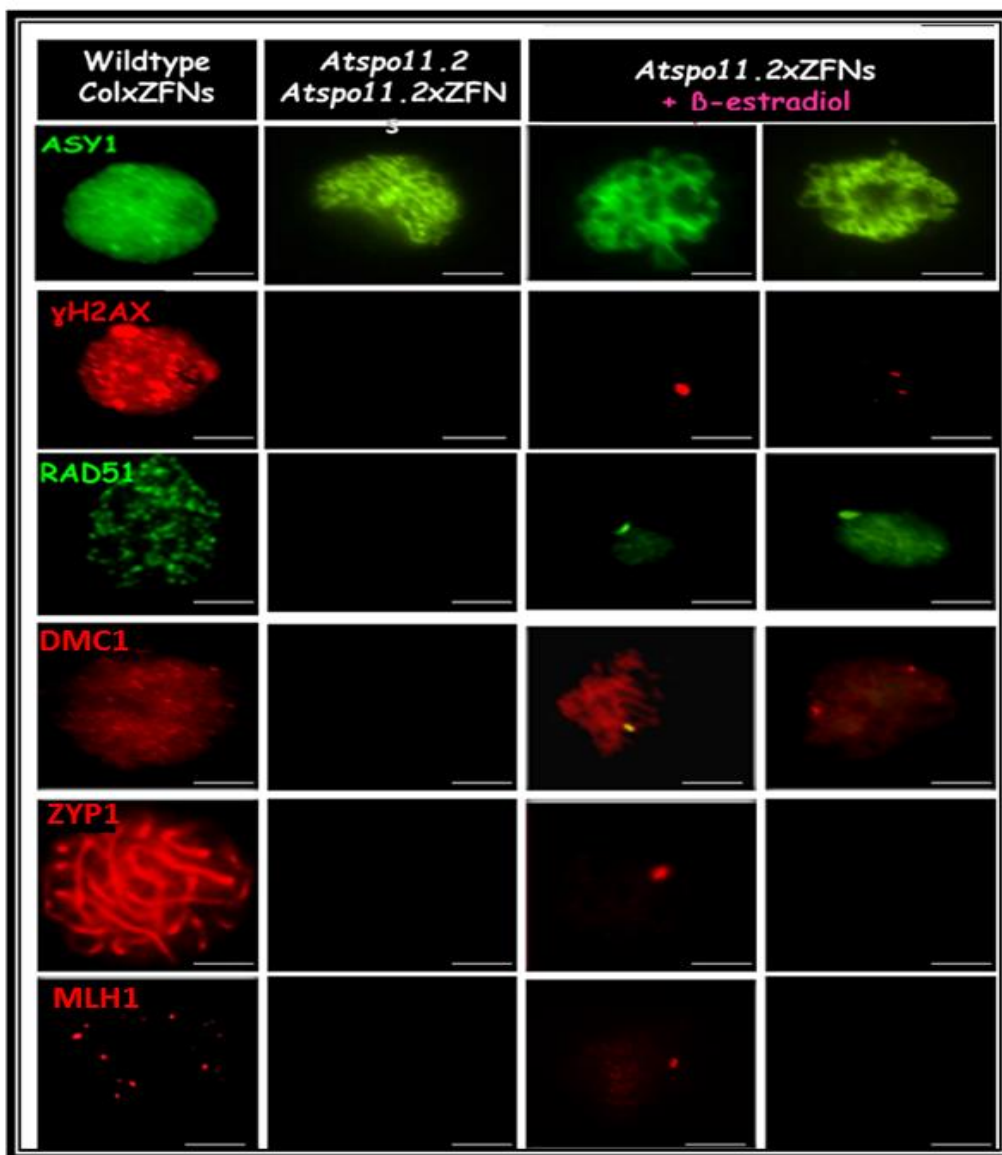


Figure 68: Immunocytoslocation of meiotic recombination machinery in *MAD1 X ZFN* at 500 μ M of β -estradiol.

AtASY1 (green), γ -H2AX (red), AtRAD51 (green), AtDMC1 (red), AtZYP1 (red)
AtMLH1 (red) Scale bar is 5 μ m.

Analysing meiotic outcomes of artificial DSBs induced by ZFNs

The numbers of meiotic DSBs have been estimated based on the number of γ -H2AX at early stages of meiotic prophase I. To check the right concentration of β -estradiol to induce DSB by ZFN during meiosis in *A.thaliana*, we treated the flower buds in *MAD1/2* at different concentrations (50 μ M, 100 μ M, 200 μ M, 300 μ M, 500 μ M). The number of experiments for each concentration was 50 experiments, and the duration of treatment was 48 hours. The total number of γ -H2AX signals was zero at 50 μ M β -estradiol which means this concentration may not be enough to produce meiotic DSB. We started to account γ -H2AX signals with increasing the concentration of β -estradiol. We managed to account some meiocytes with 1 signal of γ -H2AX at 100 μ M (4 γ -H2AX signals out of 536 zygotenes). With increasing the concentration to 200 μ M, the number of DSBs increased to 6 DSBs (6 γ -H2AX signals out of 547 zygotenes). Also with increasing the concentration to 300 μ M, the chance of increasing more DSB was possible (9 γ -H2AX signals out of 611 zygotenes). At 500 μ M which was the higher concentration that we could use to induce meiotic DSB, not all the meiocytes managed to get one DSB, but we accounted higher number of DSBs, comparing with previous concentrations (30 γ -H2AX signals out of 1174 zygotenes). Also, at this concentration, we managed to see 2 DSBs which could indicate that the ZFNs could be targeting both sister chromatids or both homologous chromosomes (4 cells with 2 DSBs out of 1174 zygotenes) (**Figure 69**).

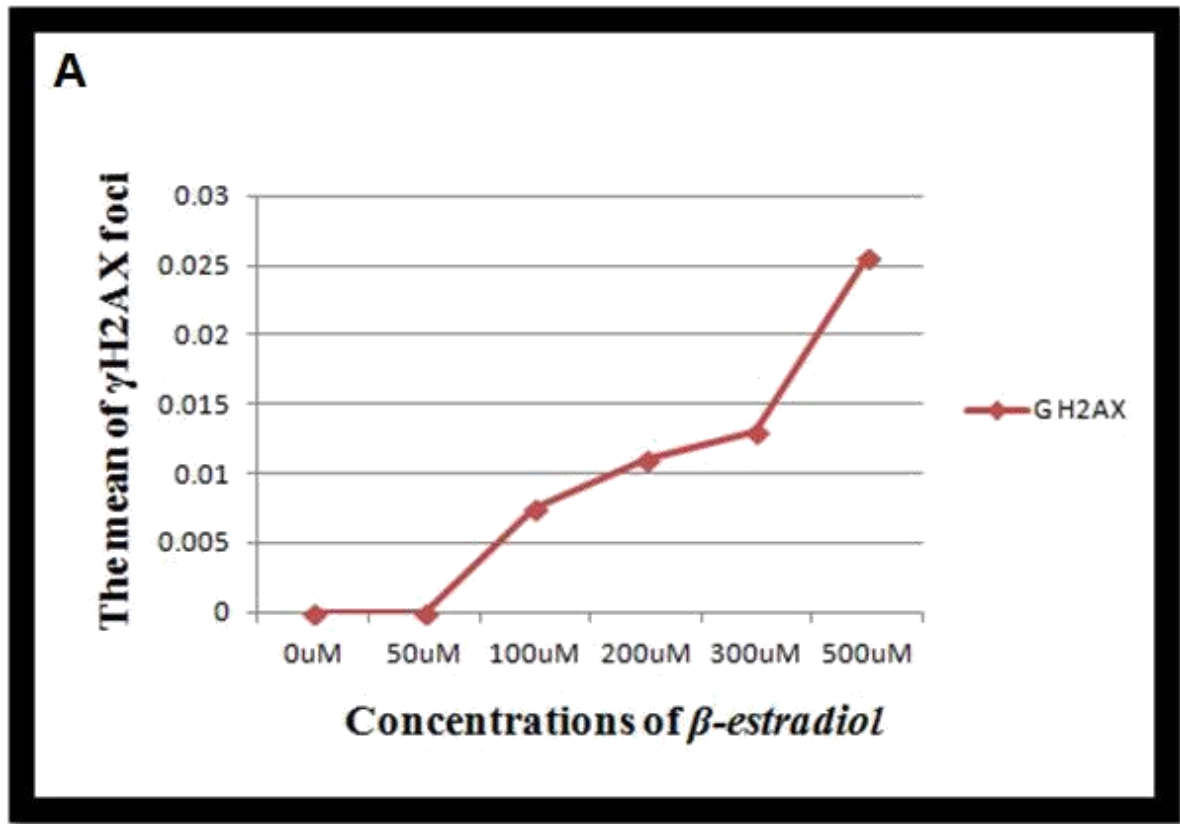


Figure 69: The total meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

This figure shows the total of γ -H2AX signals which indicates to creating DSB at the early stage of prophase I after 48 hours of treating the flower buds at different concentrations of β -estradiol.

Y-axis indicates to the mean of cells with γ -H2AX signals (cells with γ -H2AX signals /total number of cells at the early stage of prophase I). X-axis indicates to the different concentration of β -estradiol .

Our result showed that DSBs could be induced artificially by ZFNs during meiosis to produce only one DSB by ZFNs at a specific region on the genome (*MAD 1/2*). After 72 hours of treating the flower buds with 50 μ M and 100 μ M of β -estradiol, we could not account any chiasmata at MI. We were able to account only one MI cell with one chiasma of 1126 MI cells at 200 μ M. Also, only one MI cell with one chiasma was accounted at 300 μ M (1 rod bivalent of 1136 MI). The frequency of bivalent formation is increased at 500 μ M, ten MI cells with one bivalent were accounted (11 cells with one bivalent of 2434 MI cells) (Figure 70).

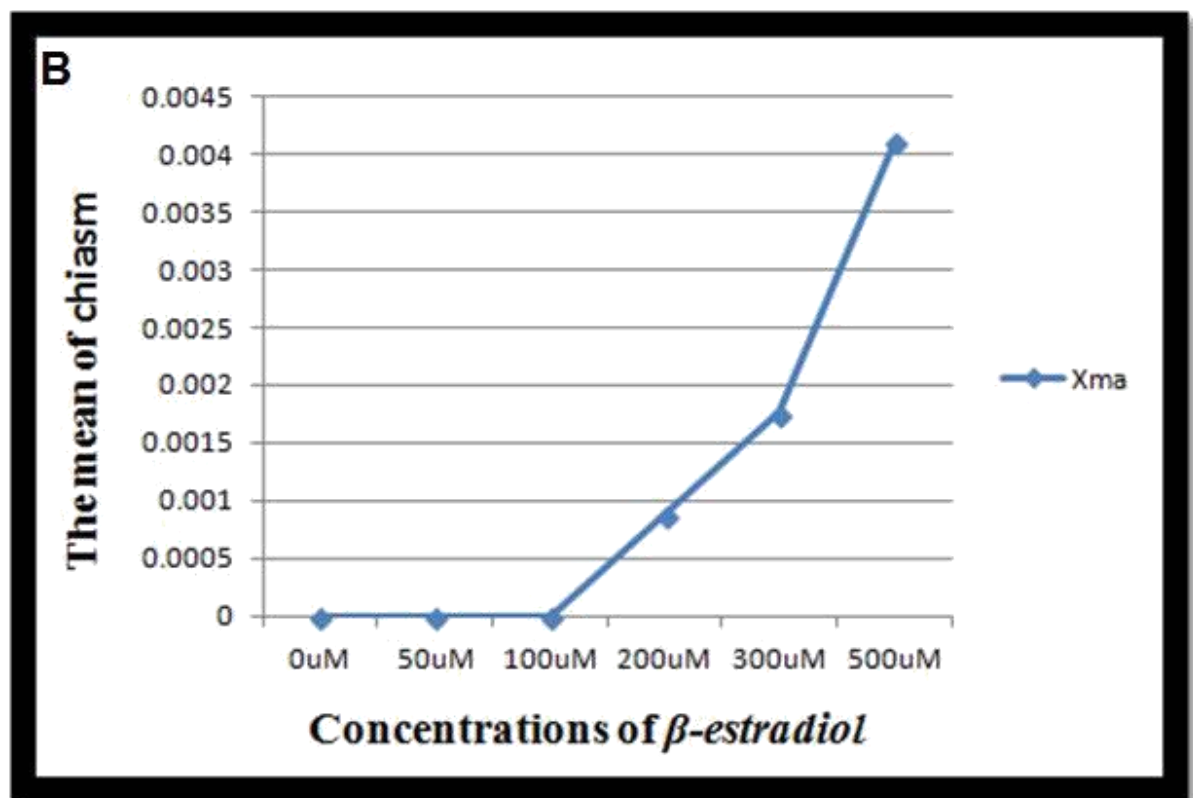


Figure 70: the total of producing bivalent from meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

This figure explains the total of bivalent formation at MI after 72 hours of treating the flower buds at different concentrations of β -estradiol.

Y-axis indicates to the mean of MI cells with one bivalent (MI cells with one bivalent /total number of all MI cells). X-axis indicates to the different concentration of β -estradiol.

We have managed to account some DNA fragments in some prophase II cells in treated *MADI/2 X ZFN*. The number of fragmentation was low at 200 μ M (2 cells with one fragment out of 1,126 cells analysed). The number of cells with DNA fragments at 300 μ M was similar (3 cells with one fragment of 1,136 cells). At 500 μ M (7 cells with one fragment and 4 cells with 2 fragments out of 2,414 cells was found) (**Figure 71**).

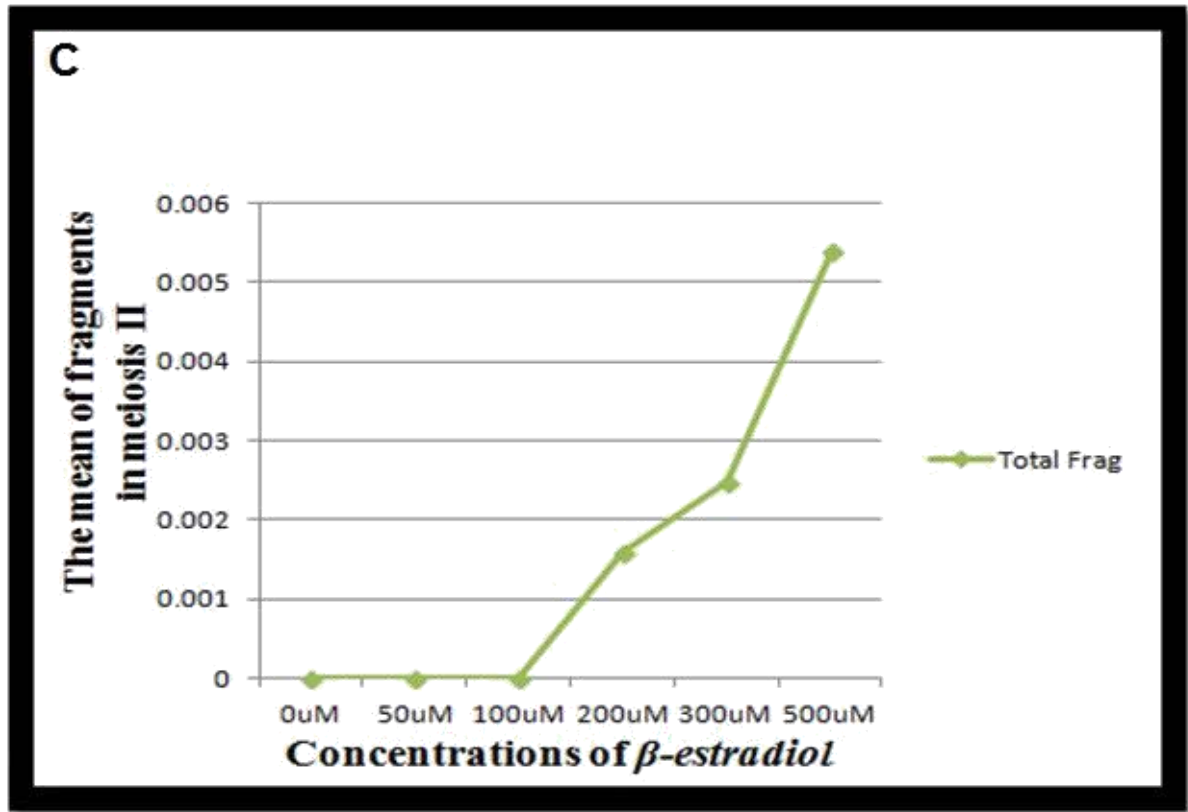


Figure 71: the total of chromosome fragmentation from meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

This figure explains the total of chromosome fragmentation at prophase II after 72 hours of treating the flower buds at different concentrations of β -estradiol.

Y-axis indicates to the mean of prophase II cells with chromosome fragmentation (prophase II cells with chromosome fragmentation /total number of all prophase II cells). X-axis indicates to the different concentration of β -estradiol .

This study has shown that the number of cells with DSBs increased as the concentration of β -estradiol increased in *MAD1/2 X ZFN* lines. The percentage of γ -H2AX at an early stage of prophase I was 0% at 50 μ M and increased consequently up to 2.5% at 500 μ M. Some of these artificial DSBs could be processed by meiotic HR as the bivalent formation at some MI cells proof. The formation of bivalent started to be observed at 200 μ M with just a 0.08% rate, and it increased up to 0.4% at 500 μ M treatment. Observation of some cells with DNA fragmentation in meiosis II stages could mean that ZFNs could be targeting both sister chromatids and both homologs chromosomes. Some fragmentation showed at 200 μ M, and it was around 0.1 %.It has increased with increasing

the concentration of β -estradiol where the percentage of fragmentations at 500 μ M around 0.5% (Figure 72).

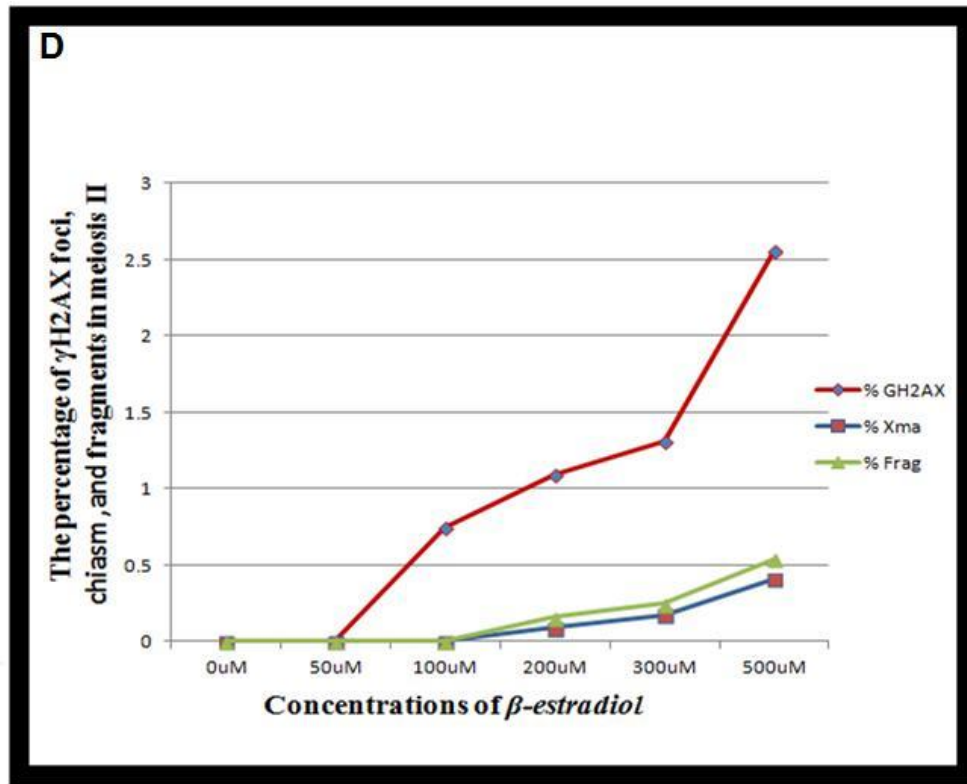


Figure 72: the proportion for the outcome of meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

This figure explains the percentage of meiotic artificial DSB by ZFN after 48 hours of treating the flower buds at different concentrations of β -estradiol. Also, it explains the percentage of bivalent formation at MI and the percentage of chromosome fragmentation at prophase II after 72 hours of treating the flower buds at different concentrations of β -estradiol.

Y-axis indicates to the mean of cells with γ -H2AX signals*100 at each used concentration of β -estradiol
Y-axis = indicates to the mean of MI cells with one bivalent*100 at each used concentration of β -estradiol
Y-axis = indicates to the mean of prophase II cells with chromosome fragmentation *100 at each used concentration of β -estradiol

X-axis indicates to the different concentration of β -estradiol.

At the lower concentration of β -estradiol (50 μ M) not induction of DSBs in *MAD1/2 X ZFN* was achieved. While, 100 μ M may able to produce a specific meiotic DSB in *MAD1/2 X ZFN*, but no chance to form chiasmata which mean the DSB did not repair by HR pathway. 8% of specific DSB at 200 μ M repaired by the HR pathway, because we were able to observe bivalent formation. Also, 14% of specific DSBs produce fragmentations at prophase II by which means ZFN could be targeting both sister chromatids or both homologs chromosome to make DSB. The percentage of repairing

DSB by HR pathway was higher at 300 μ M which was 13%, and also the percentage of fragmentations was increased to 18%. The frequency of producing specific meiotic recombination from specific DSB was higher at 500 μ M; our analyses showed that 16% of specific DSB repair by HR by showing chiasmata at MI, also at this concentration the percentage of creating fragmentation at prophase II was increased to 21% (**Figure73**).

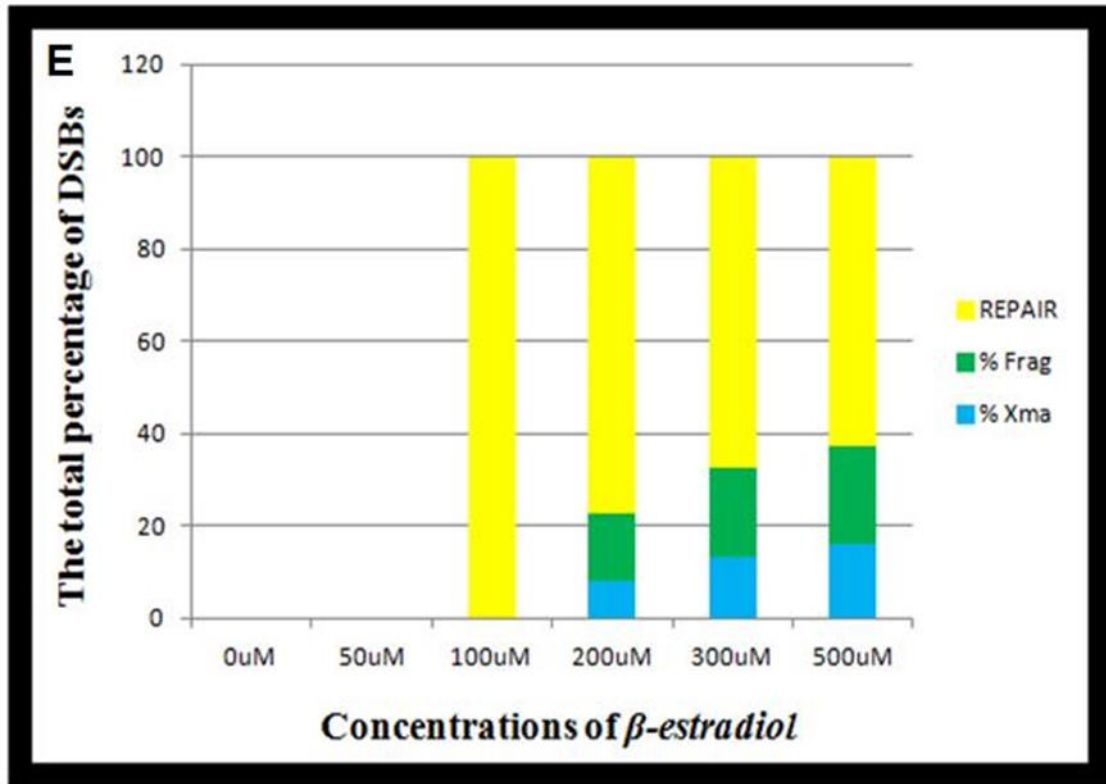


Figure 73: The proportion of repairing meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

This figure explains the percentage of repairing meiotic artificial DSB by ZFN after 72 hours of treating the flower buds at different concentrations of β -estradiol.

Yellow part indicates to the percentage of repaired DSBs that induced by ZFNs = the percentage of repairing meiotic artificial DSB by ZFN (the percentage of MI cells with one bivalent + the parentage of prophase II cells with chromosome fragmentation) – (the percentage of cell with γ -H2AX signals) / (the percentage of cell with γ -H2AX signals)*100

Blue part indicates to the percentage of MI cells with one bivalent that resolved as CO by ZFNs (the percentage of MI cells with one bivalent/ the percentage of the cell with γ -H2AX signals)*100

Green part indicates to the percentage of prophase II cells with chromosome fragmentation that could mean ZFNs targeted either homologous chromosomes or both sister chromatids (the percentage of perophase II cells with chromosome fragmentation/ the percentage of the cell with γ -H2AX signals)*100.

Discussion

Meiotic DSBs catalysed by SPO11

The initiation of meiotic recombination is catalyzed by the SPO11 complex (Keeney, 2001). The ability to form DSBs at specific sites also known as hotspot regions are 100-1000 times greater compared to other chromosome regions (Arora *et al.* 2004). Meiotic DSBs takes place between the axis association sites, in chromatin loop regions and SPO11 and associated accessory proteins do associate to these regions. The accessory proteins including Rec114, Mer2, and Mei4 binds to axis site within chromosome followed by formation of a complex with DSBs thereby generating pre-DSBs recombinosome (**Figure 74**). For the polymerization of SPO11 accessory proteins some proteins are necessary, Rec102, Rec104, (Arora *et al.* 2004; Panizza *et al.* 2011). According to the research study conducted by Panniza *et al.* (2011), Spo11 accessory proteins known as RMM are a group of protein including Rec114, Mer2, and Mei4, forming a sub-complex that partially localize on the chromatin and favor the binding of Spo11 to the DNA cleavage sites. The SPO11-accessory proteins such as Rec102 and Rec104 also prefer to associate with axis-association sites. MRN complex is composed of Mre11-Rad50-Nbs1 proteins (Williams *et al.*, 2007). The complex interacts as a DSB sensor, co-activator of DSB-induced checkpoint signaling during the cell cycle and DSB repair effector in HR and NHEJ pathways (Lamarche *et al.*, 2010).

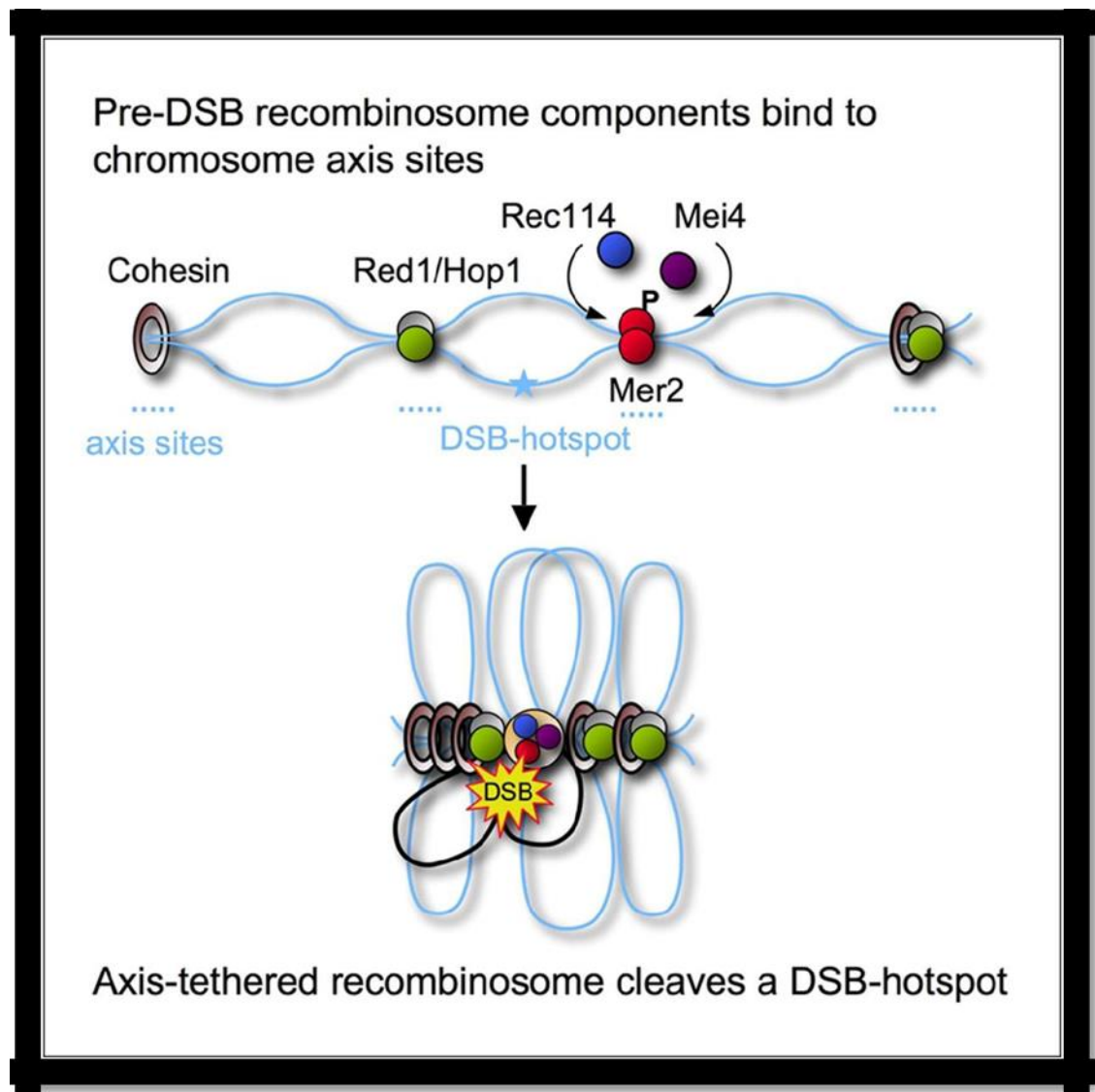


Figure 74: The proportion of repairing meiotic artificial DSB by ZFN in *Arabidopsis thaliana*

Rec114, Mer2, and Mei4 are pre-DSB recombinosome components which link to chromosome axis sites. Binding needs S stage Cdk and axial parts and are balanced by cohesion, Loss of axis sites restricting relates with loss of DSBs in budding yeast, The DSB machinery may bind hotspots to axis sites before DSB formation. Adapted from Panizza and colleagues (2011).

DSBs are important to start meiotic recombination. The DSB formation is formed by SPO11 and other proteins in loop sequences within promoters, but the DSB proteins resides on the chromosome axes. Spp1, i.e., H3K4 methyltransferase Set1 complex is essential not only for DSB formation but also connected to chromosome axes during Meiosis. During the mechanism physically interaction with the Mer2 DSB protein is also

Observed. The PHD finger module of Spp1, detect the H3K4 methylation in the vicinity of promoter regions, promotes DSB formation by binding to regions of the chromosome axes and thus activate the cleavage by proteins within the DSB (Sommermeyer *et al.*, 2013)(Figure 75).

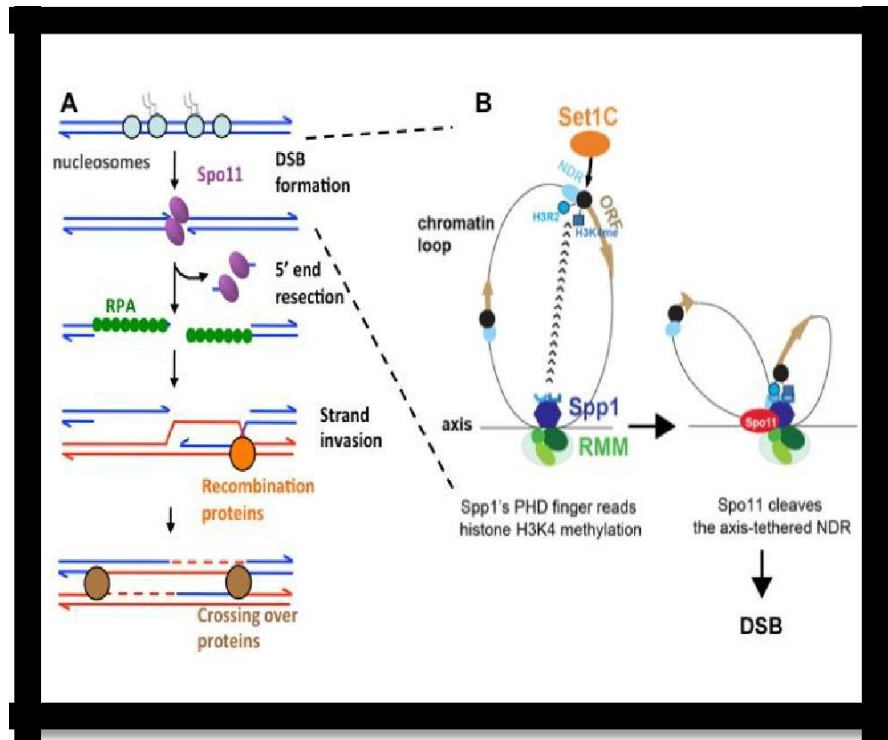


Figure 75: Molecular structure of the DSBs.

Chromatin modifications and proteins involved at meiotic recombination hotspots. A: Overview of the process of DSB formation and repair by homologous recombination. At sites of DSB formation, histones are modified, and specific proteins are bound at each step of DSB repair. B: Model to illustrate how chromatin regions that bear H3K4me3 modification are selected by a specific PHD finger module to experience DSB formation by interaction with the proteins located on the chromosomal axis. Adapted from Sommermeyer and colleagues (2013).

Meiotic time course is important for induction artificial DSB by ZFN

There are two important factors for producing DSB during meiosis artificially, to fix the material for immunolocalisation or cytological analysis. This first import point is that the timing for the maximum expression of the ZFNs induced by β -estradiol which seems to be around two days as published by Schlucking *et al.*, 2013. The second point is that the timing for forming DSBs to COs which might take up to 1 day. In *A.thaliana*, DSB formation by SPO11 is restricted to early meiotic prophase in most organisms. Spo11-

dependent γ H2AX appears at the G2/early leptotene step. Immunolocalisation studies of wild-type meiocytes at G2/early leptotene presented the of spread γ H2AX foci throughout the chromatin. Whereas nine hours is the maximum duration for G2, the duration of the meiotic S-phase is similar. Leptotene has a duration of six hours while zygotene/pachytene is 15.3 hours. The majority of the critical molecular meiosis occurrences, inclusive of chromosome replication and DNA, in addition to the formation of sister-chromatid cohesion and the complicated occurrences of chromosome pairing and also recombination and synapsis and occur in the course of these prolonged steps. Completion of the remainder of the meiotic division steps is attained particularly quickly, in fact inside three hours which includes the diplotene/diakinesis of the SC disassembles, so that the homologous chromosomes continue to be linked only by chiasmata, being the cytologically visible frameworks corresponding to the reciprocal crossovers sites' (COs). Consequently, the period from the meiosis from the termination of the meiotic S-phase to the tetrad stage lasts for 33 hours. (Bennett, 1971; Sanchez-Moran *et al.*, 2007). In our study, different samples were treated at various β -estradiol concentrations at different times, and it was discovered that the optimal time during which to fix material was three days (**Figure 62**). The *Atspo11.2* mutant and the wild type were utilised as a means of identifying the difference between these in the course of the meiosis stages. As well as a treated *Atspo11.2*, which is applied as a control to ensure that no natural DSBs were offered (**Figure 64B1-B8 & Figure 64C1-C8**).

Inducible promoter system in transgenic plants

In plant biotechnology, many systems are used for controlling gene function by chemically inducible systems which activate or inactivate gene expression with precise timing and regulation of gene expression being significant facets of such applications. Many systems have been initiated and subsequently applied to analyse the following: marker-free plant transformation, site-specific excision, gene function, activation tagging, and conditional

genetic complementation. Systems appropriate for field application which have possible applications in plant biotechnology are especially helpful for empirical systems (Padidam, 2003). In recent times, description has been given to many systems for inducing transgene expression in plants, and the principal usage of inducible systems was in *A.thaliana*, maize, rice, tomato, and tobacco. Inducible systems permit the promotion of local variations in gene expression levels but do not generate major changes in the development of the entire plant. The inducible systems such as heat shock induction; AlcR/AlcA (ethanol-inducible); GR fusions, GVG, pOp/LhGR (dexamethasone inducible); and XVE/Olexa (β -estradiol inducible) being a chemical which is utilised to control transgene expression (Borghi, 2010). In transgenic tobacco and Arabidopsis plants, XVE activated by estradiol may induce expression of a GFP reporter gene comprising eight copies of the LexA operator fused upstream of the -46 35S minimal promoters. When they are induced by estradiol, GFP expression levels may be eight times higher than the expression by the 35S promoter. There has been no observation of adverse physiological or toxic influences on the XVE system in Arabidopsis plants under all conditions of the test. Several genes in tobacco and Arabidopsis plants have been expressed by the XVE system. Over 75 percent of the transgenic lines investigated by Northern blot analysis (totaling 500 lines) upon induction, demonstrated a high transgene expression level. However, these did not demonstrate any recognisable background. Consequently, it is apparent that the XVE The system is a dependable and effective chemical-inducible process for the regulation of transgene expression in plants (Zuo *et al.*, 2000).

ZFNs specificity in *Arabidopsis thaliana*

ZFNs become a significant tool for gene targeting, which is the procedure of using homologous recombination (HR) to replace a gene within a genome of cells. ZFN-mediated gene targeting will have the capacity to site specifically and permanently by stimulating HR using a targeted genomic DSB. ZFNs are utilising to cut at a specific

chromosomal locus. Subsequently, the DNA break is repaired by HR. ZFN targeting was applied effectively to tobacco plants to promote the frequency of repairing DSBs by HR. Ten plants selected to target gene at different locations were utilised to prepare the protoplasts. This produced an enhancement of 104 to 105 times as compared with the usual rate of 105 to 106 illegitimate events per HR. This also meant that one out of five characterised gene targeting events were devoid of mutations, consequently indicating that the fidelity of gene targeting was an estimated 20 percent (Wright *et al.*, 2005). As we observed with induced DSBs by ZFNs, γ -H2AX phosphorylated after two days from treating flower buds with β -estradiol (**Figure 68**). A lot of foci homogeneously distributed on the chromosomes in the WT. In *Atspo11-2* mutant, no signal of γ -H2AX observed as also expected at 50 μ M β -estradiol no signal of γ -H2AX observed. Starting to see a γ -H2AX signal with increasing the concentration of β -estradiol. At 100 μ M in *MAD1* were managed to see some meiocytes with 1 signal of γ -H2AX. Only 4 out of 536 zygotenes was observed (1 DSB in every 134 zygotenes), 6 γ -H2AX signals out of 547 zygotenes at 200 μ M (1 DSB in every 91 zygotenes), 9 γ -H2AX signals out of 611 zygotenes at 300 μ M (1 DSB in every 68 zygotenes), 20 γ -H2AX signals out of 590 zygotenes at 500 μ M in *MAD1* (1 DSB in every 29.5 zygotenes), 10 γ -H2AX signals out of 584 zygotenes at 500 μ M in *MAD2* (1 DSB in every 58 zygotenes). The total number of at *MAD1/2 X ZFN* with 500 μ M was 30 signals in 1176 zygotenes. So not all the meiocytes managed to get one DSB. ZFNs could be targeting a DSB in both sister chromatids or both homologous chromosomes, because 2 DSBs at 500 μ M in *MAD1 X ZFN*, 2 cells with 2 DSBs out of 590 (2 DSBs in every 295 zygotenes in *MAD2*). 2 cells with 2 DSBs out of 584 (2 DSBs in every 292 zygotenes in *MAD2*). The total of one specific DSB in *MAD1/2 X ZFN* was increased with increasing the concentration of β -estradiol, and as shown in the analysis of the outcome of *MAD1/2 X ZFN* (**Figure 69**) the percentage of γ -H2AX signals in *MAD1/2 X ZFN* at early stage of prophase I was increased consequently up to 2.5% at 500 μ M (**Figure 70**).

Artificial induced DSBs in meiocytes can initiate Synapsis

ZYP1 is a synaptonemal complex protein which is located in the central element (Sym *et al.*, 1993). At zygotene, the AtZYP1 signal begins to linearize and stretching out along, and between the homologous axes to making them in close opposition. At pachytene, a near signal of AtZYP1 is formed between the synapsed homologs. The lacking of AtZYP1 in Arabidopsis shows an effect on the frequency of chiasma. At metaphase I the multivalent, homologous and nonhomologous bivalents, univalent have observed which indicates to the loss of AtZYP1 resulting in losing of Cos control. The lacking of the protein in the rice showed the opposite result comparing to Arabidopsis with increasing the frequency of chiasma. Thus, the different effects indicate that the protein has an important role in controlling the formation of COs, there is a contribution between the protein and the structural organization of the SC, and also the protein plays an important role in meiotic Recombination (Storlazzi *et al.*, 1996; Borner *et al.*, 2004, Wang *et al.*, 2010).

In inducing one DSB by using ZFNs, the initiation of the Synapsis in the artificially induced DSBs has localised by AtZyp1 polymerisation (**Figure 68**). In the WT, Zyp1 polymerise between each pair of homologs and at pachytene is fully polymerised or synapsed. In the absence of *AtSPO11.2*, there is not a signal of ZYP1 as we expected. *Atspo11.2* mutant with the induction of DSB with ZFNs is possible to see one signal with some partial polymerisation.

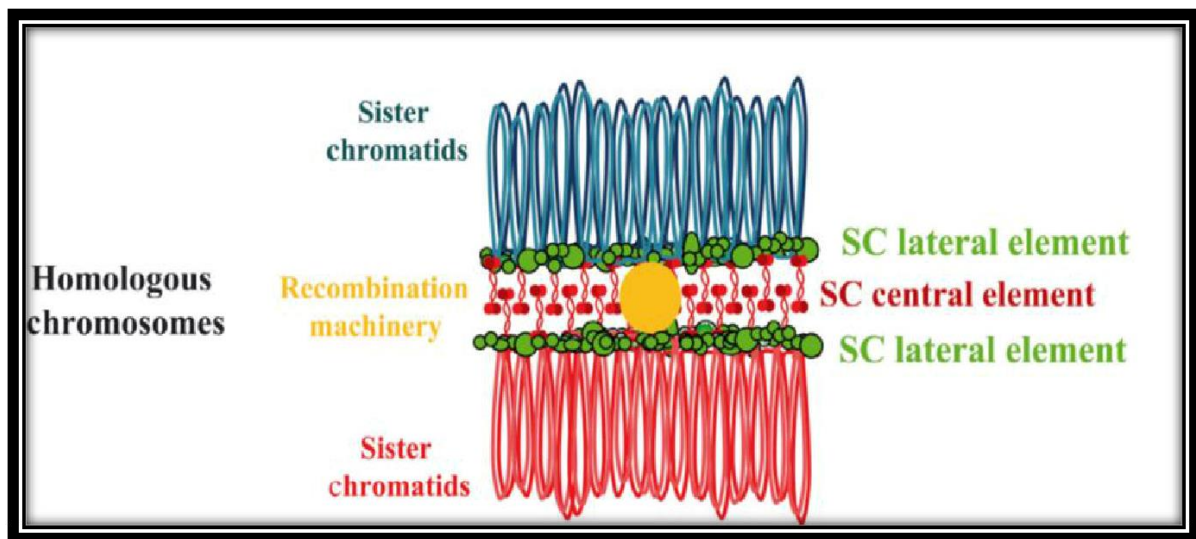


Figure 76: Synaptonemal complex formation.

At zygotene, the synaptonemal complex (SC) is shaped by bringing the homologous chromosome. SC polymerization is completely completed along the homologs at pachytene; SC polymerization might be restricted by immunolocalization of AtZYP1 which is a part of the SC focal site (image from Osman *et al.*, 2011).

Artificial DSBs by ZFNs could produce crossovers

Mammals, plants, and fungi follow common conserved processes for meiotic recombination. For recombination initiation, double-strand breaks (DSBs) are required and developed as chromosome structural axes (Zhang *et al.* 2014). In various organisms, at least two pathways are followed for the production of crossovers. In *A.thaliana*, yeasts, and humans one pathway constitutes the majority of COs which are sensitive to interference along with secondary pathway producing interference-insensitive COs (Berchowitz *et al.*, 2009 and Zhang *et al.* 2014). The association among crossover and DSB frequencies has studied with differences in terms of the SPO11 genotype, and that could quantify the crossover frequencies in eight separate intervals covering 484 kb over three chromosomes in the yeast genome. Numerous studies have been conducted on crossover formation and interference. Separation of the marks was examined in 7750 four-spore-viable tetrads for every SPO11 genotype, thus, measuring genetic linkage and the distance between chromosome positions., measured in centimorgans (cM), where one cM

is associated with a crossover frequency of 1 %. Regarding four out of the five intervals in the larger-sized chromosomes (VII and VIII), no statistically meaningful reduction was observed in the crossover frequency of the mutants in comparison to the wild type or each other. In the fifth interval as well as each of the three intervals in chromosome III, there was a significant reduction in the crossovers for a minimum of one of the mutants, although this was consistently lower than the decrease in the DSBs (de Los Santos *et al.*, 2003; Malkova *et al.*, 2004). In the majority of organisms, it is highly uncommon to find non-exchange chromosomes. For instance, they can occur at a rate of <1% in *C. elegans* oocytes, which contain an average of only one crossover for each chromosome per meiosis (Dernburg *et al.*, 1998; Hillers and Villeneuve, 2003). The convention that almost all bivalent obtains a minimum of one crossover is defined as the obligate crossover. A minimum of one DSB should be created for each bivalent, and the appropriate recombination partner should be activated, which involves a process of homologous pairing and a preference for the utilization of the homolog instead of a sister chromatid. Crossover homeostasis is a reflection of the drive in the direction of crossover generation rather than non-crossovers, thus indicating that one of the main purposes of this procedure is to contribute to the creation of the obligate crossover. This concept implies that an obligate crossover is an event that is genetically programmed. If the efficiency of crossover homeostasis is optimal, then the only requirement to guarantee a crossover would be one DSB. It is evident that this assertion does not hold for yeast, where it appeared that the maximum probability of DSB forming a crossover was ~60%. The inability for crossover homeostasis to achieve 100% crossover formation could be caused by several factors. This may be because the molecular mechanism behind the decision between crossover and non-crossover is itself constrained in terms of the enforcement of crossover generation, or as there are multiple types of DSB, implying that DSBs are created on a pairing-only pathway and are therefore unable to form crossovers. On the other hand, the indirect impact of decreased DSBs could antagonize the standard functionality of crossover management. For

instance, reduced DSB levels are associated with abnormalities in the formation of the synaptonemal complex (SC), which could be the result of homologous pairing abnormalities (Henderson and Keeney, 2004). Ionizing radiations like cosmic radiation, radionuclides are energetic as well as penetrating principle source of damage generating bystander effect generating, double-strand breaks, DNA damage, and single-strand breaks, mutation, and chromosomal instability. Similarly, chemicals agents such as Alkylating agents are also known to produce DSBs artificially within the lab environment (Helleday *et al.* 2014). The induction of DSB in Arabidopsis was made via treatment of seedling with gamma radiations and chemical treatment. The γ -irradiations were provided using a ^{137}Cs source. Further treatment with zeocin may also be associated with DSBs in a number of organisms including Arabidopsis, mammals and yeast cells by DNA cleavage via intercalating with DNA (Choi *et al.*, 2013 and Hirakawa *et al.*, 2015). The DNA double-strand breaks were induced following metals exposure such as cadmium (Cd), copper (Cu), and zinc (Zn) in mammalian cells (Haldrud and Krøkje, 2009). Our result showed that one specific DSB could be induced artificially by ZFNs during meiosis to produce one CO at a specific region on the chromosome (*MAD 1/2*). at 200 μM of ***β -estradiol*** was possible to one rod bivalent and in 1126 metaphases I cells and at 300 μM also 1 rod bivalent in 1136 metaphase I. The frequency of bivalent formation is increased at 500 μM , the total number of bivalent with *MAD1 X ZFN* was 8 bivalent of 1256 MI cells (1/ 157). In *MAD2 X ZFN* was 5 bivalent of 1178 MI cells (1/236) (**Figure 65 & Figure 67**). Recombination proteins (DMC1 and RAD51) were localized at 500 μM . Both proteins appeared in WT, but in *Atspo11.2* mutant, they are absent as expected because of the absence of DSBs catalysed by SPO11. One and two signals for both proteins are presented in *MAD1 X ZFN* in some meiocytes which could indicate that the ZFNs could be targeting a DSB in both sister chromatids or both homologous chromosomes (**Figure 68**). The percentage of bivalent formation in *MAD1/2 X ZFN* increased from 0.08% at 200 μM up to 0.4% at 500 μM (**Figure 70**). Repairing a specific DSB in *MAD1/2 X ZFN* by HR pathway increased by increasing the concentration

of β -estradiol. At 200 μ M the percentage of repairing DSB by HR pathway was 8 %, at 300 μ M was 13%, and at 500 μ M was 16% (Figure 73). Also, some fragmentations showed in *MAD1 X ZFN* at 500 μ M in the meiosis II (3 prophase cells with one fragmentation and two cells with two fragmentations out of 1234 prophase II cells) in *MAD2 X ZFN*, 4 cells with 1 fragmentation and 2cells with 2 fragmentations out of 1200 cells). So, chromosome fragmentations at prophase II increased from 0.15% at 200 μ M up to 0.5% at 500 μ M (Figure 73). 14% of specific DSBs produce fragmentations at 200 μ M, 18% at 300 μ M, and 21% at 500 μ M (Figure 73). It means that increasing the concentration of β -estradiol to induce one specific meiotic DSB leads to increasing the frequency of targeting both sister chromatids or both homologs chromosome.

Conclusion.

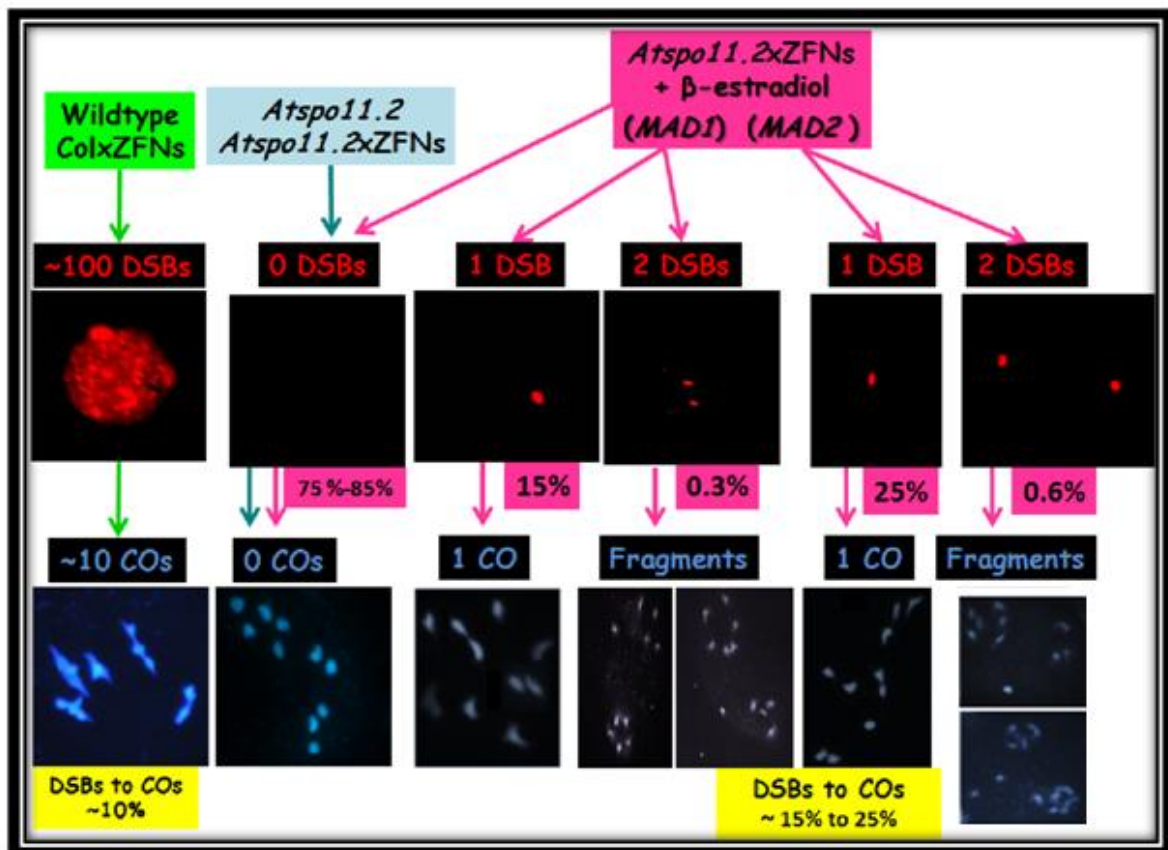


Figure 77: Summary of the results of the artificial induced DSBs using ZFNs

In the WT, around 100 DSBs are formed from which only about 10 will produce chiasmata or COs. In the mutant of no DSBs are catalysed so not recombination is initiated and therefore

not chiasma or CO is observed. In the mutant background of *AtSPO11.2* with the expression of ZFNs at 500 μ M in *MAD1/2 X ZFN*. We observed that the majority (about 63%) of early prophase I cells did not have DSBs probably due to the limitations of the induction technique by brushing the flower buds with the solution of β -estradiol. Some cells (16%) showed the presence of one DSB and others (21%) showed two DSBs; these later ones could have been explained by the fact that the ZFNs could cut both sister chromatids or cut both homologous chromosomes. So in the WT, the percentage of DSBs converted to COs is about 10% (10/100). However, the frequency of DSBs converted to COs has increased up to about 16% with using ZFN to target meiotic DSB in specific genomic sequence targeted DSBs, These results show that Genome Targeting technologies could be used to manipulate meiotic recombination localization in plants and maybe recombined genomic regions that would not have been possible to recombine.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

Importance of DNA-dependent protein kinases in plants (*Arabidopsis thaliana*)

The integrity of DNA in cells is sustained by repair pathways for DNA breaks, which include certain regulatory protein families. The most repair pathway for double-stranded breaks in DNA (DSB) that has been investigated is non-homologous end joining (NHEJ), which repairs breaks by direct re-ligation of DNA strands at the broken site (Jette & Lees-Miller 2015). Non-homologous end joining utilises a significant number of proteins that interact efficiently to undertake DNA repair and result in the formation of a ligated and sustainable DNA strand. One such family of protein that has been identified is DNA protein kinase and its associated catalytic subunits (DNA PKcs). DNA PKcs are involved in binding the broken DNA site with their heterodimer Ku, resulting in the recruitment of other proteins for the re-ligation of the DNA break (Jette & Lees-Miller 2015).

During lymphocyte development, V(D)J recombination employs DNA PKcs, which is responsible for the recruitment of other regulatory proteins that are required to complete the pathway (Underwood *et al.* 2017). Studies on invertebrates have identified the association of DNA PKcs with NHEJ and V(D)J recombination, indicating their potency regarding phosphorylation, which results in the formation of multiprotein complexes for DSB (Jette & Lees-Miller 2015).

A study by Malu *et al.* (2012) identified NHEJ as an integral part of V(D)J recombination and that the protein-protein interactions associated with these mechanisms primarily incorporate the regulatory functions of DNA PK and DNA PKcs. According to Yang *et al.*'s (2016) study, transgenic mice with Ku mutant DNA PKcs resulted in decreased NHEJ and poorly repaired DNA breaks, thereby affecting through age-related genomic disability. Moreover, research indicates that defects in DNA PKcs regulatory genes that result in the production of downregulated or non-functional DNA PKcs also result in poor NHEJ and affect the integrity of V(D)J recombination (Amiard *et al.* 2013).

The outcomes of studying putative AtDNA-PKcs in *A.thaliana* indicated that AtDNA-

PKcs had been discovered in plants, particularly in *A.thaliana*, as a result of the presence of a high degree of commonality between the human sequence and the Dictyostelium discoideum sequence with AtDNAPKc with the plant. This could be interpreted as indicating that AtDNAPKcs can contribute to the repair of DSBs in plants (*Arabidopsis thaliana*). Two AtDNAPKc genes have found in *A.thaliana* —AtDNA-PKa, which is located on chromosome 2 at a locus (AT2G17930) with a total of 33 introns and 34 exons, and AtDNA-PKb, which is on chromosome 4 at a locus (AT4G36080) with a total of 33 introns and 34 exons.

The expression of both putative AtDNA-PKcs genes in WT was higher in the flower buds, particularly in opened flowers, but it was very low in other parts of the plant. The expression of both AtDNA-PKcs genes was slightly higher in WT female organs (ovaries) than in male organs (anthers). Studying the double homozygous mutants of AtDNAPKcs has found that AtDNA-PKcs do not play any role in meiosis, but some anaphase bridges have been observed during mitosis. Also, some DNA damage agents have affected the growth of AtDNAPKcs plants. All these results could mean that putative AtDNA-PKcs are found in plants (*Arabidopsis thaliana*) and play an important role after meiosis or in somatic cells to repair DSBs by NHEJ.

The role of putative recombination initiation defects 2 (AtPRD2) in meiosis.

Recombination is the phenomena of the controlled exchange of genetic sequences and occurs with the assistance of some protein molecules that act as regulators and enzymes. The initiation of genetic recombination is performed by the formation of DSBs. Studies have identified and characterised the protein families that play a role in DSBs formation. Tang *et al.* (2017) identified and characterised the proteins involved in DSB formation in *Arabidopsis* as AtPRD1, AtPRD2, AtPRD3, AtSPO11-1, AtSPO11-2 and AtDFO. In the same study, a topoisomerase (Topo) VI-like complex was also reported as playing an important role in initiating recombination in DSB formation (Underwood *et al.* 2017).

First extracted and identified in *Arabidopsis thaliana* as Putative Recombination Initiation Defects 2 (AtPRD2), this protein has also been termed ‘protein multipolar spindle’ due to its function in meiotic spindle organisation to accurately regulate chromosome segregation (Brooks et al. 2014). In addition to its role in DSB formation for recombination initiation, AtPRD2 has also been reported to be involved in plant-type sporogenesis (in yeast), embryo sac development, chiasma assembly and male meiotic nuclear division (UniProt Database). AtPRD2 is strongly expressed in meiocytes and stems, leaves, roots, seedlings and inflorescences (Jette & Lees-Miller 2015). AtPRD2 homologs were identified within the plant kingdom. In our study of the PRD2 gene at the At5g57880 locus for the first time in *A. thaliana*. A previous study by De Muyt (2009) considered the AtPRD2 mutant as a knockout mutant, but the absence of expression in the AtPRD2 homozygous mutant proves that it is a null mutant. Investigations reported by De Muyt and colleagues (2009) indicated that no DSBs were formed in cells that were transformed with mutant AtPRD2 and AtPRD3 proteins, which reflects the significance of these proteins in recombination. Moreover, slow or defected vegetative growth was observed with reduced silique formation in AtPRD2 mutant plants (Lambing *et al.* 2017). Alexander staining confirmed that the AtPRD2 mutant is semi-sterile with reduced fertility. Cytological analysis by Jiang and colleagues (2009) showed the mis-segregation of chromosomes in meiosis, as we found in the cytological analysis for the same mutant in *Arabidopsis*. These investigations indicate the potential role played by the AtPRD2 protein in the segregation of chromosomes as well as well-balanced chromosome numbers in male and female gametophytes in *Arabidopsis thaliana* (Manova & Gruszka 2015).

Sanchez-Moran and colleagues (2007) found that meiotic recombination is instigated by 150 DSBs: 10 DSBs resolved as COs and 140 resolved as NCOs. According De Muyt and colleagues (2009), mutations or defects in AtPRD2 and associated proteins led to the abortion of female and male gametophytes of *Arabidopsis thaliana*, aberrant meiotic

products in the male gametophyte development and an absence of chiasma. In our cytological analysis for an AtPRD2 mutant, the number of DSBs was significantly reduced, and the full polymerization and formation of SC was defective, which indicated that some DSBs lead to form a crossover.

The majority of the DSB formation proteins identified in budding yeast are orthologues of AtPRD2 and ATPRD3. Approximately ten proteins have been identified in *Saccharomyces cerevisiae* as responsible for DSB formation, including Rec104, Rec102, Spo11, and Ski8 proteins. Moreover, complexes formed by proteins Mre11/Rad50/Xrs2 are involved in DSB formation as well as processing (Lambing *et al.* 2017). Observations based on evidence from several studies report that Spo11 together with Rec102, Rec104, and Ski8 proteins are required for chromosome localisations, and interaction between these proteins is required for the accurate localisation of chromosomes through the assembly of a higher order complex. These investigations indicate the significance of DSB formation proteins for meiotic recombination, and defects in these orthologues result in infertile male or female gametes, defective growth patterns and aberrant chromosome numbers in gametes (Davis 2014).

Importance of reduction in DSBs and crossover in meiosis to control meiotic recombination and crossover

The segregation of homologs requires different stages of protein-protein interaction, protein–DNA interaction and interaction with certain potent regulators to undergo the formation of crossovers and thus execute the process of meiotic recombination. Crossovers are necessary to allow the meiotic recombination process, and the formation of crossovers is carefully controlled (Fernandes *et al.* 2018). The formation of DSBs has also been recognized as a significant governing step that initiates and leads to accurate crossover formation. The controlled mechanisms of these two potential processes—i.e., DSB and crossover—promote meiotic recombination in cells, thereby allowing the appropriate

segregation of homologs (Amiard *et al.* 2013). Various strategies have been proposed to control meiotic recombination; these predominantly involve a process of controlled or reduced DSBs introduction followed by a reduction in the levels of crossover formations. According to Phadnis and colleagues (2011), the process of crossover homeostasis in animal and plant cells maintains the controlled number crossovers per cell required for meiotic recombination. Moreover according to a similar study, the intricate phenomena of crossover variance is associated with maintaining the level of crossover density, which includes the sustainability of crossovers per kb of DNA. Therefore, crossover control and maintenance is a rather complex phenomenon, and reduction protocols in crossover formation are further complicated (Yang *et al.* 2016).

Research on two species of yeast— *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*—has shown that DNA DSBs is a complex phenomenon that results in accurate crossover formation at specific sites, also termed ‘hotspots’ (Ohta *et al.* 1994; Wu and Lichten 1994; de Massy *et al.* 1995; Liu *et al.* 1995). The proteins involved in these mechanisms can be targeted to reduce DSBs generation and crossover formation, and the downregulation of these regulatory proteins may result in controlled meiotic recombination (Fernandes *et al.* 2018). DNA binding proteins for DSB generation, DNA unwinding proteins, and proteins involved in DNA stabilisation can be targeted to control the phenomena of meiotic recombination (Malu *et al.* 2012).

Advances devised to control meiotic recombination are significant for some reasons. Decreasing the number of crossovers by disrupting the genes involved in crossover regulation has been portrayed as a novel approach. In this context, genes identified in *Arabidopsis* include FANCM, RECQ4A, RECQ4B, FIG1, TOP3 α , FLIP and RMI1 (Choi 2017). Epigenetic modulation has also been proposed to affect and control the crossover mechanisms in *Saccharomyces cerevisiae*, and molecular techniques that control chromatin condensation, methylation and demethylation patterns can affect the frequency of

crossover formation. Anti-crossover epigenetic factors have been reported by Choi (2017) to control the mechanism of meiotic recombination (Gray & Cohen 2016).

Importance of gene editing for meiotic recombination in plants

With the rapid growth of the human population, food shortage has been recognised as a major global threat; researchers are persistently addressing this problem through advanced genetic engineering practices (Jette & Lees-Miller 2015). Gene editing tools are continuously advancing, and they are being applied to improve crop growth, improve the quality of crops, improve the variety of crops by interbreeding, and reduce plant infections and diseases. Understanding the deep-seated genetic and epigenetic phenomena associated with crop yield and disease can result in the identification and characterisation of the most appropriate gene editing tools for each species (Lambing *et al.* 2017).

Due to the threats associated with climate change and food security, the knowledge and applications associated with crossover formation and manipulation have increased in the past few decades. Advances in genetic engineering are utilising approaches that employ the understanding of meiotic recombination to manipulate the mechanism of crossover formation (Yang *et al.* 2016). Moreover, identifying crop related diseases and reducing the frequency by targeting meiotic recombination mechanisms has also been proposed as a novel and effective means of ensuring the sustainability of food security. Mammalian cells share common genetic regulatory features with certain species of yeast cells, and genetic manipulations of yeast cells indicate the significance of outcomes in mammalian cells (Sunder *et al.* 2012).

Moreover, identifying the hotspots for gene editing in *Arabidopsis* has also led to the discovery and identification of mechanisms required to control meiotic DSB formation and crossover formation in other plants. According to Lambing and colleagues (2017), approximately 80 meiotic genes have been identified in *Arabidopsis*, the majority of which

are involved in the regulation of meiotic recombination, crossover formation, and DSB.

Formation. According to their study, repetitive sequences in cereal genomes were found in larger quantities as compared to other edibles and possessed much larger genomes than *Arabidopsis*, and the presence of polyploidisation results in the presence of duplicated chromosomal segments. These aspects can be targeted to study mechanisms to prevent homologous pairing, dynamics and the processing of giant chromosomes and functional regulation as well as specialisation followed by gene duplication in cereal genomes (Lambing *et al.* 2017).

In eukaryotic genomes, meiotic recombination is responsible for the phenomenon of gene shuffling resulting in variations in the genes of each generation. This gene shuffling is similar in plants and animals and results in the sustainability of biodiversity due to its role in heredity and evolution. In genetic engineering mechanisms, the interbreeding of species results in the creation of chimeras, which are characterised by unique capabilities at genotypic as well as phenotypic levels (Davis 2014). The mechanism adopted by Fernandes and colleagues (2018) resulted in an eightfold increase in meiotic recombination in plant hybrids, thereby creating novel ways to efficiently increase the plant breeding processes to overcome food shortages and threats to food security in the future. Novel mechanisms introduced by recent evidence and investigations regarding meiotic recombination are serving as the mechanisms of choice for future generations for the preservation of natural resources, sustainability and the protection of food security parameters. Moreover, targeted genetic editing tools (CRISPR, ZFN, and TALEN) are emerging as breakthrough technologies to knock out the genes responsible for disease development in crops and other edible plant products (Brooks *et al.* 2014).

Importance of gene editing in food security

In light of different environmental, economic and climatic factors and overpopulation on a

global scale, global food security is emerging as a challenging debate. Limited land availability for agricultural expansion and the detrimental effects of climate change on crop yield, growth and quality has been of concern to agriculturalists and researchers in the field and has led them to identify and devise innovative tools to cope with these circumstances (Manova & Gruszka 2015). Genome editing technology—in the form of gene cloning technology—has been employed as a novel approach to optimise the quality, growth, and yield of crops while preserving natural resources (Ma *et al.* 2018).

Genome editing mechanisms employ sequence-specific nucleases (SSN), which include transcription activator-like effector nucleases, Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR) to induce targeted mutations in the genes of crops. The outcome of these induced mutations in crops includes the formation of DSBs at specific sites that may contain either a gene responsible for a certain disease or infection in the crop or a gene that regulates the growth of the crop (Slomka Pilpel 2018). Moreover, the DSBs introduced by these induced mutations trigger DNA repair mechanisms, which may involve either homologous recombination or NHEJ. The emergence of CRISPR as a gene editing tool has resulted in cutting the high costs as well as providing accurate and required outcomes (Gray & Cohen 2016). The breeding of crops based on genetic engineering tools can reduce the heavy requirements for high-cost production facilities while improving the yield and nutritional quality of the crop (Jette & Lees-Miller 2015). Due to decreases in the arable land area available for cultivation and the practise of deforestation, crop yield has halved; this has they have been accelerated by the emergence of a variety of crop pathogens. Genetically improved and competitive crop varieties are required to be cultivated and grown at higher rates to ensure sustainable food security parameters in the future (Choi 2017).

Gene editing as a tool for DSB induction in meiosis to target meiotic recombination and crossover in a specific region

In the past few decades, innovative approaches using DNA based technologies have highlighted and explained in depth the genetic and epigenetic mechanisms in plants and animal genomes. DNA sequencing has emerged as an efficient technological approach that employs bioengineering protocols and facilitates the in-depth exploration of genetic phenomena (Malu *et al.* 2012). These practices have led to further advances in plant and animal breeding practices by employing intricate high-throughput genotyping and mapping methodologies. Advances in genetic engineering and genome editing protocols have facilitated the understanding of genetic and epigenetic control mechanisms and has improved crop quality and health (Gray & Cohen 2016).

The manipulation of meiotic recombination through advanced genetic engineering technologies is conducted predominantly through gene editing mechanisms—manipulations performed at the molecular and genetic levels to control gene expressions and regulation. In our study to target a specific meiotic DSB, ZFN was activated by an inducible promoter system (XVE activated by estradiol). Flower buds were treated with β -estradiol at different times. ZFN was highly expressed at 500 μ M of β -estradiol; γ -H2AX phosphorylated two days after treatment. The optimal time for localising recombination proteins (AtDMC1 and AtRAD51) and fixing material for observing bivalent at MI was three days. DSBs induced by ZFN initiated synapsis with some partial polymerisation of AtZYP1. Thus, ZFN produced DSBs in specific regions of the *Arabidopsis thaliana* genome during meiosis and undergo meiotic recombination to produce CO. These results show that genome targeting technologies could be used to manipulate meiotic recombination localization in plants and maybe recombined genomic regions that would not have been possible to recombine. The most potent gene editing technology discovered in the past few years is a CRISPR associated system (CRISPR/Cas) (Ma *et al.* 2018). The

high-throughput CRISPR/Cas technology was discovered as a bacterial adaptive defense mechanism and has now been manipulated and used for different purposes. Gene editing performed using CRISPR/Cas technology is accurate, specific and targeted, and it utilises a complex of endonuclease proteins (Cas proteins) armed with a guide RNA to target a specific gene (Ma *et al.* 2018).

Controlling meiotic recombination by manipulating (downregulating or knocking out) the genes involved in DSB and crossover formation results in downregulating the mechanism of meiotic recombination. Brooks and colleagues (2014) used a CRISPR based gene editing approach in the tomato to efficiently achieve gene knockout, which served as the basis for reverse genetics. After characterising the genes involved in the tomato's reproductive development, the research utilised CRISPR based gene editing technology to target meiotic recombination and successfully knocked out specific genes (Fernandes *et al.* 2018). Similar evidence indicates the potential of gene editing technology for crop quality improvement and manipulation (Fernandes *et al.* 2018). The introduction of site-specific DSBs has also emerged as a novel approach to target meiotic recombination and crossover formation. Sunder and colleagues (2012) used I-PpoI endonuclease in their research and found it to be capable of inducing defined and targeted cleavage, thereby promoting efficient cell cycle arrest in *Schizosaccharomyces pombe*. Due to similarities in the chromatin condensation mechanism and chromatin organisation in *Schizosaccharomyces pombe* and mammalian cells, the results, and findings of the research are significant, highlighting hotspots that can be targeted for site-specific cleavage in mammalian cells for genetic manipulations through gene editing tools (Fernandes *et al.* 2018).

In our study of residual DSBs during Meiosis in *A.thaliana*, the number of DSBs and the polymerization of SC formation are significantly reduced in AtPRD2 mutant. Also, DSBs and meiotic recombination are partially rescued in AtSPO11-2 mutant by Cisplatin. Some γ -H2AX, AtRAD51, AtDMC1, and AtZYP1 were detected, which proves the formation of

Cisplatin-induced DSBs, meiotic recombination, and synapsis and indicates that some DSBs could be enough for SC formation. Cytological results indicated that some bivalents are observed at MI. Thus, residual meiotic DSBs can be required and enough for stabilizing homologous synapsis and undergoes recombination to produce COs.

General conclusion

In this thesis, AtDNAPKc has been discovered in plants, specifically *Arabidopsis thaliana*, along with the presence of a high level of commonality between the human sequence and the Dictyostelium discoideum sequence with AtDNAPKc in the plant. In *A.thaliana*, AtDNAPKc has two genes (AtDNA-PKa and AtDNA-PKb). AtDNA-PKa is located on chromosome 2 at a locus (AT2G17930) and contains 33 introns and 34 exons, while AtDNA-PKb is on chromosome 4 at a locus (AT4G36080) with a total of 33 introns and 34 exons. DNA-PKcs are expressed in different plant parts such as the root, leaf, flower bud, seeds, and silique in WT. Expression was high in the flower buds and very low in the other parts of the plant. Regarding the flower buds, the expression of AtDNA-PKcs was higher in opened buds. The expression of AtDNA-PKcs in *A.thaliana* was slightly higher in female parts than in male parts. Seeds production was significantly reduced in the double homozygous mutant of AtDNA-PKcs plants. Some anaphase bridges showed during mitosis in the double homozygous mutants for AtDNA-PKcs, and some DNA damage agents affected the growth of the AtDNAPKcs plants. AtDNA-PKcs and γ -H2AX proteins were localised in the wild-type pollen grain. Finally, the data from the study of AtDNA-PKcs in *A.thaliana* Arabidopsis indicated that AtDNA-PKcs might play an important role after meiosis or in somatic cells to repair DSBs by NHEJ in plants.

This thesis presented the outcome of AtPRD2 homozygous mutant during meiosis in *A. thaliana*. The results confirmed the involvement of AtPRD2 in meiosis in forming DSBs.

A small number of DSBs are formed in the mutant of AtPRD2 by showing some γ -H2AX

singles at the early stage of prophase I and presenting very clear bivalent formation at metaphase I. The limited number of DSBs can be synapsed and undergo meiotic recombination to produce COs. Another study confirmed that some DSBs could produce some COs by using Cisplatin in AtSPO11-2 mutant in *A.thaliana* to induce artificial meiotic DSBs. Limited γ -H2AX signals appeared 24 hours after Cisplatin treatment, and partial chromosome synapsis was restored as small sections. Recombination proteins (AtDMC1 and AtRAD51) appeared at 72 hours of post-Cisplatin treatment. Also, some bivalents are observed at MI in addition to some small chromosome fragmentations during the meiotic stages. This data indicates that induced artificial meiotic DSBs can undergo a homologous recombination pathway to repair and produce some COs. This thesis appeared a good prove for targeting meiotic DSBs in a specific region on the chromosome, such as the Alcohol Dehydrogenase 1 (ADH1) gene, which is on chromosome 1, and the Transparent Testa 4 (TT4) gene on chromosome 5. XVE-ZFNs inducible plants treated with β -estradiol by paint brushing can be successfully used to produce specific meiotic DSBs in the absence of AtSPO11-2. *A.thaliana* flower buds were treated with β -estradiol at different times to induce DSB by ZFN. The optimal time to localise recombination proteins (AtDMC1 and AtRAD51) and to fix material for observing bivalent at MI was three days. ZFN was highly expressed at 500 μ M of β -estradiol after 72 hours of application. Phosphorylation of γ -H2AX was detected two days after application. Induced DSBs by ZFN partially synapsed with some small polymerisation of AtZYP1. Artificial DSBs could be used by the meiotic recombination machinery and initiate homologous pairing and recombination in that genomic region. Nevertheless, because the sequence recognised by the ZFNs is present in both homologous chromosomes and both sister chromatids, it could produce DSBs in all these regions; it would not be possible for these to be processed by the meiotic recombination machinery and would produce some fragmentation.

CHAPTER SEVEN

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CHAPTER EIGHT

APPENDIX

Appendix 1: Protein sequence for *AtDNAPKcs* for Homo Sapiens (Human)

>NP_008835.5 DNA-dependent protein kinase catalytic subunit isoform 1 [Homo sapiens]
MAGSGAGVRCSLLRLQETLSAADRCGAALAGHQLIRGLGQECVLSSSPAVLALQT
SLVFSRDFGLLVFVRKSLNSIEFRECREEILKFLCIFLEKMGQKIAPYSVEIKNTCTS
VYTKDRAAKCKIPALDLLIKLLQTFRSSRLMDEFKIGELFSKFYGELALKKKIPDTV
LEKVYELLGLLGEVHPSEMINNAENLFRAFLGELKTQMTSAVREPKLPLVLAGCLK
GLSSLLCNFTKSMEEDPQTSREIFNFVLKAIRPQIDLKRYAVPSAGLRLFALHASQF
STCLLDNYVSLFEVLLKWCAHTNVELKKAALSALLESFLKQVSNMVAKNAEMHK
NKLQYFMEQYGIIRNVDSNNKELSIAIRGYGLFAGPCKVINAKDVDFMYVELIQRC
KQMFLTQTDGTGDRVYQMPSFLQSVASVLLYLDTVPEVYTPVLEHLVVMQIDSFP
QYSPKMQLVCCRAIVKVFLALAAKGPVLRNCISTVVHQGLIRICSKPVVLPKGPES
ESEDHRASGEVRTGKWKVPTYKDYVDLFRHLLSSDQMMDASILADEAFFSVNSSSE
SLNHLLYDEFVKSVLKIVEKLDLTLEIQTVGEQENGDEAPGVWMIPTSDPAANLHP
AKPKDFSAFINLVEFCREILPEKQAEFFEPWVYSFSYELILQSTRPLISGFYKLLSIT
VRNAKKIKYFEGVSPKSLKHSPEDPEKYSCFALFVKFGKEVAVKMKQYKDELLAS
CLTFLLSLPHNIIELDVRAYPALQMAFKLGLSYTPLAEVGLNALEEWISIYIDRHV
MQPYYKDILPCLDGYLKTSALSDETENNWEVSALSRAAQKGFNKVVLKHLKKT
NLSSNEAISLEEIRIRVVQMLGSLGGQINKNLLTVTSSDEMMKSYVAWDREKRLSF
AVPFREMKPVIFLDVFLPRVTELALTASDRQTKVAACELLHSMVMFMLGKATQM
PEGGQGAPPMYQLYKRFPVLLRLACDVDQVTRQLYEPLVMQLIHWFTNNKKFES
QDTVALLEAILDGIVDPVDSTLRDFCGRCIREFLKWSIKQITPQQQEKSPVNTKSLF
KRLYSLALHPNAFKRLGASLAFNNIYREFREEESLVEQFVFEALVIYMESLALAH
DEKSLGTIQCCDAIDHLCRIIEKKHVSLNKAKKRRLPRGFPPSASLCLLDLVKWL
LAHCGRPQTECRHKSIELFYKFVPLLPGNRSPNLWLKDVLKEEGVSFLINTFEGGG
CGQPSGILAQPTLLYLGRPFSLQATLCWLDLLLAALCYNTFIGERTVGALQVLGT
EAQSSLLKAVAFFLESIAMHDIIAAEKCFGTGAAGNRTSPQEGERYNYSKCTVVVR
IMEFTTTLLNTSPEGWKLLKKDLNTHLMRVLVQTLCEPASIGFNIGDVQVMAHL
PDVCVNLMKALKMSPYKDILETHLREKITAQSIEELCAVNLYGPDAQVDRSRLAA
VVSACKQLHRAGLLHNILPSQSTDLLHHSVGTLLSLVYKGIAPGDERQCLPSLDLS
CKQLASGLLELAFAFGGLCERLVSLLLNPAVLSTASLGSSQGSVIHFSHGGEYFYSLF
SETINTELLKNLDLAVLELMQSSVDNTKMVSAVLNGMLDQSFRERANQKHQGLK

LATTILQHWKKCDSWWAKDSPLETKMAVLALLAKILQIDSSVSFNTSHGSPFEVFT
TYISLLADTKLDLHLKGQAVTLLPFFTSLTGGSLEELRRVLEQLIVAHFPMQSREFP
PGTPRFNNYVDCMKKFLDALELSQSPMLLELMTEVLCREQQHVMEELEFQSSFRRI
ARRGSCVTQVGLLESVYEMFRKDDPRLSFTRQSFVDRSLLTLLWHCSLDALREFFS
TIVVDAIDVLKSRFTKLNSTFDTQITKKMGYYKILDVMMYSRLPKDDVHAKESKIN
QVFHGCITEGNELTKTLIKLCYDAFTENMAGENQLLERRRLYHCAAYNCAISVIC
CVFNELKFYQGFLFSEKPEKNLLIFENLIDLKRRYNFPVEVEVPMERKKKYIEIRKE
AREAANGSDSGPSYMSSLSYLADSTLSEEMSQFDFSTGVQSYSSYSSQDPRPATGRF
RRREQRDPTVHDDVLELEMDLNRHECMAPLTALVKHMHRS LGPPQGEEDSVPR
DLPSWMKFLH GKLG NPIVPLNIRLFLAKLVINTEEVFRPYAKHWLSPLLQLAASEN
NGGEGIHVMVVEIVATILSWTGLATPTGVPKDEVLANRLLNFLMKHVFHHPKRAVF
RHNLEIIKTLVECWKDCLSI PYRLIFEKFS GKDPNSKDNSVGIQLLGIVMANDLPPY
DPQCGIQSSEYFQALVNNMSFVRYKEYVAAAAEVLGLILRYVMERKNILEESLCE
LVAKQLKQH QNTMEDKFIVCLNKVTKSFPPLADRFMNAVFFLLPKFHGVLKTLCL
EVLVLCRVEGMTELYFQLKSKDFVQVMRHRDDERQKVCLDIYKMMPKLKPVELR
ELNPNVVEFVSH PSTTCREQMYNILMWIHDNYRDPES ETDNDSQEIFKLAKDVLIQ
GLIDENPGLQLIIRNFWSHETRLPSNTLDRLLALNSLYSPKIEVHFLSLATNFLLEMT
SMSPDYPNPMFEHPLSECEFEY TIDSDWRFRSTVLT PMFVETQASQGT LQTRTQE
GSL SARWPVAGQIRATQQQHDFTLTQTADGRSSFDWLTGSSTDPLVDHTSPSSDSL
LFAHKRSERLQRAPLKSVGPDFGKKRLGLPGDEV DNKVKGAAGRTDLLRLRRRF
MRDQEKLSLMYARKGVAEQKREKEIKSELKMKQDAQVVL YRSYRHGDLPDIQIK
HSSLITPLQAVAQRDP IIAKQLFSSLFSGILKEMDKFKTLSEKNNITQKLLQDFNRFL
NTTFSFFPPFVSCIQDISCQHAALLSLDPAAVSAGCLASLQQPVGIRLLEEALLRLLP
AELPAKRVRGKARLPPDVLRWVELAKLYRSIGEYDVLRGIFTSEIGTKQITQSALL
AEARSDYSEAAKQYDEALNKQDWVDGEPTAEKDFWELASLDCYNHLAEWKSL
EYCSTASIDSENPPDLNKIWSEPFYQET YLPYMIRSKLKL LLQGEADQSLLTFIDKA
MHGELQKAILELHYSQELSLLYLLQDDVDRAKY YIQNGIQSFMQNYSSIDVLLHQ
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QGCSEQVLTVLKTVSLLDENNVSSYLSKNILAFRDQNILLGTTYRII ANALSSEPAC
LAEIEEDKARRILELSGSSSEDSEKVIAGLYQRAFQHLSEAVQAAEEEAQPPSWSCG

PAAGVIDAYMTLADFCDDQQLRKEEENASVIDSAELQAYPALVVEKMLKALKLNS
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MSGKHDVGAYMLMYKGANRTETVTSFRKRESKVPADLLKRAFVRMSTSPEAFLA
LRSHFASSHALICISHWILGIGDRHLNFMVAMETGGVIGIDFGHAFGSATQFLPVP
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LGHEKAPAFRDYVAVARGSKDHNIRAQEPESGLSEETQVKCLMDQATDPNIGRT
WEGWEPWM

Appendix 2: Protein sequence for *DNA-PKcs* in *Dictyostelium discoideum*

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LKSLNLSLFKFSEKLMVVKIENIDEITLHLSSFINAFACILYELNELEFWYLDHIEQV
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SDFTKLLFDKPLSECTFKEVQVDSSWQHRTLNMNPLFSSSQYGIDDAT TDESNGDS
MQLDEIRATQAPNFTLTQTAFSEFY PSSSQSYGGTNNNTGSSQLSSSSSSSSGSQSSS
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ALYKNTPKESIRTFQCNIKKLIDSIVERCKFNSTLVSSILNISEKNLEFSPEFSKIKDIS
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TQWKS RFPTKLDDIMVWDDLIFYRSVLLEKIYERFSSFTSDKSSDEKVKSTLIQERA
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SPSPIETLDRFVKALKFIESKKDEESIINSNENLQKYLMMHGDIFWDIYQLDKKLG S
TLVIDSFKKNSLPINLTSIPINSLKDELFNSTFKCYSESIKLHQKSTNLLNTTSSSPSLS
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QSTDPNILSRAWVGWNGAL

Appendix 3: Full genomic DNA for the putative AtDNA-Pka in *Arabidopsis thaliana*

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>SALK_096167.53.55.x
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GGTCTCAGGTGATTTTTTATACCATGAATTGCAGCCCGGGC
SALK_096167 PRODUCT SIZE 1027 BP+RP_PRODUCT_SIZE 460-760
N666820_LP GGTCCTACCTCTTTGGTTGG Length 21 TM 59.85 GC
52.38
N666820_RP TCATTTTCGTGCACAGTGATTC Length 21 TM 59.71 GC
42.86

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AtDNA-PKa gene is 17,951bp long. Exons are shown in dark blue boxes. The left primer (LP) for T-DNA insertion genotyping is represented in a turquoise colour. The right primer (RP) is represented in a pink colour. The T-DNA insertion location is represented in a yellow box.

Appendix 4: Full genomic DNA for putative DNA-PKb in *Arabidopsis thaliana*

CGTTGCACTGCTACGCTCGAGTTCTCAAAGGTTCTCCTCGCTTTCTCTTTTCAGGGGAGTCTCGCAGA
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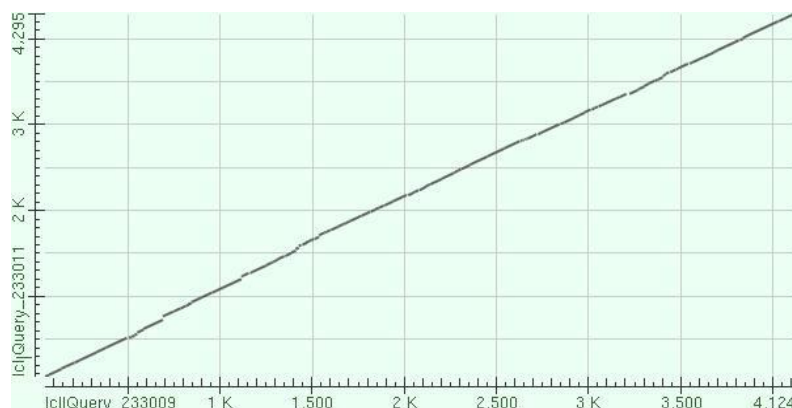
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N655803_LP TTGAAGATGGTCTTCATTGCC Length 21 TM 60.07 GC 42.86

N655803_RP GCCAACGGATACCTATCAGTG Length 21 TM 59.47 GC 52.38

Appendix 5: Comparison of a Human Protein Sequence with Dictyostelium discoideum Protein Sequence (NCBI)

DNAPKcs human vs DNAPKcs Dicty



Range 1: 63 to 4295 Graphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
1457 bits(3772)	0.0	Compositional matrix adjust.	1244/4405(28%)		
	2073/4405(47%)		503/4405(11%)		

Query 51 LALQTSLVFSRDFGLLVFVRKS--

LNSEFRECREEILKFLCIFLEKMGQKIAPYSVEIK 108

+ L SL+F+ D LL ++ KS +++ E ++ IL + F+E ++ Y++ IK

Sbjct 63

IGLVASLLFTGDHSLKYLEKSSTISNKENVKIKVSILNLIAEFIEILQVRTDDYAIAI K
122

Query 109 NTCTSVYTKDRAAKCKIPALDLLIKLLQTF-----

RSSRLMDEFKIGELFSKFYGELALK 163

NTC V+ KD+++ A + K+L + S ++ F + E+ F + Sbjct

123

NTCVLVFRKDKSHSVQAAAFGPIKKILHLMSRVLKQGSIVAESFGVSEMTQLFLLQ
FTCG 182

Query 164

KKIPDTVLEKVVYELLGGLGEVHPSEMINNAENLFRAFLGELKTQMTSAVREPCLPV
LAGC 223

K + TV ++ +LGL E S M + + L F+ L Q+ S + P+ ++ C

Sbjct 183 K-LTQTVRGEIIVVLGLFTEYFSSNMCDRNQQLSFIFMETLGGQLRS--
KSPESTLIQSC 239

Query 224 LKGLSSLLCNFTKS-

MEEDPQTSREIFNFVLKAIRPQIDLKRYAVPSAGLRLFALHASQF 282

LKGL+SLL +F++ + DP+ + I+ +V + P +R+ +P A +++ A HA

Sbjct 240

LKGLNSLLVHFSGDFIASDPKNVQLIYQYVYICLDPASSTQRFEIPRAAMKIVARHA
VLL 299

Query 283 STCLLDNYVSLFEVLLKWCAHTNVELKKAALSALESFLKQVSNMVA---
KNAEMHKNKLQ 339

L + + + + WC H N + A +A++SF +Q+S + + + E + + + Sbjct

300

RQYLAEHSQPFYTRIEHWCNHINKLNRDIAFTAVDSFFQQISKELTSGNRSLEADQS
TFK 359

Query 340 YFMEQFYGIIRNVDSNNKELSAIRGYGLFAGPCKVINAK-
DVDFMYVELIQRCKQMFLT 398

+F+ +FY I N +S+ ELSIAIRG G FA P K + + + + L + + + + +

Sbjct 360

FFIRKFYSIFENNNSSRFELSIAIRGCGRFASPVKSFMGEWELKSLLNSLFKFSEKLM
VV 419

Query 399

QTDGDDRVYQMPNFLQSVASVLLYLDTPVEVYTPVLEHLVVMQIDSFPQYSPKM
QLVCC 458

+ + D + + SF + + A +L L + + Y +E + + FP K + Sbjct 420

KIENIDEITLHLSSFINAFACILYELNELEFWYLDHIEQVLETYFIIFPHLFVKSRDRY
F 479

Query 459

RAIVKVFLALAAKGPVLRNCISTVVHQGLIRICKPVVLPKGPESSEDHRASGEVR
TGK 518

+A + + + +L G L + +S V +GL + SKP + + T

Sbjct 480 KAVNRLISSLFYHGEYLLKILLSRFVKKGLLITFSKP-----
NDSIKNLITTAN 527

Query 519 WKVPPTYKDYVDLFRHLLSS-----DQMMDSILADEAF-----
FSVNSSSE 558

P Y + Y DL + +LL + D + + +S + E + S +

Sbjct 528 --

TPFYQVYKDLWYNLLNPTIDTIDKDLNSTIKKENNNNNNNNNKNNNNNNNQTLTKE
EISK 585

Query 559 SLNHLLYDEFVKSVLKIVEKLDLTLE-----
IQTVEGEQENGDEAPGVWMIPTSDPA 609

S + L +YDE V S +L I + +KLDL + + N + + +I

Sbjct 586

SIKKLVYDEIVSSILSIKKLDLQYQKDENDNNSNSNSNNNNNNNDQDNNKISIHYES
EI 645

Query 610

ANLHPAKPKDFSAFINLVEFCREILPEKQAEFFEPWVYSFSYELILQSTRPLISGFY
KL 669

L P KD F + LVEF + + + E F W +Y F E +I S + PLISGFYKL

Sbjct 646

NQLKPVTSKDIELFLGLVEFFKLFISKYNTSELFVQWIYIFGKEMISFSKKYPLISGFY
KL 705

Query 670 LSITVRNAKKIKYFEGVSPKS----- 690

+ I ++ K KYF +

Sbjct 706

VQIVMQICKSQKYFNELQQDDNINSVIFNNQIIDEQLQQQQDIMDIDDTDNNSNSN
NKIN 765

Query 691 --

LKHSPEDPEKYSCFALFVKFGKEVAVKMKQYKDELLASCLTFLLSLPHNIIELDVR
AY 748

L +S E+ +K +CF LF K+ KEV++ YKDELL+SC+ LLSLP +I ++

Sbjct 766 KKLTNSLEE-

DKNNCFILFKKYIKEVSNFISHYKDELLSSCIELLSLPKQLISIPL--L 822

Query 749

VPALQMAFKLGLSYTPLAEVGLNALEEWISIYIDRHVMQPYKIDILPCLDGYLKTS
ALSDE 808

VP +AF+ G+SY PLA VGLNA+E W++ + V ++ +LP L+ YLK S S +

Sbjct 823 VPIANLAFQYGISYPLAHVGLNAIEYWNLVVPDQVHK-

HLDQLLPSLNDYLYKISTNSSD 881

Query 809 TKNNWEVSALSRAAQKGFNKVV-----LKHLKKTKNLSSNEAI-----
SLEEIRI 853

+ + + + + G VV +H +K K S++ I S+E+++

Sbjct 882 STSGGDID-IDSGGSMGGGGVPPPPSSSRH-

RKMKFKSNSSLIDPKQLQFKTSIEDLQN 939

Query 854

RVVQMLGSLGGQINKNLLTVTSSDEMMKSYVAWDREKRLSFAVPFREMCPV-
IFLDVFLP 912

R++++LG LGG L V+ E + + VAWD E ++ +PF + + I+LDV LP Sbjct

940

RIIRLLGQLGGDNVHFLGKVSLVGEEIVNGVAWDTEPKVLIKIPFADQNNIDIYLDV
ILP 999

Query 913

RVTELALTASDRQTKVAACELLHSMVMFMLGKATQMPEGGQGAPPMYQLYKRT
FPVLLRL 972

V LA ++RQ KVAA ELLHS+++ M+G + + Q + +LY++ FP L+ L Sbjct

1000 NVVNLAEKSPNRQIKVAAGELLHSVLLMMIGNSANQSQANQIS--

YSRLYRKIFPSLICL 1057

Query 973

ACDVDQVTRQLYEPLVMQLIHWFTNNKKFESQDTVALLEAILDGIVDPVDSTLRD
FCGRC 1032

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Sbjct 1058
STDVEQVTKQLFQPLVFQMIHWFTRNKKQESDDTMNLLNAIVDAVGNPLDGARR
DFAGQC 1117

Query 1033
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REE 1092

+ EF KWS+K + +QQEK+ N KS+ KR+YSLA HP+ +KRLGA+++FN +YR
FREE
Sbjct 1118
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FREE 1177

Query 1093 ESLVEQFVFEALVIYMESLALAH-----ADEKSLGTI
1124

+SLV+QF+FE L SL + E S+ I
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1237

Query 1125 -
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CGRPQTE 1182

+++ L ++I K+ LNK R L V+W++ C R ++
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Query 1183
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R++S+ LF + V LLP ++P W+K+ +K G+ F+++ E G G G T L Sbjct
1291
VRYESMILFTQLVTLLPNIKTPLQWVKERIKSNGIQFIVSIAEQGGGVGGGSSNGN
TSL 1350

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+ + W L +L Y F E +Q+L + E +SSLL A F
Sbjct 1351
GRIPKIEIGKHKDIEFWFRNLSTSLNIYLWFFSEGFFEPIQILTSREFKSSLLSSAFHTF
1410

Query 1298 LESIAMHDIIAAEKC-----
FGTGAAGNRTSPQEGERYNYSKCTVVVRIMEFTTTLLNTS 1352
+M ++ K ++ +P+E +++N KC V+ + LL

Sbjct 1411
TTQFSMLNFNSNLKTNQQQQQQQVSMFSSMTPKEIDQFNRLKCNVITNLFGLYILL
L--- 1467

Query 1353 PEGWKLLKK-DLCNTHLMRVLVQTLCEPASIGFNIG-----
DVQVMAHLPDVCVNLM-KA 1405

E +K + D +++LV+ L +P S+GF +V++ NLM +

Sbjct 1468 -
EKYKFEQVIDYGGVAFVQLLVKCLMDPQSVGFVNNYSEEREVELSKSSRQDIRNL
MSRT 1526

Query 1406 LKMSP-----YKDILETHLREKITAQS-----I 1428
K P Y DIL L E I + I

Sbjct 1527
FKFPPLLEIINNIKLLIQIKPKYNDILHKELMEYILPNNSNNNNNSSSSGNNKSMFSL
I 1586

Query 1429 EELCAVNLYGPDAQVDRSRLAAVVSACKQLHRAGLLHNIL----PSQS-
---TDLHHSVG 1480

+ +N G D RL + + + + L++ LL +IL QS DL S

Sbjct 1587 DIRQLINTEGTD-----
RLIHLHSYQILYKYNLLDSILLENYNEQSEQGIQDLPKS-S 1639

Query 1481
TELLSLVYKGIAPGDERQCLPSLDLSCKQLASGLLELAFAFGGLCERLVSLLLNPA
V--- 1537

E ++ + E PS L K+L ++LA G +L+SL++NP V Sbjct

1640 LEFSEFIFDYLFNNCENLS-PSKLLISKEL----
IQLALTIGIKPIKLLSLIINPNVQNT 1694

Query 1538 -----
LSTASLGSSQGSVIHFSHGGEYFYSLFSETINTELLKNLDLAVL 1580

ST + + I H ++ +LF+E IN + N L L

Sbjct 1695 TTTTTTTSTTTTTTTTTSTNNNDNNNEFIKDIHDIFYKTLFTE-
INNYISNNFQL-FL 1752

Query 1581
ELMQSSVDNTKMVSAVLNGMLDQSFRERANQKHQGLKLATTILQHWKKCDSW
WAKDSPLE 1640

L+ + + T + VLN D ++ Q ++G K + + S W +S L+ Sbjct

1753 PLLANRIIETNQIHKVLN---
DVCIMKQQQQPNRGFKDIIESIVLFLNSISEWTLNSNLK 1809

Query 1641 --
TKMAVLALLAKILQIDSSVSFNTSHGSFPEVFTTYISLLADTKLDLHLKGQAVTLLP
F 1698

K ++ +++ID F H+ V+ + L + L K ++ LLP+

Sbjct 1810 LVEKENIIEFTKNLIKIDPIQFFENKH-CYEFVYNIITNYLCRSN-
PLTFKNKVLVLLPY 1867

Query 1699 FTSL-
TGGSLLEELRRVLEQLIVAHFPMQSREFPPGTPRFNNYVDCMKKFLDALELSQSPM
1757

S + + + + L + + + V FP+ S + + + P + N Y+ C + + + L+ EL + + + P +

Sbjct 1868
LLSYPKHNNFDLIKQKLNEIVVYDFPLNSKDLTVKSPIYNEYITCIERLLET FELTKN
PL 1927

Query 1758
LLELMTEVLCREQQHVMEELEFQSSFRRIARRGSCVTQVGLLESVYEMFRKDDPRL
SFTRQ 1817

+ + + + VL + E H S R + + + + E + F L T

Sbjct 1928 VIDTLLHVL-
KETDHTHINYINLSIERSILSTNDIEAKEIFSHCFELFLNHYDELKIT-- 1984

Query 1818 SFVDRSLLTLLWHCSLDALREFFS---
TIVVDAIDVLKSRFTKLNSTFDTQITKKMGYY 1874

+ D + + L + H + L + FS + + + I L + + + + + I + K + +

Sbjct 1985 -LIDKFCVPLISHMKENILVQIFSQHLSSLMSIIPQLPKYLSDSQER-
KSSIIEKICCF 2042

Query 1875
KILDVMYSRLPKDDVHAKESKINQVFHGSCITEGNETKTLIKLCYDAFTENMAGE
NQLL 1934

+ + + + Y LP + + KIN F+ + G ELT + + K + A + E + + + +

Sbjct 2043 HLIEALYQSLPSAII---
KEKINPFFYDKPDGKGTELTAAIMKAAHSAKSEKLTSDDKYV 2099

Query 1935 ERR--
RLYHCAAYNCAISVICCVFNELKFYQGFLFSEKPEKNLLIFENLIDLKRRYNFPV
1992

R YH AA + + SVI + F + F F E + KN + + EN + ID + + NF Sbjct

2100
TRSLCTKYHGAAFSALASVIVSTQTKENFFHVFFFKENKDKNEYLWENIIDTDQVF
NFQP 2159

Query 1993
EVEVPMERKKKYIEIRKEAREAANGSDGPSYMSLSYLADSTLSEEMSQFDFSTG
---V 2049

E + + R Y + SS YL DS + LS + + + F G

Sbjct 2160 ETNFLVSYSTPQSLFANDLR-----YLSS-
QYLVDSLSQDFIAKNFLEGDNNS 2207

Query 2050

QSYSSYSSQDPRPATGRFRRREQRDPTVHDDVLELEMDELNRHECMAPLTALVKH
MHRS-- 2107

+++D G++++ +D +E+D++N + M +++ +

Sbjct 2208 TTNNNNGDDGIIMQGGQSQSGDNENQISND----
IEIDQVNSNPSPMIAMLKIIDFYQKKFV 2263

Query 2108 LGPPQ----GEEDSVPR----

DLPSWMKFLHKGKLGNIPLNIRLFLAKLVINTEEVFRP 2159

+ PP G++ P+ D+P WM ++ KL + P NI +F+ K++IN + F

Sbjct 2264 VTPSSILFGQQSQQPKPTVGDMPKWMFEIYQKLQQGVHP-
NIVIFMIKIIINRPQYFER 2322

Query 2160 YAKHWLSPLLQLAASENNGGEGIHVMVVEIVATILSWTGLATPT---
GVPKDEVLANRLL 2216

++ W+ L+ SE NGG G+HY V ++ +L W + G E +++

Sbjct 2323
FHELWIPILMDYVVSEGNNGNGLHYFVRDVCFILLKWPNIYKSQDGGGGTTLEKH
LSKFV 2382

Query 2217 NFLMKHVFHPKRAVFRHNLEIHKTLVECWKDCLSIPYRLIFEKFSGK-
DPNSKDNSV--- 2272

N LMK++ R ++ NL I+K+ VE WK + +I E S K D SK +

Sbjct 2383
NHLMKNTYCTDRRILKSNLNIVKSFVERWKSFLKVDKSIILELLSTKGDFKSKSRQI
KTT 2442

Query 2273 GIQLLGIVMANDLPPYDPQCG---

IQSSEYFQALVNNMSFVRYKEYVAAAAEVLGLILRY 2329

G+ LL I+++N P YD + I +++Q L++++ +KE+Y AA+EV G+IL Y Sbjct

2443 GLVLLSILLSNGYPAYDKEYDSNTISEFKFYQVLLDHLQ--
DFKELYDAASEVCGMILLY 2500

Query 2330 VMERKNILEESLCELVAKQLKQHQNMTM-----

EDKFIVCLNKVTKSFPLADRFMNAVFF 2384

+ +++ ++ ++ LK N++ + CL + +P F +F Sbjct 2501

LSKQQQSQQQQQQSIFPQLLKDKINSILNNNENQRAFSCLYFIGLHYPLFLQGFYGK
IFN 2560

Query 2385

LLPKFHGVLKTLCLEVVLCRVEGMTELYFQLKSKDFVQVMRHRDDERQKVCLDII
YKMMP 2444

LLP+ + + L ++ VE + +LY +LKS + ++R R+ E Q V L I+YK++

Sbjct 2561
LLPQISNETRLIALNIIHWCVEDIQDLYTKLKSNNIENLIRIREGEIQVVILRILYKLV Q
2620

Query 2445 KLKPV--
ELRELLNPVVEFVSHPSTTCREQMYNILMWIHDNYRDPESETDNDSQEIFKLA
2502

K + ++L+ ++ S E ++ I + E + S E K Sbjct 2621

KRDTTFGTVNQILSLLLSNNWFTSNVTNEYSRSLYYDIIYIYNNFIEYQDQSNENS
KDL 2680

Query 2503 KDVLIQGLIDENPGLQLIIRNFWSHETRLPSNTLDRLLA--
LNSLYSPKIEVHFLSLATN 2560

L+ GL DE+ + + FW + ++ S + ++ L + +YSP+ E +++ +

Sbjct 2681

VLSLLIGLSDESDESINKKLLQFWDNNSKTLSSASSNQRLQQFFDIMYSPETESKWVA
NSCC 2740

Query 2561

FLLEMTSMSPDYPNPMFEHPLSECEFQEYITIDSDWRFRSTVLTMPFVETQASQGTL
QTRT 2620

L ++ + S D+ +F+ PLSEC F+E +DS W+ R+ + P+F +Q T Sbjct

2741

LLFQLCNRSSDFTKLLFDKPLSECTFKEVQVDSSWQHRTLNMNPLFSSSQYGIDDA
TTDE 2800

Query 2621

QEGSLSARWPVAGQIRATQQQHDFTLTQTADGRSSFWDWLTGSSTDPLVDHTSPSS
DSLLF 2680

G +IRATQ +FTLTQTA F SS+ + +++++

Sbjct 2801 SNGDSMQ----LDEIRATQAP-NFTLTQTA-----FSEFYPPSSSQ---
SYGGTNNNTGSS 2847

Query 2681 AHKRSERLQRAPLKSVGPDFGKKRLGLPGDE--

VDNKVKGAAGRTDLLRLRRRFM----R 2734

S + S K+++D ++N LRRRF R

Sbjct 2848 QLSSSSSSSGSQSSSQNNSSSKRKQKITNDPKLINNS-----
ELRRRFKKIDDR 2896

Query 2735

DQEKLSLMYARKGVAEQKREKEIKSELKMKQDAQVVLYRSYRHGDLPDIIQIKHS
SLITPL 2794

++ + +AR V + + K ++ Q+ + R YR G+LPDIQIK +I PL Sbjct

2897

GADERIVKFARLQVKRNIEREAYFEKSKQARENQITMIRKYRVGELPDIIQIKLQDII
KPL 2956

Query 2795

QAVAQRDPPIAKQLFSSLFSGILKEMDKFKTLSEKNNITQKLLQDFNRFLNTTFSFFP
PF 2854

Q + QRD I +FSSLF+ + K K + + NI + + R F

Sbjct 2957 QLLCQRDSTIGVNVFSSLFTALYKNTPKESIRTFQCNIKKLIDSIVER-----
CKFNSTL 3011

Query 2855

VSCIQDISCQHAALLSLDPAAVSAGCLASLQQPVGIRLLEEALLRLLPAELPAKRVR
GKA 2914

VS I +IS ++ S + + + L S P + I L E + + L L + + +

Sbjct 3012 VSSILNISEKNLE-

FSPEFSKIKDISLNSNNHPMAILCEKLILSLQNTTKASTTNQAQK 3070

Query 2915 RLPPDVLR--WVELAKLYRSIGEYDVLRGIFTSEIGTK-
QITQSALLAEARSDYSEAAKQ 2971

L W L +LY+S+ E D++ G+ ++G T+ AL E + D+ K

Sbjct 3071

LQQQQQLNQGWDSLRELYKSLKEDDIIMGLIEKQMGGDIPYTKKALEFELKGDW
VNVLKV 3130

Query 2972 YDEALNK-----

QDWVDGEPTAEKDFWELASLDCYNHLAEWKSLEYCSTASIDSEN-- 3024

YDEA +K + G +E+E WE L+C+ L W +L+ D+ N

Sbjct 3131 YDEATSKLESGELNQYQGNLSESETALWENGRLECFTKLCNWSALK--
-----DNFNSYY 3183

Query 3025

PDLNKIWSEPFYQETYPYMIRSKLKLQLQGEADQSLLTFIDKAMHGELQKAILEL
HYSQ 3084

P+ N+I+ E + L Y LK+ E L FI ++Q LE +

Sbjct 3184 PNPNQIFKEK-NCDLLLSYFFEFNLKV---KENWNYLYQFIADLANTK-
QYQYLENKFP 3238

Query 3085

ELSLLYLLQDDVDRAKYIYQNGIQSFMQNYSSIDVLLHQSRLTKLQSVQALTEIQE
FISF 3144

EL+ L + + D ++A YY+ Q+F +SS L +SR LQ +Q + E++EF++ Sbjct

3239

ELAFLEVTRSDHNKASYVSKFYQAFKHQWSSSHPLAIESRHRILQPLQKIVEVEE
FLNL 3298

Query 3145

ISKQGNLSSQVPLKRLNTWTNRYPDAMDPMNWDDIITNRCFFLSKIEEKLTPLP
EDN 3204

S + + + L LL W +R+P K+D + +WDD+I R L KI E+ + D

Sbjct 3299 TSTP--IINTLKLDTLLTQWKS RFP-

TKLDDIMVWDDLIFYRSVLLEKIYERFSSFTSDK 3355

Query 3205

SMNVDQDGDPSDRMEVQEQEEDISSLIRSCKFSMKMKMIDSARKQNNFSLAMKL
LKELHK 3264

S +E + S + + + KM ARK N ++ ++ K Sbjct 3356 S-----

SDEKVKSTLIQERAILYHKMSKGARKLGNIVVSEIYFRQAVK 3398

Query 3265 E-SKTRDDWL-----VSWVQSYCRLSHCRSRSQGCSEQVLTVLKTV-----
-SLLDEN- 3310
KT+D+ L S + YC + ++ + LK + S+++ N
Sbjct 3399
SYPKTKDNDLAFPLVTSLIDIYCTKARNSPSPIETLDRFVKALKFIESKKDEESIINSN E
3458

Query 3311 NVSSYLKSNILAFRDQNIL---
LGTTYRIIANALSSEPACLAIEEDKARRILELSGSSS 3367
N+ YL + F D L LG+T I + +S P L I + + EL S+
Sbjct 3459
NLQKYLMMHGDIFWDIYQLDKKLGSTLVIDSFKKNSLPINLTSIPINSLKD--
ELFNSTF 3516

Query 3368 EDSEKVIAGLYQRAFQHLSEAVQAAEEEAQPPSWSCG-----
PAAGVID 3411
+ + I L+Q++ L+ + S
Sbjct 3517 KCYSESIK-
LHQKSTNLLNTTSSSPSLSISSSSSPYSSTSSSSQPKLSNEILIEKTKTKS 3575

Query 3412 AYMTLADFC-----
QQLRKEENASVIDSAELQAYPALVVEKMLKALKLNSNEARL 3463
A++ A+FCD QQ + N VID A V+ L+A+K + Sbjct 3576
AHLKFANFCDNILKDKISQQTPTPTQFNEEVIDLA-----
TSVIVSTLEAIKEEIPGSVD 3629

Query 3464 KFPRLLQIERYPEETLSLMTKE----
ISSVPCWQFISWISHMVALLDKDQAVAVQHSVE 3519
KFPRLL+I+ ++ E S++ +E +S++PCW FI WIS M LD Q + +
Sbjct 3630 KFPRLLEILTQF--
EQYSVIAREFKSRVSTIPCWMFIRWISQMFYLDLPQGPLILPILL 3687

Query 3520
EITDNYPQAIYYPFIISSESYSFKDTSTGHKNKEFVARIKSKLDQGGVIQDFINALDQ
LS 3579
EI YPQAI +PF ISSE + G K+ A ++ +L ++ + +L+ Sbjct 3688
EIAKWYPQAIYFPFKISSEQF-----GPLAKKISAPLEKEL-
INPLDTLVIEFQRLT 3739

Query 3580
NPELLFKDWSNDVRAELAKTPVNKKNIEKMYERMYAALGDPKAPGLGAFRRKFI
QTFGKE 3639
+PE FKD+ +++++ + TP + I K+ +Y +P G + KF +

Sbjct 3740 HPEHRFKDYMEEMKSLIKATPKDLNAIAKLNNEIYDDSFNPSTVS-
GDYNLKFKEHEAS 3798

Query 3640 FDKHFGKGGSKLLRMKLSDFNDITNMLLLKMNKDSKPPGN----
LKECSPWMSDFKVEFL 3695

++FGK +KL RM F +I + MNK+ KP LK+ S W++DF Sbjct
3799
YLSNFGKDSTKLARMDQKKFLEIFAEMSGNMKNMKPNSTASMKLKDFSGWLA
DFDRSNY 3858

Query 3696 R--
NELEIPGQYDGRGKPLPEYHVRIAGFDERVTVMASLRRPKRIIRGHDEREHPFLVK
3753

+ +++EIPGQYDG GKP PE HV I+ FD V VM SLR+PKR+ I G+DE ++PFL+K
Sbjct 3859
QTSHQMEIPGQYDGIGKQPETHVTISSFDTNVLMGSLRKPKRVKIHGNDELDP
FLIK 3918

Query 3754
GGEDLRQDQRVEQLFQVMNGILAQDSACSQRALQLRTYSVVPMTSRLGLIEWLE
NTVTLK 3813

GGEDLR DQR++QLF +MN IL +D+AC++R+L + TY VVPMTS++G+IEWL +T
L+
Sbjct 3919
GGEDLRDQRIQQFLGIMNEILKRDTACNKRSNLVTTYQVVPMTSKVGIIEWLND
TKPLR 3978

Query 3814 DLLLNTMSQEEKAAYLSDPRAPPC-----EYKDWLTKMSG----
KHDVGA-YMLMYK 3860

++L +E+ A L PR+ + DW+ + VG Y M+
Sbjct 3979 EIL-----
EEQLAHQLKTPRSNVSISKLESTKYHNDWINSFAKYLKPNSPVGPLYQQMFI 4033

Query 3861
GANRTETVTSFRKRESKVPADLLKRAFVRMSTSPEAFLALRSHFASSHALICISHWI
LGI 3920

A R + K+ SKVP +LL+ +S+SPE++L +R+ FA S A + +++GI Sbjct
4034
HATRDDCAKKLEKQHSKVPENLLQNGIWSLSSSPESYLFIRNSFARSLASFSVCSY
VIGI 4093

Query 3921
GDRHLNFMVAMETGGVIGIDFGHAFGSATQFLPPELMPFRLTRQFINLMLPMK
ETGLM 3980

GDRHL NF+++ G +IGIDFGHAFG+ATQFLP+PELMPFRLTRQF + + P+ GL+
Sbjct 4094
GDRHLENFLISQRDGRLLIGIDFGHAFGTATQFLPIPELMPFRLTRQFTSFLRPLDSVG LL
4153

Query 3981

YSIMVHALRAFRSDPGLLTNTMDV FVKEPSFDWKNFEQKMLKKGGSWIQEINVA
EKNWYP 4040

M + L A ++ +L TMDV FVKEP DW +++K+ G ++ + W+P Sbjct

4154

NHNMTYTLTALQNQKEILLTTMDV FVKEPLLDWSKLATRLVKEQGKHPKD---
TKNVWFP 4210

Query 4041 RQKICYAKRKL AGANPAVITCDELLLG-

HEKAPAFRDYVAVARGSKDHNIRAQEPESGLS 4099

+QKI AK+KL NPA IT +EL H P + + +G HNIRA+ + S Sbjct

4211

KQKIQA KKKLELVNPAYITLEELSGSVHSGLPYEKALSEIHKGDPKHNIRAKVNKV
CSS 4270

Query 4100 EETQVKCLMDQATDPNILGRTWEGW 4124

+ Q+ CL+DQ+TDPNIL R W GW

Sbjct 4271 VKEQIDCLIDQSTDPNILSRAWVGW 4295

Appendix 6: Comparison of a DNAPKcsa Protein Sequence with DNAPKcsb Protein Sequence (NCBI)

DNAPKcsa vs DNAPKcsb

Range 1: 1 to 3839GraphicsNext MatchPrevious Match

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
6784 bits(17601)	0.0		Compositional matrix adjust.	3343/3879(86%)	
	3532/3879(91%)		124/3879(3%)		

Query 1

MSPIQNFEQHSRRLVDLDLPIPTRLEMVVEVRDSLEIAHTAEYLNFLKCYFPAFSVI
LLQ 60

MSPIQNFEQHSRRLV+ DLPI RL MVVEVRDSLEI HTAEYLNFLKCYF A
SVILLQ

Sbjct 1

MSPIQNFEQHSRRLVEPDLPIERLAMVVEVRDSLEITHTAEYLNFLKCYFRASSVI
LLQ 60

Query 61

ITKPQFIDNPEHKLRNIVVEILNRLPHSEVLRPFVQDLLKVAMQVLTADNEENGLIC
IRI 120

ITKPQF DN EHKLRNIVVEILNRLPHSEVLRPFVQDLLKVAMQVLT
DNEENGLICIRI

Sbjct 61

ITKPQFTDNIEHKLRNIVVEILNRLPHSEVLRPFVQDLLKVAMQVLTADNEENGLIC
IRI 120

Query 121

IFDLLRNFRPTLENEVQPFLDFVCKIYSIFRFTVSHFFDNVKMEEVKPMEMPTSSDQ
SLT 180

IFDLLRNFRPTLENEVQPFLDFVCKIY FR TVSHFF+NVKMEEVKP+E+PT
SDQSL+

Sbjct 121

IFDLLRNFRPTLENEVQPFLDFVCKIYQNFRLTVSHFFENVKMEEVKPVEIPTPSDQ
SLS 180

Query 181

PTPPIGNVQLNPSTRSFKIITESPLVVMFLFQLYSRLVQTNIPHLLPLMVAAISVPGPE
N 240

T P N Q+NPSTRSFKI+TESPLVVMFLFQLYSRLVQ
NIP+LLPLMVAAIS+PGPE

Sbjct 181

ITAPSRNGQINPSTRSFKIVTESPLVVMFLFQLYSRLVQINIPNLLPLMVAAISIPGPE
K 240

Query 241 VPSHLKPQFIELKGAQVK----- 258

V SH+KPQFIELKGAQVK

Sbjct 241

VSSHMKPQFIELKGAQVKTVSFLTYLLKSCAEYIKPHEESICKSIVNLLVTCSDSASI
RK 300

Query 259

ELLVSLKHVLGTDFKRGLFPLIDTLLDERVLVGTGRACFESLRPLAYSLLAEIVHH
VRGD 318

ELLVSLKHVLGTDFKRGLFPLIDTLL+ERVVLVGTGRACFESLRPLAYSLLAEIVHHV
R D Sbjct

301

ELLVSLKHVLGTDFKRGLFPLIDTLLLEERVVLVGTGRACFESLRPLAYSLLAEIVHHV
RAD 360

Query 319

LSLAQLSRIIYLF SRNMHDSTLSLSIHTTCARLMLNLVEPIFEKGVDQQSMDEARIL
LGR 378

LSL+QLSRIIYLF SRNMHDSTLSL+IHTTCARLMLNLVEPIFEKG+DQQSMDEARILL
GR

Sbjct 361

LSLSQLSRIIYLF SRNMHDSTLSLNIHTTCARLMLNLVEPIFEKGIDQQSMDEARILL
GR 420

Query 379

ILDAFVGKFSTFKRTIPQLLEEGEVGKDRVTLRSKLELPVQVPVEHSKEVNDCKNLI
KTL 438

ILDAFVGKF+TFKRT+PQLLEEG+

GKD++TLRSKLELPVQVP EHSKEVNDCKNLIKTL

Sbjct 421 ILDAFVGKFNTFKRTVPQLLEEGD-

GKDQITLRSKLELPVQVPAEHSKEVNDCKNLIKTL 479

Query 439 VMGMKTIIWSITHAHLPRPQG-----

FKGMREDEVWKASGVLKSGVHCLA 483

VMGMKTIIWSITHAHLPRPQG

FKGMREDEVWKASGVLKSGVHCLA

Sbjct 480

VMGMKTIIWSITHAHLPRPQGMHPQALASQSSVTQVFKGMREDEVWKASGVLKS
GVHCLA 539

Query 484

LFKEKDEEKEMLNLF SQILAIMPRDLMDMFSLCMPELFESMINNNQLVQIFAALL
QAPK 543

LFK+KDEEKEMLNLF SQILA+MEPRDLMDMFSCMPPELFE +I+N QLVQIFA

LLQAPK

Sbjct 540

LFKDKDEEKEMLNLF SQILAVMEPRDLMDMFSCMPPELFEIIDNTQLVQIFATLLQ
APK 599

Query 544

VYKPFADVLINLLVSSKLDVLKNPDSAATKLVLHLFRCIFGAVTKTPSDFERILQH
HVPV 603

VYKPFADVLIN LVSSKLDVLKNPDSAATKL+LHLFRC+FGAV+K
PSDFERILQ VP+
Sbjct 600
VYKPFADVLINFLVSSKLDVLKNPDSAATKLILHLFRCLFGAVSKAPSDFERILQPQ
VPL 659

Query 604
IMEVCMKNATEVEKPLGYMQLLRTVFRGLAGCKYELLRLDLIPMLLPCLNILLTM
LEGPA 663

IMEVCMKNATEVEKPLGYMQLLRTVFRGLAGCK+ELLRLDL+PMLLPCLNILLTM
LEGPA
Sbjct 660
IMEVCMKNATEVEKPLGYMQLLRTVFRGLAGCKFELLRLDLVPMLLPCLNILLTM
LEGPA 719

Query 664
GEDMKDLLLELCLTLPARLSSLLPYLPRLMKPLVFCLRGSDDELVSGLRRTLEFWVD
SLNP 723
GEDM+DLLLEL LTLPARLSSLLPYLPRLM+PLV
CLRGSDDELVSGLRRTLEFWVDSLNP Sbjct 720

GEDMRDLLLELSLTLPARLSSLLPYLPRLMRPLVSCLRGSDDELVSGLRRTLEFWVD
SLNP 779

Query 724
DFLEPSMANVMSEVILALWSHLRPVPYPWGKKALQILGKLGGRNRRFLKEPLTLE
CKDNP 783
DFLEPSMA VMSEVILALWSHL+PVPYPWG
KALQI+GKLGGRNRRFLKEPLTLECKDNP Sbjct 780

DFLEPSMATVMSEVILALWSHLKPVPYPWGGKALQIVGKLGGRNRRFLKEPLTLE
CKDNP 839

Query 784
EHGLRLVLTTFEPSTPFLVPLDKFINLAVAAVIQRNHGMDIYYRKQALKFLRVCLLS
QLNL 843
EHGLRLVLTTFEPSTPFLVP+DKFINLAVAAV+Q+N
+IYY+KQALKFLRVCLLSQLNL
Sbjct 840
EHGLRLVLTTFEPSTPFLVPMDKFINLAVAAVMQKNLTTEIYYKKQALKFLRVCLLS
QLNL 899

Query 844
PGCVTDVGQTPRQLSTLLRSSVDSSWHRSEAVEIKADLGVKTKTQLMAEKSIFKTL
LITI 903
PGCVTD GQT +QLSTLL SSVDS W RSE+
EI+ADLGVKTKTQL+AEKSIFKTLLITI

Sbjct 900
PGCVTDEGQTTKQLSTLLLSSVDSFWRRSESTEIEADLGVKTKTQLIAEKSIFKTLLI
TI 959

Query 904
LAASSDPDLSDTDDDFVENICRHFMAILHVDYTSSNASTSTSSLGGSVISTSSRSKSN
QS 963

+AASSDPDLSD+DDDFV NICRHFMAILH DYTSS STS LGGS+ISTSS+ K+N
S

Sbjct 960
IAASSDPDLSDSDDDFVVICRHFMAILHGDYTSSYTSTSAGPLGGSLISTSSKPKNN
WS 1019

Query 964
SNLKQLDPLIFLDALVDVLADENRLHAKAALNALNVFAETLLFLARVKHADVLM
ARGGHN 1023

+ LKQLDPLIFLDALVDVLADENRLHAKAAL
+LNVFAETLLFLAR+KHADVLMARG H+

Sbjct 1020
TYLKQLDPLIFLDALVDVLADENRLHAKAALTSLNVFAETLLFLARIKHADVLM
RGAHS 1079

Query 1024
ASMIVSSPSTNPVYSPHPSVRIPVFEQLLPRLHGCYGSTWQAQMGGVMGLGALV
GKVVN 1083

ASMIVSSPSTNPVYSPHPSVRIPVFEQLLPRLH

CYGSTWQAQMGGVMGLGALVGKVVN Sbjct 1080

ASMIVSSPSTNPVYSPHPSVRIPVFEQLLPRLHCCYGSTWQAQMGGVMGLGALV
GKVVN 1139

Query 1084
ETLCYFQVKIVRGLVYVLKRLPVYASKEQEETSQVLMQILRVVNNVDEANSEARR
KSFQD 1143

ETLC FQVKIVRGLVYV
KRLPVYASKEQ+ETSQVL+QILRVVNNVDEAN++ARR+SFQD

Sbjct 1140
ETLCLFQVKIVRGLVYVQKRLPVYASKEQDETSQVLIQILRVVNNVDEANNDARR
QSFQD 1199

Query 1144
VVEYLATELFNPNASIPVRKNVQNCLALLASRTGSEVTELLEPLYQLLLQPLIMRPL
RSK 1203

VVEYLATELFN NASI VRKNVQNCLALLASRTGSEV+ELLEPLYQ
LLQPLIMRPLRSK

Sbjct 1200
VVEYLATELFNSNASITVRKNVQNCLALLASRTGSEVSELLEPLYQPLLQPLIMRPL
RSK 1259

Query 1204

TVDQQVGTVAALNFCLALRPPLLKVTPELVNFLQEALQIAEADETVWAVKLMNP
KVLTSL 1263

T+DQQVGTV ALNFCLALRPPLLKVTPELVNFLQEALQIAEADE
+WAVKLM+PKVLTSL

Sbjct 1260

TIDQQVGTVTALNFCLALRPPLLKVTPELVNFLQEALQIAEADEALWAVKLMSPK
VLTSL 1319

Query 1264

NRLRTACIELLCTTMAWTDFTQTTHNELRAKIISMFFKSLTCRAPEIVAVAKEGLR
QVIN 1323

NRLRTACIE+LCTTMAW DFRTQ+HNELRAKIISMFFKSLTCRAPEIV
VAKEGLRQVIN

Sbjct 1320

NRLRTACIEILCTTMAWADFRTQSHNELRAKIISMFFKSLTCRAPEIVTVAKEGLRQ
VIN 1379

Query 1324

QQRMPKELLQSSLRPILVNLAHTKNLSMPLLQGLARLLELLSNWFNVTLGKELLE
HLKKW 1383

QQRMPKELLQSSLRPILVNLA TKNL+MPLLQGLARLLELLSNWFNVTLG
KLEHLKKW

Sbjct 1380

QQRMPKELLQSSLRPILVNLAQTKNLNMPLLQGLARLLELLSNWFNVTLGCKLLE
HLKKW 1439

Query 1384

LEPEKLAQSQKAWKAGEEPKIAAAIIELFHLLPHAASKFLDELVTLTIDLEAALPPG
QVY 1443

LEPEKLAQSQK+WKAGEEPKIAAAIIELFHLLP

AASKFLDELVTLTIDLEAALPPGQVY Sbjct 1440

LEPEKLAQSQKSWKAGEEPKIAAAIIELFHLLPLAASKFLDELVTLTIDLEAALPPG
QVY 1499

Query 1444

SEINSPYRLPLTKFLNRYAALAVDYFLSRLSEPKYFRRFMYIIRSDAGQPLREELAK
SPQ 1503

SEINSPYRLPLTKFLNRYA

LAVDYFLSRLSEPKYFRRFMYIIRSDAGQPLREELAKSP

Sbjct 1500

SEINSPYRLPLTKFLNRYATLAVDYFLSRLSEPKYFRRFMYIIRSDAGQPLREELAK
SPH 1559

Query 1504

KILSYAFPEISPKDPPTLSTTASTPPATSSGDENHISVKLESSNVASTKANIASDAYF
QG 1563

KILSYAFPEI PK D LS ASTPPA SSGDE +K ESSN STK+N+ASDAYFQG

Sbjct 1560

KILSYAFPEILPKSDAILSAAASTPPAASSGDEKPTPMKSESSNTPSTKSNVASDAYF
QG 1619

Query 1564

LYLIKTMVKLIPSWLQSNRSVFDLTVLIWKSPARISRLQNEQELNLVQVKESKWL
KCFL 1623

LYLIKTMVKLIPSWLQSNR++FD L +WKS AR SRLQNEQ L
LVQVKESKWLVKCFL

Sbjct 1620

LYLIKTMVKLIPSWLQSNRTIFDALAHLWKSHARTSRLQNEQNLT
LVQVKESKWL
VKCFL 1679

Query 1624

NYLRHEKSENVNLFDFILSIFLFHSRIDYTFLKEFYIIEVAEGYPPNMKRALLHFLNL
FH 1683

NYLRHEKSE+NVNLFDF L IFLFHSRIDYTFL+EFYIIEVAE
YPPNMK+A++LHFLNLF

Sbjct 1680

NYLRHEKSEMNVNLFDFVLLIFLFHSRIDYTFLREFYIIEVAEEYPPNMKKAIVLHFLN
LFQ 1739

Query 1684

SKQLGHDHLVQAMQMLILPMLAHAFQNGQTWEVIDPDIVKTIVERLLDPPEEVSA
EYDEP 1743

SKQLGHDHLVQAMQMLILPMLAHAFQNGQTWEVIDPDIVKTIVERLLDPPEEVSA
EYDEP

Sbjct 1740

SKQLGHDHLVQAMQMLILPMLAHAFQNGQTWEVIDPDIVKTIVERLLDPPEEVSA
EYDEP 1799

Query 1744

LRIELLQLATLLLKYLQSDLVHHRKELIKFGWNHLKREDSASKQWAFVNVCHFLD
AYQAP 1803

LRIELLQLATLLLKYLQSDLV
HRKELIKFGWNHLKREDSASKQWAFVNVCHFLDAYQAP

Sbjct 1800

LRIELLQLATLLLKYLQSDLVQHRKELIKFGWNHLKREDSASKQWAFVNVCHFLD
AYQAP 1859

Query 1804

EKIIQVVFALLRTCQPENKMLVKQALDILMPALPKRLPLGDSRMPWIWIRYTKKIL
VEEG 1863

EKIILQVFVALLRTCQPENKMLVKQALDILMPALPKRLPLGDSRMPIWIRYTKKIL
VEEG

Sbjct 1860

EKIILQVFVALLRTCQPENKMLVKQALDILMPALPKRLPLGDSRMPIWIRYTKKIL
VEEG 1919

Query 1864

HSIPNLIHIFLLVVRHSDLFYSCRAQFVPQMVNSLSRLGLPYNTTAENRRRLAIELAG
LVV 1923

HSIPNLIHIF

L+VRHSDLFYSCRAQFVPQMVNSLSRLGLPYNTTAENRRRLAIELAGLVV

Sbjct 1920

HSIPNLIHIFQLIVRHSDLFYSCRAQFVPQMVNSLSRLGLPYNTTAENRRRLAIELAGL
VV 1979

Query 1924

SWERQRQNEKMMVTD TDGTSQITDEMHTSSGADPKRSTDG SATSEDPSKRVKIEP
GLQSI 1983

SWERQRQNE KMMVTD D TS+++D +H SSG DPK ST GS+ SEDPSKRVKIEPGL

S+

Sbjct 1980

SWERQRQNESKMMVTDGDATSEVSDGLHPSSGVDPKLSTAGSSISEDPSKRVKIEPG
LPSL 2039

Query 1984 CVMSPGGASSIPNVETPGSATQPDEEFKPNAAMEEMIINFLIR-----

----- 2026

CVMSPGGASSIPNVETPGSATQPDEEFKPNAAMEE+IINFLIR

Sbjct 2040

CVMSPGGASSIPNVETPGSATQPDEEFKPNAAMEELIINFLIRVRLYTSHLLPWGML
ILA 2099

Query 2027 -----

VALVIEPKDRETNTMYKQALDLSQALEVWP SANVKFNYLEKLLSSMPPSQS
2078

VA+VIEPKDRE NTMYKQALD

LSQALEVWP+ANVKFNYLEKLLSSMPPSQS

Sbjct 2100

GFDLLGGGVAVVIEPKDREANTMYKQALDFLSQALEVWP NANVKFNYLEKLLSS
MPPSQS 2159

Query 2079

DPSTALAQGLDVMNKKVLEKQPHLFIRNNINQISQILEPCFKHKMLDAGKSLCSLLK
MVFT 2138

DPSTALAQGLDVMNKKVLEKQPHLFI+NNI+QISQ

LE FKHKMLDAGKSLCSLLKMVF

Sbjct 2160
DPSTALAQGLDVMNKVLEKQPHLFIKNNISQISQFLELSFKHKMLDAGKSLCSLLK
MVFI 2219

Query 2139
AFPLDAANTPPDIKLLYQKVNELINKHVNTVTAPQTSGDDNSFGSISFVLLVIKTLA
NVH 2198

AFP D A+TPP+IKLLYQKVNELI KHV+ VTA Q SGDDNS
GS+SFVL+V+KTLA V

Sbjct 2220
AFPQDGASTPPEIKLLYQKVNELIQKHVHVVTASQASGDDNSLGSVSFVLVVLKTL
AEVQ 2279

Query 2199
KNFVDSYVLVRILQRLARDLGSAVGSHPRQGQRTDSDSAVTSSRQTADVGAICN
IKSVL 2258

K+F+D YVLV ILQRL+RDLG A G+HPRQ QR +S+S ADVGAV+ NIK VL
Sbjct 2280 KHFLDPYVLVHILQRLSRDLGLAAGAHPRQSQRIESES-----
ADVGA VVSNIKLVL 2331

Query 2259
ELIDETVMLIADCKRSVTQILNTLLSEKGTDA SVLLCILDMIKRWVEDDFSKTGAS
GLSG 2318

ELIDE VML+ADCKR VTQILNTLLSEKGTD+S+LLC+LDM+KRW EDDF K
G+SG SG

Sbjct 2332
ELIDERVMLLADCKRPVTQILNTLLSEKGTDSLLLCVLDMLKRWAEDDFGKKGS
SGSSG 2391

Query 2319
SFLTQKDVLTFLNKLSYIDKQHFSSALEEWDQKYLQLLYGLCADSTKYPLGLRQ
EVSLK 2378

+FLTQKD+++FL KLS +DKQHFSS AL+EWD+ YLQLLYGLCADSTKYPL
LRQE+SLK

Sbjct 2392
AFLTQKDIVSFLQKLSQVDKQHFSSVALDEWDKVYLQLLYGLCADSTKYPLALRQ
EISLK 2451

Query 2379
VERHFMLGLRASHPGMRRKFFLLYHESLGKTLFARLQYIIQIQDWEALSDVFWLK
QGLDL 2438

VERH MLGLRA P MRRKFFLLYHESLG LFARLQYIIQ
QDWEA+SDVFWLKQGLDL

Sbjct 2452
VERHSMGLLRARDPDMRRKFFLLYHESLGNNLFARLQYIIQNQDWEAMSDVFWL
KQGLDL 2511

Query 2439

LLAILVEDKPISLAPNSARVLP LLPSDNPGIQHQAPANLEGPEEVTSMFDSIVMKHA
QFL 2498

LLAIL+E+KPI+LAPNSARV+PLLPS NPG+ HQ P EGPEEV
SMFDSIVMKH+QFL

Sbjct 2512

LLAILIEEKPITLAPNSARVVPLLPSQNPQGVHHQPPVMPEGPEEVASMFDSIVMKHS
QFL 2571

Query 2499

SATSKLQVADVVIPLRELAHTDANVAYHLWVLVFPIVWVTLLKEEQVALAKPMIS
LLSKD 2558

SA SKLQVADVVIPLRELAHTDANVAYHLWVLVFPIVW TL
KEEQ+ALAKPMISLLSKD

Sbjct 2572

SAASKLQVADVVIPLRELAHTDANVAYHLWVLVFPIVWATLHKEEQIALAKPMIS
LLSKD 2631

Query 2559

YHKKQQGHRPNVVQALLEGQLSHQPQPRMPSELIKYIGKTYNAWHLALALLES
H VMLFMN 2618

YHKKQQGHRPNVVQALLEGQLSHQPQPRMPSELIKYIGKTYNAWHLAL
LLE+HVMLF N

Sbjct 2632

YHKKQQGHRPNVVQALLEGQLSHQPQPRMPSELIKYIGKTYNAWHLALTLETH
VMLFTN 2691

Query 2619

DSKCAESLAELYRLLNEEDMRFGWLKRSITAETRAGLSLVQHGFQWQRAQSLFY
QAMVKA 2678

DSKCAESLAELYRLLNEED RFGLWK RSIT E+RAG
S+VQHGFQWQRAQSLFYQAMVKA Sbjct 2692

DSKCAESLAELYRLLNEEDRRFGWLKRSITTESRAGFSMVQHGFQWQRAQSLFYQ
AMVKA 2751

Query 2679

TQGTYNNTVPKAEMCLWEEQWLHCASQLSQWDALVDFGKSIENYEILLDSLWKL
PDWAYL 2738

TQGTYNNTVPK EMCLWEEQWLHCA+QL QWDALVDFGKS
ENYEILLDSLWK PDW YL

Sbjct 2752

TQGTYNNTVPKTEMCLWEEQWLHCATQLGQWDALVDFGKSTENYEILLDSLWK
APDWTYL 2811

Query 2739

KDHVIPKAQVEETPKLRLVQSYFALHDRNSNGVGDAENTVGKGVDLALEQWWQ
LPEMSVH 2798

KDHVIPKAQVEETPKLRLVQ+
F+LH++N+NGVGDAEN VGKGVDLAEQWWQLPEMS+H
Sbjct 2812
KDHVIPKAQVEETPKLRLVQACFSLHEKNANGVGDAENIVGKGVDLAEQWWQ
LPEMSLH 2871

Query 2799
ARVPLLQQFQQQLVEVQESARIHVDIANGNKVSGNTAVGGLGNRYADLKDILETW
RLRTPN 2858
ARVPLLQQFQQQLVEVQES+RI+VDIANG+KV GN AVGG GN
YADLKDILETWRLRTPN
Sbjct 2872
ARVPLLQQFQQQLVEVQESSRIYVDIANGSKVPGNAAVGGQGNLYADLKDILETWR
LRTPN 2931

Query 2859
EWDNMTVWYDMLQWRNEMYNVVIDAFKDFATSNSPLHHLGFRDKAWNPNKL
ARIARKQGL 2918
EWDNMTVWYDMLQWRNEMYNVVIDAFKDF
TSN+PLHHLG+RDKAWNPNKLARIARKQGL Sbjct
2932
EWDNMTVWYDMLQWRNEMYNVVIDAFKDFVTSNTPLHHLGYRDKAWNPNKL
ARIARKQGL 2991

Query 2919
YDVCVQILEKMYGHSTMEVQEAQAFVKIREQAKAYLEMKGGERASGLNINSTNLEYF
PDKIK 2978
YDVCVQILEKMYGHS MEVQEAQAFVKI+EQAKA+LE KGE
A+GLNL+NSTNLE+F K K
Sbjct 2992
YDVCVQILEKMYGHSQMEVQEAQAFVKIKEQAKAHLETGELATGLNLVNSTNLEF
FLAKNK 3051

Query 2979
AEIFRLKGDFHLKLNDTESANIAYSNAITLTKNLPGWISWGSYCDMAYQETQEEI
WLEY 3038
AEIFRLKGDFHLKLNDTE
AN+AYSNAITLTKNLPGWISWG+YCDMAYQ+TQ+EIWLEY
Sbjct 3052
AEIFRLKGDFHLKLNDTEGANLAYSNAILTKNLPGWISWGNLYCDMAYQDTQD
EIWLEY 3111

Query 3039
AVSCFLQGIRFGVSNSRSHIARVLYLLSFD TANEPVGRVFDKHLQVPHWVWLSW
IPQLL 3098
AVSCFLQGIRFGVSNSRSH+ARVLYLLSFD NEPVGR+FDKHLQVPHW

Sbjct 3112
AVSCFLQGIRFGVSNRSRSHMARVLYLLSFDPNTNEPVGRIFDKHLDQVPHW-----
3161

Query 3099
LSLQRTEAPHCKLVLLKIAAVFPQALYYWLRTYLLERRDAVNKSELGRLVLAQR
MQQNAT 3158

RTEAPHCKLVLL+KIAAVFPQALYYWLRTYLLERRDAVNKSEL
R+VLAQRMQQN
Sbjct 3162 ----
RTEAPHCKLVLMKIAAVFPQALYYWLRTYLLERRDAVNKSELSRVVLAQRMQQN
VP 3217

Query 3159 G--
AGHGGSNLPSENQIHQGAQTSGAGGTHDSGNPHGQESERSTTENNHLPGSDQPMH
QS 3216

G AGHGG NLPSE QIHQG+QTSGA GTHD GN H QESER+T NN+H
G+DQPM+QS
Sbjct 3218
GVSAGHGGGNLPSETQIHQGSQTSGAVGTHDGGNLHVQESERATMINNVHSGND
QPMNQS 3277

Query 3217
SSAINDNNENTVRRNGASLAISAAGAFDAAKDIMEALRGKHNNLASELEVLLTEIG
SRFV 3276

S S+AISAAGAFDAAKD+MEALR KHNNLASELEVLLTEIGSRFV
Sbjct 3278 S-----
SMAISAAGAFDAAKDVMEALRSKHNNLASELEVLLTEIGSRFV 3321

Query 3277
TLPEERLLAVVNALLHRCYKYPTATTAEVPQPLKKELSGVCACFSADAVTKHVE
FVKEY 3336

TLPEERLLAVVNALLHRCYKYPTATTAEVPQPLKKELSGVCACFSADAVTKHV
FV+EY

Sbjct 3322
TLPEERLLAVVNALLHRCYKYPTATTAEVPQPLKKELSGVCACFSADAVTKHVA
FVREY 3381

Query 3337
KQDFERHLDPESTTTTFPATLAELTARLKKWKNILQSNVEDRFPVLRLEDESRVLR
DFNV 3396

KQDFER LDPES +FP TLA+LT +LK WKNILQSNVEDRFP
+LRLEDES+VLRDFNV
Sbjct 3382 KQDFERDLDPE-
NSFPVTLADLTKKLKDWKNILQSNVEDRFPVLLRLEDESKVLRDFNV 3440

Query 3397

VDVEIPGQYFADQEVAPDHTVKLDRVGADVPIVRRHGSSFRRLTLIGSDGSQKHFI
VQTS 3456

VDVEIPGQYFADQEVAPDHTVKLDRVGAD+ IVRRHGSS
RRLTLIGSDGSQKHFI VQTS

Sbjct 3441

VDVEIPGQYFADQEVAPDHTVKLDRVGADIQIVRRHGSSCRRLTLIGSDGSQKHFI
VQTS 3500

Query 3457

LTPNARSDERILQLFRVMNQMFDPKHKESRRRHIGIHTPIIPVWSQVRMVEDDLMY
NTFL 3516

LTPNARSDERILQLFRVMNQMFDPKHKESRRRH+G+HTPIIPVWSQVRMVEDDLM
YNTFL

Sbjct 3501

LTPNARSDERILQLFRVMNQMFDPKHKESRRRHGLHTPIIPVWSQVRMVEDDLM
YNTFL 3560

Query 3517

EVYENHCARNDREADLPITHFKEQLNQAISGQISAE AIGDLRLQAYIDITKTLVND
IFS 3576

EVYENHC RN RE+DLPIT+FKE+LNQAI+GQIS EAIGDLRLQAY
+ITK +VND+IFS

Sbjct 3561

EVYENHCGRNGRES DLPITYFKEKLNQAITGQISPEAIGDLRLQAYGEITKNIVND
IFS 3620

Query 3577

QYMYKTLMSGSHMWAFKKQFAVQLAVSSFMSFMLQIGGRSPNKVLFAKNTGKM
FQTD FHP 3636

QYMYKT
MSGSH+WAFKKQFAVQLAVS+FMSF+LQIGGRSPNK+LFAKN+GKMFQTD FHP

Sbjct 3621

QYMYKTSMSGSHLWAFKKQFAVQLAVSNFMSFILQIGGRSPNKILFAKN SGKMFQ
TDFHP 3680

Query 3637

AYDANGMIEFNEPVPFRLTRNMQAFFSQFGVEGLLMSSMC SAAQAVISSKQNEHL
RYQLA 3696

+YD+NGMIE NEPVPFRLTRNM AF S FGVEG LMS+MCSA+QAV
SSKQNEHLRYQLA

Sbjct 3681

SYDSNGMIELNEPVPFRLTRNMHAF LSHFGVEGPLMSNMCSASQAVFSSKQNEHL
RYQLA 3740

Query 3697

MFFRDELLSWFGRRPLGVPIPPVGGIATLNPAELKHKVNANVEDVIKRIRGIAPQYF
SEE 3756

MFFRDELLSWFGRRPLGVPIPPV GIATL+ ELKHKVN+NV+DVI
RIRGIAPQYFSEE

Sbjct 3741

MFFRDELLSWFGRRPLGVPIPPVAGIATLSSPELKHKVNSNVDDVIGRIRGIAPQYF
SEE 3800

Query 3757 DENTVEPPQSVQRGVNELVEAALSPRNLCMMDPTWHPWF 3795

DEN+VEPPQSVQRGV+ELVEAALSPRNLCMMDPTWHPWF

Sbjct 3801 DENSVEPPQSVQRGVSELVEAALSPRNLCMMDPTWHPWF 3839

Appendix 7: shows the map for T-DNA shows the location of T-DNA insertion for DNAPKa and has been obtained from Salk institute



The sequence of the T-DNA insertion:

>SALK_096167.53.55.x

CTGAAAACAATAACTCATTAACATTTCAATAAATAATGTTACTCATTAATTAGTGT
GTGTGTTTTATGAATATAATTTGAGTATTTAATTCTCTAATTTTTTTAAGAATTATT
AGAGCGCATAATCTCCCGTCTATATAAATATCCTCCGGCGAATTGAGAANCNNCT
TCNGGCGGNTGACTCTTATTGGCTCAGATGGCTCGCAAAAGCACTTTATAGTCC
AGACATCATTGACGCCTAATGCTAGAAAGTGATGAACGCATCTTGCAGCTTTTCC
GTGTAATGAACCAAATGTTTGATAAGCATAAGGAATCAAGAAGACGGCACATTG
GAATCCACACTCCAATCATCATTCTGTCTGGTCTCAGGTGATTTTTATACCATG
AATTGCAGCCCCGGGC

Appendix 8: shows the map for T-DNA shows the location of T-DNA insertion for DNAPKb and has been obtained from Salk institute

