Exploring the Role of HOP2 in the Double Strand Break Repair Pathway beyond Meiosis in *Arabidopsis thaliana*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Cell and System Biology
University of Toronto

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Abstract

The purpose of this study was to investigate the role of HOP2 protein in non-meiotic cells in *Arabidopsis*. HOP2 is known to be important to meiotic chromosome pairing and homologous recombination, yet the role of HOP2 outside of meiosis is far from being fully elucidated. My study focused on the mitotic chromosome events with and without the application of radiation. In the absence of radiation, no fragments and chromatin bridges were found in *hop2-1* plants, but they did seem to experience a modest chromosome separation delay. When irradiated, both genotypes had significant decreases of mitotic indices and increases of bridges. The decrease in mitotic indices were comparable for the two genotypes, suggesting they accomplish repair at similar rates. Irradiated *hop2-1* had significantly more incorrectly repaired breaks, as determined by the bridges. My findings suggest that HOP2 is also important for the fidelity of the exchange process in non-meiotic HR repair.
Acknowledgements

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<tr>
<td>aNHEJ</td>
<td>alternative NHEJ</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia Mutated</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CO</td>
<td>Crossover/Crossing Over</td>
</tr>
<tr>
<td>D1</td>
<td>Distance 1</td>
</tr>
<tr>
<td>D2</td>
<td>Distance 2</td>
</tr>
<tr>
<td>D3</td>
<td>Distance 3</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>dHJ</td>
<td>Double Holliday Junction</td>
</tr>
<tr>
<td>D-loop</td>
<td>Displacement Loop</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>G1 Phase</td>
<td>Gap 1 Phase</td>
</tr>
<tr>
<td>G2 Phase</td>
<td>Gap 2 Phase</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HJ</td>
<td>Holliday Junction</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
</tr>
<tr>
<td>M phase</td>
<td>Mitotic/Meiotic Phase</td>
</tr>
<tr>
<td>Mb</td>
<td>Mega-basepairs</td>
</tr>
<tr>
<td>BA</td>
<td>Buffer A</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule-Organizing Center</td>
</tr>
<tr>
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<td>Nonhomologous End Joining</td>
</tr>
<tr>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RGE</td>
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<tr>
<td>SC</td>
<td>Synaptonemal Complex</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDSA</td>
<td>Synthesis-Dependent Strand Annealing</td>
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<tr>
<td>S Phase</td>
<td>Synthesis Phase</td>
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<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>T-DNA</td>
<td>Transfer DNA (in <em>Agrobacterium tumefaciens</em> transformation system)</td>
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<td>Ler</td>
<td>Landsberg <em>erecta</em></td>
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<td>Single-stranded DNA (ssDNA)</td>
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<td>Landsberg <em>erecta</em></td>
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<td>Ionizing Radiation</td>
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x
1 Introduction

1.1 Overview of my research

My research project has focused on the role of Homologous-pairing protein 2 (HOP2) in the dicotyledonous model plant, *Arabidopsis thaliana*. The *HOP2* gene is known to be highly conserved across eukaryotes, and it is important to meiotic chromosome behavior. HOP2 protein has a role in meiotic reciprocal genetic exchange (RGE) via its involvement in the homologous recombination (HR) Double Strand Break (DSB) repair pathway. DNA DSBs occur within and outside of meiosis. Therefore, the HR DSB repair pathway is important, not only for reciprocal genetic exchange during meiosis, but also for maintaining genomic integrity outside of meiosis. For my thesis, I focused on the possible role of HOP2 in *Arabidopsis*, outside of meiosis.

1.2 Cell cycle and cell division

Cell division is an important process for eukaryotes. In order for eukaryotes to grow, many types of cells need to interact with one another. The maintenance of cell number and number of cell types is achieved through cell division. Cell division is important for replacing old, dead and damaged cells; the most typical example is epidermal cells. Cell division is also important for reproduction: mitotic division is done for asexual reproduction and meiotic division is associated with sexual reproduction.

When cells divide, both nuclear division and cytokinesis (division of cytoplasm) occur. In eukaryotes, there are two types of nuclear division: mitosis and meiosis, depending on what type of cells are dividing. The first type of nuclear division, mitosis, is an important biological process that allows cells to duplicate. Mitosis occurs in all eukaryotic cells. During mitosis each parental cell divides to produce two identical daughter cells. The second type of nuclear division is meiosis. Meiosis occurs in sexually reproducing eukaryotes, and it is an important nuclear division for gamete production. For meiosis, each diploid parental cell is divides twice and produces four haploid germ cells. Meiosis differs from mitosis in two main ways: mitosis results in two daughter cells that are genetically identical whereas the daughter cells produced in meiosis contain different genetic combinations. Additionally, there is a net reduction of chromosome number in meiosis, while in mitosis the number of chromosomes stays the same.
Cell division falls within an ordered series of events known as the cell cycle; specifically, cell division occurs during the stage known as the M phase (Figure 1). After each round of M phase, the newly created daughter cells enter Gap 1 (G1) phase of the cell cycle. Figure 1a shows two pairs of homologous chromosomes in a diploid organism at G1 phase. Diploid organisms have one pair of each chromosome, and each pair of chromosomes with the same length, shape, and gene order is known as a pair of homologous chromosomes. During G1 phase, each chromosome contains only one, double stranded DNA molecule (Figure 1a). Diploids, with their pairs of homologous chromosomes, have 2 versions of each chromosome, and the DNA content of the genome in a diploid at G1 is described as 2C. To move out of the G1 phase, the cell must pass through a barrier known as the G1 checkpoint. If DNA damage has occurred, it must be repaired to get past this checkpoint. Once the cell’s internal and external conditions are appropriate for cell division, signaling occurs that allows passage through the checkpoint and the cells enter the next stage called Synthesis (S) phase.

During S phase DNA replication, transcription and translation all occur. The replication of DNA is semiconservative; the DNA helix unwinds locally and each original DNA strand serves as a template for its replication such that the newly synthesized DNA helices each contain one original strand and one new strand. Thus after replication, the genetic material has doubled, and each one of these two DNA helices is packaged with chromosomal proteins into a chromatid. The two genetically identical chromatids are held together in their centromere region by cohesin proteins. By the end of S phase each chromosome is composed of two genetically identical sister chromatids. As illustrated by Figure 1b, the DNA content is now described as 4C.

During DNA replication spontaneous DNA damage and stalled replication forks can occur. Both can lead to DSBs. DSBs and other DNA damage activate the S phase checkpoint and halt normal cell cycle progression (Reviewed by Branzei and Foiani, 2007) until the lesions are repaired. Once DNA replication is completed and all damage is repaired, the cells will leave S phase and enter Gap2 (G2) phase (Figure 1). In the G2 phase, the chromosomes are not yet condensed, and even with a microscope, the chromosomes appear as an ill-defined mass of threads that fills the nuclear space. During G2 phase, the cells grow and synthesize the proteins that are needed for the subsequent phase. For nuclear division both chromosomal proteins and the proteins that will comprise the spindle apparatus need to be produced to allow chromosome segregation during M
phase. Cells in G2 can also sustain DNA damage which must be repaired before the cells can pass the G2 checkpoint and enter M phase (Figure 1).

![Cell cycle progression and chromosome and chromatid numbers during cell cycle.](image)

**Figure 1 Cell cycle progression and chromosome and chromatid numbers during cell cycle.**

The circle represents the complete cell cycle progression, and each subsection of the circle represents one phase. The dashed lines within the circle represent the boundaries between each cell cycle stage. The arrows indicate the direction of the cell cycle. The blue bar represents the G1 checkpoint, the green bar represents the S phase checkpoint and the red bar represents the G2 checkpoint. The letter “C” within each bracket is a unit of DNA amount. During G1 phase, two pairs of homologous chromosomes are shown (one pair shown in light blue/dark blue and one pair shown in red/light red). The yellow circle in the middle of each chromosome represents the centromere/kinetochore region. During G2 phase, two pairs of homologous chromosomes are shown. Note: the chromosomes of G1, S and G2 phases are not visible as distinct chromosomes, but are shown that way to illustrate key features.

1.3 The first type of nuclear division - Mitosis

During M phase nuclear division occurs, followed by division of the cytoplasm (cytokinesis). The first type of nuclear division is mitosis. Figure 2 provides an overview of the progression of
M phase in plant cells. Mitosis consists of five stages: prophase, prometaphase, metaphase, anaphase and telophase. At the onset of M phase, the chromosomes are not condensed and appear as slender threads; the nuclear envelope is fully intact (Figure 2a). As prophase stage progresses the chromosomes begin to condense and the nuclear envelope undergoes changes that will eventually lead to its disassembly. While still attached in their centromere regions, each sister chromatid develops its own functional kinetochore. The kinetochore is a structure with kinesin motor proteins and the ability to capture microtubules. In parallel to the events in the nucleus, events are also occurring in the cytoplasm. In the cytoplasm a structure known as the microtubule-organizing center (MTOC) duplicates, and each MTOC moves toward the opposite poles. Individual MTOC seeds the growth of an array of microtubules (Figure 2a).

Before entering metaphase, the cells progress through a stage known as prometaphase (Figure 2b). By prometaphase the fully condensed chromosomes are visible using light microscope. During prometaphase the nuclear envelope is disassembled. This disassembly allows the microtubules of the spindle to enter the nuclear area and allows individual microtubule to interact with the kinetochore of each chromatid.

The next stage is metaphase. During this stage the kinetochore of each sister chromatid attaches to the microtubules. Because the sister kinetochores face opposite directions, the microtubules attached to them originate from opposite poles. Ultimately these microtubule interactions orient the attached sister chromatids to the middle of the cell (in a region called the metaphase plate), facing opposite poles (Figure 2c). Once all of the chromosomes are aligned on the metaphase plate with sister chromatids facing opposite poles, a signal occurs to transit to anaphase (Figure 2d). In anaphase, the cohesins that hold sister chromatids together are broken down. This allows the kinesin motor proteins of the kinetochores to move along the attached microtubules. Since sister chromatids have been oriented to opposite directions, this segregates them to opposite poles. Additionally, as the segregating chromosomes are moving to opposite poles, the poles themselves are also moving farther apart (Figure 2d).

By telophase, the sister chromatids (now called sister chromosomes) reach opposite poles (Figure 2e). The mitotic spindles disassemble and the nuclear envelope reforms. The chromosomes start to decondense into threads of chromatin. In plant cells during telophase
vesicles with cell wall building materials, called the phragmoplast, aggregate in the region that was the metaphase plate. Finally, in cytokinesis, the division of cytoplasm occurs (Figure 2f). For cytokinesis in plants, vesicles containing cell wall materials fuse and the new cell wall surface forms between the two newly formed daughter nuclei.

Because mitosis separates sister chromatids, it can work correctly for cells of any ploidy. If a haploid cell (with only one copy of each chromosome) undergoes mitosis, then it will produce two genetically identical haploid cells. If a diploid cell (with two copies of each chromosome) undergoes mitosis it will produce two genetically identical diploid cells.
Figure 2 Diagram showing the progression of mitosis in a plant cell.

The arrows show the direction of mitosis. Figure a) shows prophase stage. The blue dashed circle represents the nuclear envelope. The red and blue lines represent chromatin threads. The two green crosses represent the microtubule-organizing center (MTOC) and the green lines represent the microtubules. b) Plant cell at prometaphase stage, note the two pairs of homologous chromosomes, one pair with red color and one pair with blue color. Each yellow circle represents one kinetochore. c) Plant cell at metaphase stage, the black dash line in the middle represents the metaphase plate. d) Plant cell at anaphase stage. e) Plant cell during telophase, the green dashed line in the middle represents the cell plate. f) Plant cells at cytokinesis. The green line in the middle represents the newly formed cell wall.
1.4 The second type of nuclear division - Meiosis

The second type of nuclear division is meiosis. In sexual reproducing eukaryotes, each parental cell has, in diploids, two copies of each chromosome but gives only one copy of its genetic material to the offspring. Therefore, it is crucial that a mechanism exists to reduce the number of chromosomes in gametes to half so that the genome size can be maintained in the next generation. The reduction in the number of chromosomes is achieved through meiosis.

Meiosis is a special type of nuclear division that occurs solely in reproductive cells (Figure 3). Thus, the purpose of meiosis differs from that of mitosis. The goal for mitosis is to create two copies of the parental cell that are identical to each other and the parental cell. In contrast, the goal for meiosis is to produce nuclei that contain only half the parental number of chromosomes. This reduction is accomplished by having one round of premeiotic DNA replication be followed by a meiotic division that has two successive rounds of nuclear division - Meiosis I and Meiosis II. During the first meiotic division, meiosis I, the homologous chromosomes segregate, therefore meiosis I is also known as the reductional division. Meiosis I can be divided into four stages: prophase I, metaphase I, anaphase I and telophase I.

Meiosis I begins with prophase I. Most of the unique meiotic events occur during this complex phase. At the onset of prophase I the chromosomes are not yet condensed and are not visible under the microscope; the chromosomes are located within the nucleus that is separated from the cytoplasm by the nuclear envelope. Each chromosome consists of two sister chromatids that are attached along their length. Prophase I consists of five substages: leptotene, zygotene, pachytene, diplotene and diakinesis (Figure 3a-3e).

Leptotene is the first substage of prophase I (Figure 3a). Each chromosome has developed one functional kinetochore that is shared by sister chromatids. The sister chromatids of each chromosome are held together along their longitudinal axis by the cohesin complex (Lee and Orr-Weaver, 2001). The cohesin complex with ASY1 form the axial elements (Klein et al. 1999; Armstrong et al. 2002). The axial elements become the axial components of the synaptonemal complex (SC) along with the central element and transverse filaments (defined by the ZYP1 protein) that form during the process of synapsis (Jones et al. 2003; Higgins et al. 2005).
Chromosome condensation also occurs during leptotene; however, the chromosome condensation occurs at this stage is moderate compared to mitosis (Hasenkampf et al. 1998). During leptotene, the telomeres of the chromosomes are attached to the nuclear envelope, forming a structure that resembles the bouquet of a flower (Figure 3a) (Scherthan, 2001).

The next stage of prophase I is zygotene (Figure 3b), at this stage, chromosomes become more visible under the microscope. The hallmark feature of zygotene is synapsis, the process by which the homologous chromosomes become intimately associated with each other. The formation of the bouquet (Figure 3a) has been suggested to facilitate the initial contacts between homologous chromosomes as it restricts telomere regions of the chromosomes to a limited nuclear region (Armstrong et al. 2001). From these telomeric region contacts and additional interstitial ones, homologs are thought to form contacts at which homology is tested. The homologous chromosomes first become roughly aligned (Figure 4a) and then closely aligned (Figure 4b), and the homologous contacts are further stabilized through the formation of axial associations (Figure 4c) between the homologous chromosomes (Rockmill et al. 1995). Formation of axial associations are then followed by the establishment of an additional protein framework known as the synaptonemal complex (SC) all along the pairing face of homologous chromosomes (Figure 4d) (Roeder et al. 1997). The formation of SC holds the homologous chromosomes together along the entire pairing axis, allowing a precise, juxtaposing of homologous chromosome regions.

In pachytene, the homologous chromosomes are completely synapsed (Figure 3c). The synapsed homologous chromosomes are known as bivalents. The synapsis between homologous chromosomes provides the close juxtapositioning needed to allow reciprocal genetic exchange (RGE) to occur. During the process of RGE, non-sister, homologous chromatids will swap homologous chromosome segments. This reciprocal exchange process as it occurs during meiosis is also called crossing over (CO). The reciprocal genetic exchange (RGE) between homologous chromosomes results in recombinant DNA helices. RGE plus the cohesions between sister chromatids form physical linkages known as chiasmata (Figure 3c), that collectively are critical for holding the two chromosomes of each bivalent together until the time of their segregation at anaphase I. Thus, the process of RGE is critical for proper chromosome segregation. Additionally it creates recombinant chromosomes and thus is a source of genetic
diversity. The molecular events leading to RGE will be further discussed in detail in later chapters. There are two categories of crossovers (Hollingsworth and Brill, 2004). Class I crossovers exhibit interference, meaning the occurrence of one crossover inhibits another nearby; it ensures that every chromosome will have at least one crossover (Hollingsworth and Brill, 2004). Class II crossovers are a type of noninterfering crossover and are generally less abundant compared to Class I crossovers (Hollingsworth and Brill, 2004).

It is thought that RGE is completed by the end of pachytene. At the next stages (diplotene and diakinesis: Figure 3d and 3e), chromosomes are further condensed and begin to coil (Hasenkampf et al. 1998). The SC starts to disassemble, except in the regions of the chiasmata (Reviewed by Schmekel and Daneholt, 1995).

While critical chromosome events have been occurring in the nucleus, the spindle has also been forming in the cytoplasm. As cells leave diakinesis and enter metaphase I, the nuclear envelope breaks down, allowing the developing spindle and its microtubules to enter the nuclear region. At the beginning of metaphase I (Figure 3f), each bivalent consists of paired homologous chromosomes that are connected at chiasmata. The two sister chromatids of each chromosome are connected in the centromere region, and unlike mitosis, share one functional kinetochore. Free microtubules from opposite poles of the MTOC are now captured by the kinetochores of the chromosomes. RGE plus sister chromatid cohesion produce the chiasmata that holds the bivalent together and allows it to interact with the spindle microtubules in a way that leads to their orientation to opposite poles. Therefore, once each kinetochore attaches to a microtubule, the resulting balanced mechanical force exerted from opposite directions ensures that homologous chromosomes will face opposite poles (Figure 3f). Other free microtubules push these chromosomes to the midzone, known as the metaphase I plate. The orientation of all of the bivalents on the metaphase I plate is the critical set of events that signals the end of metaphase I and progression to anaphase I. It should be noted that different pairs of homologs orient independently of each other.

During anaphase I (Figure 3g), the cohesin complex between sister chromatids is released, except at the centromere region. Removal of cohesins along the arms of sister chromatids releases the chiasmata that were holding the homologous chromosomes together (Cai et al.
Separation of the homologous chromosomes occurs, allowing the motor proteins of the kinetochores to move along the attached microtubules toward the poles (Banks and Heald, 2001). The separation of the homologs from each other is supplemented by the fact that spindle microtubules are sliding apart, further separating the two poles from each other (Gramont and Cohen-Fix, 2005). Therefore, while the chromosomes are moving to opposite poles, the two poles are also separating. Separating the homologous chromosomes to the opposite poles is required to ensure the production of balanced sets of chromosomes in each daughter cell.

Once the chromosome sets are optimally apart, telophase commences. In telophase I (Figure 3h), the chromosomes decondense and the nuclear envelope begins to reform. By the end of meiosis I, one parental meiotic cell is divided to produce two daughter nuclei that each has the haploid number of chromosomes (if the starting cell was diploid) (Figure 3i). From telophase I, the two nuclei immediately move into meiosis II. The mechanism of meiosis II is very similar to a mitotic division because, like mitosis, it is the chromatids that are separated. In prophase II (Figure 3j), chromosomes condense and now the sister chromatids each develop its own functional kinetochore. At the end of prophase II the nuclear envelope breaks down, allowing the two newly forming spindle apparati to invade the nuclear regions and interact with the chromosomes.

During metaphase II (Figure 3k), the kinetochores of sister chromatids attach to microtubules facing opposite poles and are driven to the metaphase II plate facing opposite poles. In anaphase II (Figure 3l), the sister chromatids segregate to opposite poles, and it is also analogous to the mitotic anaphase. In telophase II (Figure 3m), the sister chromatids reach the opposite poles. Once the nuclear division is completed, cytokinesis occurs. In cytokinesis (Figure 3n), chromosomes are decondensed and nuclear envelope reforms. In plant cells, the cell walls produced from the secretion of vesicles extend from the middle of the metaphase plates. By the end of meiosis there are a total of 4 daughter cells produced. Because the starting nucleus only replicated once but divided twice, each daughter cell has half the number of the chromosomes as the parental cell. Also, the four nuclei produced are genetically different from one another due to the independent alignment on the metaphase I plate of the nonhomologous chromosomes and the reciprocal genetic exchange of genes on homologs.
Figure 3 Diagram showing the progression of meiosis in a plant cell.

a) A plant cell at leptotene stage, the dark blue and light blue parallel lines represent a pair of homologous chromosomes and the red and light red parallel lines represent a different pair of homologous chromosomes. The yellow circles represent kinetochores. The purple segments represent telomeres. The grey rings are the cohesin complexes. The blue circle represents the nuclear envelope. The green pluses represent MTOCs and the green lines represent the microtubules. b) A plant cell at zygotene stage, note the chromosomes become more condensed compared to figure a). Note that only one pair of homologous chromosomes is completely synapsed, the other pair of homologous chromosomes is still in the process of pairing. Details of the synaptonemal complex (SC) formation can be seen in Figure 4. c) A plant cell at pachytene stage. Note that the crossing over occurs for each pair of homologous chromosomes. The chiasmata is the visible evidence that crossing over has occurred. d) Diplotene. Note that the chromosomes become even more condensed. e) Diakinesis. The dash line representing the nuclear envelope becomes lighter, indicating that it is gradually being broken down. The chromosomes are completely visible under the microscope. f) Metaphase I, note that there are two pairs of bivalents. The light blue and dark blue chromosomes together are referred to as one bivalent and the red and light red chromosomes are the two chromosomes of a different bivalent. The green lines extending from the MTOCs represent the microtubules. The black dash line represents the metaphase plate. g) Anaphase I h) Telophase I, the light green dash line in the middle represents the cell plate. i) Cytokinesis, the light green line in the middle represents the newly formed cell wall. j) Prophase II, note that after cells finished meiosis I, two daughter cells are produced and undergo meiosis II simultaneously. k) Metaphase II. l) Anaphase II. m) Telophase II. n) Cytokinesis.
Figure 4 Diagram showing chromosomal behavior during homologous pairing.

a) Rough alignment. The light blue and dark blue circles represent loops of chromatin of the two sister chromatids of each chromosome. The two chromosomes are a pair of homologous chromosomes. b) Close alignment. Note that the pair of homologous chromosomes are closer compared to a). c) The purple lines represent axial association. d) The black lines represent the lateral elements, the orange line represents central element and the red wavy lines represent the transverse filaments. The lateral elements, central element and transverse filaments form a structure known as the synaptonemal complex (SC).
1.5 DSB repair pathway in meiosis

One of the key events that occurs in meiosis is the swapping of chromosome segments between homologous chromosomes during prophase I, producing regions of reciprocal genetic exchange (RGE). The reciprocal exchange of genetic information between homologous chromosomes requires a specialized pathway that first induces DNA double-strand breaks (DSBs) in chromatids and then repairs them using the homologous recombination (HR) pathway of DNA repair.

Figure 5 illustrates the DNA events in the meiotic HR DSB repair pathway. The pathway is triggered by the programmed initiation of DSBs in the DNA (Keeney et al. 1997). SPO11 proteins bind to the dsDNA, forming a covalent SPO11-DNA complex (Figure 5a) (Symington and Gautier, 2011). SPO11 proteins then cleave the dsDNA (Keeney et al. 1997) and remain bound to the cleaved segments (Figure 5b). The two SPO11 proteins (each with a few oligonucleotides) are then removed via a resection process (Keeney et al. 2001), generating two 3’ ended single stranded DNA overhangs (Figure 5c) (Reviewed by Keeney et al. 2001). This resection process is initiated by a protein complex known as the MRN complex (Symington and Gautier, 2011). The MRN complex is composed of Mre11, Rad50 and Nbs1 (Yin and Smolikove, 2013). The Mre11 protein is a sensor of the DSBs (Park et al. 2017), and it serves as a DNA binding site (Hopfner et al. 2001); it also possesses the nuclease activity that releases SPO11 proteins along with the short oligonucleotides (Buis et al. 2008). Rad50 forms a complex with Mre11 and enhances the nuclease activity of Mre11 (Paull and Gellert 1998). The Nbs1 protein functions in localizing the MRN complex to the nucleus and activating the Ataxia-telangiectasia Mutated (ATM) protein kinase after DSBs are initiated (Williams et al. 2010). The Nbs1 protein is also important for the subsequent loading of RAD51 (a protein important for subsequent steps in the pathway) (Girard et al. 2018). Both Nbs1 and Mre11 have also been shown to play a role during meiosis in blocking the Nonhomologous End Joining (NHEJ) pathway of DSB repair (Yin and Smolikove 2013; Girard et al. 2018). NHEJ is an alternative repair pathway that repairs DSBs, and it will be further discussed in later chapter.

Strand invasion of the ssDNA occurs next (Figure 5d). The 3’ ends of the ssDNAs are bound by two RecA-like proteins: RADiation sensitive 51 (RAD51) and Disrupted Meiotic cDNA1
(DMC1) (Kurzbauer et al. 2012). The RAD51 and DMC1 proteins each binds to one ssDNA, forming nucleofilaments (Kurzbauer et al. 2012). The two proteins help to promote strand invasion of the ssDNA into the unbroken dsDNA of the homologous chromosome, allowing the formation of a DSB intermediate known as the displacement loop (D-loop) (Figure 5d) (Ma, 2006). It has been suggested that DMC1 and RAD51 are not functionally equivalent (Kurzbauer et al. 2012). DMC1 is thought to be critical for having strand invasion occur between the broken chromatid and a chromatid from the homologous chromosome; this is known as the interhomolog strand invasion (Pradillo et al. 2012). RAD51, is thought to be critical for all strand invasions, and has been reported to play a role in ensuring the fidelity of the process of DSB repair (Pradillo et al. 2012). In the absence of DMC1, RAD51 can repair DSBs, typically using only sister chromatids as the template for invaded strand (Siaud et al. 2004).

After formation, the D-loop is extended when DNA synthesis initiates from the invading strand using the homologous chromosome as a template (Figure 5e). The synthesis occurs at the 3’ end of the invading DNA to recover the information lost by the removal of the SPO11/DNA segments. After this DNA synthesis, multiple possible pathways can occur depending on how D-loop is further processed.

The first pathway is known as the Synthesis-Dependent Strand Annealing (SDSA) pathway (Figure 5f-5h). During this pathway, the D-loop is only extended slightly before being displaced (Figure 5f), returning the invading strand to its original base pairing partner (Figure 5g); subsequent DNA synthesis and ligation does not lead to a crossover, but the repair of the break is accomplished (Figure 5h) (McMahill et al. 2007).

In the second pathway (Figure 5f-5k) branch migration occurs only at one end of the D-loop (Cromie et al. 2006). The left side of the D-loop is cleaved (Figure 5i) and the cut end anneals with the cut end of the homolog, which was generated at the very beginning, forming a single Holliday Junction (HJ) (Figure 5j) (Cromie et al. 2006). The top and bottom strands are also cleaved and part of the segments are switched (Figure 5j), this results in the production of Class II crossovers (Figure 5k) (Cromie et al. 2006).

For the third pathway (Figure 5e-5n), DNA synthesis is first initiated on the top helix (Figure 5e), extending the size of the D-loop region. While the D-loop is still present, the invading strand
keeps using its new partner’s sequence to extend its length significantly, allowing it to ligate to the top strand (Figure 5l). This second ligation constitutes the second-end capture that produces a double Holliday junction (dHJ) (Figure 5l) (Cromie et al. 2006). This pathway produces dHJ dissolution. For dissolution, the two sides of the dHJ gradually approach one another (Figure 5m) and the structure is decatenated by a topoisomerase (Bizard and Hickson, 2014), yielding a non-crossover product (Figure 5n).

The fourth pathway completes DSB repair by the resolution of dHJ (Figure 5l-5r). In this pathway, if both sides of the dHJ are cleaved horizontally as indicated in Figure 5o (Wyatt and West, 2014), the ligation leads to the formation of a non-crossover product (Figure 5p). Instead, if one side of the dHJ is cleaved horizontally and other cleavages occur vertically at the top and bottom strands (Figure 5q), subsequent ligations will produce Class I crossovers (Figure 5r) (Wyatt and West, 2014).
Figure 5 The homologous recombination DSB repair pathway in meiosis.

a) The light blue parallel lines represent one dsDNA and the dark blue parallel lines represent another dsDNA, during meiosis, the two dsDNAs come from a pair of non-sister homologous chromosomes. The orange circles represent the SPO11 proteins. b) DSB is induced, as indicated by the gaps on the blue lines. c) The extended light blue lines represent the ssDNA tails. d) The light blue line is inserted into the dark blue parallel lines, representing the process of strand invasion and formation of displacement loop (D-loop). e) The red dashed line represents DNA synthesis. Note that after the DNA synthesis occurs, multiple possible pathways can occur depending on how D-loop is further processed. f) The second end is not captured. g-h) The invading strand returns back. SDSA occurs, producing a non-crossover repair of the DSB. i) The left end of the D-loop is cleaved, as indicated by the triangle. j) The cleaved end of the D-loop anneals with the left end of the light blue DNA, forming single Holliday Junction (HJ). j-k) Resolution of HJ yields the production of Class II crossover, the triangles and numbers indicate the sites of cleavage during HJ resolution. l) After D-loop formation, if second end capture and strands ligation occur, double Holliday Junction (dHJ) forms. m) The two sides of the dHJ move towards each other, the green arrows indicate the direction of the migration. The black curved arrow indicates the decatenation of the dHJ. n) Dissolution of the dHJ yields a non-crossover repair of the DSB. o-p) If the dHJ is cleaved at site 3 and 4 horizontally, the ligation yields a non-crossover repair of the DSB. q-r) If the dHJ is cleaved horizontally only at site 3, and the strands at site 1 and 2 are cleaved vertically, the ligation yields a Class I crossover (Derived from Keeney et al. 1997; Cromie et al. 2006; McMahlil et al. 2007; Bizard and Hickson, 2014; Wyatt and West, 2014).
SPO11 induces DSBs

MRN complex initiates the resection process

RAD51 and DMC1 catalyze the strand invasion, forming displacement loop

DNA synthesis extends from the invading strand

Second end capture does not occur

Second end capture and the broken DNA strands ligation yields a double Holliday junction (dHJ)

SDSA

HU formation

dHJ Dissolution

Cleavages on both sides of dHJ

Cleavage on one side of dHJ

Non-crossover

Resolution of HJ

Non-crossover

Non-crossover

Class I Crossover

Class II Crossover
1.6 The HOMOLOGOUS PAIRING PROTEIN 2

There are many proteins involved in the HR repair pathway during meiosis. My research has focused on one such protein known as the HOMOLOGOUS-PAIRING PROTEIN 2 (HOP2). It has been implicated in the strand invasion steps preceding dHJ formation. HOP2 was first characterized in budding yeast (Leu et al. 1998), and was subsequently studied in other eukaryotic organisms.

HOP2 protein has been shown to be involved in the meiotic HR DSB repair pathway. Yeast two-hybrid assays conducted on Arabidopsis show that HOP2 interacts with a protein known as the MEIOTIC NUCLEAR DIVISION PROTEIN 1 (MND1) (Kerzendorfer et al. 2006). Experiments with yeast have found that the HOP2 and MND1 proteins coimmunoprecipitate from meiotic extracts (Tsubouchi and Roeder, 2002), and it is suggested by in vivo studies that HOP2 and MND1 form a heterodimer in meiosis (Chen et al. 2004; Petukhova et al. 2005).

In vitro studies in Arabidopsis suggest that the HOP2-MND1 protein complex facilitates DMC1 and RAD51 in promoting the strand invasion activity of the ssDNA (Vignard et al. 2007). It has also been suggested by the in vitro studies in mammals that the HOP2-MND1 complex prefers binding to the dsDNA and it increases DMC1 and RAD51-mediated D-loop formation (Petukhova et al. 2005).

The importance of HOP2 in meiosis can be seen in the mutant studies in different organisms. In yeast, hop2 mutants show deficiency in sporulation; the meiotic cells in hop2 mutants are arrested at prophase I without entering metaphase (Leu et al. 1998). Extensive amounts of illegitimate synapsis between nonhomologous chromosomes and tangled masses of chromatin are also seen in hop2 yeast (Leu et al. 1998). The meiotic arrest observed in hop2 mutants is eliminated when SPO11 (protein responsible for initiating DSBs) is absent (Leu et al. 1998). Thus it is thought that in a normal meiosis, HOP2 is required to repair DSB breaks induced by SPO11.

In mouse hop2 mutants are sterile (Petukhova et al. 2003). Spermatogenesis is stalled at prophase I, resulting in cell cycle arrest and subsequent programmed cell death (Petukhova et al. 2003). A small amount of incorrect synapsis between nonhomologous chromosomes is observed
in hop2 mice, and unrepaired DSBs persist until apoptosis occurs (Petukhova et al. 2003). Similar results have been shown in another study in mouse conducted by Pezza et al. (2014); decreases of synapsis in both hop2 and hop2 mnd1 mutants are observed (Pezza et al. 2014).

HOP2 also plays an important role in plant meiosis. Unlike yeast or mammals, the absences of strict meiotic checkpoints in plants allow hop2 mutants to progress through meiosis (Schommer et al. 2003), so the downstream effects can be observed. The Arabidopsis hop2-1 null mutant was generated using transfer DNA (T-DNA) insertion on chromosome 1 at the gene At1g13330. In Arabidopsis, hop2-1 null mutants are both male and female sterile; the siliques in hop2-1 Arabidopsis are significantly smaller compared to wild-type (Schommer et al. 2003). The stamens in mature flowers are shorter in hop2-1 due to the failure of cell elongation, and non-viable pollen is found inside the anthers (Schommer et al. 2003). Cytological analyses show that in the absence of HOP2 the close alignment between homologous chromosomes is not stabilized, and there are greatly reduced amounts of synaptonemal complex formation (Pathan et al. 2013), except in the regions of the nucleolus organizers (Stronghill et al. 2010). Time course experiments show that hop2-1 mutants have a shortened leptotene stage (Stronghill et al. 2014) and a delayed exit from zygotene stage (Stronghill et al. 2010). Like in yeast and mammals, hop2-1 meiosis also shows a tangled mass of chromosomes (Stronghill et al. 2010). Meiosis is not arrested in prophase I in Arabidopsis, therefore subsequent stages can be examined; chromosome bridges and fragments are seen during chromosome segregation in hop2-1 mutants (Stronghill et al. 2010). It is important to note that in hop2-1 mutants, chromosome bridges are found in both anaphase I and anaphase II meiocytes (Stronghill et al. 2010).

In summary, when lacking HOP2 protein, yeast, mammals and plants all are sterile, showing severely defective meiosis. Illegitimate synapsis and tangled chromosomes are seen in all three groups. Evidence of incorrectly repaired DSBs are found in hop2 mutants of plants, but in yeast and mammals, meiosis does not progress far enough to allow studies to determine if the breaks would have been eventually repaired. HOP2’s primary role is thought to be in the intermediate stages of DSB repair in the HR pathway. Failure of its function also somehow leads to reduced levels of SC formation. Additionally, the presence of illegitimate chromosome rejoining in hop2-1 Arabidopsis plants leads to chromosome bridges and fragments.
1.7 DSB repair pathways outside of meiosis

DNA double strand breaks (DSBs) are lesions that occur on both strands of a DNA double helix. During meiosis DSBs are induced intentionally by the SPO11 protein. In addition to the programmed DSBs (Keeney et al. 1997) that are induced during meiosis, dsDNA breaks can also be produced by various DNA damaging agents including radiation (Terradas et al. 2012). These breaks are considered highly toxic as they can lead to genomic instability (Reviewed by McKinnon, 2009), and if left unrepaired could be lethal or tumorigenic (Amiard et al. 2010). The HR DSB repair pathway is important not only for repairing the programmed DSBs during meiosis, but also to repair DSBs created via DNA damaging agents and in response to stalled DNA replication forks (Budzowska and Kanaar, 2009; Iliakis et al. 2015).

In eukaryotes, there are two main systems responsible for repairing DSBs in the DNA: 1) The homologous recombination (HR) pathway and 2) the non-homologous end joining (NHEJ) pathway (Mahaney et al. 2009; Mazon et al. 2010). The HR pathway repairs DSBs using homologous DNA sequences derived either from a homologous chromosome or a sister chromatid as the recombination partner (Shrivastav et al. 2008). In the cell cycle, other than at meiotic M phase, the HR pathway works mainly in late S, G2 and early M phase, during which times sister chromatids are available to recover the lost genetic information (Shrivastav et al. 2008). The NHEJ pathway does not use DNA sequence homology to repair the break, but instead it joins the DNA ends from both sides of the break (Brandsma and Gent, 2012). Because NHEJ does not require a homologous sequence it may be used throughout the cell cycle, but must be used during G1 when homologs are not associated and there are no sister chromatids (Brandsma and Gent, 2012).

Many meiotic proteins involved in the HR repair pathway during meiosis have been found to function in DSB repair outside meiosis. Mutations in these proteins can cause abnormalities in genomic integrity. As previously mentioned, the Mre11-Rad50-Nbs1 (MRN) complex functions to detect DSBs and initiates the DSB repair during meiotic HR pathway (Amiard et al. 2010). In Arabidopsis, mrn mutants also exhibit genomic instability, and are more sensitive to replication stress (Amiard et al. 2010). The MRE11 protein plays an important role in binding to DNA
(Williams et al. 2010) and resectioning DSBs during the repair; mre11 mutants of Arabidopsis show mitotic chromosome fragments and bridges (Puizina et al. 2004).

In the meiotic HR pathway, the RecA homologs RAD51 and DMC1 promote strand invasion of the single-stranded DNA (ssDNA) into the homologous double stranded DNA (Uanschou et al. 2013). RAD51 is required not only for meiotic recombination, but also mitotic recombination in most eukaryotes (Rodrigue et al. 2013). Studies that knocked out RAD51 paralogs (RAD51C and RAD51B) in HeLa cells have found G2/M checkpoint arrest (Rodrigue et al. 2013). The partial loss-of-function rad51-2 mutants in Arabidopsis are hypersensitive to both γ-irradiation and cisplatin (Pradillo et al. 2012). The mutant plants experience severe vegetative growth defects when treated with these two damaging agents (Pradillo et al. 2012). In contrast to rad51 mutants, dmc1 mutants only show deficiencies in meiosis (Bishop et al. 1992; Fukushima et al. 2000); suggesting that the DMC1 protein is meiosis specific, with no reported roles outside of meiosis (Andersen and Sekelsky, 2010).

1.8 Research objectives

Many studies have analyzed HOP2 in meiosis, yet the possible role of HOP2 outside of meiosis has not been investigated. My research project focused on the possible role of HOP2 in genomic integrity. Specifically, I investigated the possibility that HOP2 might also have a role in repairing DSBs in other parts of the cell cycle where HR can occur (late S, G2 and early M phase).

There are several observations that make this a reasonable study. In Arabidopsis, HOP2 expression can be found not only in reproductive tissues, but also in vegetative tissues during periods of rapid cell division (Schmid et al. 2005). Previous studies have found anaphase bridges not only in hop2-1 anaphase I but also in anaphase II of meiosis (Stronghill et al. 2010). The chromosome bridges found in anaphase I could be caused by unresolved DSB intermediates between homologous chromosomes, but the anaphase I bridges should be broken before the cells enter meiosis II (Stronghill et al. 2010). The presence of bridges at meiotic anaphase II suggests that incorrect DSB repair occurred when a sister chromatid was used for the repair. Since sister chromatids are the usual substrates for HR repair during a vegetative cell cycle, I wanted to see if HOP2 might be important for vegetative DSB repair.
*Arabidopsis* is a powerful model organism to study HOP2 due to its short generation time, small size and sequenced genome (Koornneef and Meinke, 2010). While *hop2-1* mutants in *Arabidopsis* are sterile, no obvious vegetative phenotype has been reported by previous studies for mutants grown under growth chamber conditions. In my study, to investigate the role of HOP2 outside of meiosis, mitotic cells from *Ler* and *hop2-1* *Arabidopsis* were compared both under growth chamber conditions and when plants were exposed to DNA damaging ionizing radiation (IR). Specifically, I examined the mitotic chromosome behavior in the mitotically active petal cells of *hop2-1* and its parent line, *Ler*. To probe for abnormalities in *hop2-1* plants, I assessed the ability of cells to get past the G2 checkpoint and enter mitosis by examining the mitotic indices in *Arabidopsis* petals. Additionally, I measured and compared the distances between segregating chromosome sets in *Ler* and *hop2-1*. I also focused on cells undergoing chromosome segregation and counted the number of cells with chromosome fragments and bridges, which indicates incorrectly repaired DSBs.
2 Materials and Methods

2.1 Plant material

In this study, I used *Arabidopsis thaliana* Landsberg erecta (Ler) lines for chromosome analysis. A total of two lines were used. The Ler plants were planted and used as a positive control. Seeds of the Ler ecotype were provided by the *Arabidopsis* Biological Resource Center, Columbus, Ohio. The hop2-1 mutants were recovered from a segregating population of hop2-1+/− heterozygotes. The original hop2-1+/− seeds were obtained from R. Sablowski, John Innes Centre, Norwich, UK. The hop2-1 mutant was generated via T-DNA insertion that interrupted the nucleotide sequence (Schommer et al. 2003). For my study, the progeny of self-crossed hop2-1/HOP2 heterozygotes were planted. The hop2-1 homozygous mutant plants were identified by their sterility; homozygous hop2-1 mutants did not have pollen on their stigma, and the anthers were short, never reaching the stigma.

2.1.1 Seed preparation

Both Ler and hop2-1 heterozygous seeds were stored in microfuge tubes in the freezer (at 0°C) overnight prior to planting to kill insect pests’ eggs. The seeds were taken out the next day, and approximately 1mL of distilled water was added to the microfuge tube. The tube was placed on a vortexer for 30 minutes, and then the seeds were surface sterilized by replacing the water with 70% ethanol and doing a 5-minute incubation with agitation. After sterilization, the seeds were washed 3 times using sterile distilled water, and for every wash a fresh sterile tip was used to add distilled water or remove discard.

A second sterilization was performed by adding 1mL of a bleach and wetting solution containing 0.7% sodium hypochlorite and 0.2% Sodium Dodecyl Sulfate (SDS) with agitation. The solution was made by adding 10uls of 20% SDS, 66uls of chloro-12-bleach (12% sodium hypochlorite) and 924uls of distilled water. The seeds were incubated in the solution for 5 minutes and were washed 6 times using sterilized distilled water. For every wash the microfuge tube was placed into the centrifuge for 5 seconds and then the water was removed and replaced with clean sterile
distilled water. After the last wash approximately 1mL of sterile distilled water was added into the microfuge tube before the seeds were planted.

2.1.2 Soil preparation

The soil used for planting was Promix ‘Plug and Germination Growing Medium’. The pots used for planting each holds 140mL of fully hydrated soil mix. To plant 5 pots, equivalent 5 packed-pots worth of Promix were mixed with two solutions. The first solution was made by mixing 1/4tsp of fertilizer with 500mLs of distilled water. The second solution was prepared by mixing 1/8tsp of nematode paste (Natural Insect Control) with 100mLs of tap water. The soil-solution components were well mixed until a homogenously moist soil was achieved. The final volume of fully hydrated soil mix was 700mLs. The mixture was allowed to set and a little more water was added as necessary until the soil was moist and would not hold more water. When the soil was ready, the lower third of each pot was first filled and the soil was packed down with a spoon. The pot was then filled to approximately 2cm from the top.

2.1.3 Planting

For each pot, approximately five well separated seeds were planted. 5 pots of Ler and 20 pots of hop2-1 heterozygous Arabidopsis were planted every week. The pots containing newly planted seeds were covered with a plastic lid for protection, and the lid was left in place until two cotyledon leaves and two true leaves were seen. The plants were grown in the growth chamber with the temperature of 22°C, humidity of 69% and 170μMol of light with 16-hour light/8-hour dark cycle per day. After seven days, excess plants in each pot were removed, leaving only two well separated plants.

2.1.4 Watering

Plants were watered with tap water. Before germination each pot was sprayed five times at the soil surface using a spray bottle every day; the plastic lid was also squirted, then was placed back over the plants to provide a moist environment. Once germination had occurred and plants had two true leaves, the lids were removed. After the lids were removed the plants were watered
from below by filling the tray with approximately 1-2cm of water every 2-3 days, as soon as the soil at the top of the pot dried out.

2.2 Cytology

2.2.1 Bud preparation

Previously, buds with diameters of 0.4-0.5 mm were identified as buds with high mitotic index in the petals (Smyth et al. 1990). Hence buds in the size range of 0.4-0.5 mm were harvested for the experiment. Each appropriate size bud was placed on a double sided, fabric tape for sepal removal. The outer two sepals were first peeled back and removed with a fine scalpel, and then the inner two sepals were removed using the same technique.

Bud fixation was performed after sepal removal. Each bud was harvested and processed separately to make sure that each bud had the same incubation time. The ‘De-sepaled’ bud was placed and incubated in a depression slide containing 1mL of 3.7% paraformaldehyde (PFA) in buffer A (Appendix 1 for recipes) for 2 hours, followed by 3 rinses using 10mM Sodium Citrate (NaCitrate) buffer with pH4.5 (Appendix 1 for recipes). The depression slides containing buds were covered with a plastic lid to prevent evaporation and placed inside the fume hood during the fixation process.

2.2.2 Cell wall softening

Cell wall softening was performed using 20ul of 0.3% Pectolyase (Sigma-Aldrich) dissolved in 10mM NaCitrate buffer (pH4.5). Each de-sepaled bud was individually incubated in one drop of freshly made 0.3% Pectolyase (approximately 50uls) on a clean segment of black hydrophobic table cloth fabric for 1 hour at room temperature. To prevent the drop of enzyme from evaporating, a cover was put on top of the enzyme.

2.2.3 Post-fixation and permeabilization

Buds were transferred from the cell wall enzyme mixture to buffer A in a depression slide and incubated for 30 minutes. Post-fixation and permeabilization was performed simultaneously via transferring the buds into depression slides containing 500ul of 3.7% PFA and 500ul of 0.5%
Triton X in Phosphate Buffered Saline solution (PBS) (Appendix 1 for recipes); each bud was incubated for 15mins.

2.2.4 Petal isolation

Buds were rinsed three times using 3% Bovine Serum Albumin (BSA) (BioShop) in PBS buffer in a depression slide. Each rinsed bud was transferred to a drop (approximately 200uls) of 3% BSA in PBS on a hydrophobic table cloth. Individual bud was dissected with a fine scalpel, and the petals from each bud were isolated and individually transferred onto a slide with 10ul of Vectashield Mounting Media containing 4’,6-Diamidino-2-Phenylindole (DAPI) (1.5ug/mL) (Vector Laboratories, Canada). A 22X22 mm coverslip was placed over the sample area. After the location of the petal was identified with phase contrast using a Zeiss AxioStar microscope, a circle was drawn on the underside of the slide to indicate the location of the petal. Pressure was slightly applied onto the coverslip to spread the petal cells, then the coverslip edges were sealed using nail polish.

2.3 X-ray treatment

X-ray treatment was carried out at the animal facility at the University of Toronto Scarborough using a RAD SOURCE 2000 device. Prior to irradiation, plants with 1-2 open flowers were selected and their heights were measured. The height was measured from the base of the weighing boat (the small tray that holds the pot) to the top of the primary inflorescence. The plant height had to be factored in such that all irradiated inflorescences were the same distance from the radiation source. For example, if the tallest plant had a height of 4.6cm, then the distances of the shorter plants from the radiation source were adjusted by placing an appropriate number of sheets of paper under the weighing boat, such that all the plant’s inflorescences were the same distance from the X-ray source. The distance selected was such that 2.9 Gy/min were delivered and the exposure was for 8.62 minutes, so that the dosage received for each plant was 25Gy.

Irradiated plants were returned back to normal lighting conditions (growth chamber) after the treatment, and the buds were collected and processed after a recovery period. For the
experiments, two different recovery periods were used; 70mins was used for one set of experiments and 24hrs was used for the other set of experiments.

2.4 Microscopic analysis

Cytological analysis of the petal preparations was done using an Axioplan Fluorescence Microscope with a Zeiss 63X oil immersion PLAN-APO CHROMAT (NA 1.4) lens for the detailed imaging. An image capturing software Axiovision Ver. 4.8 was used for image captures.

2.5 Data collection and three dimensional reconstructions

The first set of experiments were done without radiation. For these un-irradiated plants, the mitotic index was determined and anaphase and telophase stages were inspected for the presence or absence of normal chromosomal segregation. As well, when conducting the anaphase distance measurements a z-stack image acquisition was first conducted using Axioplan Fluorescence Microscope and Axiovision Ver. 4.8. Next the images of the chromosomes were used for three dimensional reconstructions using Image Analysis Software Volocity (Figure 6). D1, D2 and D3 distances were measured from the 3D reconstructions, as shown in Figure 7. Distance 1 (D1) is a measure of the closest inner distance between the separating chromosome sets, distance 2 (D2) is a measure of the farthest inner distance observed and distance 3 (D3) is a measure of the farthest outer chromosome distance (Figure 7). All ‘normal vs. mutant’ measurements and assessments were done ‘blind’, where slide number and genotype were only matched after the primary data was collected.

2.6 Statistical analysis

Statistical software R project was used to conduct data analysis, “Two sample” T-Test and 2X2 Chi-square tests were used to determine whether there were any significant differences between Ler and hop2-1 Arabidopsis data.
Figure 6 3D reconstruction of the chromosomes using the software Volocity.

The diagram shows two chromosome sets separating from one another during anaphase stage. The blue line represents the inner distance measured between the separating chromosome sets. Note that all the distances between chromosome sets were measured in 3D as illustrated in the diagram.
Figure 7 Illustration of the measurements of three chromosome distances.

The diagrams show a symmetric a), and an asymmetric anaphase b), and in each diagram two chromosome sets are separating from one another. The double headed arrows represent the way the distances were measured. Note that all the distances between chromosome sets were measured in 3D as illustrated in Figure 6. Distance 1 (D1) (shown in white) represents the minimum inner distance between the separating chromosome sets, distance 2 (D2) (shown in blue) is the maximum inner distance and distance 3 (D3) (shown in red) is the maximum outer distance between the chromosome sets. Note that in b), D1 is close to zero. Scale bar = 10μm.
3 Results

3.1 No abnormal vegetative phenotypes were found in hop2-1 homozygous mutant plants.

As previously reported no obvious abnormal vegetative phenotypes were found in hop2-1 mutant Arabidopsis (Figure 8a and 8b). However, abnormal reproductive phenotypes were seen in hop2-1 plants (Schommer et al. 2003; Stronghill et al. 2010). First the stamens were shorter in hop2-1 (Figure 8d) when compared to Ler (Figure 8c). Also, while the Ler stigma contained viable pollen (Figure 8e) no pollen was found in hop2-1 stigma (Figure 8f). The siliques in hop2-1 (Figure 8h) were significantly smaller, when compared to Ler (Figure 8g).

Figure 8 Images of Ler and hop2-1 plants of A.thaliana.

Images from Fig 8a and 8b were captured using a digital camera. Figure 8c-8h were captured by a digital camera attached to a dissecting microscope.

a) A mature Ler plant. b) A mature hop2-1 mutant plant. c) Stamens from a Ler plant. d) Stamens from a hop2-1 mutant plant, note the short stamens compared to Ler, as indicated by the arrows. e) Pollen on the Ler stigma, as indicated by the arrow. f) No pollen found on hop2-1 stigma, as indicated by the arrow. g) Siliques from a Ler plant. h) Siliques from a hop2-1 mutant plant, note the small silique phenotype, as indicated by the arrows.
3.2 The *hop2-1* plants exhibited no major differences from *Ler* plants when grown under normal conditions

The first set of experiments was conducted by comparing mitotic cells from petals between *Ler* and *hop2-1* plants grown under normal conditions. For assessing the chromosome segregation phenotypes 300 *Ler* ‘anaphases+telophases’ and 300 *hop2-1* ‘anaphases+telophases’ were collected, fixed and stained using DAPI. The mitotic index for each petal was calculated by dividing the total number of cells undergoing mitosis by the total number of cells counted (1500 cells per petal), as is provided in Table 1.

**Table 1 Comparisons of the average mitotic index, total number of normal and chromatin bridge segregations in un-irradiated *Ler* and *hop2-1* plants**

<table>
<thead>
<tr>
<th></th>
<th>Mitotic Index</th>
<th># of chromatin bridge segregation</th>
<th># of Normal segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ler</em></td>
<td>2.4%</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td><em>hop2-1</em></td>
<td>2.2%</td>
<td>0</td>
<td>300</td>
</tr>
</tbody>
</table>

Petals taken from *Ler* plants (Figure 9a) had an average mitotic index of 2.4% and petals taken from *hop2-1* plants (Figure 9b) had an average mitotic index of 2.2%. The statistical analysis showed a p-value of 0.1329, indicating no significant difference between these two mitotic indices. No abnormal anaphases (fragments or chromatin bridges) were found in either genotype, but there were some suggestions that the two separating chromosome sets in *hop2-1* nuclei were generally not as uniformly far apart as those in *Ler* nuclei. To check this quantitatively, three key distances were measured in reconstructed nuclei; results are presented in the next section.
Figure 9 DAPI-stained mitoses prepared from un-irradiated *A.thaliana* petals. 
*Ler* and *hop2-1* petals were shown in a) and b), respectively. Anaphase (arrows) and metaphase (triangles) stages chromosomes are distinctly visible. *Scale bar* = 10μm.
3.3  *hop2-1* plants showed a slight tendency to asymmetric segregation of the chromosomes at anaphase

For distance measurements, a total of 300 *Ler* anaphases and 300 *hop2-1* anaphases were measured. For each anaphase, three distances were measured (Figure 7). Distance 1 (D1) is the closest inner distance between two separating chromosome sets, distance 2 (D2) is the farthest observed inner distance between two separating chromosome sets, and distance 3 (D3) is the farthest outer distance between two separating chromosome sets.

The average D1, D2 and D3 distances for each genotype are shown in Table 2. The “Two sample” T-test was conducted to determine whether there were any significant differences between the distances in *Ler* and *hop2-1* plants. When comparing the mean D1, D2 and D3 distances, *hop2-1* nuclei had a higher average D3 but a lower average D1 and D2 distances than *Ler*. But the T-Tests showed no significant difference between *hop2-1* and *Ler* nuclei for distances D1 and D3, with a p-value of 0.0782 and 0.202 respectively. The average D2 in *hop2-1* was significantly shorter than the average D2 distance in *Ler*, with a p-value of 0.0374. Thus, for one of the three distances measured, the segregating chromosome sets for *hop2-1* nuclei were somewhat closer together than in *Ler* nuclei, suggesting a slight delay in chromosome segregation in *hop2-1* nuclei. I also compared the (D3-D1) difference between the two genotypes. I used the D3 distance as a measure of how far into anaphase the nucleus was and the D1 distance was an indicator of the chromosome region that had separated the least amount. I took the D3-D1 differential distance as a measure of the slowness of the chromosome segregation. Therefore, the D3-D1 value is an attempt to measure the difficulties in chromosome segregation. Data analysis of the D3-D1 distances in *hop2-1* and *Ler* revealed a p-value with a value of 0.0001. Indicating that grown under optimal growth chamber conditions, *hop2-1* anaphase nuclei had some difficulties segregating the chromosomes, even though they did eventually completely segregate.

Thus even though no obvious morphological phenotypes were observed, *hop2-1* plants cultivated in growth chambers under optimal conditions only had mild difficulties in chromosome segregation during mitosis. In the next set of experiments *hop2-1* mutants were compared with *Ler* when both genotypes were exposed to double strand break inducing X-radiation.
Table 2 Comparisons of the mean D1, D2, D3 distances between 300 un-irradiated Ler and 300 hop2-1 anaphases

<table>
<thead>
<tr>
<th></th>
<th>Mean D1 (μm)</th>
<th>Mean D2 (μm)</th>
<th>Mean D3 (μm)</th>
<th>Mean (D3-D1) (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler</td>
<td>6.26531±2.98</td>
<td>9.2845±3.28*</td>
<td>19.0299±4.40</td>
<td>12.76459±2.80*</td>
</tr>
<tr>
<td>hop2-1</td>
<td>5.85367±2.74</td>
<td>8.762067±2.86*</td>
<td>19.43217±3.25</td>
<td>13.57849±2.29*</td>
</tr>
</tbody>
</table>

\[D1\] The shortest inner distance between the separating chromosome sets
\[D2\] The farthest inner distance between the separating chromosome sets
\[D3\] The farthest outer distance between the separating chromosome sets

* Statistical analysis showed a significant difference between Ler and hop2-1

3.4 hop2-1 plants showed a significant increase of “incorrectly repaired” DSBs, when compared to Ler after ionizing radiation

For my next set of experiments (X-ray treatment 1), both Ler and homozygous mutant hop2-1 plants were irradiated; 70mins after irradiation the buds were collected and processed. For the last set of experiments (X-ray treatment 2) the buds were collected and processed 24hrs after irradiation with the same dosage.

3.4.1 X-ray treatment 1: 25Gy X-irradiation and 70mins of post exposure recovery

For X-ray treatment 1 both Ler and hop2-1 Arabidopsis were irradiated by X-ray with a dosage of 25Gy, after irradiation the plants were kept in the growth chamber for 70mins before the buds from primary inflorescences were collected and processed. For this set of experiments, 1,500 cells were analyzed for each petal. A total number of 32 petals were used, so a total sample of 48,000 nuclei were analyzed for each genotype. For each petal the mitotic index was calculated, and the number of normal and abnormal segregations were recorded. Examples of abnormal segregations are illustrated in Figure 10.
Figure 10 Abnormal chromosome segregations were found in both Ler and hop2-1 plants 70mins after irradiation.

a) Mitotic cells prepared from X-ray treated Ler Arabidopsis petals; an anaphase with chromatin bridge is shown in the middle. b) Mitotic cells prepared from X-ray treated hop2-1 petal, an anaphase with chromatin bridge is shown. c) Mitotic cells prepared from X-ray treated Ler Arabidopsis petal, anaphase with two chromatin bridges is shown in the middle. d) Mitotic cells prepared from X-ray treated hop2-1 Arabidopsis petal, an anaphase with two bridges is shown. e) Mitotic cells prepared from X-ray treated Ler petal, a telophase with chromatin bridge is shown in the middle. f) Mitotic cells prepared from X-ray treated hop2-1 Arabidopsis petal, a telophase with chromatin bridge is shown in the middle. Scale bar = 10μm.
The results for X-ray treatment 1 are summarized in Table 3.

**Table 3 Comparisons of the average mitotic index and the total number of normal and chromatin bridge segregations for Ler and hop2-1 plants 70mins after irradiation**

<table>
<thead>
<tr>
<th></th>
<th>Mitotic index</th>
<th>Normal Segregation</th>
<th>Chromatin bridge Segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ler</strong></td>
<td>0.3%</td>
<td>60/86 (69.8%)</td>
<td>26/86 (30.2%)*</td>
</tr>
<tr>
<td><strong>hop2-1</strong></td>
<td>0.27%</td>
<td>43/85 (50.6%)</td>
<td>42/85 (49.4%)*</td>
</tr>
</tbody>
</table>

* Statistical analysis showed a significant difference between Ler and hop2-1

The mitotic indices for both Ler and hop2-1 nuclei were reduced dramatically 70mins after irradiation, when compared to the un-irradiated cells of both genotypes. In un-irradiated Arabidopsis, Ler plants had an average mitotic index of 2.4% and hop2-1 plants had an average mitotic index of 2.2%. In contrast the irradiated Ler and hop2-1 plants had a reduced average mitotic index of 0.3% and 0.27% respectively. A T-test revealed no significant difference of mitotic indices between Ler and hop2-1 (with a p-value of 0.6279). 70mins after X-ray exposure, the small difference of mitotic indices observed between the two genotypes was not significant, but the large reduction seen between radiation and no radiation for both genotypes was significant. Thus, the mitotic indices for both genotypes had dramatically decreased compared to un-irradiated plants, and both genotypes equally had significant and similar difficulties in entering mitosis 70mins after irradiation.

For those cells that were in mitosis, I looked for evidence of aberrant chromosome segregation. Specifically, I looked for chromosome fragments and chromatin bridges that would indicate segregation abnormalities due to incorrect repair of the induced double strand breaks. The typical aberration I found consisted of one or more chromatin bridges between sets of segregating chromosomes (Figure 10). The data is summarized in Table 3. Ler petals had an increased number of chromatin bridge segregations when compared to un-irradiated Ler, and hop2-1 nuclei also exhibited an increase of chromatin bridge 70mins after irradiation. The irradiated hop2-1 nuclei in X-ray treatment 1 had a greater number of chromatin bridge segregations compared to irradiated Ler nuclei (Table 3). The 2X2 Chi-square analysis showed a p-value of 0.01513, indicating a significant difference of chromatin bridge segregations between Ler and hop2-1 nuclei 70mins after irradiation.
3.4.2 X-ray treatment 2: 25Gy X-irradiation and 24hrs of post exposure recovery

For X-ray treatment 2 both Ler and hop2-1 Arabidopsis were irradiated by X-ray with a dosage of 25Gy, and after irradiation the plants were kept in the growth chamber for 24hrs before the buds from primary inflorescences were collected and processed. Examples of abnormal chromosome segregations can be seen in Figure 11.

![Figure 11](image)

**Figure 11** Abnormal chromosome segregations were found in both Ler and hop2-1 plants 24hrs after irradiation.

a) Mitotic cells prepared from X-ray treated Ler petal, an anaphase bridge is shown by the arrow. 
b) Mitotic cells prepared from X-ray treated hop2-1 petal, an anaphase with chromatin bridge is shown by the arrow. 
c) Mitotic cells prepared from X-ray treated Ler petal, an anaphase with two bridges is shown by the arrow. 
d) Mitotic cells prepared from X-ray treated hop2-1 petal, an anaphase with remnant of bridge is shown by the arrow. *Scale bar = 10μm.*
The results for X-ray treatment 2 are summarized in Table 4.

**Table 4 Comparisons of the average mitotic index and the total number of normal and chromatin bridge segregations for *Ler* and *hop2-1* plants 24hrs after irradiation**

<table>
<thead>
<tr>
<th></th>
<th>Mitotic index</th>
<th>Normal Segregation</th>
<th>Chromatin bridge Segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ler</em></td>
<td>1%</td>
<td>260/278 (93.5%)</td>
<td>19/278 (6.8%)*</td>
</tr>
<tr>
<td><em>hop2-1</em></td>
<td>1.3%</td>
<td>280/316 (88.6%)</td>
<td>38/316(12.0%)*</td>
</tr>
</tbody>
</table>

*Statistical analysis showed a significant difference between *Ler* and *hop2-1*.

Similar to X-ray treatment 1, the mitotic indices and the number of normal and abnormal segregations were recorded. The mitotic indices for both *Ler* and *hop2-1* nuclei increased significantly 24hrs after irradiation (up to 1.0% for *Ler* and 1.3% for *hop2-1* mutant petals) when compared to the first X-ray treatment, but were still lower than the average mitotic indices found in un-irradiated *Arabidopsis* (Table 4). A T-test revealed no significant difference of mitotic indices between *Ler* and *hop2-1* plants 24hrs after irradiation (with a p value of 0.0693).

In the second X-ray treatment both genotypes still had difficulties entering mitosis. The *hop2-1* petals had a higher percentage of chromatin bridge segregations compared to *Ler* petals 24hrs after irradiation; the Chi-square analysis showed a p-value of 0.04511, indicating a significant difference of chromatin bridge segregation between *Ler* and *hop2-1*.

Therefore, the *hop2-1* mutants had more incorrectly repaired breaks than was seen in *Ler* plants for both X-ray treatments 1 and 2.
4 Discussion

4.1 A reliable staining and squash protocol was developed to visualize the chromosomal mitotic behavior in petal cells

A staining and squashing protocol has been developed, allowing the visualization of the chromosomal mitotic behavior in petal cells. Petals were used in my experiment because petals from buds with diameter of 0.4-0.5mm (Buds at stage 9) are undergoing rapid growth and have high mitotic indices (Smyth et al. 1990). Also the petals are very thin, having only three cell layers (Irish, 2008); this allows the chromosomes to be readily stained and visualized without the need to disrupt the chromosome organization significantly.

For my first set of experiments, petals from Ler and hop2-1 Arabidopsis growing under normal conditions were collected and stained; I did not find any fragments or chromatin bridge threads in either genotype. This was not totally unexpected, since there was no vegetative phenotype observed in hop2-1 plants, but it did raise concerns that the DAPI staining protocol might not be sensitive enough to detect chromatin bridges. However, in the second and third set of experiments in which I irradiated the plants and then checked for bridges, bridges of various chromatin thicknesses were detected in both genotypes. Thus the DAPI staining technique I used was sensitive enough to detect even slender chromatin bridges when they occurred.

4.2 Probing the role of HOP2 outside of meiosis

Chromatin bridges are seen during meiotic M phase in hop2 mutant Arabidopsis (Stronghill et al. 2010), thus HOP2 is important for the correct repair of the DSBs that are intentionally induced during meiosis. The HR pathway in meiosis shares some features with the HR used in the vegetative cell cycle, but also has features unique to meiosis. To understand the possible role of HOP2 protein outside of meiosis it is crucial to identify the differences between meiotic and non-meiotic HR.

Meiotic and non-meiotic HR pathways differ in four main aspects. Meiotic HR only occurs in prophase I whereas non-meiotic HR occurs during late S, G2, and early M phase, whenever sister chromatids are available as templates to repair DSBs. The meiotic and non-meiotic HR pathways
occur for different purposes: in meiotic HR, DSBs are intentionally induced and then repaired; the reciprocal genetic exchanges that produce crossovers (COs) formed during meiotic HR are crucial for proper chromosome alignment and segregation in metaphase I and anaphase I. The non-meiotic HR is used for repairing DSBs caused by DNA damaging agents (Reviewed by Andersen and Sekelsky, 2010), thus its main role is to prevent genomic instability. While meiotic HR that produces crossovers preferentially uses the homologous chromosome as a template to repair the breaks, non-meiotic HR uses the sister chromatid to repair DSBs. Additionally, some proteins are important for both meiotic and non-meiotic HR while some of the proteins in meiotic HR are unique to meiosis; two examples are SPO11 and the RecA homolog DMC1.

There is evidence that in meiotic HR, both RAD51 and DMC1 are important in the formation of D-loops. The interhomolog-biased repair process in meiotic HR is promoted by DMC1 (Schwacha and Kleckner, 1997), as indicated by the absence of interhomolog recombination in dmc1 meiocytes (Schwacha and Kleckner, 1997). But in yeast and in Arabidopsis, when DMC1 is absent, RAD51 repairs DSBs using sister chromatids (Siaud et al. 2004), and RAD51 overexpression mutants in yeast can replace DMC1 in the production of crossovers (Tsubouchi and Roeder, 2003).

What is not clear from previous studies is whether the role of HOP2 is in only assisting DMC1. If that was the case it would be expected that HOP2’s role would be meiosis-specific. In contrast if HOP2 worked with both RAD51 and DMC1 or with just RAD51, then it might also have an important role in HR that occurs in the vegetative cell cycle.

4.2.1 Impact of HOP2 in unchallenged nuclei

Given that the $HOP2$ gene is expressed in vegetative cells in Arabidopsis (Schmid et al. 2005), in non-meiotic HR, I looked for abnormalities in the vegetative cell cycle, as could be revealed during mitosis in Ler and hop2-1 mutant plants.

As expected for a mutation with no obvious morphological phenotype, there were no major mitotic abnormalities detected in hop2-1 mutant plants grown under normal growth chamber conditions. There were no significant reductions in mitotic indices and there were no detected chromosome fragments or bridges that would provide evidence of incorrectly repaired breaks.
The one observed difference was a tendency in the hop2-1 mutants to have one or more chromosome regions lagging in its segregation. An ideal symmetric anaphase can be seen in Figure 7a. However, even in Ler, not all anaphases are symmetric across the metaphase plate. The five pairs of chromosomes in Arabidopsis have different lengths, with the longest chromosome (chromosome 1) having approximately 30Mb of DNA and the shortest chromosome (chromosome 4) having approximately 18Mb of DNA (Giraut et al. 2011). Therefore, observing some regions with smaller degrees of separation at anaphase (Figure 7b) is not surprising. But the hop2-1 mutant had more of these, as determined by the statistically significant, greater D3-D1 distances in hop2-1 nuclei. It is important to note that eventually the mutant nuclei did seem to separate the chromosome sets since no chromatin bridges were observed in hop2-1 nuclei, and its average mitotic index was similar to that of the Ler plants.

4.2.2 The impact of HOP2 on DSB repair in plants challenged with radiation

In plants, G1 and G2 cell cycle checkpoints are activated until DSBs are repaired. The activation of cell cycle checkpoints stimulates a rapid cellular response to DSBs (Carballo et al. 2006). Previous studies that applied different dosages (5, 10, 20, 40Gy) of X-rays onto Allium cepa L. root meristems found an inverse relationship between the mitotic index and the accumulation of G2 cells, regardless of the dosage applied, suggesting that the cells respond to X-rays by delaying the progression into M phase (Carballo et al. 2006). After a certain amount of recovery time, the mitotic index gradually recovers back to normal (Carballo et al. 2006). Cells receiving a lower dosage requires a shorter time to recover its mitotic index; cells receiving 20Gy of X-ray treatment require approximately 24hrs to resume their mitotic index back to the original state (Carballo et al. 2006).

In my second set of experiments, I irradiated the plants with 25Gy of X-rays, and I processed the buds 70mins after the irradiation. The analysis of my experiment only focused on cells at M phase when it would be possible to visualize the chromosomes using imaging techniques. As shown in Figure 12, the cell cycle in Arabidopsis root meristem are lasts for 17hrs, and the duration of S and G2 phase combined is 8hrs (Yin et al. 2014). My experiment used rapidly dividing Arabidopsis petals (Smyth et al. 1990); they are expected to have similar cell cycle time as root meristems. In order for the cells to be in M phase after only 70mins, they must have been
in late G2 or M phase when they were irradiated. Thus any cells that were hit during G1 or S phase were not assessed in my mitotic analyses done only 70mins after irradiation.

Figure 12 Cell cycle time in cells of *Arabidopsis root epidermis.*

The duration of G1 phase is 6 hours, the duration of S and G2 phase combined is 8 hours. The duration of M phase is 3 hours, with the first hour representing the transition from late G2 to early M phase (As indicated by the double-headed arrow). The solid lines represent the boundaries between stages, but the dashed line indicates that the boundary between S and G2 phase was not determined and together G2 and S are 8 hours in duration (Modified from Yin *et al.* 2014).
The average mitotic index in un-irradiated Ler and hop2-1 plants was 2.4% and 2.2% respectively (Table 1); 70mins after X-ray treatment, the mitotic indices dropped significantly in both genotypes. Ler petals had an average mitotic index of 0.3% and hop2-1 petals had an average mitotic index of 0.27% (Table 3), these small differences in mitotic indices between the two genotypes were not statistically significant. Thus both genotypes did not have time to repair many of the double strand breaks created by the radiation, and thus could not enter M phase. My results for the mitotic indices for irradiated plants was consistent with the study conducted by Carballo et al. (2006), but it is noteworthy that of those breaks that were repaired, the hop2-1 mutant had significantly more incorrectly repaired breaks than in Ler. This conclusion is supported by the fact that 49.4% of the observed chromosome segregations had bridges in the hop2-1 mutant, compared to 30.3% in Ler.

In order to analyze the cells that were irradiated in all parts of the cell cycle, I conducted my last set of experiments by irradiating the plants using 25Gy of X-ray and waiting for 24hrs before I processed the buds for mitotic analysis. With a 24hrs recovery time both genotypes had significantly higher mitotic indices (~1%) when compared to the samples processed 70mins after irradiation (Table 4), but both genotypes still had significantly lower mitotic indices than the un-irradiated plants. As seen in the 70min-recovery experiment, the 24hrs-recovery experiment yielded no difference in mitotic indices between Ler and hop2-1 plants. Thus the rate of repair of DSBs is similar in the Ler and hop2-1 petals.

In the samples processed 24hrs after irradiation, a reduction in abnormal DSB repairs was seen for both Ler and hop2-1 nuclei when compared to samples collected 70mins after irradiation, but the hop2-1 plants still had significantly more incorrectly repaired as shown by the number of mitotic segregations with bridges.

When a 24hrs recovery time was allowed after irradiation, not only did the cells have a longer time to repair the breaks, but also the cells had more than one type of repair mechanism that might be used (HR and NHEJ). Thus the proportion of breaks that are repaired via homologous recombination might be expected to be less. The DNA damage response is mediated by ATM when DSBs are caused by radiation (Williams et al. 2010). The activated ATM then induces a signal cascade that initiates DSB repair and cell cycle checkpoints (Deriano and Roth, 2013).
non-meiotic cells, two main pathways are responsible for repairing DSBs during the cell cycle: the NHEJ pathway and the HR pathway. The contribution of NHEJ and HR depends on various factors including the stage of the cell cycle and the type of DNA damage (Knoll et al. 2014).

NHEJ is a relatively simple pathway (Brandsma and Gent, 2012), and can be classified into two main categories based on the type of joints made at the DSB sites: the “canonical” or “classical” NHEJ (cNHEJ) pathway and the “alternative” NHEJ (aNHEJ) pathway (Mladenov and Iliak, 2011; Knoll et al. 2014). In cNHEJ, the DSB ends are first bound by KU70 and KU80, protecting the ends from degradation (Knoll et al. 2014). The broken ends are then ligated without using any homologous template. The early steps in aNHEJ resembles the HR pathway in that 3’ end resection occurs after the induction of DSB, producing two 3’ ended ssDNA tails, one on each side of the break (Knoll et al. 2014). These two single strands are processed until a short segment of nucleotides is found on complementary strand such that the overhangs can base pair along this segment, with subsequent trimming and ligation (Knoll et al. 2014). Therefore, in aNHEJ, deletion of short sections of nucleotides can occur (Knoll et al. 2014).

The NHEJ system can be used at any time in the cell cycle, except that it is blocked during meiotic prophase I by Nbs1 and Mre11 protein (Yin and Smolikove 2013; Girard et al. 2018). In contrast HR can only occur when either a homologous chromosome is present nearby (during meiosis) or when a sister chromatid is present (only during late S, G2 and early M phase).

It is generally agreed that NHEJ plays the bigger role in repairing simple DSBs caused by various factors in somatic cells (Brandsma and Gent, 2012), possibly due to the fact that it does not require sequence homology to repair the breaks, thus can occur throughout the cell cycle. Yet, it should not be suggested that HR is not important.

HR also plays an important role in repairing ionizing radiation (IR) induced DSBs, as evidenced by the increase of radiosensitivity and abnormal chromosome phenotypes observed in mammalian HR mutants (Tamulevicius et al. 2007). Therefore, when a sister chromatid is available, the IR induced DSBs can be repaired by either HR or NHEJ. Decisions on which pathway to use is based on the complexity of the break (Jeggo et al. 2011). It has been shown that HR is responsible for repairing complex DSBs whereas NHEJ is responsible for repairing the breaks that are less complex (Brandsma and Gent, 2012). Here, the “complexity” refers to
both the chromatin structure and the DNA lesion complexity (Shibata et al. 2011). DSBs located in heterochromatin are more likely to be repaired by HR, whereas the breaks located at regions of euchromatin are predominantly repaired by NHEJ (Jeggo et al. 2011; Shibata et al. 2011). Experiments have shown that DNA lesions that cannot be repaired rapidly undergo resection via HR, whereas the lesions that can be repaired rapidly do not undergo resection, and are repaired by NHEJ (Shibata et al. 2011). One of the models states that whenever DSBs are induced, NHEJ will try to repair the breaks, but if the repairs are not achieved rapidly, NHEJ will be aborted and the repairs will be performed by HR (Jeggo et al. 2011).

In my 70min-recovery experiment all the cells that made it into mitosis must have been in irradiated either in G2 or mitosis itself. But in my 24hr-recovery experiment many of the cells that made it into mitosis could have been hit during G1 and early S phase and might then have been repaired by the NHEJ system. In my 24hr-recovery experiment the numbers of incorrectly repaired breaks in both genotypes were dramatically reduced compared to the 70min-recovery experiment. Whether or not this is due to the fact that 24hr-recovery allows the optimum time in G2 (when HR is possible) or just more time when NHEJ can be used, is not addressed in my study. But in both the 70min and 24hr-recovery experiments the hop2-1 mutant plants had significantly more incorrectly repaired breaks than the Ler plants. Therefore, this strongly suggests that HOP2 is important for the repair of DSBs outside of meiosis.

While the mitotic chromosomes I observed did not show the extensive prophase entanglements similar to those reported during meiotic prophase I, I did observe anaphase bridges similar to those observed in anaphase I and II of meiosis (Stronghill et al. 2010). Combining my observations with the Arabidopsis meiotic work, I conclude that HOP2 is important in repairing breaks produced by radiation in non-meiotic cells via the HR pathway.

4.3 Possible role of HOP2 in non-meiotic HR pathway

DMC1 has the meiosis specific role of ensuring that strand exchange occurs between homologous chromosomes instead of between sister chromatids (Schwacha and Kleckner, 1997), but RAD51 is important for both meiotic and non-meiotic HR DSB repair. The importance of RAD51 protein in non-meiotic HR can be seen in rad51 mutants. While no defects are seen in
Ler, the *C. elegans* rad51 vulva shows abnormalities after being irradiated by 20Gy of γ-rays, suggesting that *rad51* somatic cells are hypersensitive to γ-rays (Rinaldo et al. 2002). In plants, deletion of the RAD51 paralog RAD51B has shown an increased sensitivity to DNA damaging agents including UV-B and bleomycin C, as indicated by the decrease of survival rate after treatment with these agents (Charlot et al. 2014). Thus, their experiments show that RAD51 is important for repair of DSBs in non-meiotic cells. The significant increase of hypersensitivity to radiation also was observed in my experiment, suggesting that HOP2 is also important for the non-meiotic repair.

*In vitro* experiments done on yeast show that in non-meiotic cells, when DNA damage is caused by γ-rays, RAD51 is assembled at the break sites (Gasior et al. 2001). Given its well-defined role in meiosis, it is reasonable to infer that when ssDNA is generated at the site of vegetative DSBs, RAD51 will bind to ssDNA, facilitating inter-sister strand invasion and D-loop formation. The meiotic HOP2 forms a tetramer with MND1 that is thought to assist RAD51 and DMC1 in accomplishing effective strand exchange (Chen et al. 2004; Petukhova et al. 2005; Vignard et al. 2007; Pezza et al. 2014). Given the meiotic data from previous studies, it is highly likely that HOP2 is associated with RAD51 for HR outside of meiosis.

The similar mitotic indices observed in both Ler and hop2-1 mutants after irradiation suggest that the irradiated hop2-1 mutants have similar rates of eliminating IR-induced DSBs as the Ler when either 70mins or 24hrs of recovery is allowed. But a significant increase of incorrectly repaired breaks was found in hop2-1 in both X-ray treatments. I interpret this to mean that HOP2 does not play a crucial role in strand invasion and D-loop formation per se, instead, it ensures accuracy in the subsequent steps of the repair after the initial D-loop formation.

Both my study and the meiosis study conducted by Stronghill et al. (2010) observed chromatin bridges in the absence of HOP2; such bridges are indicative of the presence of dicentric chromosomes. The formation of a dicentric chromosome is illustrated in Figure 13. When DSBs occur on two different helices (Figure 13a), a dicentric chromosome and an acentric chromosome will be formed if the two left sides of the breaks are incorrectly rejoined to each other and the two right sides are rejoined (Figure 13b-c) (Charbonnel et al. 2011). In contrast, a correct repair ligates one left side of the breaks with one right side such that each helix ends up having only
one centromere (Figure 13d-e). Thus, one possible role for HOP2 is that HOP2 is important in the pathway of dHJ resolution in some manner ensuring that ligations are done correctly.

Figure 13 Dicentric and acentric chromosomes form if improper ligation occurs.

a) The light and dark blue parallel lines represent two helices. The yellow circles represent centromeres. DSBs occur on two helices, ssDNA tails are generated after resectioning. b) The dashed curved double-headed arrows represent the incorrect fusion of the two broken helices. c) Formation of dicentric and acentric chromosomes, the red dashed lines represent the ligation of the broken helices. d) The correct fusion of the two broken helices, as indicated by the dashed double-headed arrows. e) Formation of two helices, each with one centromere.
As shown earlier in Figure 5, there are four major pathways that branch after D-loop formation. Two of these pathways (SDSA, Figure 5f-5h and dHJ dissolution, Figure 5m-5n) do not involve crossing over and therefore cannot generate the dicentric chromosomes that would produce bridges. Thus, another possibility is that HOP2 has a role in driving nonDMC1-associated D-loops formed outside of meiosis toward SDSA and/or dissolution pathways.

Interestingly, experiments done on tobacco cells reveal that in the vegetative cells, SDSA is favored over the pathways involving formation of Holliday Junctions (Puchta, 1998). It has been suggested that in vegetative cells, HR via SDSA is the primary model of repairing DSB (Reviewed by Knoll et al. 2014). As COs can lead to dicentric and acentric chromosome formation in somatic cells, the preference of SDSA ensures that COs are rare, thus maintaining genomic stability (Reviewed by Knoll et al. 2014).

Information combined from meiotic and non-meiotic studies prompts me to propose two possible roles of HOP2 in HR. HOP2 could function in driving the repair pathway towards SDSA or dissolution pathways after D-loop formation, unless DMC1 and any accessory proteins are present to drive the D-loop toward the HJ or dHJ resolution pathways. Indeed, in Arabidopsis there are an average of ~250-300 induced DSBs and only an average of 8.9 crossovers produced (Copenhaver et al. 1998; Reviewed by Serrentino and Borde, 2012).

Another possibility is that HOP2 could be involved in the fidelity of processing Holliday Junction (HJ or dHJ), ensuring that subsequent ligations always combine the left side of the break with the right side of the break (Figure 13d-e).
5 Summary and Future Direction

The developed cytological technique allows visualization of the chromosome morphology in M phase nuclei. I assessed the DSB repair events in Ler and hop2-1 plants both when they were grown under normal conditions and when they were challenged by X-irradiation. Plants grown under normal conditions are well protected and are less likely to experience any DNA damage. Indeed, no fragments or chromosome bridges were found in either genotype under normal conditions. When plants were irradiated, the mitotic indices in both genotypes dropped with the same rate, but a significant increase of abnormal (chromatin bridge) chromosome segregations was found in irradiated hop2-1 plants. Furthermore, an increased amount of recovery time after irradiation allowed the mitotic indices in both genotypes to recover to a greater extent, but hop2-1 plants still had significantly more abnormal (chromatin bridge) chromosome segregations than Ler. Therefore, HOP2 seems to have a role in the accuracy of non-meiotic HR repair because when it is absent, there is a significant increase of incorrectly repaired breaks in mitotic anaphase and telophase that can be seen as chromatin bridges.

Chromatin bridges found in mitosis and meiosis are due to the formation of dicentric chromosomes. Thus, the absence of HOP2 led directly or indirectly to creation of dicentric chromosomes both within and beyond meiosis. This suggests that HOP2 is performing the same or at least overlapping functions in meiotic and non-meiotic HR. The effect might be direct- the lack of HOP2 leads to incorrect ligation of the broken ends (Figure 13a-c). Alternatively, the effect could be indirect, where HOP2 drives DSB repair to the SDSA or dHJ dissolution pathways where there is no crossing over and hence a greatly reduced chance of creating dicentric chromosomes.

Previous studies have confirmed RAD51's role in strand exchange and D-loop formation in non-meiotic HR pathway. Comparing the rate of repairs and incorrect-repairs in hop2 and rad51 single and double mutants might allow for a more exact determination of the relationship between HOP2 and RAD51, as well as help in determining which step(s) in HR requires the presence of HOP2.

Additionally, as previously described, HOP2 works together with MND1 and facilitates strand invasion during meiotic HR. The mnd1 mutants in Arabidopsis meiocytes resemble the
phenotypes found in \textit{hop2}; chromosome fragments and bridges are found in anaphase I and II (Kerzendorfer \textit{et al.} 2006) of \textit{mnd1} mutants. In addition to its known function in meiosis, \textit{mnd1} mutants in \textit{Arabidopsis} also showed increased sensitivity to $\gamma$-irradiation, as suggested by the severe growth deficiency observed in irradiated \textit{mnd1} plants (Domenichini \textit{et al.} 2006). This observation provides evidence for MND1’s function in non-meiotic HR pathway (Domenichini \textit{et al.} 2006). Investigation of the mitotic cells in \textit{Arabidopsis mnd1} plants with the exact same cytological methods used in my experiment would be helpful to further the understanding of the relationship between HOP2 and MND1 in non-meiotic HR pathway.
References


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Appendices

Appendix 1 Recipes for Buffer A, Sodium Citrate Buffer and Phosphate Buffered Saline solution (PBS)

Recipes of Buffer A (BA)

<table>
<thead>
<tr>
<th></th>
<th>Initial Stock Concentration</th>
<th>Final Concentration</th>
<th>For 40mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipes-NaOH (pH6.8)</td>
<td>150mM</td>
<td>15mM</td>
<td>4mL</td>
</tr>
<tr>
<td>KCl</td>
<td>0.8M</td>
<td>80mM</td>
<td>4mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>200mM</td>
<td>20mM</td>
<td>4mL</td>
</tr>
<tr>
<td>EGTA (pH6.8)</td>
<td>5mM</td>
<td>0.5mM</td>
<td>4mL</td>
</tr>
<tr>
<td>EDTA (pH6.8)</td>
<td>20mM</td>
<td>2mM</td>
<td>4mL</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>1M</td>
<td>0.32M</td>
<td>12.8mL</td>
</tr>
<tr>
<td>Distilled Water</td>
<td></td>
<td></td>
<td>7.2mL (bring volume to 40mL)</td>
</tr>
</tbody>
</table>

10mM Sodium Citrate Buffer pH=4.5

Dissolve the following in 90mLs of sterile distilled H2O

1. 4.45mL of 0.1M sodium citrate buffer
2. 5.55mL of 0.1M citric acid
3. Adjust the pH

1X Phosphate Buffered Saline solution preparation

Dissolve the following in 800mL distilled water:

1. 8g of NaCL
2. 0.2g of KCl
3. 1.44g of Na2HPO4
4. 0.24g of KH2PO4
1. Using HCl, adjust the pH value to 7.4
2. Using H2O, adjust the total volume to 1L
3. Autoclave the solution