Investigation of the Function of the Meiotic Protein AHP2 in
Arabidopsis thaliana

by

Patti Elizabeth Stronghill

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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Abstract

The ultimate purpose of this study was to investigate AHP2 protein function in *Arabidopsis*; AHP2 protein is known to form a heterodimer with MND1. But to do this an existing chromosome spreading protocol had to be modified to reproducibly provide large numbers of well preserved chromosomes and a multi-criteria meiotic staging method was developed to accurately identify chromosome spreads at specific prophase I substages. As well a technique for combined immuno-cytochemistry and fluorescent *in situ* hybridization (FISH) had to be developed. Coimmuno-localization of AHP2 and MND1 proteins, in wild type meiocytes, revealed synchronous temporal organization (signal initially peaks, for both, during zygotene) but spatially the AHP2 signal appeared exclusively as chromosome axis-associated foci whereas the MND1 signal was diffuse with some foci suggesting that MND1 may also be localizing to loop regions. This finding strongly suggests that MND1 also functions independently of AHP2 during meiosis.

We coupled transmission electron microscopy (TEM) analysis of ultrathin sections of meiotic nuclei with light microscope (LM) analysis of chromosome spreads and demonstrated
that *ahp2* meiocytes fail to stabilize chromosome close alignment that normally occurs during zygotene. My method of combining 2-Bromo-5-deoxyUridine (BrdU) - determination of meiosis duration and the relative durations of each substage allowed us to calculate the absolute duration of each prophase I substage in wild type and revealed that early to mid-zygotene was prolonged in the *ahp2* mutant. This finding was consistent with the *ahp2* mutant’s overall lack of stabilized chromosome alignment.

Scanning electron microscopy (SEM) showed that the *Arabidopsis ahp2* short stamen filament was due to reduced cell elongation in filament parenchyma (epidermal) cells. Detection of illegitimate connections between chromosomes at anaphase I may trigger cell-to-cell signals that result in reduced epidermal cell elongation in the stamen filament.

Finally, I report that the short arms of NOR-bearing chromosomes 2 and 4 homologously pair and synapse in the *ahp2* mutant despite an overall lack of stabilized pairing. This NOR phenomenon has been observed in *Drosophila* but ours is the first report of the ability of NORs to locally induce pairing and synapsis in plants.
Acknowledgements

First and foremost I would like to thank my family; this includes my husband Peter, my children James and Jess, and my parents Alf and Ruby Collins (but most especially I thank my mom). I know that the pursuit of my PhD, at times, negatively impacted them. I thank them for their patience, understanding and encouragement.

I thank God for health of body and mind that was needed to meet the challenges of trying to balance family responsibilities and career. I thank Dr. Hasenkampf for her mentorship and for her understanding as she let me pursue my research at a pace that did not compromise my commitment to family. I thank Dr. Riggs, Dr. Vanlerberghe and Dr. Siddiqui for their molecular biology expertise. I also thank Esther, Helena, Gaya and Nazia for their help on various aspects of my research.

I have immensely enjoyed working with the past and current members of the Hasenkampf and Riggs’s labs; their youthful exuberance has made me feel much younger than my actual years. In particular I would like to thank Esther, Paul, Rashida and Amy for helping me, many times, see the humorous side of things.
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<td>ABRC</td>
<td><em>Arabidopsis</em> Biological Resource Center</td>
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<td>AGI</td>
<td><em>Arabidopsis</em> Genome Initiative</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<td>CT</td>
<td>chromosome territory</td>
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<td>DHJ</td>
<td>double holiday junction</td>
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<td>DIG</td>
<td>digoxigenin</td>
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<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
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<td>DSB</td>
<td>double strand break</td>
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<tr>
<td>DAPI</td>
<td>4′6-diamidino-2-phenylindole</td>
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<td>ds-DNA</td>
<td>double stranded DNA</td>
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<td>FISH</td>
<td>fluorescent <em>in situ</em> hybridization</td>
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<td>fluorescein isothiocyanate</td>
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<td>HtH</td>
<td>helix-turn-helix</td>
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<td>interference insensitive crossover</td>
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<td>ISC</td>
<td>interference sensitive crossover</td>
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<td>LM</td>
<td>light microscope</td>
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<td>Ler</td>
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<td>NASC</td>
<td>Nottingham <em>Arabidopsis</em> Stock Center</td>
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<td>pollen mother cell</td>
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<td>RGE</td>
<td>reciprocal genetic exchange</td>
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<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<td>synaptonemal complex</td>
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<td>SCE</td>
<td>sister chromatid exchange</td>
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<td>SDSA</td>
<td>synthesis dependent strand annealing</td>
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</tr>
<tr>
<td>CDC</td>
<td>Cell division cycle</td>
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<tr>
<td>DMC</td>
<td>Disruption of meiotic control</td>
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<tr>
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<td>Histone H2A variant</td>
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<tr>
<td>MLH</td>
<td>MutL protein homolog</td>
</tr>
<tr>
<td>MND</td>
<td>Meiotic nuclear division</td>
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<tr>
<td>MSH</td>
<td>MutS protein homolog</td>
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<tr>
<td>NBS</td>
<td>Nijmegen breakage syndrome</td>
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<tr>
<td>PAM</td>
<td>Plural abnormalities of meiosis</td>
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<tr>
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<td>Pairing defective</td>
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<td>PTD</td>
<td>Parting dancer</td>
</tr>
<tr>
<td>RAD</td>
<td>Recombination defective</td>
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<td>Rock-n-rollers</td>
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<tr>
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<td>Replication protein A</td>
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<td>SGS</td>
<td>Suppressor of gene silencing</td>
</tr>
<tr>
<td>SMC</td>
<td>Structural maintenance of chromosomes</td>
</tr>
<tr>
<td>SPO</td>
<td>Sporulation</td>
</tr>
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</table>
Glossary of Plant and Meiotic Terminology

Abaxial surface – An example would be the surface of an anther, which faces away from the axis of the flower.

Adaxial surface – An example would be the surface of an anther, which faces toward the axis of the flower.

Achiasmate meiosis - A meiosis where four genetically equivalent gametes are created, in the absence of chiasma between homologous chromosomes.

Acrocentric – The centromere is placed closer to one end of a chromosome compared to the other resulting in a short and long chromosome arm.

Axial association – A protein structure that forms perpendicularly between homologous chromosomes thus stabilizing the association.

Autotetraploid - A diploid organism that has doubled its chromosome complement giving it two equivalent sets of homologs for each of its chromosomes.

Allotetraploid – A hybrid of two closely related species that has two sets of homologous chromosomes, one set from each parent.

Bivalent – Homologous chromosomes joined by one or more chiasmata.

Bouquet – The telomeres of chromosomes localize to a small region of the inner surface of the nuclear envelope (nucleolus for Arabidopsis) thus having the shape of a floral bouquet.

Chiasma – A point along a chromosome where two homologous non-sister chromatids exchange genetic material during chromosomal crossover that occurs during meiosis; this event occurs in pachytene but is not visible until diplotene.

Chromosome close alignment - Homologous chromosomes are considered to be closely aligned when they run parallel to each other and are separated by approximately 200 nm.

Cis-acting DNA - Generally this means ‘acting from the same molecule’; an example would be the NOR of a chromosome facilitating the pairing of that chromosome to its homolog.
Crossover interference – Crossovers (reciprocal genetic exchange) between homologous chromosomes is not random; the presence of one crossover negatively affects the occurrence of a coincidental crossover in proximity to the other.

Dicot - Herbaceous or woody plants that have two seed leaves, net-like leaf vein pattern, circularly arranged vascular bundles in the stem and a taproot system.

Ecotype – A genetically distinct geographic variety of organism that exists within a species; genetic difference mainly relate to adaption to a specific environment.

Epistasis - A phenomena where the effects of one gene are modified by one or more other genes; the gene whose phenotype is expressed is said to be epistatic.

Euchromatin – Chromatin which is lightly packed and is usually transcriptionally active.

Gene conversion - Non-reciprocal genetic exchange, between two homologous chromosomes, that does not result in chiasma formation.

Gynoecium - The female portion of plant anatomy comprised of ovary, style and stigma.

Heterozygous - Two different alleles, of a gene, are present at a specific chromosome locus of homologous chromosomes.

Heterochromatin – Chromatin that is tightly packed and as such is not typically available for transcription.

Heteroduplex DNA – DNA that involves base pairing between homologous single strands derived from different parental duplex molecules; this DNA contains some mismatched base pairs.

Holoenzyme – An enzyme that is comprised of many subunits.

Homologous – The pair of chromosomes in a diploid that contain the same sequence of genes and only differ at some loci with regards to the allele represented.

Homozygous - Identical alleles, of a gene, are present at a specific chromosome locus of homologous chromosomes.
‘Hot spot’ - A specific region along a chromosome where meiotic events such as DSBs or crossovers occur with a frequency greater than the average for the overall chromosome.

Inflorescence – This is a group or cluster of flowers that are arranged on a stem; this stem is called a peduncle.

Karyotype – The characterization of a chromosome complement, of an organism, providing descriptive information for each of the chromosomes eg. length, centromere position, etc.

Kinetochore – A protein structure located at the centromere of each chromosome that provides an attachment point for the spindle apparatus.

Lateral element – The chromosomal axis of the two homologous chromosomes become the two lateral elements of the synaptonemal complex upon SC formation during synapsis.

Locule - The compartment within an anther that contains meiocytes.

Meiotic filament - A collection of meiocytes held together in a callose matrix within an anther locule.

Meiotic synchrony – An example would be a group of meiocytes within a meiotic filament that are approximately at the same meiotic stage.

Meristem – Regions of undifferentiated cells in a plant that are capable of division and growth.

Monocot – Mainly herbaceous plants with long narrow leaves whose major veins run parallel to each other, its vascular bundles are scattered within the stem and these plants have a fibrous root system.

Non-disjunction – The unequal segregation of chromosomes that can occur in meiosis either during anaphase I or II that, in plants, typically results in non-viable pollen grains or ovules.

Non homologous end joining (NHEJ) - A major mechanism for the repair of un-programmed double strand breaks that does not require homologous chromosomes as templates for the repair.

Nucleolus – A non-membrane bound structure made up of proteins and nucleic acids that is found in the nucleus and is involved in ribosome formation.
Nucleolus organizer regions (NOR) - DNA regions on one or more chromosomes, that contain numerous, tandemly arranged repeats of rRNA genes.

Pairing of chromosomes – In light microscopy, chromosome threads that look ‘double thickness’ are paired but whether they are just closely aligned or synapsed is not known at this level of magnification.

Paralog – A gene that has been duplicated and has diverged in sequence; the protein product of this new gene has an altered function within the same species.

Pedicel – The part of a plant that is the stalk of a single flower or silique from an inflorescence.

Peduncle – The part of a plant that is the stalk of an inflorescence.

Pollen mother cell (PMC) – This is the male meiocyte within plants.

Polysomic – An organism that has one or more chromosomes in addition to the normal haploid or diploid complement.

Prophase I - A stage of meiosis I that itself is organized into various substages as follows: leptotene, zygotene, pachytene, diplotene and diakinesis.

Ortholog – A gene, present in different species that has evolved from a common ancestor and has retained the same function.

Reciprocal Genetic Exchange (Crossover) - The exchange of genetic material, reciprocally, between two homologous chromosomes during meiosis; this exchange of DNA results in the formation of chiasmata (a temporary connection) between these chromosomes.

Recombination nodule – This is a transient structure present in prophase I between homologous chromosomes; some nodules may be associated with sites of recombination.

Silique – The seed pod of a plant containing the fertilized ovules.

Sister chromatids – The two chromosomes that result from DNA replication; these chromosomes are temporarily glued along their length by cohesin proteins.
Stamen – The male portion of the plant made up of the stamen filament terminating at one end with the anther which ultimately contains pollen grains.

Svedberg unit – A unit used to measure the speed of sedimentation during ultracentrifugation; this unit is commonly used to describe ribosomal subunits.

Synaptonemal complex (SC) – A tripartite protein-based structure made up of a central element and two lateral elements; this structure secures the alignment of homologous chromosomes along their entire length until reciprocal genetic exchange is complete.

Synizetic knot – The aggregation of centromeres into one or two groups observed during zygotene.

Synthesis dependent strand annealing (SDSA) – This is a type of DNA double strand break repair that requires single strand invasion of duplex DNA but not DNA second end capture.

Tapetum – A layer of cells that surround a group of meiotic cells providing them with nourishment.

Telocentric - The positioning of the telomere at the terminus of the chromosome.

Univalent – Sister chromatids that are not paired with their homologous partners.
Preface

The data in Results – Part II has been published in the American Journal of Botany
The original publication/PDF is available at:
http://www.amjbot.org/cgi/reprint/94/12/2063

The data in Results – Part III has been published in Chromosoma
Stronghill P, Pathan N, Ha H, Supijono E, Hasenkampf C (2010) AHP2 (HOP2) function in Arabidopsis thaliana (Ler) is required for stabilization of close alignment and synaptonemal complex formation except for the two short arms that contain Nucleolus Organizing Regions.
Chromosoma 119: 443-458
The original publication/PDF is available at:
http://www.metapress.com/content/68k02q346836x361/fulltext.html

The bioinformatics data from Results Parts I and IV was retrieved by Patti Stronghill; all corresponding websites and algorithms have been acknowledged
1.0 Introduction

1.1 A very brief overview of my research
The absence of the meiotic protein HOP2 (AHP2) is known to cause sterility in yeast, mouse and Arabidopsis. The goal of my research, in Arabidopsis, is to better understand AHP2 protein’s role in meiosis; more specifically to determine where and how it functions in the context of the double strand break (DSB) repair pathway. This pathway functions during meiosis to repair programmed DSBs in a very specific manner that can result in reciprocal genetic exchange (RGE) between homologous chromosomes. Successful RGE is critical to plant fertility and the generation of genetic diversity.

1.2 Arabidopsis reproductive morphology and meiotic cytology

1.2.1 Why is Arabidopsis a good model organism?
There are several reasons why Arabidopsis is particularly amenable to plant research. Arabidopsis has a relatively small genome ~146 X 10^6 bp. The entire Arabidopsis genome has been sequenced; physical and genetic maps are available for all five Arabidopsis chromosomes (TAIR 2010).

Arabidopsis has a short life cycle; seed to seed requires 4 to 6 weeks depending on ecotype and growing conditions. This is particularly useful when multiple crosses have to be made involving several generations of plants. The plants are small and easily cared for and each plant can produce hundreds of seeds. Arabidopsis thaliana can self fertilize facilitating the maintenance of homozygotes. Selective out crossing to produce double mutants is easily accomplished.

Arabidopsis is easily transformed with the Ti plasmid from Agrobacterium tumefaciens. The engineered T-DNA within this plasmid is randomly inserted into the Arabidopsis genome in an illegitimate manner creating mutations. Thousands of seed lines that contain a single mapped mutation are available at stock centers [Arabidopsis Biological Resource Centre (ABRC) or the Nottingham Arabidopsis Stock Center (NASC)]. Information on these seed lines can be browsed using various criteria (gene name or AGI annotation) and this allows for a reverse genetics approach to the analysis of specific proteins that have already been characterized in other organisms.

1.2.2 Arabidopsis reproductive anatomy
Arabidopsis is a member of the mustard or crucifer family and grows as a small ground rosette whose stems can extend upward to a height of 30-70 cm and whose buds are grouped as inflorescences (Schommer et al. 2003) (Fig. 1a). Siliques or seed pods each contain approximately 50 fertilized ovules
(Fig. 1b, c). As siliques mature and dry out they turn brown and separate along their false septum thus releasing their ripened ovules or seeds. There are numerous ecotypes but the most commonly used in research are the rapid flowering ecotypes Columbia and Landsberg erecta. Flowers arise on the flank of the apical meristem; a dome of cells that is initially about 45 um in diameter. Flowers can be placed in order of age and developmental stage by their position on an inflorescence. The flowers consist of four petals, four sepals, six stamens (2 lateral 4 medial) and a superior gynoecium that consists of two fused carpels (ovary and style) and stigma (Fig. 2a).

1.2.3 Arabidopsis stamen and microspore development
Flowers of eudicots are organized into four concentric whorls of organs (sepals, petals, stamens, and carpels) that arise sequentially from the floral meristem (Fig. 2a). Stamen primordia first differentiate at the surface of the apical meristem and extend slightly with a wider upper region which will ultimately develop into the anther. Cell replication establishes the width of the stamens followed by cell expansion. Stamen cell expansion occurs in two stages and primarily involves the phytohormone gibberellin (Cheng et al. 2003). The terminus of the stamen filament differentiates into the anther that is the receptacle of pollen grains. The cells within the anther differentiate into the various layers; the innermost is the microsporocytes which will ultimately develop into pollen. The next layers, from the inside to the outside are the tapetum, the middle cell layer, the endothecium and the epidermis (Zhao et al. 2002) (Fig. 2b).

During microsporogenesis, archaesporial cells develop into microsporocytes and tapetal cells (Bhatt et al. 2001). The tapetal cells are arranged in a single layer within the anther locule and surround the microspores. The tapetum is metabolically active and plays a nutritive role during microspore development. As meiosis progresses a thick layer of callose (beta-1, 3-glucan polymer) is deposited between the primary cell wall and the cell membrane of the microsporocytes. Callose is also deposited around each microsporocyte and essentially glues them together (temporarily). Each of the four chambers of the anther contains a meiotic filament comprised of approximately 30 pollen mother cells (PMC) connected via a callose matrix (Scott et al. 2004) (Fig. 2c).
Fig. 1 *Arabidopsis* gross morphology. The following are shown: a) a picture of a wild type *Arabidopsis thaliana* plant (Landsberg erecta) ecotype, b) an enlargement from image a) that shows a mature inflorescence with many elongated siliques or seed pods, c) wild type and *ahp2* mutant siliques that have been cut open to expose fertilized and unfertilized ovules respectively.
Fig. 2 *Arabidopsis* reproductive morphology. The following are shown: a) *Arabidopsis* flower anatomy, b) an *Arabidopsis* anther cross section showing the four chambers or locules: M microsporocytes, T tapetum, ML middle layer, C connective tissue, V vascular bundle, E epidermis, EN endothecium, S septum (modified from Scott et al. 2004), c) a fluorescent microscope image of a meiotic filament (a cluster of microsporocytes) extracted from an anther locule; the DAPI-stained DNA appears white.
During the later stages of meiosis the tapetal cells produce and secrete callase that dissolves the callose wall of tetrads allowing individual microspores to separate. The microspores freely reside in the locule and continue their development into immature pollen grains. The immature pollen undergoes two rounds of mitosis to become mature pollen grains (Scott et al. 2004; Enns et al. 2005).

1.2.4 *Arabidopsis* genome

The *Arabidopsis* genome size was originally reported to be 125 Mbp that included an approximation of 10 Mbp of heterochromatic regions (AGI 2000). More recently Hosouchi et al. (2002) report the *Arabidopsis* genome to be approximately 146 Mbp and the larger estimate is based on a more accurate measurement of centromeric DNA. *Arabidopsis*, like many diploid plants, is thought to be an ancient tetraploid; this assessment is partly based on the presence of segmental duplications. Twenty-four large, duplicated segments of 100 kb or more have been identified in *Arabidopsis*. Functional redundancy has to be considered in the study of gene function in *Arabidopsis* because of the large number of segmental duplications present (AGI 2000). Despite significant amounts of gene redundancy the *Arabidopsis* genome is considered to be small (by plant standards). This is mainly due to the relatively small amount of repetitive DNA present; ~ 60% of *Arabidopsis* nuclear DNA is protein coding (AGI 2000).

1.2.5 *Arabidopsis* karyotype

Despite its tetraploid origins, from a chromosomal perspective, *Arabidopsis* is a diploid (n = 5) and a schematic of its karyotype is shown in Figure 3. The lengths of the short and long arms of metacentric chromosome 1 are 14.2 and 14.8 Mbp (Hosouchi et al. 2002). The short and long arms of sub-metacentric chromosomes 3 and 5 are 9.3 and 13.7 Mbp, 11.1 and 14.4 Mbp respectively (Hosouchi et al. 2002). The short and the long arms of acrocentric chromosomes 2 and 4 are 3.0 and 14.7 Mbp and 3.5 and 13.5 Mbp respectively (Hosouchi et al. 2002). Centromere lengths were determined via physical mapping and the lengths for chromosomes 1, 2, 3, 4 and 5 are 9.0, 4.0, 4.0, 5.3 and 4.7 Mbp respectively (Hosouchi et al. 2002). Both chromosomes 2 and 4 have nucleolus organizer regions (NORs) estimated to be 3-3.5 Mbp and are located adjacent to the telomeres of their short arms. The NORs were localized using a 45S rDNA probe and despite the apparent similarity in the number of base pairs, the signal for NOR 4 (when stained with the DNA-dye 4’6-diamidino-2-phenylindole) is always considerably larger than that observed for NOR 2 (Lysak et al. 2001; Siroky 2008).
**Fig. 3 The *Arabidopsis* karyotype.** The five *Arabidopsis* chromosomes are shown. The approximate lengths in megabases (MB) of the short arms, long arms, centromeres and NORs are given. Also the total lengths (MB) are shown beside each chromosome. Chromosome and centromere lengths were obtained from Hosouchi et al. (2002). The NORs are marked in red and the centromeres in black.

<table>
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<th>Total Lengths (MB)</th>
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</thead>
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<td>38.0 MB</td>
</tr>
<tr>
<td>chr 2</td>
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<tr>
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</tr>
<tr>
<td>chr 4</td>
<td>3.5 3.5 5.3 13.5</td>
<td>25.8 MB</td>
</tr>
<tr>
<td>chr 5</td>
<td>11.1 4.7 14.4</td>
<td>30.2 MB</td>
</tr>
</tbody>
</table>
1.2.6 *Arabidopsis* nucleolus organizer region (NOR)

In general NORs consist of multiple tandem repeats of ribosomal RNA genes; the number of repeats is species- and chromosome-dependent. The number of repeats can vary from 50 copies to approximately 500 and NORs can be situated on one or more chromosomes.

In *Arabidopsis* there is a NOR situated sub-terminally at the short ends of chromosomes 2 and 4 (Fig. 3). The organization of *Arabidopsis* NORs is shown in Figure 4 (Neves et al. 2005). Each NOR is approximately 3 to 3.5 Mbp in length and appears to be organized into rDNA gene units; each unit is made up of a heterochromatic intergenic spacer followed in tandem by the genes for 18S, 5.8S and 25S ribosomal RNAs (Neves et al. 2005). Besides being the site where ribosomal RNA genes are located, NORs may have other roles during meiosis, as will be considered later.

1.3 Nucleolus structure and function

The nucleolus is found in both lower and higher eukaryotes. The nucleolus is a prominent subnuclear domain and is the site of transcription of rDNA, processing of pre-rRNAs and the formation of pre-ribosomal particles (Figure 5). In eukaryotes the small 40S ribosomal subunit is comprised of 18S rRNAs and 33 ribosomal proteins. The 60S ribosomal subunit is comprised of 5S, 25S, 5.8S rRNAs and 49 ribosomal proteins. Both ribosomal subunits are assembled in the nucleolus. The 40S and 60S ribosomal subunits are escorted to the cytoplasm by non-ribosomal proteins. In the cytoplasm, the subunits combine to form the 80S ribosome.

The size and number of nucleoli per cell is variable and largely depends on the needs of a specific cell at a specific time. The nucleolus is comprised of rDNA, transcriptional factors and enzymes, pre-rRNA, processed rRNA and pre-ribosome assemblies. It is essentially a cloud of macromolecules surrounding the nucleolus organizer region(s). Using fluorescence recovery after photo-bleaching (FRAP), the nucleolus has been shown to be highly dynamic with a constant exchange of material with the nucleoplasm (Olson 2004). There are three main ultra-structural components to the nucleolus; the fibrillar center (FC), the dense fibrillar component (DCF) and the granular component (GC) (Schwarzacher and Wachtler 1993). The prevalence of each of these sub-structures within the nucleolus is dependent on the transcriptional activity of the cell as demonstrated by Hernandez-Verdun (2006).

The movement of the nucleolus within the nucleus during meiosis has traditionally been used to aid in the identification of the various substages of prophase I of meiosis. During the earliest part of meiosis, the nucleolus occupies a central position in the nucleus, and then moves to the nuclear periphery at the next stage. Later it moves away from the nuclear membrane. Cyclin dependent kinases control the disappearance of the nucleolus as the chromosomes condense and its reappearance is coordinated with the reformation of the nuclear envelope as chromosomes decondense (Olson 2004).
At NORs = ~ 350 rRNA gene units repeated in tandem

rRNA gene unit = --intergenic spacer--18S---5.8S---25S---

~ 10-10.5 kb

Fig. 4 Arabidopsis Nucleolus Organizer Regions (NORs). A schematic is shown of the molecular makeup of the Arabidopsis NORs. The 18S, 5.8S and 25S rRNAs are produced by the splicing of a single 45S pre-rRNA encoded by the rRNA gene unit. The promoter for each 45S pre-rRNA transcript is located within the intergenic spacer of each rRNA gene unit (Neves et al. 2005). S = Svedberg sedimentation coefficient
Fig. 5 The steps in ribosome production. A schematic of the nucleolus and the cellular organization of the progressive steps involved in the production of functioning ribosomes is given. The snoRNA are involved in the methylation of specific nucleosides of 45S pre-rRNA prior to its cleavage by exo- and endonucleases into 18S, 5.8S and 25S rRNAs. The 40S ribosomal subunit is comprised of 18S rRNAs and 33 ribosomal proteins. The 60S ribosomal subunit is comprised of 5S, 25S, 5.8S rRNAs and 49 ribosomal proteins. The *A. thaliana* 5S rRNA (in many ecotypes) is transcribed from rDNA genes located in the pericentric regions of chromosomes 4 and 5. NOR = nucleolus organizer region, snoRNA = small nucleolar RNA; 40 S = small ribosomal subunit; 60 S = large ribosomal subunit; 80 S = mature ribosome.
The nucleolus is now recognized as being complex both in structure and in function. The nucleolus appears to be a highly complex and multi-functional regulatory compartment that functions in diverse biological processes. In addition to being a ribosome factory the nucleolus has recently been associated with a number of alternate functions. Although the primary function of this prominent nuclear sub-organelle is in ribosome biogenesis, a growing body of evidence indicates that it also participates in other aspects of RNA processing, as well as in the regulation of mitosis, cell growth and death, stress responses and the cell cycle (Fig. 6).

1.4 Techniques for examining meiotic nuclei and chromosomes

There are various cytological methods for the examination of chromosomes during meiosis or mitosis. The simplest method is the chromosome squash that relies on gentle physical pressure to flatten meiocytes and in a percentage of the cells to partially extrude nuclear material/chromosomes for better viewing. Squashes typically work well for organisms with large chromosomes and/or with no cell walls or easily displaced cell walls.

Chromosome spreading, to various degrees, more completely removes chromosomes from meiocytes by first breaking down the cell wall with weak acid or detergent (in plants and fungi) and then disrupting both the plasma and nuclear membranes by osmotic pressure and/or detergent lysis. The more complete separation of chromosomes from other cellular components allows for better immuno-staining and a more detailed examination of chromosome morphology. This is particularly important in organisms such as yeast and Arabidopsis that have small chromosomes. However the disadvantage is that spatial organization is lost and weaker types of chromosome associations may be disrupted.

Albini et al. (1994), using a detergent-based spreading protocol, examined Arabidopsis spreads using both light and electron microscopy. The microscopy data produced a karyotype for this model plant and confirmed the existence of NORs at the terminus of the short arms of chromosomes 2 and 4.

Ross et al. (1996), using DAPI stained chromosome spreads, provided the first detailed cytological image-based account of all the substages of meiosis from pre-leptotene to telophase II; a cytological ‘atlas’ of Arabidopsis meiosis. The positioning of chromosomes within the Arabidopsis nuclei has been investigated using an acid-based spreading protocol and FISH (Pecinka et al. 2004). Fransz et al. (1998) have estimated the overall lengths of Arabidopsis wild type pachytene bivalents; these measurements have provided a baseline for comparison to meiotic mutants of this species.
**Fig. 6** Nucleolus-associated cellular processes  Here is a schematic illustrating various nucleolus-associated cellular processes that occur in addition to the nucleolus functioning as a factory for ribosome production (modified from Stark & Taliansky 2009).  snoRNA = small nucleolar RNA, snRNA= small nuclear RNA, siRNA= small interfering RNA
The meiotic staging of chromosome spreads can be difficult and the differentiation of zygotene and diplotene is particularly challenging due to the presence of both paired and un-paired chromosomes at both prophase I substages. A multi-criteria approach to the correct meiotic staging of wild type chromosome spreads from *Arabidopsis* is described in section 2.8.1.

Examination of chromosome ultra-structure such as axial elements and recombination nodules requires electron microscopy. Electron microscope examination of anther ultrathin sections preserves the presence, if any, of un-stabilized chromosome close alignment and spatial relationships within the cell. Digital three-dimensional serial reconstruction of sectioned meiotic nuclei is now possible with the aid of computer programs (Zickler et al. 2006).

1.5 Meiosis overview

Meiosis is a specialized form of nuclear division in which pre-meiotic DNA replication is followed by two divisions of nuclear material. Meiosis is required to halve the chromosome number in meiocytes prior to the fertilization event which restores the diploid number of chromosomes. In a standard meiosis, in a diploid organism, the reduction is done in such a way that each meiotic product receives exactly one of the two homologous chromosomes that a diploid has; thus producing a haploid cell with one of each chromosome of the complement. In the first reductional division, termed meiosis I, homologous chromosomes segregate and in the second equational division, termed meiosis II, sister chromatids are separated. The prophase of meiosis I is extended compared to the prophase of the second meiotic division or to that of mitotic prophase. The extended duration of prophase I is not surprising given the complexity of events that must occur; homologs must come into proximity, recognize each other as their homolog, intimately associate, do reciprocal genetic exchange and stabilize the association in the form of chiasmata. Chiasmata ‘tie’ the homologs together such that they will orient to opposite poles after prophase I is completed. Proper segregation of homologous chromosomes at anaphase I relies on chiasmata dissolution and on cohesin complex release from sister chromatid arms (Cai et al. 2003). Subsequent release of the cohesin complex from sister chromatid centromeric regions is a prerequisite of the equational division of chromosomes at anaphase II (Chelysheva et al. 2005).

Failure of either division is referred to as non-disjunction, an event which has serious genetic consequences for the meiotic products. The entire meiotic process involves a myriad of proteins, many of which interact and some of which have dual roles. Some of these proteins may even have pre-meiotic functions, particularly during S phase (DNA replication), which are important to the meiotic events that follow.
1.6 *Microscopist’s* view of a ‘standard’ meiosis

1.6.1 Light microscopy view

DNA replication occurs during pre-meiotic S phase. After replication each chromosome consists of two sister chromatids that remain attached along their length via the cohesin complex (Nasmyth 2005). These attached sister chromatids are considered to be one chromosome until they become detached during meiosis II. Individual chromosomes are not visible with light microscopy during pre-meiotic interphase. After DNA replication the cell progresses through G2 of interphase and then enters prophase I of meiosis I. The first substage of prophase I is leptotene and during leptotene the chromosomes become visible as thin threads in standard preparations viewed with the light microscope. The ends of chromosomes, called telomeres, attach to the same small region of the inner surface of the nuclear envelope in most eukaryotes. This clustering of telomeres causes the collection of chromosomes to resemble a bouquet of flowers; it is at this time, in many organisms, that homologous telomeres pair up (reviewed by de La Roche Saint-Andre 2007).

During the next stage of prophase I, zygotene, homologous chromosomes typically pair up all along their length. As cells progress to the next substage of prophase I, the ‘bouquet’ arrangement of chromosomes is lost as telomeres of homologous chromosomes remain paired but these ‘pairs of ends’ disperse from each other. The stably associated homologous chromosomes are referred to as bivalents and the process by which they become intimately associated is known as synapsis (Ross et al. 1996).

Chromosomes continue to condense in the next substage known as pachytene. The formation of the structure known as the synaptonemal complex (SC) is completed as meiocytes enter pachytene but synapsed chromosomes appear as one double-thick mass in the light microscope. It is during pachytene that commitment to reciprocal genetic exchange occurs between synapsed homologous chromosomes and chiasmata usually form marking the sites of RGE. The chiasmata cannot be seen microscopically until the next prophase I substage (diplotene) when homologous chromosomes move slightly apart except at these points of attachment (Ross et al. 1996).

During the next substage diakinesis, chromosome condensation continues. Prophase I ends as the nuclear envelope breaks down. The invading microtubules of the spindle apparatus attach to kinetochores (located at the centromere of each chromosome) and bivalents line up in the central region of the meiocyte; this stage is called metaphase I. Once chiasmata dissolve, homologous chromosomes move to opposing cellular poles during anaphase I, and in telophase I two polar groups of chromosomes are observed (Ross et al. 1996).

During meiosis II, which is much like mitosis, sister chromatids each have their own kinetochore. At this point sister chromatids are connected via cohesin proteins only at their centromere. In metaphase II
these sister chromatids can be seen with light microscopy lined up at the metaphase plate with sisters facing opposite poles. Sister chromatids lose their centromeric connections and move to opposing poles of the cell during anaphase II along the microtubules of the spindle apparatus (Nasmyth 2005).

At the end of meiosis II, in plants, the four microsporocytes (male meiocytes) that result from the two meiotic divisions remain attached and are seen with the light microscope as a tetrad of microspores. These microspores are eventually liberated from the tetrad and subsequently undergo two mitotic events to ultimately form the mature gamete or pollen grain (Lui and Qu 2008). These pollen grains are genetically different from the parental cells due to new allelic combinations introduced by reciprocal genetic exchange and the independent assortment of homologous chromosomes during anaphase I. Pollen grains are easily recognizable in the light microscope.

1.6.2   Electron microscopy view
At the resolution of the light microscope each chromosome appears as one entity in leptotene. However during leptotene electron microscopy techniques reveal that each chromosome is composed of two sister chromatids. Sister chromatids appear to share a common chromosome axis, as shown in Figure 7, comprised of two intimately associated parallel sister cores/axes (Dresser and Moses 1980). The cohesin complex that temporarily establishes linkage of sister chromatid axes is likely put in place shortly after S-phase DNA replication. Stack and Anderson (2001) have proposed a model (Fig. 7) whereby chromatin loops of sister chromatids, attached at their bases to their common axis, extend outward in opposite directions. Another model places chromatid sister loops folded back in the same direction (Roeder 1990). In both models the common axis is exposed and free to associate with its homologous counterpart. The common axis is called the axial element.

In early prophase I homologous chromosomes closely align and this alignment, in most organisms, is stabilized by the formation of protein structures called axial associations. Electron and light microscopy examples of axial associations are shown in Figure 8. Typically axial associations are most easily seen in zip1 mutants that closely align their chromosomes but do not follow this with synaptonemal complex (SC) formation; transverse filaments of the SC normally mask the presence of associations (Rockmill et al. 1995).

Normally stabilization of the close alignment of homologous chromosomes is immediately followed by SC formation. The SC as seen in electron microscope preparations is a tripartite structure composed of a central element flanked on both sides by a lateral element (formerly an axial element) (Heyting 1996). A schematic of SC ultrastructure is shown in Figure 9 (Higgins et al. 2005).

Anderson and Stack (1988) upon staining chromosomes of *Psilotum nudum* with uranyl acetate – lead citrate were able to visualize numerous darkly stained nodules associated with the forming SC.
Fig. 7 Schematics of sister chromatid ultrastructure. Three schematics are shown: a pre-meiotic S-phase chromosome (after DNA replication), a G2 chromosome (immature chromosome axis including cohesin complex) and a leptotene chromosome (mature chromosomal axis and loops of chromatin have become further condensed); SAR is the scaffold attachment region of DNA located at the base of chromatin loops. In this model, sister chromatid loops extend out in opposite directions from their common chromosome axis; this arrangement would favor the inter-homolog repair bias that is required in meiosis for reciprocal genetic exchange.
Fig. 8  Electron and light microscope images of axial associations.  Examples are shown of axial associations seen in chromosome spread preparations: a) an electron microscope image of *P. nudum* chromatin (Anderson and Stack 1988); the open arrowheads indicate axial associations and closed arrowheads indicate recombination nodules.  *Scale Bar* 1um ,  b) a light microscope image of triploid lily chromatin (George et al. 2002).  Note how the third DNA strand associates with the bivalent at the axial associations (open arrowheads).  *Scale Bar* 10 um
These nodules had a variable size and shape ranging from ~80 nm spherical particles to 100 by 200 nm ellipsoidal particles. Some of these nodules were also seen associated with unsynapsed axial elements. There appear to be two classes of nodules; early nodules and late nodules observed during zygotene and pachytene respectively. Early nodules are very numerous, but the number and distribution of late nodules along chromosomes appears to correlate with chiasma formation in most organisms (reviewed by Zickler and Kleckner 1999).

1.7 Molecular biologist’s view of meiosis – an overview
The DNA of chromosomes is replicated during pre-meiotic S-phase. Very early in prophase I numerous programmed double stand breaks occur in the DNA of meiocyte chromosomes (Zickler and Kleckner 1999). The 5’ ends of these DSBs are digested by exonucleases to create 3’ single stranded DNA overhangs. These single strand overhangs invade the counterpart duplex DNA regions of homologous chromosomes. By mid- prophase I DSBs are repaired using homologous DNA as their template; an inter-homolog repair bias established at the onset of prophase I favours homologs as repair templates instead of sister chromatids (Zickler and Kleckner 1999). At some sites of DSB repair chiasma are formed and mark the sites of successful reciprocal genetic exchange. These chiasmata are critical to the proper segregation of homologous chromosomes at anaphase I.

1.8 Chromosome preparation for meiosis
1.8.1 Pre-meiotic S-phase and DNA replication
In all organisms examined, the S-phase immediately preceding meiosis is known to be considerably longer than mitotic S-phase in the same organism; for example in C. elegans it is known to be twice as long (Jaramillo-Lambert et al. 2007). It is during S-phase that DNA replication occurs. A possible reason for a prolonged meiotic S-phase may be that the replication process itself is slower as fewer replication origins may be utilized and/or replication forks may advance more slowly. Extra time may be needed for specific chromosomal features to be put in place to ensure that both reductional and equational segregation events are successful. The prolonged pre-meiotic S-phase may be controlled by checkpoints. These checkpoints could involve cyclins, some of which are common to both meiotic and mitotic S-phase progression; an example is the CHK2 protein in C. elegans. This protein is a member of the conserved CDS1/CHK2 family of checkpoint protein kinases that function in a variety of signaling contexts. The C. elegans CHK2 protein possibly functions to delay the transition into G2 until meiosis-specific chromatin modifications, occurring in conjunction with DNA replication, are complete (MacQueen and Villeneuve 2001). In contrast to the CHK2 protein there are some cyclins
Fig. 9  A schematic of the synaptonemal complex. The synaptonemal complex forms between two homologous chromosomes during prophase I of meiosis. The chromatin loops in the schematic represent folded back sister chromatids. The lateral element is a further elaboration of the axial element of un-synapsed chromosomes. The central element is primarily made up of the ZYP1-like protein whose carboxy-terminus is embedded in the lateral element and amino-terminus extends into the central region; these interdigitating proteins make up the central region of the SC (modified from Higgins et al. 2005). The red oval represents a recombination nodule.
that specifically control meiotic S-phase progression; an example is yeast CLB 5/6 proteins (Stuart and Wittenberg 1998). Some Arabidopsis proteins that are required for a successful meiosis appear to have both pre-meiotic S-phase and meiotic functions. AtSPO11-1 and AtREC8 proteins appear to have an effect on pre-meiotic S-phase duration and later meiotic events (Cha et al. 2000). The demonstration of dual roles for meiotic proteins will probably become more commonplace with the advancement of meiotic research.

1.8.2 The cohesin complex and sister chromatid cohesion

The cohesin complex functions in mitosis and meiosis to tether sister chromatids together along their arms and at their centromeres as DNA replication progresses. This tether ensures that the binding and subsequent release of sister chromatids from each other occurs in a controlled manner. The plant mitotic cohesin complex is comprised of four main proteins SMC1, SMC3, SCC1 and SCC3 but during meiosis instead of SCC1 the SYN1/REC8 protein is involved (Lam et al. 2005).

During meiosis in Arabidopsis the cohesin complex localizes to the nuclear matrix (a 3-dimensional filamentous protein network) during interphase and associates with chromosomes by early leptotene (Lam et al. 2005). The complex dissociates from chromosomes in a two stage process. In the first stage cohesin is released from chromosome arms allowing homologous chromosomes to separate at anaphase I. The second stage is the release of cohesins from centromeres at anaphase II, which allows sister chromatids to separate (Lam et al. 2005).

In Arabidopsis the presence of SYN1 is required for the correct localization and organization of the SMC and SCC cohesin proteins (Bai et al. 1999). SYN1 is a cohesin protein that localizes to both chromosome arms and centromeres and is required for the localization of the remainder of the cohesin complex. In the syn1 mutant problems in chromosome cohesion, condensation and bivalent formation were observed (Bai et al. 1999). The SYN1 protein, located at the centromere is protected by the protein shogosin (SGO) during the first division (Watanabe et al. 2004). This is important as SYN1 must dissociate from sister chromatid arms to allow the separation of homologous chromosomes but sister chromatids must remain attached at their centromeres until the initiation of the 2nd meiotic division. In the sgo1 mutant SYN1 is lost at the centromere at anaphase I and attachment of spindles to kinetochores becomes disorganized; as a result non-disjunction occurs (Watanabe et al. 2004).

In Arabidopsis additional proteins are required for the proper assembly and disassembly of the cohesin complex. The AtSWI1 protein that is involved in sister chromatid cohesion is expressed in G1 and S phase of meiosis (Mercier et al. 2003). In the Arabidopsis swi1 mutant the SYN1 cohesin does not associate or dissociate correctly from chromosomes.
Arabidopsis AESP, a separase protein, functions at both the first and second division to cleave SYN1 (Liu and Makaroff 2006). Shogosin protein dissociates from chromosomes during the second division which exposes SYN1 protein to cleavage by the AESP protein (Nakajima et al. 2007). The cleavage of SYN1 initiates the disassembly of the remaining cohesin proteins.

The cohesin complex, in addition to tethering sister chromatids together, is involved in monopolar (anaphase I) and bipolar (anaphase II) kinetochore orientation (reviewed by Watanabe 2004). In Arabidopsis the two non-SMC proteins SYN1 (AtREC8) and AtSCC3 are necessary not only to maintain centromere cohesion at anaphase I, but also for the monopolar (same pole) orientation of the kinetochores during the first meiotic division (Chelysheva et al. 2005). The release of SYN1 from centromeres appears to facilitate the transition to bipolar (opposing pole) sister kinetochore orientation that occurs prior to sister chromatid separation at anaphase II. Interestingly, in Arabidopsis, the cohesin SMC3 localizes to meiotic spindles but the reason for this is unknown (Lam et al. 2005).

The cohesin complex is also thought to be involved in axial element formation and establishing the interhomolog repair bias for reciprocal genetic exchange. In most organisms it appears that axial element formation depends on the prior establishment of sister chromatid cohesion (reviewed by van Heemst and Heyting 2000). The orientation disruptor (ORD) protein is a meiosis specific protein identified in Drosophila that is involved in sister chromatid cohesion. In the ord1 mutant there is an increased incidence of inter-sister DNA exchange that demonstrates a role for cohesin proteins in maintaining an interhomolog repair bias (Weber et al. 2004).

1.8.3 Inter-homolog DSB repair bias
Both un-programmed DSBs and programmed DSBs (as occurs in meiosis), require timely repair that preserves the integrity of the chromosome. Failure to repair meiotic DSBs has lethal genetic consequences. DSB repair can be accomplished by recovering genetic information from a sister chromatid during G2 or from a homologous chromosome during the other stages of the cell cycle or during prophase I of meiosis.

Sister chromatids are also available for DSB repair during meiosis but it is a hallmark of meiosis that repair mainly involves homologous chromosomes. The use of sister chromatids does not give rise to reciprocal genetic exchange. The use of homologous chromosomes as DNA repair templates can lead to the crossovers (reciprocal exchange of genetic material) between homologs that are important for proper chromosome segregation at anaphase I of meiosis. Therefore it is important that an inter-homolog repair bias be established and maintained, during meiosis I, to ensure the formation of an adequate number of crossovers for proper chromosome segregation. The homolog repair bias, in most organisms, likely arises from mechanisms that suppress inter-sister events (eg. proper axis formation) and/or mechanisms
that promote homologous recombination (e.g. a functioning DMC1-mediated DSB repair pathway) (Zickler and Kleckner 1999).

Proper chromosome axis formation is an important contributor to the establishment of the inter-homolog DSB repair bias. In Arabidopsis ASY1 protein localizes to chromatin proximal to the developing chromosome axis (Sanchez-Moran et al. 2007). The ASY1 protein in Arabidopsis, its orthologs, HOP1 protein in yeast and HIM-3 protein in C. elegans, appear to play an important role in maintaining the inter-homolog repair bias in their respective organisms (Sanchez-Moran et al. 2007; Hollingsworth and Ponte, 1997; Zetka et al. 1999). These proteins all contain a HORMA (HOP1, REV7, MAD2) domain (Aravind and Koonin 1998), found in proteins that function as part of a surveillance system that monitors interactions between homologs (Fukuda et al. 2009). ASY1 protein localizes to Arabidopsis chromatin as foci in G2 and during leptotene localization becomes continuous along the chromosome axis (Armstrong et al. 2002). In asy1 mutants it has been suggested that RAD51 replaces DMC1 within the DSB intermediate and this shifts the repair bias to sister chromatid exchange (Sanchez-Moran et al. 2007).

Vignard et al. (2007) have suggested that inter-homolog DSB repair is favored upon physical interaction of DMC1 protein with RAD51 protein. In Arabidopsis the RAD51, XRCC3 and MND1 proteins normally promote repair of DSBs from homologous chromosome templates but in the absence of DMC1 these proteins appear to repair DSBs from sister chromatid DNA templates (Vignard et al. 2007). The 10 univalents observed in both anaphase I and II dmc1 meioocytes, reported by Couteau et al. (1999), suggest that this mutant’s DSBs are mainly repaired by sister chromatid exchange as a consequence of ‘unchaperoned’ RAD51 protein and an impaired DMC1-mediated DSB repair pathway.

In summary it appears that normal sister chromatid cohesion, proper chromosome axis formation, axis-associated localization of ASY1 protein and a functioning DMC1-mediated DSB repair pathway are all important contributors to the establishment and maintenance of the inter-homolog chromosome DSB repair bias in Arabidopsis.

1.8.4 Double strand break (DSB) formation
The entire process of meiosis involves key molecular and chromosomal events many of which occur during prophase I of meiosis I. The first key molecular event of the DNA double strand break (DSB) repair pathway shown to be common to yeast, Sordaria, Drosophila, C. elegans, mice and Arabidopsis is the formation of DSBs in nuclear DNA created by the topoisomerase like protein SPO11; the spo11 mutant meioocytes of these organisms lack DSB intermediates and RGE (Keeney et al. 1997; Storlazzi et al. 2003; McKim et al. 1998; Dernburg et al. 1998; Romanienko et al. 2000; Grelon et al. 2001). DSB formation has been determined to occur in the G2 (pre-meiotic) or leptotene interval in yeast and
Sordaria (Keeney et al. 1997; Storlazzi et al. 2003). SPO11 is an ortholog of the archaeabacterial DNA topoisomerase VIA subunit. In archaea it is involved, together with its B subunit (topo VIB), in DNA replication. The topoisomerase VIA subunit can bind to DNA non-specifically and is thought to cleave DNA via a 5’-phosphotyrosyl linkage (Bergerat et al. 1997).

In budding yeast at least nine other proteins have been identified as being required for DSB formation. The roles and interactions of these proteins are not well understood but it is unlikely that they form a holoenzyme. Rather it appears that they organize into subgroups that functionally interact with one another over time. There are at least four functionally distinct subgroups among the yeast DSB proteins: SPO11-SKI8; REC102-REC104; MER2-MEI4-REC114; and MRE11-RAD50-XRS2 (Maleki et al. 2007). Interestingly yeast SPO11 is involved in DNA replication during S-phase and mutation of this gene decreases the duration of S-phase by 25% (Cha et al. 2000).

Most eukaryotes have only one SPO11 protein but in Arabidopsis there are 3 paralogs; SPO11-1, SPO11-2 and SPO11-3 (Stacey et al. 2006). The three Arabidopsis SPO11 paralogs seem to function in two distinct processes; SPO11-1 and SPO11-2 in meiotic recombination and SPO11-3 in DNA replication. AtSPO11-1 and AtSPO11-2 are involved in DSB formation and thus the initiation of genetic recombination. AtSPO11-3 has a role in DNA endo-reduplication and the Atspo11-3 mutant exhibits an extreme dwarf phenotype (Stacey et al. 2006).

Plants homozygous for spo11-2 exhibit a severe sterility phenotype. Both male and female meiosis are severely disrupted in the Atspo11-2 mutant; this is associated with severe defects in synopsis during the first meiotic division and reduced meiotic recombination (Grelon et al. 2001). This finding suggests that AtSPO11-1 cannot alone compensate for the loss of AtSPO11-2 function. AtSPO11-1 and AtSPO11-2 proteins might function as a heterodimeric complex (Hartung et al. 2007). Additional genetic analysis revealed that plants heterozygous for both Atspo11-1 and Atspo11-2 are also sterile (Stacey et al. 2006). Together the two genetic studies indicate a ‘dosage effect’ in which AtSPO11-1 and AtSPO11-2 contribute to wild type function or that a non-functional complex is formed in double heterozygous plants.

The approximate number of DSBs that occur per Arabidopsis meiocyte nucleus has been estimated using phosphorylated γH2AX protein localization as a marker. γH2AX is a meiosis-specific isoform of histone H2A and upon phosphorylation it localizes rapidly to sites of DNA double stand breaks (Sanchez-Moran et al. 2007). γH2AX foci begin to accumulate in G2 reaching a maximum >50 foci at 5 hours post S-phase and this level is maintained another 13 hours but there is a gradual decrease to 10-20 foci at pachytene (24 hr post S-phase). The pachytene number of γH2AX foci numerically approximates chiasma frequency in wild type Arabidopsis and therefore suggests that these foci mark the site of crossovers. By 30 hrs post S phase γH2AX foci are no longer visible on chromatin (Sanchez-
Moran et al. 2007). γH2AX foci were absent in the spo11-1-3 mutant thus validating the use of γH2AX immuno-labeling to monitor DSB formation in Arabidopsis (Sanchez-Moran et al. 2007).

1.9 Rough alignment of homologous chromosomes

1.9.1 Pre-meiotic influences on rough alignment

Prior to the onset of meiosis homologs are not associated. By mid-prophase I homologs are intimately associated. Several types of non-DSB pathway organizing factors may contribute to the ultimate close alignment of homologous chromosomes seen during prophase I of meiosis; these include somatic/vegetative association, Rabl orientation and telomere/centromere clustering.

1.9.1.1 Somatic/vegetative chromosome association

Somatic homologous pairing occurs in both yeast and Drosophila, at multiple interstitial sites, during both G1 and G2 and therefore can occur in the absence or presence of sister chromatids (Burgess et al. 1999). Somatic pairing, in yeast, is independent of the RecA homologs RAD51, RAD55, RAD57 and DMC1 that are normally required during meiosis (Burgess et al. 1999). The pairing of homologous chromosomes in somatic cells is thought to play an important role in the repair of un-programmed DSBs due to DNA damage; this is the case in Drosophila (Burgess et al. 1999). There also is mounting evidence that sister chromatid exchanges are a prominent double-strand break repair mediated pathway in somatic G2 mammalian cells (Johnson and Jasin 2000).

There is no apparent vegetative pairing of homologous chromosomes in Arabidopsis. Pairing between homologous alleles is possible if they are in close proximity but pairing of entire chromosomes has not been seen (Pecinka et al. 2004). McGuire (1966) observed a loose alignment of homologous chromosomes in maize tapetal and root tip cells. In this case there was a loose association of homologous heterochromatin. Subtle loose arrangement of homologous chromosomes is not easily detected and may be more prevalent in plant vegetative cells than existing evidence would suggest.

1.9.1.2 Nuclear organization of chromosomes

In most organisms, there is evidence of differing degrees of chromosome organization within pre-meiotic/mitotic nuclei (Zickler and Kleckner 1999); one organizing principle is the Rabl orientation. Rabl (1885) was the first to report on a polarized arrangement of interphase chromosomes observed in the nuclei of salamander larvae. During anaphase of mitosis, centromeres (via their connection to microtubules) are the most poleward portion of each chromosome and the telomere is the least. If
retained beyond mitosis the Rabl orientation is one contributor to non-random chromosome organization. The retention of the Rabl organization is species dependent and can even vary among tissues or developmental stages of the same organism. Yeast demonstrates Rabl orientation of its chromosomes in pre-meiotic nuclei; centromeres are clustered at one pole and chromosome arms are extended toward the other pole (Lorenz et al. 2003).

In plants the Rabl organization, beyond mitosis, seems to be limited to those organisms that have a particularly large genome size (>4,000 Mbp) and/or long chromosome arms, eg. wheat (Martinez-Perez et al. 1999). The Rabl organization has been observed in wheat, rye, barley, and oats, all of which have haploid genome sizes (C values) above 4,800 Mbp (Dong and Jiang 1998). In wheat the Rabl organization most likely facilitates homologous pairing of centromeres prior to the onset of leptotene (Martinez-Perez et al. 1999). Chromosomes of sorghum and rice, both with genomes under 1,000 Mbp, do not retain the Rabl configuration beyond mitosis (Dong and Jiang 1998). It is important to again note though that creation of the Rabl organization does not rely on large genomes but perhaps its retention correlates with the length of individual chromosome arms within a genome.

Retention of the Rabl orientation is not seen in Arabidopsis pre-meiotic nuclei (genome size ~150 Mbp). Chromosome painting in Arabidopsis root tip and leaf interphase nuclei has shown that the Rabl orientation is not retained and chromosomes tend to occupy individual territories within the nucleus (Pecinka et al. 2004). In these nuclei the territories occupied by chromosomes 1, 3 and 5 demonstrated homologous associations at a random frequency level, as determined by computer simulation. Only the NOR-bearing short arms of chromosomes 2 and 4 demonstrated a ‘higher than random’ frequency of chromosome territory (CT) association; this association is likely due to the organization of these NORs around the same nucleolus (Pecinka et al. 2004), and is not related to the Rabl orientation.

1.9.1.3 Pre-meiotic telomere and/or centromere associations

Arabidopsis telomeres cluster onto a small region of the nucleolus during interphase; pre-meiotic telomere clustering has not been observed in most organisms (Armstrong et al. 2001). Homologous telomeres are paired within the cluster during interphase then disperse (as pairs) throughout the nucleus at the onset of meiosis but loosely cluster again during zygotene as a modified bouquet.

Arabidopsis centromeres are scattered during interphase. In contrast the centromeres in several organisms yeast, wheat, onion, female Drosophila are clustered as they enter meiosis and this may facilitate rough alignment of homologous chromosomes (Stewart and Dawson 2008); but the centric heterochromatin of Drosophila males may not pair at all (reviewed by McKee 2005). In both yeast and wheat the centromeres are randomly associated pre-meiotically but as meiosis proceeds the random
associations are resolved in favor of the pairing of homologous centromeres. This partner switching appears to be driven by the association of homolog arms; it is not centromere driven (Stewart and Dawson 2008).

1.9.2 Meiotic events of rough alignment

Homologous chromosome regions loosely aligned and separated by 500-1000 nm are considered to be in rough alignment (Wilson et al. 2005). Once roughly aligned, close alignment and SC formation usually quickly follows. But how is rough alignment accomplished? Different species may have evolved varying rough alignment strategies and more than one alignment mechanism may be involved. One mechanism implicated in alignment, in most species, is known as ‘bouquet’ formation. In most organisms bouquet formation, involves the clustering of telomeres in a small region of the nucleus, at the nuclear membrane (reviewed by Zickler and Kleckner 1999). In Arabidopsis, bouquet formation involves telomere clustering on a small region of the nucleolus (Armstrong et al. 2001).

Bouquet formation appears to be independent of the DSB pathway and is normal in spo11 mutants of N. crassa, S. macrospora, S. cerevisiae, C. cinereus, A. thaliana and mouse (Bowring et al. 2006; Storlazzi et al. 2003; Trelles-Sticken et al. 1999; Celerin et al. 2000; Grelon et al. 2001; Romanienko et al. 2000). Normal bouquet formation was also observed in S. pombe rec24, rec25 and rec27 mutants that lack DSBs (Martin-Castellanos et al. 2005).

In Arabidopsis the general facilitation of rough alignment occurs with the clustering of telomeres (bouquet formation) at a specific region of the nucleolus during G2/leptotene (Armstrong et al. 2001). As meiosis progresses telomeres more closely associate with their homologous counterparts (Armstrong et al. 2001). DNA sequences adjacent to telomere regions or chromosome specific telomere-associated proteins must be involved in the correct matching of homologous telomeres as the telomeres themselves are comprised of many copies of the consensus sequences 5’ TTTAGGG 3’. The formation of the bouquet does not require the presence of homologs. Monoploid rye forms a normal bouquet during leptotene despite the absence of homologous chromosomes (Santos et al. 1994). This finding strongly suggests that bouquet formation is not driven by DNA-DNA interactions of homologous chromosomes. Carlton and Cande (2002) have shown, that in maize, telomere sequence not the terminal position at the ends of chromosome arms drives bouquet formation and that overall chromosome behavior is telomere not centromere-mediated.

The pairing of homologous telomeres and in some instances centromeres most likely would not be sufficient to roughly align entire chromosomes, especially the relatively long chromosomes of higher eukaryotes. Wilson et al. (2005) have proposed a model for rough alignment that incorporates both the ‘bouquet’ configuration and transcription centers; this model offers a plausible explanation for the
juxtaposition of entire homologs. This model is based on the premise that the transcription of a gene relies on a physical collaboration of a specific set of several polymerases and transcription factors at a ‘transcription center’ and that the coordinated transcription of alleles on homologous chromosomes would bring them to the same center at the same time. The movement of homologous regions to a common center would serve to roughly align the interior regions of homologous chromosomes whose ends and centromeres are already in register. Jackson et al. (2003) using 5-bromo-2-deoxyuridine (BrdU) incorporation into newly synthesized transcripts coupled with RNA polymerase II localization, has demonstrated that such transcriptionally active centers appear to exist in nuclei. In addition their resistance to removal by nucleolytic digestion (that removes most of the chromatin) is suggestive of attachment to a nuclear ultrastructure such as the nuclear matrix (Jackson et al. 2003).

There still remains the question of how specific alleles find their respective transcription centers. A ‘thermodynamic’ model for chromosome alignment proposed by Nicodema et al. (2008) may provide a mechanism for the directed movement required. Assemblage of transcription centers could conceivably create ‘cytoplasmic trails’ or concentration gradients for its component proteins; relevant chromosome regions could move along these gradients and eventually associate with homologous genes that dock at the same center. Chromosomes would be presented with multiple trails but ultimately regions of a particular chromosome would follow the trail for which they have the greatest affinity.

An alternative model for the rough alignment suggests that random collisions between chromosomes are involved and that when collisions occur between homologous regions a mildly stabilizing association would occur. Progressive accumulation of many adjacent interactions would ultimately roughly align homologous chromosomes; non-homologous and homologous but ectopic associations give way to interactions that reflect more global homology (Weiner and Kleckner 1994).

1.10 Close alignment of homologs and their subsequent stabilization

1.10.1 Possible close alignment mechanisms

Generally homologous chromosomes are considered to be closely aligned when the distance seen for rough alignment (500-1000 nm) is closed to 100-120 nm (Wilson et al. 2005). The process of transitioning from rough to close alignment may involve 3’ DNA single strand overhangs that exist at the sites of double strand breaks and are the result of the 5’ DNA end exonuclease digestion orchestrated by the MER3/RAD50/NBS1 (MRN) protein complex (van den Bosch et al. 2003). The DSB DNA 3’ end single strand invasion from a non-sister chromatid into homologous duplex DNA is well accepted as a means of probing for complementary DNA in most organisms (Zickler and Kleckner 1999). In addition strand invasion could serve to bring chromosomes in close enough juxtaposition for the formation of axial associations (if the homolog check is successful). Evidence for the involvement of
DSB single strand DNA is seen in the confocal analysis of spo11 meiocytes from N. crassa and S. macrospora; these are two filamentous fungi that lack close alignment of chromosomes in the absence of double strand breaks (Bowring et al. 2006; Storlazzi et al. 2003). Incrementally increased doses of ionizing radiation (creating DSBs) applied to N. crassa spo11 meiocyte nuclei resulted in concomitant increases in chromosome close alignment from 10 to 60%; these findings provide evidence of DSB involvement in chromosome close alignment (Bowring et al. 2006). In S. cerevisiae spo11 mutants (no DSBs), the close alignment of homologous chromosomes was reduced to approximately 22% of that observed in wild type meiocytes. Celerin et al. (2000) also reported that chromosome pairing was delayed in the C. cinereus spo11 mutant but the reduction in pairing was not as severe; the mutant had ~67% of wild type pairing levels.

But how might single strand DNA invasion of homologous duplex DNA draw roughly associated homologous chromosomes into close alignment? It has been suggested that the invading strand may move into the region between homologs, encounter early recombination nodules and then shorten. This shortening might draw homologous chromosomes closer together (Anderson and Stack 1988). Despite numerous theories on how homologous chromosomes might closely align, the actual mechanism(s) that ultimately bring homologs within 100-120 nm of each other is still speculative.

Not all organisms rely on the DSB repair pathway to closely align their chromosomes. The homologous chromosomes of both C. elegans and Drosophila spo11 mutants closely align and synapse in the absence of DSBs (MacQueen et al. 2002; McKee et al. 2004). Obviously these organisms do not rely on DSB presence for close alignment and have evolved alternate methods for bringing chromosomes in close enough juxtaposition for pairing stabilization and synapsis to occur. The pairing centers of C. elegans and the rDNA regions of Drosophila have specific proteins that bind to them and function with the centers to stabilize chromosome pairing during meiosis (MacQueen et al. 2002; McKee et al. 2004). The specific protein(s) associated with a particular cis-acting DNA sequence (pairing center) might also facilitate the transition from rough to close alignment as homologous chromosomes could move, in a directed fashion, along increasing concentration gradients toward these proteins for which they have a shared affinity; thus drawing them closer together.

1.10.2 Chromosome homology searching and checking
The search and subsequent check for chromosome homology is probably a multi-step process that begins during bouquet formation. The homologous telomere pairing that occurs during bouquet formation likely reduces homology search complexity as chromosomes progress from rough to close
alignment. Wilson et al. (2005) have proposed that the search for chromosome homology occurs region by region with a requirement for positive recognition at two sites bracketing a region (most likely axial association sites) prior to SC formation in that region. This homology checking mechanism would greatly reduce the possibility of ectopic associations between repetitive DNA sequences and segmental duplications found on non-homologous and homologous chromosomes. Wilson et al. (2005) also suggested that a block to SC formation might be put in place during pre-meiotic S-phase as regions of DNA replicate. This again makes sense as the journey to chromosome close alignment likely involves considerable non-homologous chromosome contact. Non-homologous associations would have the highest probability of occurrence prior to rough alignment; a block would likely be maintained until after this event.

The maize pam1 and rye sy10 meiotic mutants exhibit difficulties in rough and close alignment of chromosomes respectively; non-homologous pairing and synopsis were observed in both mutants (Golubovskaya et al. 2002; Mikhailova et al. 2006) thus implicating both wild type genes in homology seeking/checking. The pam1 mutant’s failure to achieve rough alignment resulted in considerable non-homologous synopsis most likely as a consequence of the early impact (no bouquet formation) this mutation had on the homology seeking process. The sy10 mutant that lacked close alignment, exhibited only a small amount of non-homologous pairing perhaps relating to the fact that rough alignment was accomplished, but close alignment failed (Mikhailova et al. 2006). The non-homologous synopsis found in both the pam1 and sy10 mutants was delayed and likely occurred after the normally imposed blocks to such associations had been universally removed.

In wild type organisms non-homologous associations that form (during bouquet formation – homologous telomere pairing) have a greater potential for correction while non-homologous associations detected later (eg. during strand invasion) are probably not correctable and likely result in crossover absence, non-disjunction of chromosomes and infertility. When homology checking is successful between homologous chromosomes, the subsequent formation of axial associations stabilizes the arrangement (Anderson and Stack 1988).

1.10.3 DSB-dependent alignment stabilization

Axial associations are a regular feature of closely aligned homologous chromosomes in most diploid organisms; an example from the plant P. nudum (Anderson and Stack 1988) is shown in Figure 8a. Axial associations also were observed in lily triploid meioocytes (Fig. 8b) where all three chromosomes are in stabilized alignment but only two are synapsed (George et al. 2002). Axial associations in yeast require chromosome homologs and DMC1-mediated DSB repair events. It has been suggested that, in
some organisms, synopsis initiates at the sites of axial association formation; spacing between axial associations appears to be species dependent (reviewed by Zickler and Kleckner 1999). Whether synaptonemal complex (SC) initiates at axial associations or merely requires the stabilized close association is not known, but SC only forms between closely and stably aligned chromosomes. This critical distance may be due to the constraints on the length of transverse filaments which extend from both axial elements and must overlap and inter-digitate to form the central element of the synaptonemal complex (Higgins et al. 2005).

Axial associations do not appear to be present in dmc1, rad51, and hop2 mutants in yeast (Rockmill et al. 1995; Leu et al. 1998) suggesting that these proteins have some involvement in axial association formation. DMC1, RAD51 and HOP2 proteins are known to interact with DNA (Chi et al.2007; Pezza et al.2007; Petakhova et al. 2005) and therefore are most likely not actual components of these protein structures. Their involvement in axial association formation is likely mediated via their role in the DSB repair pathway, perhaps in facilitating successful homology checks. Meiocytes of the sgs1 yeast mutant demonstrate a dramatic increase in the number of axial associations, the number of synopsis initiation complexes and the number of crossovers; providing evidence for a relationship between these meiotic structure (Rockmill et al. 2003).

1.10.4 DSB-independent alignment stabilization

*Drosophila* and *C. elegans* both can achieve close alignment of homologous chromosomes in a *spo11* mutant background (in the absence of double strand breaks) (reviewed by McKee 2004). In addition, in these organisms, orthologs of the *DMC1*, *HOP2* and *MND1* genes are absent and therefore stabilized homologous chromosome pairing is not dependent on the DMC1-mediated DSB repair pathway (Pawlowski and Cande 2005).

The sex chromosomes of *Drosophila* males, during meiosis, rely on strong cis-acting pairing sites that correspond to the intergenic spacer repeats of the rDNA arrays; these arrays are located in the heterochromatin of the X and Y chromosomes. *Drosophila* male autosomal chromosomes also exhibit cis-acting pairing sites; a strong site exists on chromosome 2. This site lies within euchromatin and overlaps with the *his* locus that contains repetitive structural genes for histones. In addition *Drosophila* males have weak pairing sites that have been identified, in euchromatin, all along autosomal chromosome 2 (McKee 1996).

The sex chromosomes in *Drosophila* females also have a strong pairing site, but it is heterochromatic and does not involve rRNA genes as seen in males. The pairing of autosomal chromosome 4 in females also relies on specific regions of heterochromatin (McKee 1996).
One common factor amongst these *Drosophila* pairing sites is that rDNA, the *his* locus and heterochromatin all contain repetitive DNA. A second factor is that rRNA genes and the histone genes are transcriptionally active during meiosis. Thus, in *Drosophila*, specific DNA regions function in a non-DMC1 related DSB repair pathway to maintain homolog pairing but the mechanism is not yet understood.

*C. elegans* also displays strong pairing sites; one terminal site per chromosome. These pairing sites typically cluster at a small region of the nuclear envelope during early meiotic prophase analogously to the telomere-mediated meiotic bouquet in other organisms (Phillips and Dernburg 2006). *C. elegans* pairing sites are better understood than pairing sites in *Drosophila*. In *C. elegans* specific proteins are involved that uniquely recognize and interact with a particular pairing site. The HIM8 protein has been shown to interact with the pairing site on the X chromosome and a family of four related C2H2 zinc-finger proteins (ZIM-1, ZIM-2, ZIM-3, ZIM-4) play a central role in pairing site mediated homologous pairing in *C. elegans*’s other chromosomes (Phillips et al. 2005). These ‘ZIM’ proteins are encoded within a tandem gene cluster and most likely evolved through a common ancestor via gene duplication and selection. These ZIM paralogs collectively mediate the pairing behaviour of the five autosomes in *C. elegans* (Phillips and Dernburg 2006).

### 1.10.5 Possible pairing centers in plants

Maguire (1986) reported evidence to suggest that pairing centers may exist in maize. This assertion was based on the findings that certain specific segments of chromosomes, when translocated into other chromosomal positions, maintained higher than average chiasmata frequencies per unit length. She speculated that this occurrence reflects an innate capacity for pairing, in these chromosome segments, possibly due to the presence of strong pairing centers. Lastly, *Arabidopsis* autotetraploid lines demonstrate low frequencies of chromosome 2 and 4 multivalent formation that may reflect a NOR-related pairing force that strongly influences the pairing choice for the entirety of these chromosomes (Santos et al. 2003).

### 1.11 Chromosome synopsis

#### 1.11.1 The synaptonemal complex

Synaptonemal complex (SC) formation is normally coupled with homologous pairing such that only homologous synapsis occurs between chromosomes. Moses (1968) was one of the first to publish a review on the synaptonemal complex and since then much more has been discovered about this proteinaceous structure that temporarily binds homologous chromosomes together during early and mid-prophase I. Electron microscope studies of prophase I chromosomes, involving numerous species,
have found that the diameter of the SC is relatively constant (90-120nm not inclusive of the lateral elements) (reviewed by Westergaard and von Wettstein 1972). Therefore SC diameter is not dependent upon chromosome size, morphology or DNA content as these parameters vary considerably amongst various species. The SC is a tripartite structure made up of two aligned axial elements (lateral elements) of the participating homologous chromosomes and a largely proteinaceous central element (Fig. 9). In yeast the central element is primarily made up of ZIP1 protein (Sym et al. 1993).

Meiotic proteins with enzymatic function have relatively conserved primary amino acid sequence but this does not hold true for structural proteins such as ZYP1. *Arabidopsis* ZYP1 protein identification relied heavily on similarities in secondary structure with yeast ZIP1 protein (Higgins et al. 2005). There is only 18-20% amino acid sequence identity and 36-40% gene sequence similarity between ZYP1 (*Arabidopsis*); ZIP1 (yeast); C(3)G (*Drosophila*), SYP1/2 (*C. elegans*) and SCP1 (mice) orthologous proteins, as reviewed by Osman et al. (2007). All ZYP1 protein orthologs had an extended coiled coil structure flanked by N- and C-terminal globular domains (reviewed by Osman et al. 2006). In *Arabidopsis* immuno-gold labeling of the ZYP1 protein determined that ZYP1’s C-terminus associates with lateral elements while the N-terminus inter-digitates in the central region of the SC (Higgins et al. 2005).

Two *ZYP1* genes have been identified in *Arabidopsis*. AtZYP1a and AtZYP1b genes appear to be functionally redundant, as single null mutants of either of these genes display identical phenotypes (Higgins et al. 2005). A *ZYP1* RNA interference construct that knocked out both versions of the *ZYP1* genes was required to entirely eliminate SC formation (Higgins et al. 2005). In *Arabidopsis* *ZYP1*RNAi null plants considerable amounts of crossing over still occurred (80% of wild type); the ratio between homologous and non-homologous RGE was approximately 2.6 to 1 (Higgins et al. 2005). Thus SC formation seems to be important in maintaining crossover fidelity in *Arabidopsis*.

### 1.11.2 The process of synapsis

Originally it was thought that synapsis was initiated at the sites of axial associations (Leu et al. 1998). However studies in budding yeast have provided new information on the mechanism of synapsis initiation. Tsubouchi et al. (2008) report on the existence of two types of budding yeast synapsis initiation events that differ in regards to location, timing, proteins involved and relationship to crossover formation. Approximately 80% of *S. cerevisiae* synapsis initiation is type one; it originates from centromeres and moves progressively outward toward the telomeres. Type two SC initiation involves a synapsis initiation complex (SIC) comprised of ZIP2, ZIP3, ZIP4, SPO22 and SPO16 proteins (Tsubouchi et al. 2008). The SC formation that arises from SIC, that are associated with axial associations, is only a minor contributor to overall synapsis but these interstitial initiation sites appear to
mark the eventual sites of crossover (Tsubouchi et al. 2008). This pattern of synapsis does not hold true for all organisms. For instance in *C. elegans* the presence of only one pairing site per homologous pair is required to allow synapsis along the entire length of chromosome due to the very progressive nature of this synapsis.

In *Arabidopsis* initial regions of synapsis tend to start close to the telomeres at early zygotene and then are supplemented with interstitial sites along chromosomes (Lopez et al. 2008). Synapsis is regional along chromosomes and does not occur in a synchronized fashion; some bivalents may have several SC stretches by mid-zygotene and other bivalents only a few (Lopez et al. 2008). The interstitial sites most likely involve axial associations that form between paired chromosomes. This pattern of synapsis is fairly consistent with respect to other plants species such as *maize*, *lily*, *Tradescantia*, *rye*, *wheat* (Gillies 1973; Hasenkampf 1984; Gillies 1985; Corredor et al. 2007).

There are a handful of organisms that achieve crossing over and successful segregation in the absence of synapsis. These asynaptic organisms include the ciliate protist *T. thermophila* (Loidl and Scherthan 2004), the filamentous fungus *A. nidulans* (van Heemst et al. 2001) and the fission yeast *S. pombe* (Olson et al. 1978).

### 1.12 Chromosome crossovers and gene conversions

#### 1.12.1 Crossover definition and importance

Reciprocal genetic exchange (RGE), also known as crossing over, can result from a specific type of DSB repair. These RGEs combined with the occurrence of sister chromatid cohesion result in the formation of the chiasmata that hold homologous chromosomes together until their programmed separation at anaphase I. Failure to form chiasmata between homologous chromosomes can result in non-disjunction and infertility. In humans non-disjunction can result in aneuploidy and subsequent medical conditions such as Down’s syndrome. In addition RGE contributes to genetic diversity (via new allelic combinations) and the overall health of the species by producing genetically distinct individuals.

#### 1.12.2 The DSB pathway and crossover formation

A model of the pathways used to repair a DSB, with its many branches, is shown in Figure 10 (Chen et al. 2007). Only one branch-path can produce crossovers. RGE begins with the formation of double strand breaks (Fig. 10a). Next the 5’ ends of the DSB undergo exonucleolytic digestion or resection to produce 3’ DNA overhangs (Fig. 10b). One of the 3’ single stranded DNA tails invades the homologous
duplex DNA; this invasion creates a displacement (D)-loop (Fig. 10c). The D-loop can be extended via DNA synthesis (Fig. 10d). D-loop extension allows the capture of the second 3’ DNA overhang via DNA synthesis and ligation; this repair intermediate is called a Double Holiday Junction (DHJ) (Fig. 10e). If the second strand is not captured then the DSB is repaired via Synthesis Dependent Strand Annealing (SDSA) (Fig. 10f); the SDSA branch does not lead to RGE but gene conversion is possible. Double Holiday Junctions can be dissolved (Fig. 10g); such dissolution can lead to gene conversion but does not produce RGE. Even if the DHJ is resolved (Fig. 10h) this does not ensure RGE because resolution of DHJ can produce both non-crossover and crossover products; this depends on how resolvase cleaves the two HJs (Fig. 10i, j respectively).

1.12.3 Gene conversion
Gene conversions represent a non-reciprocal transfer of genetic information that may occur as a result of DNA strand exchange (Chen et al. 2007). Gene conversion can occur in any of the strand invasion repair pathways and is usually dependent on how much strand exchange occurred; whether heteroduplex DNA is created and how heteroduplex mismatches are repaired. If newly generated sequence, from a homologous but not identical allele, is used as the template for mismatch repair then gene conversion can occur. In addition if the created region of DNA mismatch is intergenic then gene conversion cannot be readily detected. Gene conversions for the gene(s) involved alter the allele ratio but do not result in RGE unless it occurs in the context of branch-path Figure 10j.

1.12.4 Crossover frequency regulation
The number of crossovers per chromosome is regulated (Berchowitz and Copenhaver 2008). Tight control of crossover formation ensures that even the smallest chromosomes have at least one crossover and crossover interference ensures the spacing of crossovers.

Too many crossovers can be detrimental; this is the case in the yeast sgs1 mutant that exhibits an increase in the rate of chromosome synapsis, elevated levels of chiasmata formation and aberrant sporulation (Rockmill et al. 2003). Generally crossovers are suppressed near centromeres, as they might interfere in chromosome segregation, and in regions of highly repetitive DNA (eg. telomeres and rDNA) where there is an increased potential for ectopic exchange and/or loss of rDNA (reviewed by Anderson and Stack 2002). In contrast there are crossover ‘hotspots’, along chromosomes, where crossovers have a higher tendency to form than observed for other regions along the chromosomes. The reason why these regions have a high propensity for crossover formation is not known.
Fig. 10  Double strand break repair pathways.  This schematic depicts the various pathways used for double strand break repair (modified from Chen et al. 2007); (a-d) is shown on the first page and (d-i) continues on the next page.  Only one double helix, from each non-sister chromatid, is shown to simplify the schematic.  The common steps (a-d) of these pathways are  a) double strand break formation, b) 5’- 3’ DNA end resection, c) D-loop formation, d) D-loop extension; e) Double Holiday Junction (DHJ) formation, f) synthesis dependent strand annealing (SDSA), g) dissolution of a DHJ and h) resolution of a DHJ ; red and blue arrows indicate possible DNA cleavage sites, i) non-crossover product and j) a crossover product.  Note that gene conversion can occur in any of the repair pathways shown and is usually dependent on whether the affected region contains a gene, whether the affected gene has heterozygous alleles on each of the sister chromatid involved and how mismatch repair of heteroduplex DNA is performed.
double strand break

5'- 3' end resection

Strand invasion (D-loop)

DNA synthesis (D-loop extension)
Double Holiday Junction
Resolution
Dissolution

non-crossover + possible gene conversion

SDSA

Strand displacement

Second end capture, DNA synthesis, ligation

Strand annealing

DNA synthesis, ligation

Resolution

Dissolution

non-crossover + possible gene conversion

crossover + possible gene conversion

DNA synthesis (D-loop extension)
In wild type *Arabidopsis* the chiasma frequency, analyzed in metaphase I meiocytes was found to be 9.2 chiasmata/meiocyte and chromosomal chiasma frequencies varied according to chromosome size. Chromosome 1 had the highest mean chiasma frequency (2.14) and the short acrocentric chromosomes 2 and 4 have the lowest frequencies (1.54, 1.56; respectively) (Sanchez Moran et al. 2002).

1.12.5 Crossover interference
In most organisms, the occurrence of the first crossover inhibits the occurrence of another crossover nearby in a distance-dependent manner; this results in crossovers being more evenly spaced than would be expected if they occurred randomly. This phenomenon is known as crossover interference (Sturtevant 1915). There appears to be two types of crossovers. One type is interference sensitive and the other type is interference insensitive; the crossovers are distributed along chromosomes in a non-random and random fashion respectively (Copenhaver et al. 2002).

The presence of interference sensitive and/or insensitive crossovers is species-dependent. *Drosophila* and *C. elegans* form interference sensitive crossovers exclusively (McKim et al. 1998; MacQueen et al. 2002) whereas humans, yeast, *Arabidopsis* and mouse appear to use both types of crossovers (Housworth and Stahl 2003; Bishop and Zickler 2004; Lam et al. 2005; Holloway et al. 2008). *S. pombe* and *A. nidulans* do not form SC and only exhibit interference insensitive crossovers (Zhao et al. 1995).

1.13 The Hop2 and Mnd1 orthologs

1.13.1 *S. cerevisiae* (budding yeast) HOP2/MND1 protein complex
The *HOP2* gene was first investigated in the yeast *S. cerevisiae* where abundant synapsis was observed in the *hop2* mutant but this occurs predominantly between non-homologous chromosomes (Leu et al. 1998). In the budding yeast *hop2* mutant DSBs largely are not repaired (although some delayed DSB repair may occur) and DMC1 protein does not disassociate from chromosomes during pachytene (Leu et al. 1998). The *S. cerevisiae hop2* meiocytes do not progress beyond prophase I but this last defect is not manifest in a *spo11* mutant background where there is an absence of double strand breaks (Leu et al. 1998). Thus the block is thought to be due to unrepaired breaks in the DNA.

In wild type yeast *HOP2* gene expression is mainly meiosis-specific with little or no expression in vegetative cells. In budding yeast the HOP2 protein localizes to meiotic chromosomes from leptotene until diplotene and its DNA binding does not require DSBs as it localizes normally in a *spo11* background. The formation of axial associations was visible in *zip1* knockouts but not in *hop2 zip1* double mutants (Leu et al. 1998). This finding either reflects the absence of axial associations in *hop2*
meiocytes or that they could not survive the chromosome spreading process due to added ‘pulling’ stress from numerous associations between non-homologous chromosomes.

Over-expression of the MND1 gene identified it as a suppressor of a temperature-sensitive non-null allele of HOP2 (Tsubouchi and Roeder 2002). Several other observations in yeast meiocytes provide evidence for HOP2 and MND1 proteins’ functional and physical interaction (Tsubouchi et al. 2002). The hop2 mnd1 double mutant has the same apparent phenotype as the hop2 and mnd1 single mutants indicating that they are both in the same epistasis group with respect to meiotic chromosome behaviour. In yeast it appears that both proteins require each other’s presence in order to bind chromosomes. In addition MND1 protein coimmuno-precipitates with the HOP2 protein from yeast cell extracts (Tsubouchi et al. 2002). In vitro analysis of the yeast HOP2/ MND1 complex by Chen et al. (2004) found that these proteins form an elongated heterodimer that preferentially binds double stranded DNA and stimulates DMC1-mediated single stand invasion of homologous duplex DNA.

1.13.2 S. pombe (fission yeast) MEU13/MCP7 protein complex
In S. pombe, the HOP2 /MND1 orthologs are MEU13/MCP7 respectively (Nabeshimi et al. 2001; Saito et al. 2004). Immuno-precipitation analysis demonstrated that MEU13 and MCP7 do form a complex. Meiosis is not arrested in S. pombe meiotic mutants. But meiosis I is prolonged in meu13 single mutant meiocytes. An increase in meiosis I duration is not seen in mcp7 single or meu13/mcp7 double mutant meiocytes (Saito et al. 2004). The reason for this difference is not known. Nonetheless both meu13 and mcp7 mutants exhibit difficulties in close alignment and stabilization of homologous chromosome associations (Nabeshimi et al. 2001; Saito et al. 2004).

1.13.3 M. musculus (mouse) HOP /MND1 protein complex
Petakhova et al. (2003) identified the mouse ortholog of the yeast HOP2 gene and generated a hop2 knockout mouse. The mutant spermatocytes from this line exhibited fully developed axial elements but synopsis was very limited. Meiotic double strand breaks were formed and partially processed in hop2 mutant meiocytes but failed to be repaired. These hop2 meiocytes arrested prior to pachytene and underwent apoptosis. In contrast to budding yeast the amount of SC observed, in mouse hop2 meiocytes, was greatly reduced compared to wild type levels, but similar to yeast the observed SC formed mainly between non-homologous chromosomes (Petakhova et al. 2003).

In mouse the Hop2 and Mnd1 proteins have been shown to form a complex via co-immuno-precipitation experiments (Petakhova et al. 2005). HOP2 and MND1 co-purified as a complex from a co-expression system thus providing additional proof of their ability to physically interact (Enomoto et al. 2006). In vitro work demonstrates that this complex preferentially binds dsDNA, enhances single
stranded invasion of homologous duplex DNA and functionally interacts with a DMC1/RAD51 complex. MND1 protein, in vitro, was found to suppress the D-loop activity of HOP2 in the absence of DMC1 and RAD51 proteins (Petakhova et al. 2005). In mouse it is suggested that the HOP2/MND1 heterodimer also stabilizes the DMC1–ssDNA and RAD51–ssDNA nucleo-protein filaments against dissociation from chromosomes (Pezza et al. 2007; Chi et al. 2007).

1.13.4 Arabidopsis AHP2/MND1 protein complex
Schommer et al. (2003) identified the Arabidopsis ortholog of the yeast HOP2 gene and called it AHP2. They also identified an ahp2-1 (Arabidopsis homologue pairing 2) mutant in a forward screen of a collection of T-DNA insertions. The T-DNA construct included two selectable markers; one for kanamycin resistance and one for the constitutive expression of green fluorescent protein (GFP). The T-DNA inserted into the fourth exon of the AHP2 gene. RT-PCR failed to detect a full length AHP2 mRNA transcript in the ahp2 mutant. Primers made to amplify a truncated AHP2 cDNA (the first three exons) detected severely reduced levels of transcript as compared to wild type. This indicates that the T-DNA insertion in the AHP2 gene significantly disrupted the processing or stability of the entire AHP2 transcript. In wild type Arabidopsis the AHP2 mRNA transcript was detected in meiotic tissue and in vegetative tissue.

Schommer et al. (2003), using cross pollination, found the ahp2 mutant to be both male and female sterile. The F1 generation from a self cross of heterozygotes produced a 3:1 ratio that was expected for a typical Mendelian recessive mutation involving a single locus. The ahp2 mutant exhibited both a shortened stamen and stunted silique phenotype. Anthers had a desiccated appearance upon flower opening and contained no viable pollen as determined by Alexander staining (Schommer et al. 2003). In addition the ahp2 mutant had shortened internode distances between pedicels of siliques in flowering plants. No vegetative growth abnormalities were detected.

In contrast to budding yeast and mice, meiosis is not arrested in Arabidopsis ahp2 meiocytes. Schommer et al. (2003) found that the ahp2 anaphase I and II meiocytes exhibited extensive chromosome entanglement, fragmentation, chromatin bridging and unbalanced segregation. Yeast two hybrid analysis demonstrated that the Arabidopsis AHP2 and MND1 proteins physically interact (Kerzendorfer et al. 2006). The mnd1 mutant like the ahp2 mutant was shown to have greatly reduced pairing and synopsis and extensive entanglement, fragmentation and chromatin bridging in post prophase I meiocytes (Kerzendorfer et al. 2006).
1.14 *Arabidopsis* AHP2 protein’s function – my research

The main goal of my research was to develop robust cytological techniques for investigating events of prophase I in *Arabidopsis* and use the techniques to further our understanding of the role of AHP2, as part of the AHP2/MND1 protein complex, in the association of homologous chromosomes during plant meiosis. Bioinformatic analysis provided information on AHP2 gene expression patterns, the timing of expression and possible phosphorylation sites that may be involved in the control of AHP2 protein function.

Experiments were performed to generate and use a polyclonal antibody raised against an AHP2 protein domain to determine when AHP2 protein functions during prophase I. Additionally experiments were performed to compare AHP2 protein’s pattern of localization along the chromosome with the distribution pattern of MND1 protein. The *Arabidopsis* ahp2 mutant is sterile; it has stunted siliques containing unfertilized ovules (Fig. 1c). Meiocytes of the ahp2 mutant were examined to determine the effect of AHP2 protein absence on prophase I of meiosis; this required improvement of existing chromosome spreading and meiotic staging techniques. The effect of the ahp2 mutation on meiosis duration was examined and the involvement of a specific prophase I substage was determined. During examination of ahp2 meiocytes it became apparent that the nucleolus organizing regions (NORs) of *Arabidopsis* could act as ‘cis-acting’ pairing sites that facilitate localized pairing of homologous chromosomes. It also became apparent that MND1 may have a role in meiosis independent of its combined role with AHP2.
2.0 Materials and methods

2.1 Bioinformatic analysis of the AHP2 gene and protein

*Default parameters were set for all the algorithms used in the *in silico* analysis of AHP2 unless otherwise stated.

2.1.1 Homology search

The *HOP2* gene was originally examined in *S. cerevisiae*. The existence of a *HOP2* cognate gene in *Arabidopsis* was investigated using the National Center for Bioinformatics (NCBI) non-redundant nucleotide (nr/nt) database, the NCBI search program tblastn and the yeast HOP2 amino acid sequence (Genbank Accession AAC31823) [http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi). This failed to reveal an orthologous gene in *Arabidopsis* but instead identified the mouse Tat binding protein interacting protein (*TBPIP*) gene (Genbank Accession BC030169). Subsequently the amino acid sequence for TBPIP (Genbank Accession BAA23155) was used in a tblastn search which identified the *Arabidopsis AHP2* gene (Genbank Accession BT025172).

The *Arabidopsis* AHP2 protein sequence (Genbank Accession CAF28783) was used to search the NCBI *Arabidopsis* protein database, for AHP2 paralogs, with the search program Blastp. Additionally the AHP2 protein domain (corresponding to the 3\(^{rd}\) exon of the *AHP2* gene), that was used as an antigen to produce antibody against the AHP2 protein, was used in a blastp search of the *Arabidopsis* proteome to determine the possibility of antibody cross reactivity in immuno-blots and immuno-cytochemistry.

2.1.2 Homologous protein alignment

The AHP2 protein sequence in *Arabidopsis* was aligned with homologous sequences from plant and animal using the clustalw program at [http://www.ebi.ac.uk/Tools/clustalw](http://www.ebi.ac.uk/Tools/clustalw); some of the species included were grape, poplar, rice, moss, human, mouse and rat. The protein weight matrix used was Gonnet with gap open = 10, gap extension = 0.2 and gap distance = 5.

2.1.3 Phylogeny

The tools for the phylogenetic analysis were obtained at [http://www.phylogeny.fr](http://www.phylogeny.fr). The input data for the phylogenetic analysis of AHP2 orthologous sequences was aligned with the MUSCLE alignment tool (full mode setting; 20 iterations). The alignment was converted to Phylip format, curated using the Gblock program (stringent setting) and then analyzed using the Neighbour Joining algorithm with standard bootstrapping (500 iterations). The phylogenetic tree was rooted using *Arabidopsis* as the out group and rendered using the Treedyn drawing program.
2.1.4 Motif scan
The possible presence of known protein structural/functional motifs, in the AHP2 protein, was investigated using the search engine at http://myhits.isb-sib.ch/cgi-bin/motif_scan.

2.1.5 Primary sequence analysis
The theoretical molecular weight and isoelectric point for the AHP2 protein was obtained using the protein parameters program at http://www.expasy.ch/cgi-bin/protparam. This program also provided a breakdown of the percentage of each amino acid present in the AHP2 protein. Kyte-Doolittle hydrophobicity plots were generated for the AHP2 protein orthologs using the program protscale at http://expasy.org/tools/protscale.html.

2.1.6 Secondary structure analysis
Secondary structure of the AHP2 and MND1 proteins was investigated using the nnpredict program at http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html. The presence of coiled coils, known to be involved in protein-protein interactions, was investigated in the AHP2 protein using the algorithm at http://www.ch.embnet.org/software/COILS_form.html. The coiled coil analysis window was set to 28 amino acids. The MTIDK matrix scoring option was selected that compares the query sequence with the sequence of the following types of proteins that are known to have coiled coil structure: myosins, tropomyosins, intermediate filaments, desmosomal proteins, kinesins.

2.1.7 Expression pattern
The expression patterns for the AHP2 and MND1 genes within the various plant tissue types was examined using the electronic Fluorescent Pictograph (eFP) browser within the bio-array resource (bar) www.bar.utoronto.ca. The eFP browser pictorially displays absolute and relative gene expression information from micro-array data. Relative levels of gene expression for Histone H1, AHP2 and MND1 genes were obtained from http://www.weigelworld.org/resources/microarray/AtGenExpress.

2.1.8 Protein localization prediction
The cell eFP browser at www.bar.utoronto.ca was used to display possible AHP2 and MND1 protein subcellular localization data. The localization predictions for AHP2 and MND1 were given confidence scores based on the number of different algorithms placing them in a particular cellular compartment; protein localization algorithms that were used included subloc, wolfsport, loctrue and mitopred.
2.1.9 Phosphorylation target site prediction

Possible phosphorylation target sites, in the AHP2 and MND1 proteins, were investigated using the prediction software NetphosK 1.0 at http://www.cbs.dtu.dk/services/NetphosK.

2.2 Plant material and growth conditions

Seeds of A. thaliana, Landsberg erecta (Ler) ecotype were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds heterozygous for the mnd1 mutation (A. thaliana SALK_110052 line) were also obtained from the ABRC. Seeds for the meiotic mutant ahp2-1 (Landsberg erecta background) (Schommer et al. 2003) were kindly provided by Dr. Robert Sablowski of the John Innes Centre. The seeds of wild type Ler, the ahp2 and mnd1 mutants were sown onto autoclaved plant tissue culture media [0.44% Murashige and Skoog basal salt mixture (Sigma-Aldrich), 3.0% sucrose, 0.05% 2-morpholino-ethanesulphonic acid (MES), 0.7% Phytoagar]; the media used for the ahp2 and mnd1 seeds also contained 50 ug/ml kanamycin. From this point on the growth conditions for wild type, the ahp2 and mnd1 mutants were identical. The plates were stored for 3 days at 4°C then placed under grow lights for 7 days. Healthy looking seedlings were then transferred to a sphagnum peat moss, perlite, and vermiculite mixture (Pro-Mix) lightly watered with dilute fertilizer. For the first two weeks of growth, the distance from the plants to the overhead grow lights was 9 inches. This distance was then increased to 12 inches for weeks three and four. Plants were grown under long day conditions (16 h light: 8 h dark); the temperature during illumination was 22°C and the temperature during the dark period was 19°C. Humidity was constant at approximately 55%. The watering schedule and the amount of water (with very dilute fertilizer) given to the plants was kept constant for both wild type and the ahp2 mutant throughout their growth cycle and between successive crops. The growing area was pest free for all crops. Primary inflorescences were harvested and fixed within 5 days after the opening of the first flower. Flower opening allowed for the phenotypic identification of ahp2 and mnd1 plants via their considerably shortened stamens and desiccated anthers.

2.3 Arabidopsis AHP2 heterozygote identification (for seed collection)

Plants homozygous or heterozygous for a T-DNA insertion in the AHP2 gene were identified by their ability to grow on MS plant tissue culture supplemented with kanamycin. These plants survived because of the presence of a kanamycin resistance gene in the pROK T-DNA insert. AHP2 heterozygotes were identified by their normal fertility as compared to the sterile phenotype of ahp2 mutant plants. The presence of a T-DNA insertion in one or both of the AHP2 genes was further confirmed by the green fluorescence emission exhibited by plant material, eg. rosette leaves, when
excited by blue (≈475 nm) light and observed with a dissecting microscope [a green fluorescent protein (GFP) gene is present in the pROK T-DNA insert].

In my work seeds were collected from the first selfing of AHP2 heterozygotes grown from seeds obtained from the Sablowski lab. These seeds were used throughout my research and every planting produced the expected Mendelian ratio; 75% of germinated seedlings grew in the presence of kanamycin and 33% of these seedlings were ahp2 homozygotes. Maintenance of Mendelian ratios is an indicator of T-DNA insertion stability. Schommer et al. (2003) demonstrated that, for the ahp2 mutant, the T-DNA insertion was in the 4th exon of the AHP2 gene. We confirmed the presence of the T-DNA in the AHP2 gene using PCR and primers from the 3’ and 5’ ends of the AHP2 gene. Amplification of the AHP2 gene from wild type DNA and the lack of PCR product (using the same primers) from ahp2 DNA demonstrated the disruption of the AHP2 gene in the mutant. In all PCR work Actin gene amplification from ahp2 DNA was used as a positive control.

2.4 Preparation of chromosome squashes in unfixed anthers
This protocol was used for the meiotic staging of unfixed, mainly intact, male meiocytes (pollen mother cells) extruded from individual anthers. A bud of desired size was placed into 10 mM sodium citrate (pH 4.0) situated in the cavity of a depression slide and the sepals were teased away with a scalpel thus revealing the bud’s anthers. Individual anthers were cross-sectioned twice (two parallel cuts) across their locules and then transferred to 20 ul of 4’6-diamidino-2-phenylindole (DAPI) (10 ug/ml) in Vectashield mounting medium (Vector Laboratories, Canada) placed at the center of a clean glass slide. A glass coverslip was carefully applied and its edges were sealed with nail polish. The slide was viewed with fluorescence to check for the extrusion of meiotic cells from the anthers. The coverslip was tapped only if the number of released meiocytes was insufficient for accurate meiotic staging.

2.5 Arabidopsis bud fixation protocol
The primary inflorescences were collected into a small vial containing tap water. When the collection (usually takes 15-30 min) was finished the water was exchanged for 4% paraformaldehyde (PFA) freshly prepared; pH was adjusted to 8.2 with 0.1N sodium tetraborate. The buds were gently agitated while in PFA for 15 min. The PFA was then exchanged with a mixture of freshly prepared methanol: glacial acetic acid (3:1). The buds were gently agitated in the 3:1 mixture for 15 min. After 15 min this 3:1 fixative was discarded and replaced with fresh 3:1 fixative. This was repeated once more. At this point the inflorescences appeared white (the chlorophyll was cleared). The inflorescences were then stored in the acidified methanol at 4°C until needed. Storage time did not exceed 1 month.
2.6 Preparation of chromosome spreads from lipsol-lysed male meiocytes

Primary inflorescences were transferred from 3:1 acidified methanol into the cavity of a depression slide containing tap water (pH 7.0). The inflorescences were repeatedly rinsed with tap water until the pH of the water, in the depression slide cavity was ~ 7 (determined using pH test strips). The larger yellow (pollen containing) buds were discarded and the remaining buds were rinsed a few more times with tap water. Buds in the size range (0.3-0.6 mm) were cut away from the inflorescences and the unwanted plant material discarded. The tap water was exchanged with 10 mM sodium citrate buffer (pH 4.0) and then subsequently exchanged with 200 ul of 0.5% pectinase (Sigma) (pH 4.0). The buds were incubated in this solution at RT for 30 min. The pectinase was then replaced with 10 mM sodium citrate (pH 5.3) followed by cell wall digestion enzyme mixture [0.5% pectinase, 0.5% hemicellulase (Sigma), 0.5% cellulase (Sigma), 0.5% cytohelicase (Sigma), 0.5% polyvinylpyrrolidone MW 40,000 (PVP 40) dissolved in citrate buffer (pH 5.3)]. The depression slide with the buds was then placed in a humid chamber and the chamber placed in an oven at 37°C for 2.5 h. After bud digestion, the enzyme mixture was exchanged with ice cold bud stabilizing buffer [1000 ul 10 mM sodium citrate (pH 7.0) 10 ul 500 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 1/8 protease inhibitor tablet (Roche)] and the depression slide was placed in a covered Petri dish sitting on ice. Individual buds were transferred to a clean glass slide and tapped out in 10 ul of stabilizing buffer thus creating a cell suspension. From this point the chromosomes were ‘lipsol-spread’ according to Armstrong et al. (2002) with minor modifications as follows: 5.0 ul of cellular suspension partially expelled but retained at the end of a pipette tip was combined with 5.0 ul of 1% lipsol partially expelled and also retained at the end of a pipette tip. The cell suspension and lipsol combined were allowed to drop onto the center of another clean glass slide. The slide was viewed with phase contrast; when swollen meiotic nuclei, in the cell suspension, were beginning to rupture (2 - 5 min after lipsol treatment) 10 ul of 4% PFA was added to the suspension and the slide was allowed to air dry in the fumehood. Slides not being used in subsequent immuno-localization and/or fluorescent in situ hybridization had DAPI applied to the spread area and a coverslip was added. These DAPI stained slides were viewed for spread quantity and quality. A Zeiss axiophot epifluorescent microscope and a Plan-Neofluar 100X oil immersion were used. Images were captured with a cooled charge coupled device (CCD) camera and processed with Northern Eclipse software. Slides to be used in immuno-localization and/or FISH were stored at 4°C until needed (used within 1-3 days).

2.7 Preparation of chromosome spreads from acid-lysed male meiocytes

The spread protocol is the same as for lipsol-lysed meiocytes up to placing digested buds in bud stabilizing solution on ice; except that buds are not pre-digested in pectinase. After enzyme digestion, a
bud was selected and transferred to another depression slide containing freshly prepared 4% 
paraformaldehyde (pH 8.2) for 60 s (using a fine wire loop and a dissecting scope). The bud was then 
immediately transferred into 5 ul of 45% acetic acid situated at the centre of a clean glass slide. The bud 
was gently prodded (usually less than 1 min) to facilitate its dispersal in the acid and then the nuclear 
suspension was immediately dried down with a hair dryer (held approximately 25 cm from the 
specimen). After drying down, the slides were briefly rinsed in distilled water pH 7.5 and then 100 ul of 
4% PFA (pH 8.2) was applied to the spread area (marked with a diamond pencil on the underside of the 
slide). The PFA was tapped off the slide after 10 min incubation (room temperature) and the slides were 
allowed to air dry in the fumehood. Slides to be used in the examination and comparison of wild type 
and ahp2 mutant chromosome morphology had DAPI mounting medium applied to the spread area and 
a coverslip added. A Zeiss axiophot epifluorescent microscope and a Plan-Neofluar 100X oil immersion 
1.4) were used to examine DAPI-stained chromosome spreads. Slides to be used in future 
FISH experiments were stored at 4ºC until needed (used within 1-3 days).

2.8 Meiotic staging of chromosome spreads

2.8.1 Multi-criteria staging of chromosome spreads

A multi-criteria staging method was used to correctly stage chromosome spreads. Buds from individual 
primary inflorescences were always spread from smallest to largest. There is a rough correlation 
between bud size and meiotic stage within individual inflorescences; smaller buds are typically at an 
earlier stage of meiosis than larger buds. Spreading in this manner provided some context with respect 
to the staging of meiocytes within the buds. The morphology of the chromosome spread and the 
organization of chromatin within the nucleus next was used to identify meiotic stage. Finally substage 
associations between chromosome spreads found within individual meiocytes could be used to identify 
the more difficult to stage zygotene and diplotene spreads.

2.8.2 Meiotic substage association data analysis

The minimum number of chromosome spreads per bud (per slide), included in the substage association 
analysis, that would be representative of the large and smaller anther meiocytes within that bud was 
determined to be twenty-five using a statistical sampling formula. The statistical formula used was: n = 
Nx / [(N+1) E² + x]; where n = sample size (# of chromosome spreads obtained per bud), N = 
population size (total # of meiocytes per bud), E= error (20%) and x = confidence level (95%) 
(Danesoft statistical software).

The meiocytes per bud population size (N) was calculated to be 720 (6 anthers per bud x 4 locules 
per anther x 30 meiocytes per locule). Slides were not included in the analysis if all the chromosome
spreads appeared to come from a single anther as this would not be representative of the meiocytes within both the large and smaller anther(s) of the bud. In the *ahp2* mutant late zygotene and early pachytene could not be differentiated, therefore these prophase I spreads were combined for scoring; the same applies for pachytene and early diplotene in the mutant.

From individual buds the numbers of meiocytes of specific meiotic substages were recorded and the two most predominant meiotic substages from each bud were noted. In total, 59 wild type and 47 *ahp2* mutant buds were examined in this manner.

### 2.9 RNA isolation and RT-PCR

In the following RNA isolation autoclaved eppendorfs, pestles and pipette tips were used in order to minimize sample exposure to RNase. A falcon tube containing approximately 50 *Arabidopsis* inflorescences (~100 mg fresh weight tissue) was removed from the -70°C freezer and immediately placed into a container of liquid nitrogen. These inflorescences were then quickly transferred into a cold eppendorf tube and immediately crushed with a pestle using a turning motion. Trizol (1 ml) was then added to the crushed tissue. The pestle was used to resuspend the tissue and then the tube was inverted periodically throughout the next 5 min of room temperature incubation. Two hundred microlitres of chloroform was added to the eppendorf tube which was securely capped and then mixed vigorously for 10 s. The solution was incubated for 3 min at room temperature. The solution was then centrifuged at 12,000 rpm at 4°C for 15 min. After centrifugation the upper aqueous phase was transferred to a new eppendorf tube; care was taken not to aspirate any interphase. The RNA was precipitated by adding 500 ul of isopropyl alcohol. The contents of the tube were mixed via inversions and then incubated on ice for 10 min. The tube was then centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was carefully removed and 1 ml of 75% ethanol was added to the tube. The solution was mixed via vortexing and then centrifuged again at 12,000 rpm at 4°C for 15 min. The supernatant was removed and care was taken not to lose the RNA pellet which could be loose or floating in the ethanol. The pellet was then allowed to air dry and was resuspended in 25 ul of 1X TE (10 mM Tris and 1 mM EDTA, pH 8). Five ul of the inflorescence RNA was ran on a 1% gel (0.5 g agarose, 3 ml 10X 3-(N-morphilino) propanesulfonic (MOPS), 47 ml distilled water, 2.5 ml formaldehyde) to check for quantity and quality. The remainder of the RNA was stored at –70°C.

RT-PCR was performed using inflorescence RNA. Five ul of total RNA was first incubated with 1 ul DNase I (amplification grade) in a 10 ul reaction volume also containing 1 ul of 10X Buffer C (200 mM Tris-HCl, 500 mM KCl, 30 mM MgCl2, pH 8.3) and 3 ul of sterile distilled water. The reaction mixture was allowed to incubate at room temperature for 15 min after which the DNase was inactivated by the addition of 1 ul of 25 mM EDTA. The mixture was heated at 70°C for 15 min and then placed
on ice for 1 min. After a brief spin the following was added to the mixture: 1 ul of AHP2 exon 3 back primer (5’ CAC ACC GAA TTC CAC ATC ACT GAT AGT 3’), 2.5 ul 10X Buffer C, 2.0 ul 10 mM dNTPs, 2.5 ul 0.1M dithiothreitol, 1.0 ul superscriptTM reverse transcriptase (Invitrogen) and 5.0 ul of sterile distilled water to bring final volume to 25 ul. The mixture was heated at 42°C for 30 min and then 37°C for one hour. The reverse transcriptase was then inactivated by a 10 min incubation at 70°C.

Three microliters of the RT reaction was subsequently used as cDNA template in a PCR to identify the presence of AHP2 mRNA in Arabidopsis. The backward (described above) and forward (5’ AAA GGC TAG CCT TAA GAA GAC TGC GGT GCA A 3”) primers for the AHP2 gene’s third exon were used in the PCR reaction. The expected band was produced with a DNA fragment size of 210 base pairs (bp) indicating that the AHP2 gene is transcribed in Arabidopsis. PCR negative controls included no template, no forward primer, no backward primer and RNA template (prior to reverse transcription) that was treated with DNase.

2.10 DNA isolation and ahp2 mutant PCR genotyping

A small rosette leaf was placed into a clear eppendorf tube and ground vigorously for 60 s. To this epitube 100 ul of extraction buffer (50 mM Tris pH 8, 200 mM NaCl, 0.2 mM EDTA, 0.5% sodium dodecyl sulfate and 100 ug/ml proteinase K) was added. The contents of the tube were again ground vigorously for 60 s. The tube was tightly capped and placed in a 37°C water bath for 30 min. In the fumehood 50 ul of clear non-oxidized phenol was added to the tube. The tube was vortexed for 30 s after which 50 ul of 24:1 chloroform: isopropyl alcohol was added. The tube was again vortexed for 30 s and centrifuged at 13,000 rpm for 3 min to facilitate separation of the organic and aqueous layers. The upper aqueous phase was then transferred to a clean tube and its volume recorded. Care was taken not to aspirate any of the interphase. The DNA was precipitated by adding 0.1 volumes of 3M sodium acetate pH 5.2 and 2 volumes of ice cold 100% ethanol to the tube and placing the tube on ice for 30 min. The DNA was pelleted by centrifuging the tube at 13,000 rpm for 5 min at 4°C. The DNA pellet was washed 1X with 70% ethanol, allowed to air dry and then resuspended in 50 ul of 1X TE buffer (10 mM Tris, 1mM EDTA, pH 7.5). The DNA was stored at 4°C until needed.

The T-DNA (pROK2) integration site in the ahp2-1 mutant was determined to be in the fourth exon of the AHP2 gene (Sablowski et al. 2003). Repeated attempts to confirm the location of the insertion site using both T-DNA left and right border primers individually in conjunction with an AHP2 gene primer failed to produce any bands. As a positive control in these experiments Actin gene primers were used with the same extracted ahp2 mutant DNA; these primers produced the expected fragment length size.

The PCR genotyping of ahp2 mutants (T-DNA homozygotes) used a left border primer (5’ TGGAAACTTAGACCGACATCTTCATCGTA 3’) and right border primer (5’
TCGTCTTTGCAGCAGCTAAACCATCTTC 3’) of the AHP2 gene. As expected no band was produced due to the very large size (12.8 kb) of the T-DNA insert located within both copies of the AHP2 gene. Actin gene primers again were used as a positive control and they produced a band of the expected size from the same ahp2 DNA. DNA extracted from wild type Ler plants produced a band with the expected fragment length (1600 bp) using the same left and right AHP2 gene primers that were used with the ahp2 mutant’s DNA.

2.11 AHP2 antibody generation
2.11.1 Initial PCR amplification of the 3rd exon of the AHP2 gene
Initially the third exon of the AHP2 gene was amplified via PCR using forward and backward primers (previously described) for this exon.

2.11.2 Ligation of AHP2 gene’s 3rd exon into pGEM-T vector
Two microlitres of PCR amplified AHP2 exon 3 product were used in a ligation reaction to insert this sequence into the vector pGEM-T. PCR products typically have a 5’ ATP overhang and pGEM-T has a complementary 3’ T overhang at the insertion site. To the PCR product the following were added: 5 ul of 2X rapid ligation buffer, 50 ng of pGEM-T, 1 ul of T4 DNA ligase (3units/ul) and 1 ul of sterile water. The ligation reaction mixture was incubated overnight at room temperature.

2.11.3 Transformation of DH 10B E. coli with pGEM-T(AHP2 exon 3 insert)
Electroporation was used to transform electro-competent E. coli with the recombinant pGEM-T. Fifty microlitres of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL) and 8 ul of isopropyl β-D-1-thiogalactopyranoside (IPTG) were distributed evenly over the surface of the agar. Two microlitres of ligated DNA was pipetted into a sterile eppendorf tube containing competent E. coli cells. Forty microlitres of this mixture was placed in the electroporator. Immediately following electroporation one millilitre of LB broth was added to the cuvette. Fifty microlitres of transformed cells were steriley transferred to individual Luria-Bertani (LB) plus ampicillin (50 ug/ul) plates. Plates were allowed to sit for 10 min and then were incubated overnight in a 37°C oven.

2.11.4 Isolation of AHP2 exon 3 insert from pGEM-T
A single white colony was steriley transferred into each of several numbered tubes containing 2 ml of LB broth. These tubes were incubated overnight at 37°C. PCR was run on each of these tubes using AHP2 exon 3 forward and backward primers to confirm positive clones containing the recombinant pGEM-T. All the clones produced the expected bands with 210 bp DNA fragment lengths.
A mini-lysate method was used to remove the pGEM-T recombinant plasmid from the *E. coli*. One ml from each of two of the positive clones was steriley transferred to individual eppendorf tubes. The tubes were spun briefly to harvest the cells, the supernatant was removed and the tubes were then placed on ice. One hundred microliters of solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8) was added to each tube to resuspend the cell pellet. After briefly vortexing each tube 200 ul of freshly made solution 2 (200 mM NaOH, 1% SDS) in distilled water was added to each and the tubes were then gently inverted. The tubes were placed on ice for 5 min and during this incubation were periodically inverted. After incubation the supernatant was transferred, after its volume was measured, to new eppendorf tubes. One hundred and fifty microliter of solution 3 (5 M potassium acetate, glacial acetic) was then added to each tube and each tube was again gently inverted and then placed on ice for 5 min. After incubation the tubes were spun for 2 min. The supernatant containing plasmid DNA was collected into a new eppendorf. The DNA was precipitated by adding 2 volumes of 100% ethanol and incubating at room temperature for 10 min. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min. The pellet was washed once with 70% ethanol, dried and resuspended in 25 ul of 1X TE buffer. The insert was cut from the plasmid using the restriction enzymes *NheI* and *EcoRI* which recognized cut sites originally engineered into the 5’ and 3’ ends of exon 3 of the *AHP2* gene. The insert was separated from pGEM-T by electrophoresis followed by isolation of the insert from the agarose gel using a Qiagen gel extraction kit. The identity of the insert was confirmed by cutting a 10 ul aliquot of the insert with the restriction enzyme *DraI* which yielded bands with the expected fragment lengths of 60 bp and 150 bp. Insert DNA was kept at 4°C until needed. A glycerol stock culture of the DH10B *E. coli* containing the recombinant pGEM-T plasmid was prepared by steriley adding 300 ul of culture to 700 ul of 50% glycerol. The glycerol culture was catalogued and stored at -70°C.

### 2.11.5 Preparation, amplification and sequencing of pRSETB with exon 3 of the Ahp2 gene insert

The *AHP2* exon 3 insert was ligated into *NheI*/*EcoRI* precut pRSET B. DHB10 *E. coli* were transformed with this recombinant vector using electroporation. Clones containing recombinant pRSETB were confirmed via PCR. Once again the mini-lysate method was used to isolate our amplified recombinant vector from its *E. coli* host.

At this point it was important to confirm that the ligation of the insert into pRSETB was in frame and to do this it was necessary to have the recombinant vector sequenced. A single positive clone was used to inoculate a starter culture of 5 ml LB medium plus ampicillin (50 ug/ml) that was incubated overnight at 37°C. One milliliter of overnight culture was then used to inoculate 500 ml of LB medium plus ampicillin. A Qiagen midi-lysate plasmid isolation kit was then used to remove the recombinant plasmid from its *E. coli* host. A 1% agarose slot gel was used to isolate super coiled pRSETB with insert and this
band was subsequently cut out of the gel. The Qiagen gel extraction kit was again used to remove all traces of agarose. Twenty-five microliters of recombinant plasmid (~200 ng/µl) in sterile water was sent away for sequencing using the T7 promoter primer. Sequencing showed that the AHP2 exon 3 insert was, in frame, within the pRSETB vector.

2.11.6 Heat shock transformation of E. coli BL21 (DE3) pLysS with pRSET B plus Ahp2 exon 3

Two milliliters of LB broth were inoculated with 3 µl of BL21 (DE3) pLys E. coli stock culture (stored at -70°C) and allowed to incubate overnight on a shaker at 37°C. The next morning 30 µl of overnight culture was used to re-inoculate 2 ml of LB broth which was allowed to grow for 3 h at 37°C (with shaking). One milliliter of 50 mM CaCl₂ was freshly prepared and placed on ice. One milliliter of culture was pelleted and the pellet was then resuspended in 250 µl of cold 50 mM CaCl₂. Ten microliters of recombinant plasmid was added to the resuspended pellet. The mixture was incubated on ice for 20 min, heat shocked at 37°C for 45 s and then placed on ice again for 2 min. After adding 750 µl of LB broth the transformation mixture was incubated at 37°C for 30 min. Fifty and one hundred microliter aliquots of this mixture were then plated on LB plus ampicillin plates and these were incubated overnight at 37°C.

2.11.7 Confirmation of AHP2 exon 3 presence in transformed BL 21 E. coli

Four isolated colonies from the above-mentioned plates were used to individually inoculate tubes containing 2 ml of LB broth plus ampicillin. These tubes were incubated on the shaker at 37°C for 4 h. The contents of each tube were pelleted and then brought back up into 20 µl of 1X TE pH 8. Plasmids were isolated from each of the reconstituted pellets. The restriction enzymes EcoRI and NheI were used to cut the AHP2 exon 3 insert from the pRSETB vector. Thirty microliters of each restriction digest was run on a 1.7% agarose gel. The expected 200 bp bands were seen thus confirming the presence of recombinant plasmid in these cultures of transformed BL21 E. coli.

2.11.8 BL21 E. coli induction and expression of AHP2 exon 3

Thirty microliters of BL21 transformed culture was used to inoculate 2 ml of LB broth plus ampicillin, which was incubated on the shaker at 37°C overnight. One milliliter of overnight culture was used to inoculate 50 ml of LB broth plus ampicillin. After 4 h incubation on the shaker at 37°C the optical density of an aliquot of the culture was measured with the spectrophotometer at 600 nm. The optical density reading was 0.994 indicating that culture growth was in the log phase. Log phase growth is required for proper induction of gene expression. Prior to addition of IPTG a 1 ml aliquot of culture (T=0) was placed in an eppendorf tube (negative control), spun briefly, LB removed and the pellet
stored at -20°C. To the remaining culture IPTG was added to a final concentration of 1 mM. This culture was placed on the shaker at 37°C for 3 h. After 3 h of incubation the optical density of another 1 ml aliquot was determined to be 1.5 at 600 nm. The increase in OD from original reading indicated that the *E. coli* were not killed by the presence of Ahp2 protein domain. A glycerol stock culture of BL21 (DE3) pLys with pRSETB (*AHP2* exon 3) was prepared, catalogued and placed in the -70°C freezer. Prior to native protein extraction from the supernatant a 1 ml aliquot of induced culture (T=3 h) was placed in an eppendorf tube, spun briefly, LB removed and the pellet stored at – 20°C.

2.11.9 Bacterial cell lysis

Cells from 100 ml of transformed BL21 (50 ml into each plastic centrifuge tube; it is important that the weight of each tube is equal) were harvested by centrifuging at 6,000 rpm and 4°C for 8 min using a Sorval SLA – 1500 rotor. The supernatant was removed and the pellets were placed on ice. To each pellet 2 ml of ice cold 1X binding buffer (5mM imidazole, 4 M NaCl, 160 mM Tris-HCl pH 7.9) was added. Stock lysozyme (50 mg/ml) (Sigma) was added to each pellet to a final concentration of 0.2 mg/ml. The cell pellet was resuspended by intermittent vortexing and placing on ice for 5 min and resuspended cells were placed on ice for 10 min. Cells were lysed by 5 X 15 s sonicator pulses (BIOSONIK–III sonicator used at maximum setting with the red probe) with 1 min on ice between each pulse. Cell suspension was put through one flash freeze with liquid nitrogen followed by a thaw to further ensure lysis of the cells. After 5 min on ice the cell suspensions were centrifuged at 18,000 rpm (Sorval SS-34 rotor) 4°C for 20 min. The cell supernatants were transferred to new eppendorf tubes. The expressed Ahp2 protein domain might have been in the pellet (insoluble cell fraction), but was more likely to be found, due to its relatively low molecular weight, in the supernatant (soluble cell fraction). The pellet was stored at – 70°C until a protein gel proved the *AHP2* protein domain was indeed in the supernatant.

2.11.10 Affinity purification of His-tagged *AHP2* protein domain under native conditions

The *Arabidopsis* AHP2 protein domain was isolated from *E. coli* proteins using rapid affinity purification with the pET His-Tag System (Novagen). The six histidine residues (from the pRSET vector) expressed at the N-terminus of the AHP2 domain bind to the nickel cations immobilized on the metal chelation resin. Approximately 1 ml of resin slurry was added the column provided in the kit. When the resin had settled it was charged and equilibrated with the follow washes ( 1 volume = the settled resin bed volume) 3 volumes of sterile deionized water, 5 volumes of 1X charge buffer (50 mM NiSO₄), 3 volumes of 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris- HCl pH 7.9). Three milliliters of supernatant from the native extraction of protein from induced BL21 *E. coli* was
added slowly to the top of the resin and was allowed to flow through the column at a drip rate of 2 drops per min. As supernatant exited the column it was collected and allowed to flow through the column a second time to maximize the amount of AHP2 protein domain His-tag binding. The column was then washed with 10 volumes of 1X binding buffer and then 6 volumes of 1X wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). The bound AHP2 protein domain was then eluted with six volumes of elute buffer (1M imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). All of the above fractions were collected into individual tubes kept on ice and these fractions were run on a 20% SDS polyacrylamide gel. The majority of AHP2 protein domain was found in the elute buffer and by comparison to concurrently run protein markers had a molecular weight of approximately 11 kDa. The theoretical molecular weight of the AHP2 protein domain plus His-tag was calculated to be 10.2 kDa. A small amount of AHP2 protein domain was lost in the wash buffer. The concentration of AHP2 protein domain in the elute buffer was determined to be 1.2 ug per ul using Bradford reagent. The AHP2 protein domain was stored at 4°C.

2.11.11 Protein electrophoresis of supernatant

Proteins in 5 and 10 ul aliquots of the native extraction supernatant were separated by a denaturing polyacrylamide electrophoresis (SDS–PAGE) using a BIORAD Mini-Protean II gel apparatus. A 10% polyacrylamide gel was prepared in two steps. First the protein resolving gel was made by mixing together 2 ml of resolving buffer (1.5 M TRIS pH 8, 0.4% SDS), 3.3 ml of distilled water and 2.6 ml of resolving acrylamide (30% acrylamide, 0.15% bis-acrylamide). After gentle mixing, to avoid introduction of air bubbles which can hinder polymerization, 40 ul of 10% ammonium persulfate (APS) and 4 ul of tetramethyl ethylenediamine (TEMED) was added. The mixture was immediately injected into the space between the two glass plates using a five ml syringe. A two centimeter margin was left at the top of the two glass plates. The acrylamide was overlaid with water to exclude air and was allowed to polymerize for one hour. The water was then removed by inversion of the gel apparatus and by wicking with a kimwipe. The stacking gel was made by mixing 2.5 ml of stacking buffer (0.5 M TRIS pH 6.8 and 0.4% SDS), 1.5 ml of stacking acrylamide (30% acrylamide, 0.8% bis-acrylamide) and 6 ml of distilled water. After gentle mixing 30 ul of 10% APS and 10 ul TEMED were added. This mixture was immediately applied to the top of the resolving gel using a syringe and a comb was inserted, for the creation of sample wells. The top of the gel apparatus was covered with saran wrap to prevent evaporation and to exclude air. The stacking gel was allowed to polymerize for one hour after which the comb was removed. The protein gel was placed in the gel holder which was in turn placed in the running buffer reservoir. The gel was run using 1X SDS running buffer (25 mM TRIS, 192 mM glycine, 0.1% SDS). Samples run on this gel included proteins extracted, using a denaturing protocol,
from the T=0 and T=3 h induced BL21 E. coli and the proteins from the native extraction supernatant. The T=0 h (non-induced) BL21 E. coli was the negative control for this procedure.

2.11.12 Preparation of AHP2 antigen for injection
The concentration of AHP2 antigen in solution was determined to be approximately 1.2 ug/ul using Bradford reagent. Approximately 100 ug of antigen was delivered to the rabbit per injection to ensure an adequate immune response. One must be careful that excipients in the injection eg imidiazole from the elute buffer does not exceed 610 mg/kg (LD$_{50}$ for rabbits) or 50% of what would be a lethal dose for rabbits. It was determined that our 100 ul injection contained only 6.8 mg imidiazole. In addition our 100 ul injection contained 2.92 mg NaCl which also was not harmful to the rabbits. Four hundred ul of 1X sterile PBS (0.8% NaCl, 0.02% KCl, 0.12% Na$_2$HPO$_4$, 0.02% KH$_2$PO$_4$) and 500 ul of Freund’s complete adjuvant (containing heat killed Tuberculosis bacterium) was added to 100 ul of antigen. The total volume of injection was 1 ml; it was drawn up into a five ml plastic syringe which was fitted with a double cannula micro-emulsifying needle. The injection sample was emulsified, 5 min prior to injection, by forcing the sample back and forth between the two connected syringes. The back and forth motion was continued right up to the time of injection to prevent solidification of the injection solution.

2.11.13 Preparation and injection of the rabbits
A large patch of fur was shaved from both sides of each rabbit and then disinfectant was applied to the shaven areas. Each rabbit was injected with a total of 1 ml of antigen solution. The antigen solution was delivered subcutaneously via three injections. Care was taken not to enter any major veins. The rabbits were placed back in their cages and monitored, for any adverse reactions, for a couple of hours after injection. The rabbits received two more boosters each containing 100 ug of AHP2 protein domain antigen. Each booster was separated by six weeks. After the third set of injections, blood was taken from each rabbit to check for the presence of anti-AHP2 antibody.

2.12 Western analysis of rabbit AHP2 pre-immune serum and AHP2 serum
2.12.1 Arabidopsis bud protein extraction (denaturing method)
A 1.5 ml eppendorf tube, on ice, was filled to the half mark with collected fresh Arabidopsis buds. Two hundred microliters of 2X DB was added to the plant tissue, the tube was tightly capped and was then incubated in a 95°C water bath for 3 min. The tube was then removed from the water bath and using a pestle the plant tissue was homogenized for 3 min. The above was repeated 2 times. Finally the tube was left in the 95°C water bath for 15 min. The contents of the tube were then briefly microfuged and
the supernatant transferred to a new eppendorf tube. The protein extract was stored at -20°C until needed for testing rabbit pre-immune serum for antibody recognition of Arabidopsis bud protein.

2.12.2 Collection of rabbit AHP2 pre-immune serum

The two rabbits to be injected with AHP2 protein domain antigen were named Summer and Daisy (white New Zealand females). Prior to injecting the rabbits with the AHP2 protein domain approximately five ml of blood was drawn from each rabbit. The back of one ear was shaved and xylene was swabbed onto the major ear vein which made it swell and become more visible. A gauge 23 butterfly needle was used to collect the blood. The syringe was kept below the level of the ear such that the flow of blood would be gravity assisted. The collected blood was left to clot at 4°C overnight. In the morning the blood was stirred slightly and then centrifuged at 3,000 rpm 4°C for 10 min. The pre-immune serum was collected and sodium azide was added to a final concentration of 0.05%. The pre-immune serum was aliquoted into several epipods most of which were placed into the -70°C freezer; a couple of epipods were placed in -20°C freezer for short term storage. The pre-immune serum was subsequently used as a negative control.

2.12.3 Western analysis of Arabidopsis bud protein extract with rabbit AHP2 pre-immune serum

Both 5 and 10 ul aliquots of extracted bud protein were run on a 15% SDS polyacrylamide gel along with protein marker. The gel was cast and the protein samples were prepared as previously described. The gel was run at 110 V for approximately 2 h.

The proteins were transferred from the polyacrylamide to a nitrocellulose membrane using a BIORAD protein transfer apparatus. The proteins were transferred using Western transfer buffer (192 mM Glycine, 15% methanol, 25 mM Tris HCl, pH 8.3). Prior to transfer the far left lane of acrylamide gel containing a 10 ul aliquot of extracted protein was stained with Coumassie to ascertain the quality and quantity of the extracted proteins. The remainder of the gel comprised of 4 groupings (protein marker, 5 and 10 ul of extracted protein) was placed in the transfer apparatus. The transfer apparatus was placed in a shallow glass tray and ice was packed around the apparatus to aid in the dissipation of heat which is typically produced during transfer. The transfer apparatus was run at 100 volts for 1 h. After transfer the nitrocellulose was blocked by rocking it in a small container with 3% (w/v) gelatin in TTNaCl (10 mM Tris pH 8, 0.25% Tween 20, 0.6 M NaCl) for 1 h at room temperature. To ensure that the gelatin did not solidify during blocking a heat lamp was positioned over it during rocking. Four Petri dishes were prepared as 1:100 and 1:500 dilutions of each rabbit’s pre-immune serum. To each dish 15 ml of 1% gelatin in TTNaCl and the appropriate quantity of pre-immune serum was added and gently swirled to mix. The nitrocellulose was blocked and then cut (using gloves and clean scissors)
into four strips each containing marker protein, 5 and 10 ul aliquots of extracted protein. The strips were immediately placed individually into the four labeled Petri dishes. The nitrocellulose was gently rocked for 1 h at room temperature. The pre-immune serum was carefully removed and the nitrocellulose was washed 6 X 5 min with 10 ml of 1X TTNaCl. Four Petri dishes were again prepared. This time each dish contained 15 ml of a 1:5,000 dilution of goat anti rabbit-alkaline phosphatase secondary antibody. The Petri dishes were again gently rocked for 1 h at room temperature. After the secondary antibody incubation the nitrocellulose was again washed 6X 5 min with 10 ml of 1X TTNaCl. The strips of nitrocellulose were then placed in a Petri dish with 10 ml of freshly made colour development buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 20% NBT(Nitro-Blue Tetrazolium Chloride)/BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt)) and gently rocked for 10 min at room temperature. The colour development buffer was carefully removed, the nitrocellulose was washed 4X 1 min with 1X TE. The immuno-blot was then examined for bands related to any recognition of Arabidopsis inflorescence proteins by rabbit pre-immune serum.

2.12.4 Affinity purification and testing of rabbit AHP2 serum

The rabbit blood was affinity purified using AHP2 protein antigen immobilized on nitrocellulose. An aliquot of affinity purified serum was electrophoresed on a SDS polyacrylamide gel and after Coumassie staining the expected bands representing the light and heavy chains of IgG were observed. This affinity purified rabbit AHP2 serum was used in a Western analysis of extracted Arabidopsis bud protein to test if recognition of the AHP2 protein could be observed. The wild type bud protein was run on an SDS-PAGE gel and then transferred onto nitrocellulose as described in section 2.12.3. The secondary antibody was a 1:5,000 dilution of goat anti-rabbit antibody conjugated to horse radish peroxidase (HRP). The detection system [Pierce SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA)] was used according to kit instructions. The nitrocellulose was placed in a petri dish with 10 ml of chemiluminescent substrate and gently agitated for 5 min at room temperature. Western analysis yielded a number of non-specific bands on the exposed Kodak X-ray film (film exposure times 5, 10, 15, 20 and 30 min) in all lanes including marker protein. Blocking was changed from 3 h in 3% gelatin at room temperature to 48 h in 5% bovine serum albumin at 4°C. The new blocking regimen resulted in a strong band for the positive control (approximately 3 ng of AHP2 protein domain antigen) but no band was seen for the AHP2 protein from wild type bud protein extract. In addition no bands were seen for the ahp2 protein extract. The positive control was the only band that showed up on the exposed film (film exposure times 5, 10, 15, 20 and 30 min).
2.13 Immuno-cytochemistry

2.13.1 Immuno-cytochemistry procedure
Chromosome spreads from liposol-lysed male meiocytes were used in all immuno-cytochemical experiments. Immuno-localization experiments were carried out, on chromosome spreads, using the following antibodies: rabbit ASY1 (Armstrong et al. 2002), rabbit SYN1 (Cai et al. 2003), rabbit ZYP1 (Higgins et al. 2005), rabbit SMC3 (Lam et al. 2005), rat MND1 (Vignard et al. 2007) and rabbit AHP2 at the following dilutions ASY1 1:350, SYN1 1:500, ZYP1 1:350, SMC3 1500, MND1 1:100 and AHP2 1:100. The AHP2 antibody did not localize to the ahp2 mutant chromosome spreads that were used as a negative control. Relevant pre-immune serums were used as negative control in all other immuno-cytochemical experiments.

Slides were placed on a slide warmer at 37°C for 1 h. Slides were washed (0.1% triton-X in 1X PBS) for 2 X 5 min at room temperature (RT). For immuno-localization of ASY1 and ZYP1 no blocking was done; for AHP2, MND1, SYN1 and SMC3 the slides were blocked (1.0 % BSA in 1X PBS) for 1.5 h at room temperature. Primary antibody (200 ul per slide) was applied and the slides were incubated overnight in a humid chamber at 4°C. The slides were then washed 4 X 5 min (RT) prior to adding secondary antibody [(goat anti-rabbit FITC, (Sigma); goat anti-rat rhodamine, (Jackson Immuno-research) at 1:50 dilution in incubation buffer (1% BSA + 0.1 % triton X in 1X PBS)]. Slides were incubated for 90 min at room temperature in a darkened area. Slides were washed 3 X 5 min and then allowed to dry at room temperature. Finally slides were mounted in DAPI, cover slips applied and sealed with nail polish. Slides were examined and images were captured as previously described.

2.13.2 Estimation of the relative intensity of AHP2 immuno-signal
Both zygotene and pachytene chromosome spreads had considerable signal associated with them. Zygote and pachytene spreads appear to have more AHP2 signal association but pachytene spreads occupy a larger area which disperses signal and gives it a weaker appearance. Taking spread area into consideration, the amount of AHP2 signal associated with zygote and pachytene spreads was compared. Several microscope and software parameters were kept constant to properly compare the average AHP2 signal intensity of zygote and pachytene chromosome spreads.

Zygote and pachytene spreads from the same experiment and the same slide were compared. Individual spreads (not part of a cluster) were placed in the centre of the field of view and the optimum exposure time was determined by the Northern eclipse software. This setting was not changed throughout the comparison of AHP2 signal intensity. The minimum grey threshold was set at 40,000 and was used to distinguish background from spread associated signal. The amount of blue excitation was set at medium and the diaphragm on the 100 X oil immersion objective lens was kept wide open.
At least 10 zygotene and 10 pachytene spreads from individual slides/buds were used in the signal intensity comparison. In total 42 zygotene and 50 pachytene AHP2 immuno-stained, wild type chromosome spreads were compared. The Northern eclipse trace tool was used to delineate the outer edge of each spread and then the ‘measure’ function calculated the amount of signal above background found within the traced area. A two-tailed t-test was used to evaluate whether the differences seen in average AHP2 signal, between zygotene and pachytene spreads, were statistically significant.

2.14 Fluorescent in situ Hybridization (FISH) probe preparation

2.14.1 BACs obtained from Arabidopsis Resource Center (ABRC)
The following BACs were originally propagated in E.coli DH10B host strain and received as stab cultures from ABRC: chromosome 1 sub-terminal BACs F5I6, T21F11, F232A5; chromosome 1 interstitial BACs F14L17, T5E21, F10B6; chromosome 2 short end BACs F2I9, T8O11, T23K3; chromosome 4 short end BACs F6N15, F5I10, F6N23. These cultures were used to inoculate overnight cultures containing the appropriate antibiotic. For long term storage aliquots of the overnight cultures were combined with glycerol (300 ul BAC culture, 700 ul of 50% (v/v) glycerol). These glycerol cultures were labeled, catalogued and placed in the – 70°C freezer.

2.14.2 Plasmid isolation and purification
Plasmids containing the BACs were isolated from their E. coli hosts and purified using the Qiagen plasmid midi kit (Roche). A 50 ml volume of LB medium was prepared in a 200 ml flask, sterilized by autoclaving and added to the appropriate selective antibiotic (50 ug/ml kanamycin or 34 ug/ml chloramphenicol). A 3 ml aliquot of selective medium was transferred into a 5 ml polyethylene tube. Five microliters of BAC culture in glycerol was then added to the 3 ml of LB-antibiotic medium and incubated in the 37°C shaker for 5 h. This 3 ml culture was added to 47 ml of LB –antibiotic medium and incubated for an additional 16 h on the 37°C shaker.

Bacterial cells were harvested by centrifugation at 6000 X g for 10 min at 4°C (SorvallRC-5C Plus) in two autoclaved 30 ml polypropylene centrifuge tubes, each containing 25 ml of bacterial culture. Each bacterial pellet was resuspended in 2 ml of resuspension buffer [50 mM Tris HCl pH 8, 10 mM ethylenediamine tetraacetic acid (EDTA), 100 ug/ml Ribonuclease A (RNase A), LyseBlue reagent1:1,000 (Qiagen)] for 6 min at 22°C and then combined into one polypropylene centrifuge tube. Four milliliters of lysis buffer [200 mM NaOH, 1% sodium dodecyl sulphate (SDS) (w/v)] was added to the tube which was then inverted 6 times and incubated at 22°C for 5 min. After incubation 4 ml of cold neutralization buffer (3.0 M potassium acetate pH 5.5), the tube was inverted 8 times and then incubated
on ice for 15 min. The bacterial lysate was then centrifuged at 20170 X g for 15 min at 4°C. The supernatant contained the plasmid DNA.

The resin of the Qiagen tip column was equilibrated at 22°C with 4 ml of equilibration buffer [750 mM NaCl, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)]. A miracloth (2 inch by 2 inch area) was inserted into the top of the column to filter cell debris and prevent the column from clogging. In addition care was taken not to allow the column resin to dry out. The supernatant with plasmid DNA was poured through the miracloth and allowed to run slowly through the resin via gravity flow. The resin was then washed with 20 ml of wash buffer [1.0 M NaCl, 50 mM MOPS pH 7, 15% isopropanol (v/v)]. Plasmid DNA was then eluted from the resin using five 1 ml aliquots of 65°C elution buffer [1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol (v/v)]. The plasmid DNA in elution buffer was collected into a 15 ml Corex glass tube.

The eluted plasmid DNA was precipitated with 3.5 ml of room temperature isopropanol and then centrifuged at 9682 X g for 30 min at 4°C. The plasmid DNA pellet was then washed with 2 ml of room temperature 70% ethanol followed by centrifugation at 9682 X g for 10 min. The supernatant was decanted and the pellet was air dried in the fume hood for 40 min. The plasmid DNA pellet was redissolved in 200 ul 1X Tris EDTA (TE) buffer (10 mM Tris pH 8.0, 1 mM EDTA) by gently rolling the Corex tube at an angle on ice for 1 h. The dissolved plasmid DNA was transferred to a 1.5 eppendorf tube and then stored at 4°C until needed.

2.14.3 Qualitative and quantitative analysis of isolated plasmid DNA

The identity of the desired plasmid DNA was verified by restriction digestion with the appropriate restriction enzymes followed by SDS polyacrylamide gel electrophoresis to visualize the resultant DNA fragment lengths. Electrophoresis of uncut plasmid DNA also was used to check the quality of the DNA and to give a rough estimate of DNA concentration. DNA concentration can be more exactly measured by determining its absorbance at 260 nm using a UV spectrophotometer and a quartz cuvette. It is important that the DNA be diluted such that the absorbance reading falls between 0.1 and 1.0 where there is a linear relationship between absorbance and concentration. The plasmid DNA concentration (ug/ml) was calculated from UV spectrophotometry measurements of absorbance at 260 nm and the equation \( A_{260} \) (dilution factor) (50 ug/ml) = DNA concentration (ug/ml). The amount of protein contamination present in the plasmid DNA was estimated by measuring the DNA’s absorbance at both 260 and 280 nm. If the ratio of \( A_{260}/A_{280} \) is within the range of 1.65 – 2.0 then the DNA preparation is said to have negligible protein contamination.
2.14.4 BAC labeling
Probes were labeled with either digoxigenin (DIG) or biotin using a nick translation kit (Roche). In a
PCR tube an equivalent of 1 ug of plasmid DNA (template) was added to 4 ul DIG or biotin nick
translation mix (5X concentration stabilized reaction buffer in 50% glycerol(v/v) and DNA polymerase I
, deoxyribonuclease (DNase I), 250 uM deoxyadenosine triphosphate (dATP), 250 uM deoxycytidine
triphosphate (dCTP), 250 uM deoxyguanosine triphosphate (dGTP), 170 uM deoxythymidine
triphosphate (dTTP), 80 uM biotin-16-deoxyuridine triphosphate (dUTP) or 80 uM DIG-11-dUTP)).
The total reaction volume was brought to 20 ul with sterile distilled water. The reaction mixture was
microfuged briefly and then incubated at 15°C for 90 min in a programmable thermocycler. A 3 ul
aliquot of reaction mixture was run on a 0.8% agarose gel to determine fragment length size. Ideal
probe fragment lengths are in the range of 200 – 500 base pairs. If the desired fragment length had
been achieved then the labeling reaction was halted by the addition of 1 ul 0.5 M EDTA, pH 8.0 to the
remainder of the reaction mixture and heating the mixture to 65°C for 10 min. If the fragment length
was not in the desired range the probe reaction was allowed to continue for an additional amount of time
eg 10, 20 or 30 min. Labeled probe was stored at –20°C.

2.15 Fluorescent in situ hybridization (FISH)

2.15.1 Preparation of chromosome spreads for FISH
The spread protocol is the same as for immuno-localization up to and including placing digested buds on
ice in bud stabilizing solution. Subsequently a bud was selected and transferred to another depression
slide containing freshly prepared 4% paraformaldehyde (pH 8.2) for 60 s using a fine wire loop and a
dissecting scope. The bud was then immediately transferred into 5 ul of 45% acetic acid situated at the
centre of a clean glass slide. The bud was gently prodded (usually less than 1 min) to facilitate its
dispersal in the acid and then the nuclear suspension was immediately dried down with a hair dryer held
approximately 12 in above the sample. The slides were briefly rinsed in distilled water pH 7.5, and
then 100 ul of 4% PFA (pH 8.2) was added to the sample area of the slide. The PFA was tapped off
after 10 min and the slides were allowed to air dry in the fumehood. Slides not being used in subsequent
in situ hybridization had DAPI applied to the spread area, a coverslip added and were viewed using a
Zeiss axiophot epifluorescent microscope and a Plan-Neofluar 100X oil immersion lens. Slides to be
used for in situ were stored at 4°C until needed (usually within 1-3 days)

2.15.2 Fluorescent in situ hybridization
Selected slides were placed at 60°C for 30 min, then treated with RNaseA (100 ug/ml in 2X SSC) for 1
h at 37°C and then rinsed in 2X SSC (1X SSC = 0.15M NaCl, 0.015M sodium citrate) for 2 X 5 min.
After rinsing with 1X phosphate buffered saline (PBS) 1X 5 min. the slides were post fixed in 4% paraformaldehyde in water for 10 min then rinsed again with 1 X PBS 2 X 5 min. The slides were then dehydrated through an ethanol series (70%, 90%, 100%), 1 min each and then air dried. The multi-BAC probes were prepared for use as described by Lysak et al. (2006). The probe was thoroughly dissolved in 10 ul of hybridization buffer (HB50) (50% formamide, 2X SSC, 50 mM sodium phosphate, pH 7.0) in a 42°C water bath. To this mixture 10 ul of 20% dextran sulfate in HB50 was added. Twenty microliters of one of the following multi-BAC probes were applied to the chromosome spreads on a slide: chromosome 1 (interstitial) biotin labeled BACs F14L17, T5E21, F10B6; chromosome 1 (sub-terminal) biotin labeled BACs F23A5, T21F11, F5I6; chromosome 2 (sub-terminal) biotin labeled BACs F2I9, T8O11, T23K3; chromosome 4 (sub-terminal) biotin labeled BACs F6N15, F5I10, F6N23 and chromosome 4 (interstitial) DIG labeled BACs F6N23_ T18A10_ _ F3D13_ _ T15B16 (underscores represent unused BAC clones that are non-chromosome specific). The DIG labeled BAC clones were kindly provided by Dr. Lysak. Coverslips were then applied and their edges were sealed with rubber cement. The probe DNA and the chromosomal DNA were denatured together on the slides at 85°C for 5 min. The slides were then incubated in a moist chamber for 24 h at 37°C.

2.15.3 Post hybridization signal detection

Post hybridization washing and hybridization signal detection were performed as described by Lysak et al. (2006). The slides had their coverslips removed and were then washed in 2X SSC at 42°C for 2 min. The slides were then washed in SF50 (50% formamide in 2X SSC pH 7.0) at 42°C 3 X 5 min. The slides were then washed in 2X SSC at 42°C for 2 min. The slides were rinsed briefly in 4T buffer (0.05% Tween 20 in 4X SSC pH 7.5) and then 200 ul of blocking solution [5% bovine serum albumin (BSA), 0.2% Tween 20 in 4X SSC] was added to the spread area. The slides were incubated in a humid chamber at 37°C for 30 min and then were rinsed in 4T buffer at 42°C for 2 X 5 min.

The slides were then rinsed in TNT buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 pH 7.5) at 42°C for 5 min. DIG-dUTP labeled probe was detected with mouse anti-DIG antibody (Jackson Immuno-research), 1:200 in TNB (100 mM Tris-HCl, 150 mM NaCl, 0.5% Boehringer blocking reagent). Biotin-dUTP probe was detected with mouse anti- biotin (Jackson Immuno-research), 1:100 in TNB. Two hundred microliters of mouse anti-DIG or mouse anti-biotin was prepared and applied to the spread area on the slides. The slides were then incubated in a humid chamber at 37°C for 30 min. The slides were rinsed in TNT at 42°C for 3 X 5 min. The remaining steps in the protocol were performed in a darkened area. The secondary antibody used was goat anti-mouse rhodamine (Jackson Immuno-research) 1: 200 in TNB was prepared and placed on ice. Secondary antibody (200 ul) was applied to the spread area of each slide and the slides were incubated at 37°C for 30 min. The slides were then
rinsed at 42ºC for 3 X 5 min. The slides were dehydrated in an ethanol series of 70%, 90% and 100%, 1 min each and air dried. The negative control was treated identical to the sample except no probe was involved. Finally slides were mounted in DAPI, cover slips were applied and sealed with nail polish. Viewing involved the same microscope, lens and image capturing software as described previously for immuno-localization. Digital images were merged with image processing software (JASC Paint Shop Pro 9) and the montages were made with Photoshop.

2.16 FISH following ZYP1 immuno-localization
After ZYP1 immuno-localization several chromosome spreads with linear ZYP1 signal had their positions on the slide recorded and images of these spreads were digitally captured. The coverslips of these slides were carefully removed and the slides were washed in 1X PBS (pH 7.3) 3 X 5 min at RT. The FISH protocol was then followed as previously described. The chromosome 4 sub-terminal probe used was biotin labeled BACs F6N15, F5I10, F6N23. The biotin label was detected with mouse anti-biotin (1:50) followed by the secondary antibody goat anti-mouse rhodamine (pre-absorbed against rabbit serum) (1:50). Viewing involved the same microscope, lens and image capturing software as described previously in this report for immuno-localization. Digital images of were merged with image processing software (JASC Paint Shop Pro 9) and the montages were created with Photoshop.

2.17 5-Bromo-2-deoxyuridine (BrdU) labeling of S-phase male meiocytes for the estimation of meiosis duration
2.17.1 Pulse labeling of male meiocytes during pre-meiotic S-phase
For each post BrdU pulse time point to be analyzed at least two inflorescences were required. The stems of inflorescences were cut under water to a length of approximately 6 cm; they were cut under water to prevent the introduction of air bubbles into the plant’s vascular system that might impede BrdU uptake. The cut ends of the stems were immediately placed into a small glass vial containing 1ml of 10 mM BrdU labeling reagent (undiluted) (Roche) for 2 h at room temperature under the grow lights. Care was taken to ensure that all cut ends were submerged in the BrdU reagent. After the 2 h labeling period the BrdU labeling reagent was exchanged 10X with fresh tap water to halt any further uptake of BrdU. The inflorescences were left in tap water under normal growing conditions for the remainder of the experiment. The analysis time points for wild type were 28, 30, 32, 33 and 35 h post mid-point of the BrdU pulse. The analysis time points for the ahp2 mutant were 30, 32, 34, 36, 38 and 40 h post mid-point of the BrdU pulse. There were two negative controls: 1) fixation of non-BrdU pulsed tetrads and 2) fixation of tetrads at the 30 min time point after the 2 h BrdU pulse. Inflorescences were removed
from the water at the various time points, their stems were removed and the buds were immediately placed into 10 mM sodium citrate buffer (pH 4) on ice.

2.17.2 Chromosome squashes of unfixed buds for BrdU analysis of tetrads
The buds used for this analysis were from the primary inflorescences of well grown *Arabidopsis* with shoots just recently bolted to a length of at least six centimeters. In most primary inflorescences there was at least one bud with pollen mother cells at the tetrad stage of meiosis. At the various time points after the BrdU pulse an inflorescence was taken and one by one its buds examined for the presence of tetrads. First using a dissecting microscope a bud from the inflorescence was placed on a clean slide containing 10 ul of citrate buffer (10 mM sodium citrate pH 4.0). The remainder of the inflorescence was kept in the depression slide with citrate buffer and the slide was placed in a petri dish on ice. The bud was sliced across its base with a scalpel thus releasing the anthers contained within. Each anther was then sliced across its width at mid-length which releases the pollen mother cells. Anthers at the tetrad stage released numerous tear shaped tetrad particles as seen with the dissecting scope’s high power objective lens (6X); the total magnification including the ocular lens (10X) was 60X. In contrast released pollen grains appeared as circular particles and are considerably smaller then tetrad particles. If the presence of tetrads was confirmed using the light microscope then the anthers on the slide were further cut and pressed with micro-tools to maximize tetrad release from anther locules. After adding 5 ul more of 10 mM sodium citrate to the tetrad sample, micro-tools were used to remove as much anther cell debris as possible. The tetrad solution was then dried down with a hair dryer (held ~12 inches above sample) and then 30 ul of fixative (70% ethanol, 50 mM glycine pH 2.0) was placed over the sample area; this area was previously marked on the underside of the slide with a diamond pencil. A coverslip was applied and the slide with fixative was incubated at –20°C for 20 min. The slide was then immediately submerged for 5 s in liquid nitrogen. Immediately after removal of the slide from the liquid nitrogen the coverslip was popped off. The slide was dried with a hair dryer and then was stored at 4°C until needed for the immuno-detection of BrdU.

2.17.3 Immuno-detection of BrdU
The slides with tetrads to be analyzed were first placed on the slide warmer at 40°C for 30 min to help ensure adherence of cells during the immuno-cytochemistry procedure. The slides were then rinsed 2X each for 5 min in 1X PBS pH 7.3 at room temperature. The slides were dried off around the sample area and then 200 ul of mouse anti-BrdU (Roche) was added and a small strip of parafilm was applied over the top of the antibody. The slides were incubated within a humid chamber at 37°C for 45 min. The slides were then washed 3 X 5 min with 1X PBS pH 7.3 at room temperature. The slides were again
dried off around the sample area. The following steps were performed in a darkened area of the lab.
Two hundred microliters of goat anti-mouse FITC (Roche) was added to the sample area of the slide and then covered with parafilm. The slides were incubated in a humid chamber at 37°C for 45 min. The slides were washed 3 X 5 min in 1X PBS pH 7.3 at room temperature and then allowed to air dry. DAPI then was added to each slide, a coverslip was applied and its edges were sealed with nail polish. Slides were analyzed for the percentage of tetrads present that were labeled with BrdU.

2.18 Transmission electron microscopy (TEM) analysis of ultrathin sections of ahp2 male meiocytes (TEM work performed by MSc student Nazia Pathan)

2.18.1 Bud fixation and embedding
Chromosome squashes (methods section 2.4) were initially performed by P. Stronghill to ascertain the bud size required to examine zygotene/pachytene in wild type and ahp2 male meiocytes; then buds of the appropriate size were gathered by P. Stronghill. Prior to each experiment, all of the solutions were freshly prepared, except for the HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, which was no more than one week old. The procedure used is according to Li et al. (2004) with the following exceptions; bud sepals were opened slightly prior to fixation, vacuum infiltration was used to ensure bud submersion into fixative and bud dehydration was achieved via a graded ethanol instead of graded acetone series. For each experiment, 5-10 buds (0.2-0.4 mm) were transferred into a vial of fixative made of 2.8% EM grade glutaraldehyde (Canemco and Marivac) and 0.02% Triton X-100 added to 0.1 M HEPES buffer (pH 7.2). The buds were incubated in the same fixative for additional 1.5 h at room temperature with gentle shaking followed by overnight storage at 4°C in new fixative. The buds were rinsed with three 15 min washes in 0.1M HEPES buffer (pH 7.2) followed by overnight post fixation in 1.0% osmium tetra-oxide (OsO₄) in 0.1M Phosphate buffer (pH 7.2) at room temperature, then rinsed again three times in HEPES buffer for a period of 15 min per rinse. Dehydration was achieved by transferring the buds through an ascending alcohol series by using two, 10 min treatments in each of: 50%, 70%, 95% and 100% ethanol. The infiltration and embedding were carried out using the Spurr’s resin (Canemco & Marivac). The buds were infiltrated first for 1 to 2 h at room temperature followed by overnight infiltration at 4°C. Plastic infiltrated buds were cured overnight at 72°C, and then stored for at least three days.

2.18.2 Preparation of Formvar coated grids
0.4% formvar in 1-2-dichloroethane was prepared from Formvar 15/95 resin powder (Canemco Inc.) dissolved in the 1-2-dichloroethane solvent in a coplin jar. Glass slides were dipped into the 0.4% formvar for 5 s and the slides was allowed to dry within 1, 2-dichloroethane fumes for 30 s in the
dessicator (to promote even drying of the formvar film) and then air dried for 1 min. The formvar was floated onto the dust free surface of a volume of water and then collected onto an aluminum bridge (with holes) by lightly touching the top surface of the bridge to the film. Formvar film was transferred to a copper grid by touching the grid to film overlying one of holes in the bridge.

2.18.3 Sectioning and staining of meiotic nuclei
Prior to ultra-thin sectioning thick sections were examined with light microscopy to determine if they were the desired meiotic stage. All sectioning was carried out with the ‘Leica Ultracut UCT’ microtome, in conjunction with glass knives for thick sectioning or the Diatome™ Ultra diamond knife, 45°, 2.1 mm for ultrathin sectioning. Conventional double staining with uranyl acetate (UA) and Reynolds lead citrate was used to stain the 200 nm and 80 nm ultrathin sections for the TEM protocol. Aqueous saturated uranyl acetate stain was prepared by dissolving approximately 7.7% uranyl acetate in 50 ml of double distilled water for a period of 24 h followed by filtration. Reynolds Lead Citrate was prepared using the technique described by Reynolds (1963). Ultrathin sections were examined with a JEOL 1200 EXII transmission electron microscope.

2.18.4 Method of chromosome alignment assessment and measurement
Measurements were made on drawn outlines of the specific structures of interest. Axial elements separated by 400 nm to 1000 nm were considered to be roughly aligned and this was distinguished from chance association if alignment continued from one section to another or became more intimate. Axial elements that ran parallel and were separated by 200 nm to 400 nm (no central element) were considered to be closely aligned.

2.19 Scanning electron microscopy (SEM) examination of Arabidopsis wild type and ahp2 stamen filaments (assistance was provided by MSc student Esther Supijono)

2.19.1 Fixation of stage 14-15 mature flower (performed by P.Stronghill)
Inflorescences containing mature flowers were fixed in 10 ml of freshly made FAA fixative (5 ml 100% ethanol, 3.5 ml distilled water, 0.5 ml acetic acid, 1 ml formaldehyde) overnight at 4°C.

2.19.2 Inflorescence/flower dehydration (performed by P.Stronghill)
The fixed inflorescences were rinsed according to the following regimen. The fixative containing inflorescences was exchanged with 70% ethanol and the inflorescences sat in this solution at room temperature for 60 min.; this was repeated once. Inflorescences were then placed in 85% ethanol at
room temperature for 45 min. followed by 95% ethanol at room temperature for 45 min. Finally inflorescences were rinsed twice with 100% ethanol at room temperature; each rinse was for 60 min.

2.19.3 Critical point drying of inflorescences (performed by P.Stronghill and E. Supijono)
The Polaron E3000 was used for critical point drying. The inflorescences were placed in the drying boat with 100% ethanol. The 100% ethanol was exchanged twice with acetone, 10 min each rinse at room temperature. Care was taken not to expose the tissue to air. The boat with sample was placed within the cooled down (10°C) drying chamber. The acetone was exchanged with liquid carbon dioxide. This was repeated twice such that all acetone was removed. The sample was left to sit in liquid CO₂ for 30 min, this was exchanged for fresh CO₂ and sample was incubated for an additional 30 -60 min. The level of CO₂ was slightly lowered in the chamber. The chamber temperature was slowly increased to 33°C and pressure was increased to 1200 psi (the critical point of CO₂). Condensation was avoided by slow venting of the chamber.

2.19.4 Inflorescence mounting and sepal/petal removal from flowers (performed by P.Stronghill)
A small piece of double sided tape was cut and placed on the top of the 0.5 inch aluminum stud. Four mature flowers were placed on the tape. Using very fine tweezers and a dissecting scope the sepals and petals were carefully removed thus exposing the stamens. This was repeated using additional studs.

2.19.5 Gold sputter coating of inflorescences (performed by P.Stronghill and E. Supijono)
Gold sputter coating was performed using the Polaron E5000C sputter coater. Forceps were used to place studs with sample on the base plate of the chamber. Nitrogen gas was used in conjunction with the following sputter coater parameters: plasma current set to 19 mA, sputter coating time 100 s, vacuum pressure 0.3 mBar. After first sputter coating samples were repositioned and sputter coated again to more uniformly coat the sample and minimize subsequent charging during SEM analysis. After sputter coating a small dab of liquid silver was used to make an electrical connection between the gold and the aluminum stud.

2.19.6 SEM film development and negative scanning (performed by P. Stronghill & E. Supijono)
A Hitachi S-530 scanning electron microscope was used to view the sample at 25 kV. Pictures were taken using Nikon FG-20 camera and ultra fine grain (black and white) Neopan 100 (Fujifilm). The film was then developed in complete darkness using KODAK D-76 film developer and KODAK rapid fixer solutions. The negative film was scanned using EPSON Perfection 4490 photo scanner and Adobe Photoshop 7.0 software was used to adjust the brightness and contrast of the images.
Results (Part I) - Bioinformatic and molecular analysis of AHP2 (HOP2) genes/proteins and AHP2 antibody characterization

The work described in this section was performed by Patti Stronghill
3.1 Results (Part I) - Bioinformatic and molecular analysis of \textit{AHP2} /\textit{HOP2} genes/proteins and \textit{AHP2} antibody characterization

3.1.1 Overview

BLAST searches of NCBI databases were done to identify an \textit{Arabidopsis} cognate of the yeast \textit{HOP2} gene. An \textit{Arabidopsis} cognate was found but because of the weak sequence identity between yeast \textit{HOP2} and \textit{Arabidopsis AHP2} genes/proteins, further comparisons of the two genes/proteins were made using bioinformatic resources. Some of the protein properties compared were primary amino acid sequence, molecular weight, coiled coil tertiary structure and hydrophobicity. RT-PCR was used to assess \textit{AHP2} gene expression in \textit{Arabidopsis}. Western analysis demonstrated that the anti-\textit{AHP2} antibody used in immuno-labeling experiments did not recognize \textit{AHP2} in \textit{Arabidopsis} bud protein extracts but did recognize the \textit{AHP2} protein domain used as antigen.

3.1.2 Results

3.1.2.1 The \textit{HOP2/ AHP2} gene is present in the \textit{Arabidopsis} genome

A tBLASTn search of the non-redundant nucleotide (nr/nt) database (Genbank) using the \textit{S. cerevisiae} \textit{HOP2} protein sequence was unsuccessful in identifying a cognate gene in \textit{Arabidopsis} but did recognize an orthologous gene in mouse (\textit{TBPIP}). Mouse TBPIP will be referred to as \textit{HOP2} for the remainder of this thesis. Mouse \textit{HOP2} protein sequence was used in a subsequent search of the (nr/nt database) for the \textit{Arabidopsis} genome. The tBLASTn search identified an orthologous gene in \textit{Arabidopsis}. Schommer et al. (2003) subsequently named the \textit{Arabidopsis} cognate of the \textit{HOP2} gene, \textit{AHP2} (\textit{Arabidopsis} homolog pairing 2). The sequence for the \textit{Arabidopsis AHP2} gene (At1g13330) is shown in Figure 11. The sequence identity between the budding yeast \textit{HOP2} gene and the \textit{Arabidopsis AHP2} gene was 17\% whereas the sequence identity between mouse \textit{HOP2} gene and \textit{Arabidopsis AHP2} gene was 34\%.

3.1.2.2 The \textit{Arabidopsis AHP2} transcript

The \textit{Arabidopsis AHP2} transcript is 2664 base pairs (bp) (Genbank accession: NC_003070.9; TAIR: At1g13330) and is comprised of seven exons and six introns (Fig.11). The open reading frame (including introns) is 1403 bp and the six coding regions (minus introns) in total are 678 bp in length. An \textit{E2F}_a-DP\_a transcription factor target site sequence, ‘\textit{TTTCCCGC}’, is present 17 bp upstream of the ATG translation start site in the \textit{AHP2} gene. \textit{AHP2} transcript organization is described in Table 1.
Fig. 11 The Arabidopsis *AHP2* transcript. This is a schematic of the *AHP2* transcript including both 5′ and 3′ un-translated regions. This sequence was obtained at: [http://www.arabidopsis.org/](http://www.arabidopsis.org/). The seven exons have been highlighted, each in a different color. The light green, underscored sequence, upstream of the translation start site, is a target site for the E2F<sub>a</sub>-DP<sub>a</sub> transcription factor. The GT and AG nucleosides at the beginning and end of each intron have been underlined. The underscored portion of the third exon (red) was the sequence used to generate an antibody to the AHP2 protein. The stop codon for the coding region of the transcript is marked with a black box. The *AHP2* transcript is further described in Table 1.
<table>
<thead>
<tr>
<th>Sequence type</th>
<th>nucleotide coordinates</th>
<th># of nucleotides</th>
<th>amino acid coordinates</th>
<th># of aa in the protein domain</th>
<th>protein domain aa start .../.. aa finish</th>
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</thead>
<tbody>
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<td>5' UTR</td>
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<td>73</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E2Fa-DPa target site</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>10-18</td>
<td>9</td>
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<td>108-131</td>
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<td>132-194</td>
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<td>Coding region/exon 6</td>
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<td>195-226</td>
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<td>-</td>
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<td>259</td>
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<tr>
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<td>678</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total # of amino acids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>226</td>
<td>-</td>
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</tbody>
</table>
Vandepoele et al (2005) found that the *Arabidopsis* AHP2 gene undergoes a 16:1 induction in expression upon cells entering into S phase; its expression appears to be linked to the cell cycle. Examination of both the mouse *HOP2* and the *S. pombe MEU13* genes also revealed the presence of possible E2F$_2$–DP$_2$ transcription factor target sites.

### 3.1.2.3 AHP2 protein ortholog comparison

The weak identity of *Arabidopsis* AHP2 protein sequence with budding yeast HOP2 (23%), fission yeast MEU13 (30%) and mouse HOP2 (35%) orthologs warranted further homology checks. The yeast and mouse orthologs were chosen for comparison as corresponding mutant phenotypes have been reported for these organisms. The protein molecular weight for budding yeast, fission yeast, mouse and *Arabidopsis* AHP2 orthologs were very similar at 25.0, 24.7, 25.5 and 26.2 KDa respectively. The phylogram in Figure 12 provides an overview of the amino acid similarity of AHP2 (HOP2) primary amino acid sequence in several organisms. The *Arabidopsis* AHP2 protein appears to be evolutionarily least similar to budding yeast.

The presence of coiled coil regions (a feature of tertiary structure) in the AHP2 orthologs was compared (Fig 13) and found to be very similar. The central region (residues ~75 - ~150) of the AHP2 (HOP2) protein has largely coiled coil structure. Coiled coil regions are thought to play an important role in protein-protein interactions; so this region most likely is involved in AHP2’s physical interaction with MND1.

Hydropobicity plots for AHP2 protein orthologs were also compared and found to be similar to AHP2 (Fig 14). The AHP2 (HOP2) protein is not, in general, hydrophobic owing to its high content of positively charged and polar residues. The similarity in hydrophobicity plots is greatest for *Arabidopsis* AHP2 and mouse HOP2 proteins, as a larger percentage of residues in these orthologs have polar or charged side chains as compared to the yeast orthologs. In addition, when hydrophilicity values were compared at 11 positions along the AHP2 protein, the largest similarity was found between *Arabidopsis* and mouse orthologs; this comparison is shown in Table 2.

A possible DNA minor groove binding motif was found in the C-terminal region of the AHP2 protein in all organisms examined except budding and fission yeast. The *Arabidopsis* AHP2 protein’s bioinformatic profile (excluding plants) appears to be most similar to the profile for mouse HOP2 (for the set of parameters and organisms examined); this bioinformatic comparison is summarized in Table 3.
Fig.12  The *Arabidopsis* AHP2 protein appears to be most evolutionarily diverged from the budding yeast ortholog, in this phylogenetic analysis. The tools for this phylogenetic analysis were obtained at [http://www.phylogeny.fr](http://www.phylogeny.fr). The input data for the phylogenetic analysis of AHP2 orthologous sequences was aligned with the MUSCLE alignment tool (full mode setting; 20 iterations). The alignment was converted to Phylip format, curated using the Gblock program (stringent setting) and then analyzed using the Neighbour Joining algorithm with standard bootstrapping (500 iterations). The phylogenetic tree created by this algorithm was rooted using *Arabidopsis* as the outgroup and the estimated evolutionary distances (from *Arabidopsis* AHP2 protein) are given in bolded black at the end of each branch. Dicot and monocot plants are highlighted in red and blue respectively. NCBI accession numbers are given for each orthologous protein. The scale bar represents the degree of evolutionary divergence.
Fig. 13 The coiled coil plot for *Arabidopsis* AHP2 appears most similar to the mouse ortholog.

The plots show the predicted location of coiled coil tertiary structures in *Arabidopsis*, mouse, fission and budding yeast HOP2 (AHP2) orthologs. The x-axis represents a rolling window of 21 consecutive amino acids that start at the amino-terminus and end at the carboxy-terminus of the AHP2 protein; for example X=1 represents residues 1-21 inclusive; X=2 represents residues 2-22. The y-axis represents the averaged probability of coiled coil structure for each of the amino acid sequence windows on the x-axis [http://www.ch.embnet.org/software/COILS_form.html](http://www.ch.embnet.org/software/COILS_form.html).
Fig. 14 The hydrophobicity plot for the *Arabidopsis* AHP2 protein is most similar to the plot for the mouse ortholog. The x-axis represents a rolling window of 7 consecutive amino acids that start at the amino-terminus and end at the carboxy-terminus of the AHP2 protein; for example x=1 represents residues 1-7 inclusive; x=2 represents residues 2-8. The y-axis represents the degree of hydrophobicity/hydrophilicity for the 7 amino acid region that comprises each window on the x-axis. Positive y-values indicate the degree of hydrophobicity and negative y-values indicate the degree of hydrophilicity; the red line represents y = 0. The vertical dotted lines indicate the positions along the x-axis (11 positions labeled a-k) that were used to compare AHP2 ortholog hydrophobicity values. Points in the *Arabidopsis* plot that exceeded the hydrophobicity threshold value indicated by the green line were included in the comparison. Comparison values can be found in Table 2. This hydrophobicity plot was generated with the algorithm at [http://expasy.org/tools/protscale.html](http://expasy.org/tools/protscale.html).
Table 2  Hydrophilicity Value Comparisons for AHP2 Protein Orthologs

<table>
<thead>
<tr>
<th>Positions on the X-axis used in the comparison</th>
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<td></td>
<td>Arabidopsis</td>
</tr>
<tr>
<td>a</td>
<td>-2.5</td>
</tr>
<tr>
<td>b</td>
<td>-4.0</td>
</tr>
<tr>
<td>c</td>
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<tr>
<td>h</td>
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<tr>
<td>i</td>
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<tr>
<td>j</td>
<td>-3.2</td>
</tr>
<tr>
<td>k</td>
<td>-3.5</td>
</tr>
</tbody>
</table>

X difference in hydrophilicity values (At-mouse) = \[ \sum \text{diff}/11 = 1.4 \]

X difference in hydrophilicity values (At-fission yeast) = \[ \sum \text{diff}/11 = 2.4 \]

X difference in hydrophilicity values (At-budding yeast) = \[ \sum \text{diff}/11 = 2.4 \]
Table 3  Bioinformatics Profiles for AHP2 Orthologs

<table>
<thead>
<tr>
<th>Properties</th>
<th>Arabidopsis AHP2</th>
<th>fission yeast MEU13</th>
<th>budding yeast HOP2</th>
<th>mouse HOP2</th>
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<tr>
<td>protein hydrophobicity plot *</td>
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<td>3</td>
<td>2</td>
</tr>
<tr>
<td>protein molecular weight (kDa)</td>
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<td>24.7</td>
<td>25.0</td>
<td>25.5</td>
</tr>
<tr>
<td>1° amino acid sequence (% identity)</td>
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<td>30</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td>protein coiled coil 3° structure *</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>E2F&lt;sub&gt;a&lt;/sub&gt;-DP&lt;sub&gt;a&lt;/sub&gt; transcription factor target site **</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>DNA minor groove binding motif</td>
<td>yes</td>
<td>maybe ***</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

* 1- 3 denotes degree of similarity to *Arabidopsis*: 1 = most similar and 3 = least similar

** E2F<sub>a</sub>-DP<sub>a</sub> transcription factor target sites are found in genes that are expressed pre-meiotically

*** there may be a truncated version of the minor groove motif ‘WRKRKRM/L/I’ in fission yeast MEU13 but it is more likely that the ‘RKK’ sequence in the C-terminal region is a nuclear localization signal

Note: *Arabidopsis* AHP2 appears most similar to mouse HOP2 for the set of parameters and organisms examined
3.1.2.4 The AHP2 gene is expressed in Arabidopsis inflorescences

Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that the AHP2 gene is transcribed in Arabidopsis inflorescences. The primers used were expected to amplify a 210 bp region of RT-PCR cDNA product that corresponds to the 3rd exon of the AHP2 gene. The 210 bp band that is seen in the agarose gel (Fig. 15; lane 8) shows that AHP2 mRNA was present in the RNA extracted from the inflorescences. The 210 bp band (Fig. 15; lane 5) resulted from the amplification of the same DNA region from the genomic DNA template (present in the untreated RNA extract); this demonstrates the need to DNAase treat extracted RNA prior to reverse transcription. Negative controls included no forward primer, no reverse primer, no DNA template and DNase-treated RNA extracted from wild type Arabidopsis inflorescences.

3.1.2.5 The AHP2 antibody recognizes the intact AHP2 protein in chromosome preparations but not in Western blots

In Western blots the AHP2 antibody strongly recognized the AHP2 protein domain that was used as antigen to generate anti-AHP2 antibody in rabbits. But it does not recognize intact AHP2 protein found in protein extracts from Arabidopsis buds. The chemi-luminescent detection system used, in the western analysis, was very sensitive as it successfully detected three nanograms of AHP2 protein antigen applied to the gel. The AHP2 antibody did not cross react or non-specifically bind any Arabidopsis bud proteins as can be seen in the western blot shown in Figure 16. The AHP2 antibody did recognize AHP2 protein in wild type Arabidopsis chromosome spread preparations; this finding is discussed in section 3.3.2.4.
Fig. 15 The AHP2 gene is transcribed in Arabidopsis inflorescences. An RT-PCR experiment was done using RNA extracted from Arabidopsis inflorescences. Products were run on a 1.75% agarose gel. The RT product was run on lanes 3, 4 (negative controls) and lane 8 of the gel; lane 1 =marker DNA, lanes 2, 3, 4, 5, 6 and 7 are negative controls; lane 2 = no cDNA template, lane 3 = no forward primer; lane 4 = no backward primer; lane 5 = 1 ul of AtRNA (no DNAase treatment); lane 6 = 1 ul of DNAase treated AtRNA; lane 7 = 3ul of DNAase treated AtRNA; lane 8 = RT product (AHP2 cDNA). In lane 8 the backward primer (5’ CAC ACC GAA TTC CAC ATC ACT GAT AGT 3’) and forward primer (5’ AAA GGC TAG CCT TAA GAA GAC TGC GGT GCA A 3’) were expected to amplify a 210 bp region of the reverse transcribed cDNA product corresponding to AHP2’s 3rd exon. The band in lane 5 is the result of undesired DNA presence in the extracted RNA.
Fig. 16 AHP2 antibody did not recognize AHP2 in the extracted protein from *Arabidopsis* buds but did recognize the AHP2 protein domain that was used as antigen. Western analysis of AHP2 antibody; lane 1= marker protein, lane 2, 6= *ahp2* mutant bud protein, lane 3, 7= wild type bud protein, lanes 4, 8 = AHP2 protein domain (10 kDa) used as antigen (~3 ng was run on the gel)  Notes: 1) the theoretical molecular weight of AHP2 protein is 26 kDa,  2) the position of marker protein bands (not seen on the film) were identified by staining the corresponding nitrocellulose blot with Ponceau stain and overlaying the film on the stained blot. The AHP2 antibody did not cross react with or non-specifically bind to any *Arabidopsis* bud proteins.
Results (Part II) - *Arabidopsis* Cytology: Analysis of substage associations in prophase I of meiosis in floral buds of wild type *Arabidopsis thaliana* (*brassicaceae*)

Material in this section has been published in The American Journal of Botany.


The original publication/PDF is available at:

http://www.amjbot.org/cgi/reprint/94/12/2063

All the work described in this section was carried out by Patti Stronghill
3.2 Results (Part II) - Arabidopsis Cytology: Analysis of substage associations in prophase I of meiosis in floral buds of wild type Arabidopsis thaliana (brassicaceae)

3.2.1 Overview

We developed an improved cytological protocol for producing high quality, light microscope images of plant meiotic chromosomes. Because the technique works on species with small genomes and thick microsporocyte cell walls, it should be useful for studying the wild relatives of Arabidopsis and other eudicots with small genomes. Combining this improved fixation protocol with our new analysis of associated substages in floral buds, we can unambiguously assign individual meiotic cells to particular substages of prophase I in Arabidopsis thaliana, even for difficult distinctions such as that between late zygotene or early diplotene. In this report we provide the first estimate of the individual duration of the zygotene and pachytene substages (4.8 h and 10.0 h, respectively) in A. thaliana. We also have examined the diffuse substage of prophase I and report that during this post-pachytene substage, nuclei retain the association of homologous nucleolus organizer regions and homologous centromeres despite the generally diffuse chromatin and generally unpaired chromosome regions. Additionally, we have observed that centromeric regions of the chromosomes of diffuse-stage nuclei are highly condensed, more so than those of any other substage of prophase I.

3.2.2 Results

3.2.2.1 Arabidopsis flowers - meiotic synchrony within anthers

In a preliminary study, meiosis was examined in unfixed DAPI-stained, gently squashed individual anthers of single buds. Each bud has six anthers; four larger anthers and two smaller anthers. Each anther has four chambers. The meiocytes within a single chamber were always at the same stage; meiocytes in different chambers of the same anther were usually, but not always at the same stage. While the four larger anthers were usually at the same meiotic stage, the smaller anthers often were at an earlier stage (Fig. 17). Thus, there is often some asynchrony within an individual bud.

3.2.2.2 Substage associations in floral buds

The slight asynchrony observed in individual buds, in conjunction with an improved fixation protocol, was used to identify the substages of prophase I that were found within the same flower bud, thereby defining the substage associations in the floral buds. For identifying the floral bud substage associations, 59 wild type buds were collected and analyzed. Each bud was
**Fig. 17** The smaller anthers within an *Arabidopsis* bud are usually at a slightly earlier meiotic stage. *Arabidopsis* meiotic chromosome squashes from small anthers (a, c) and slightly larger anthers (b, d) from two individual wild type *Arabidopsis* primary inflorescence buds: (a, b) bud 1, size =0.37 mm; (a) early leptotene (large central nucleolus), (b) mid-late leptotene (off center nucleolus), (c, d) bud 2, size = 0.55 mm, (c) telophase II and (d) tetrads. Chromosomes are stained with DAPI. *Scale Bar* 5 um
Fig. 1. Male meiocyte chromosome squashes from small anthers (A,B) and slightly larger anthers (C,D) from two individual Arabidopsis (Ler) primary inflorescence buds: (A,C) bud 1, size = 0.35 mm; (C,D) bud 2, size = 0.55 mm (A) early leptotene, (C) mid-late leptotene, (B) telophase II and (D) tetrads. Scale bar: 5 um.
treated as indicated in the Materials and Methods and placed on a single microscope slide. Only slides with at least 25 recognizable meiocytes were included in the bud substage analysis; in total, 3073 nuclei were examined. For each bud, we noted which meiotic substages were found together; the associated substages are summarized in Table 4.

Twelve buds that contained meiocytes at the leptotene substage were analyzed. In three of these buds, only leptotene meiocytes were seen; in the remaining nine buds, both leptotene and zygotene substages were observed. The slides in which only leptotene meiocytes were seen were usually early to mid-leptotene (Fig. 18a, b; respectively). Those slides that were a mixture of leptotene and zygotene typically had late leptotene (Fig. 18c) and early to mid-zygotene nuclei (Fig. 18d, e; respectively). Of the buds judged to contain zygotene nuclei, five had exclusively zygotene meiocytes, nine had a mixture of leptotene and zygotene substages, and 15 contained a mixture of zygotene and early pachytene (Fig. 18f, g; respectively).

Based on the level of chromosome condensation and the presence of chiasmata, the stages of diakinesis (Fig. 18l), metaphase I, and anaphase I can be identified readily. In the five buds that contained a mixture of diakinesis, metaphase I, and anaphase I stages, four also contained meiocytes that we interpreted to be at diplotene (Fig. 18i, j). Because these diplotene meiocytes contain partially paired regions, they might have been misinterpreted as zygotene nuclei. But nuclei we judge to be at diplotene were recovered from buds in which the other substages were late pachytene or later (Table 2). Nuclei with an appearance like those in Fig. 18i and j were never found in buds with the substages bracketing the zygotene substage. On the basis of the other meiotic stages (or substages) found in the same bud (i.e., based on the associated substages), diplotene nuclei could readily be distinguished from zygotene nuclei.

Thus with knowledge gained from previous fluorescence in situ hybridization (FISH) studies (Armstrong et al. 2001; Armstrong and Jones 2003), our high quality and detailed images, and substage associations, we can present a complete sequence of prophase I substages in the bud as seen in Figure 18. A loose bouquet is seen in late leptotene (Armstrong et al. 2001) and persists during zygotene, where there is an asymmetric distribution of the chromosomes and nucleolus to one side of the nucleus. The homologous chromosomes become associated during zygotene, with the nuclei containing a mixture of paired and unpaired chromosome regions; the centromere regions of all five pairs of chromosomes are aggregated, forming one or two chromocenters (Fig. 18d, e). By late zygotene (Fig. 18f), homologous chromosomes are extensively aligned along most of their length. In early pachytene, the chromosomes are maximally paired; the centromere regions, while paired with their homolog, are no longer all aggregated in chromocenters (Fig. 18g). By late pachytene, the five paired centromere regions appear as five distinct DAPI–bright regions. The chromosomes in general are uniformly distributed
within the spread, except the nucleolus organizer regions (NORs) of chromosomes 2 and 4 are usually still associated (Fig. 18h).

By early diplotene, small regions of desynapsis begin to occur in non-centromeric regions of the chromosome spreads (Fig. 18i). Using FISH probes, Armstrong and Jones (2003) showed that centromere regions of homologous chromosomes remain associated throughout diplotene. Our improved cytological protocol and substage associations allow us to confirm this feature without the use of FISH probes. Thus, our relatively simple fixation protocol makes it possible to see that, excepting chiasmata themselves, the centromere regions of homologous chromosomes are the last regions to desynapse. By mid-diakinesis, the chromosomes have condensed, and five distinct, chiasmate bivalents are apparent (Fig. 18i). At metaphase I, all five bivalents are distinct, and at anaphase I, the chromosomes have segregated properly to opposite sides of the metaphase I plate (data not shown).

### 3.2.2.3 Duration of meiotic substages in wild type Arabidopsis

We examined the stages of meiosis in a set of primary inflorescences of wild-type Arabidopsis. Inflorescences were selected that had only one or two open flowers so that all meiotic stages would be represented (Smyth et al. 1990). Meiosis I stages were found in buds that ranged from 0.39 to 0.50 mm, 60.01 mm). To avoid sampling bias, we processed all buds in this size range, with a size distribution as follows (with the number of buds given in parentheses): 0.39 mm (6), 0.40 mm (5), 0.41 mm (5), 0.42 mm (4), 0.43 mm (4), 0.44 mm (5), 0.45 mm (6), 0.46 mm (4), 0.47 mm (5), 0.48 mm (4), 0.49 mm (6), and 0.50 mm (5). For each bud used in the analysis, the number of meiocytes at each meiotic substage was recorded. In total, 3073 meiocytes from wild type were included. We used the percentage of the total meiocytes at a particular stage to estimate the relative duration of each stage (Fig. 19). From this, we estimated that leptotene would be only slightly longer in duration than zygotene, and each would be about half as long as the pachytene substage.

Armstrong et al. (2003) used bromodeoxyuridine (BrdU) labelling of S-phase meiocytes and measured the average time until the appearance of BrdU labelling in the various meiotic stages, to estimate prophase I substage durations in Arabidopsis. They estimated that leptotene lasts an average of 6 h. They were unable to distinguish between the individual durations of zygotene and pachytene but did conclude that the combined substages of zygotene/pachytene lasted an average of 15.3 h. They also estimated the total duration of meiosis to be 24 h, of which the pooled substages of zygotene and pachytene constituted 63.7% of the total. When we combined the number of nuclei in our study in both zygotene and pachytene substages (to make them comparable to the pooled zygotene/pachytene sample of Armstrong et al. (2003), our results agreed well with theirs for the relative durations of leptotene vs. combined substages of zygotene/pachytene. However, in our study we were able to distinguish zygotene
Table 4  Prophase I Substage Associations found in Wild Type *Arabidopsis* (Ler ecotype)

<table>
<thead>
<tr>
<th>Associated meiotic stages</th>
<th>Leptotene</th>
<th>Zygotene</th>
<th>Early Pachytene</th>
<th>Late Pachytene</th>
<th>Diplotene</th>
<th>Diakinesis–Anaphase I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptotene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zygotene</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Pachytene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late Pachytene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplotene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Diakinesis–Anaphase I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

Notes: The number of wild type *Arabidopsis thaliana* buds observed to contain the meiotic substages indicated by the row and column designators. For example nine buds contained both leptotene and zygotene nuclei. The zygotene and diplotene substages (which are sometimes misinterpreted) of wild type *A. thaliana* have distinct substage associations in the floral bud. A minimum of 25 DAPI-stained meiotic nuclei were examined from each of 59 *A. thaliana* flower buds; meiosis I substage spreads observed from the same bud along with zygotene spreads are shaded blue and meiosis I substage spreads associated with diplotene spreads are shaded pink.
Fig. 18 DAPI-stained meiosis I chromosome spreads from wild type Arabidopsis (Ler ecotype) meiocytes. The substages of the wild type chromosome spreads are as follows: a) early leptotene, b) mid-leptotene, c) late leptotene, d) early zygotene, e) mid-zygotene, f) late zygotene, g) early pachytene, h) late pachytene, i) early diplotene, j) mid-diplotene, k) diffuse stage, l) diakinesis. Long white arrows indicate paired centromeres; short white arrows indicate nucleolus organizer regions.

Scale Bar 10 um
Fig. 19 The relative durations of meiosis I substages in wild type *Arabidopsis*. The distribution of meiotic substages in 3073 wild type *A. thaliana* male meiocytes is presented in the histogram. The distribution reflects the relative durations of meiosis I substages in wild type *Arabidopsis*. Pachytene has the longest duration of the prophase I substages in wild type *Arabidopsis*. The preserved, DAPI-stained microsporocytes were obtained from 59 flower buds. These meiocytes are from the same buds analyzed in Table 4.
from pachytene preparations, and we thus have data on the relative numbers of each of these two substages and the percentage of total meiocytes that each substage represents. By using our data and combining it with the estimate of Armstrong et al. (2003) that meiosis lasts 24 h in A. thaliana, we provided the first estimate of the separate durations of the zygotene and pachytene substages. Zygote nuclei represent 20.1% of all meiocytes; if meiosis lasts 24 h, we estimated the zygote substage to be 4.8 h. Similarly, because pachytene nuclei represent 41.9% of the total meiocytes, we estimated the pachytene substage to last 10 h. Our estimates of the duration of the interval spanning from diplotene-metaphase I are similar to that of Armstrong et al. (2003), but we estimated that diplotene is longer than diakinesis/metaphase I combined. In contrast, Armstrong et al. (2003) found diplotene to be shorter than diakinesis/metaphase I. This relatively small difference might be due to our classifying diffuse stage nuclei as part of diplotene.

3.2.2.4 The diffuse stage
Our substage association analysis and improved fixation protocol have allowed us to produce high quality images of the meiotic substage known as the diffuse stage (Fig. 18k) and to document a novel feature of chromosome centromeres at this stage. The diffuse stage has been reported in passing, many times in many organisms (Moens 1968; Klasterska and Ramel 1980). In typical acid-alcohol preparations the diffuse stage appears as an ill-defined stage in which the chromosomes are not highly condensed and often have sticky and web-like connections to each other (Klasterska and Ramel 1980). In our preparations, a paraformaldehyde fixation both precedes and follows the acetic alcohol treatment, yielding superior retention of chromosomal features, and producing striking diffuse stage images. Despite an overall loss of pairing of the homologous chromosome axes, the NOR regions of chromosomes 2 and 4 remain associated with each other (one DAPI bright region), and chromosomes are still associated in the region of their centromeres (five DAPI bright regions) (Fig. 18k). More striking is the fact that despite an overall reduction in the level of chromosome condensation, the centromere regions actually appear to be in a more condensed state than at any other prophase I substage. The paired centromeres are thick and ovoid in shape (Fig. 18k).

It has been suggested that the diffuse stage may be a time of chromatin remodeling (Qureshi and Hasenkampf 1995), as part of the chromosomes’ transition from mildly condensed, recombinogenic structures to the more highly condensed metaphase I chromosomes ready to be segregated. It might also be a time to resolve any residually interlocked chromosomes. Retention of the association of homologous chromosomes at the NORs and centromeres, and increased condensation in the centromere regions, may help ensure that important homologous chromosome associations at chiasmata remain intact during the period of ‘remodeling’.
Results (Part III) The short arms of NOR-bearing chromosomes 2 and 4 can pair and synapse in both wild type and the *ahp2* mutant, but widespread pre-synaptic stabilized pairing requires AHP2 (HOP2) function in *Arabidopsis thaliana* (*Ler*) during male meiosis.

Material in this section has been published in Chromosoma:


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http://www.metapress.com/content/68k02q346836x361/fulltext.html

The work related to Figs. 21, 22, 24, 25, 26, 27, 30, 34 was performed by Patti Stronghill
The work related to Fig. 20 was performed by Patti Stronghill and Esther Supijono
The work related to Fig. 23 was performed by Patti Stronghill and Gaya Raveendran (undergraduate student)
The work related to Figs. 28, 29, 32 was performed by Patti Stronghill and Nazia Pathan
The work related to Figs. 31, 33 was performed by Patti Stronghill and Helena Ha (undergraduate student)
3.3 Results (Part III) AHP2 (HOP2) function in *Arabidopsis thaliana* (Ler) is required for stabilization of close alignment and synaptonemal complex formation except for the two short arms that contain Nucleolus Organizer Regions

3.3.1 Overview

A cytological comparative analysis of male meiocytes was performed for *Arabidopsis* wild type and the *ahp2* (*hop2*) null mutant with emphasis on *ahp2*’s largely uncharacterized prophase I. Leptotene progression appeared normal in *ahp2* meiocytes; chromosomes exhibited regular axis formation and assumed a typical polarized nuclear organization. But DAPI stained *ahp2* chromosome spreads demonstrated a severe reduction in pre-synaptic stabilized pairing; fluorescent *in situ* hybridization (FISH) supported this finding. FISH in combination with ZYP1 immuno-staining revealed that *ahp2* mutants undergo homologous pairing and synapsis of the short arms of NOR-bearing chromosomes 2 and 4, despite the otherwise ‘nucleus-wide’ lack of stabilized pairing. The duration of *ahp2* zygotene was significantly prolonged and is most likely due to difficulties in chromosome pairing stabilization. AHP2 and MND1 proteins have previously been shown, *in vitro*, to form a heterodimer. Here we show, *in situ*, that the AHP2 and MND1 proteins are synchronous in their appearance and disappearance from meiotic chromosomes and that AHP2 and MND1 proteins localize along the chromosomal axis. However localization of the AHP2 protein was entirely axial and foci-based whereas MND1 protein exhibited an immuno-staining pattern with some foci along the axis and a diffuse staining for the rest of the chromosome.

3.3.2 Results

3.3.2.1 The *ahp2* short stamen phenotype is due to a cell elongation problem

Mature wild type flowers have four, long, medial stamens and two shorter, lateral ones (Fig. 20a). Mature *ahp2* flowers have stamens that are visibly shorter (Fig. 20b). Schommer et al. (2003) also observed the *ahp2* short stamen phenotype.

Using SEM we found that cell elongation was defective in the *ahp2* mutant; the epidermal cells that constitute the stamen filaments are substantially shorter than wild type (Fig. 20c, d). Average wild type stamen length was 2.3 mm +/- 0.20 mm (n= 100). The average *ahp2* stamen length was 0.98 mm +/- 0.18 mm (n = 100). In contrast to stamens, pistil lengths were very similar in wild type and *ahp2* mutants (Fig. 20e, right).
Fig. 20 The abnormally short stamens in the *ahp2* flower are due to problems in epidermal cell elongation. Images captured from a dissecting microscope are shown of (a) wild type and (b) *ahp2* stage 14 mature flowers (sepals and petals removed). Note the short stamen phenotype of the *ahp2* flower. Scanning electron microscope (SEM) images of *Arabidopsis* are shown for (c) wild type and (d) *ahp2* stamen filaments (from stage 14 mature flowers). Note that both the SEM images are from the mid-stamen filament region. (e) A histogram of the average lengths of stamens and pistils (mm) from wild and *ahp2* stage 14 mature flowers. *Scale Bar* for (c) and (d) 50 μm.
Comparison of Average Stamen and Pistil Length in Wild type (Ler) and ahp2 Mature (stage 14) Flowers

<table>
<thead>
<tr>
<th></th>
<th>Stamen</th>
<th>Pistil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>ahp2</td>
<td>1.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
3.3.2.2 Stabilized chromosome alignment is impaired in *ahp2* meiocytes

Schommer et al. (2003) reported that the *ahp2* meiocytes failed to form bivalents but did not analyze early prophase I events in the mutant. We undertook a light microscopy analysis of the *ahp2* mutant’s meiosis; in particular we looked at its prophase I to determine its ability to accomplish the prophase I substages. DAPI-stained chromosome spreads from wild type and the *ahp2* mutant were examined. Representative images from wild type are shown in Figure 21a-c, g-i and m, n. In total 3073 wild type and 2147 *ahp2* meiocytes were examined. Progressive meiotic stages of the *ahp2* mutant are shown in Figure 21d-f, j-l and o, p; these images were generated from buds of increasing size from the same inflorescence.

Early leptotene chromosome spreads from the mutant appeared normal (Fig. 21d); as leptotene progressed the *ahp2* meiocytes exhibited the same asymmetric distribution of chromatin as seen in wild type. The first observable abnormality in *ahp2* meiosis is seen in zygotene chromosome spreads. In all of the examined wild type zygotene nuclei the centromeric heterochromatin is aggregated into one or two regions whereas in some *ahp2* zygotene nuclei significant amounts of isolated, unpaired centromeric heterochromatin was observed (Fig. 21e).

From pachytene onward the *ahp2* cytological phenotype became dramatic; pachytene chromosomes reveal long tracts of unpaired chromosomes (Fig. 21f). Late pachytene/early diplotene *ahp2* chromosome spreads, unlike wild type, exhibit many unpaired centromeres (Fig. 21j). In contrast to the organized appearance of wild type diakinesis (5 distinct bivalents with 5 paired centromeres); the *ahp2* mutant diakinesis is characterized by a disordered entanglement of chromosomes exhibiting up to 10 DAPI bright regions representing unpaired centromeres (Fig. 21k).

Another striking feature of the *ahp2* phenotype is the generally ‘sticky’ and tangled appearance of the chromosomes that is evident from diakinesis onward. Schommer et al. (2003) also reported this ‘sticky’ chromosome phenotype at the same stages. Chromosomes have unresolved connections as seen by the tangled mass of chromosomes observed during metaphase I (Fig. 21l) and the clumped and fragmented chromosomes seen at anaphase I (Fig. 21o). Unequal segregation of chromatin was observed in anaphase II meiocytes (Fig. 21p). It is noteworthy that chromatin bridges also are seen at anaphase II; possibly indicating problems in sister chromatid separation (Fig. 22).
**Fig. 21 Stabilized chromosome alignment is impaired in ahp2 meiocytes.**

DAPI-stained *Arabidopsis* chromosome spreads from male meiocytes are given in a-c, g-i and m, n for wild type, d-f, j-l and o, p for the ahp2 mutant. The prophase I substages are as follows: (a, d) mid-leptotene, (b, e) mid-zygotene, (c, f) mid-pachytene, (g, j) mid-diplotene, (h, k) diakinesis, (i, l) metaphase I, (m, o) anaphase I and (n, p) anaphase II. Short white arrows indicate unpaired centromeres, long white arrows indicate paired centromeres and the arrowhead indicates the NOR. *Scale Bar* 10 μm
Fig. 22 Chromatin bridging is observed in ahp2 anaphase II meiocytes. An example of chromatin bridging in an anaphase II ahp2 meiocyte is shown (white arrows). Chromosome fragmentation can also be seen. The chromosome spread was DAPI-stained. Scale Bar 10 μm
3.3.2.3 Meiocytes of the *ahp2* mutant have an extended meiosis due to a prolonged zygotene

Problematic meioses in both fission yeast and *Arabidopsis* mutants typically results in prolonged meioses (Shimada et al. 2002; Higgins et al. 2004; Jackson et al. 2006). We wished to determine whether the severe reduction in stabilized pairing observed in the *ahp2* mutant would be correlated with a disruption of normal meiotic progression. For this we employed BrdU pulse labeling of S phase male meiocytes (Armstrong et al. 2003) to determine the duration of meiosis, in wild type and the *ahp2* mutant. The data from these six experiments (3 for wild type and 3 for *ahp2*) can be found in Figure 23. The duration of meiosis (leptotene to tetrad stage) in the *ahp2* (*Ler* background) mutant was estimated to be 30 h as compared to the 23 h required by wild type (*Ler*) male meiocytes (Fig. 23a).

Because stabilized pairing is significantly reduced in the *ahp2* mutant we wanted to determine if the duration of zygotene (or other stages) was involved in the extended duration of *ahp2* meiosis. The duration of individual meiosis I substages was estimated from the relative number of meiocytes observed for a particular substage.

The meiosis I substages of 3073 wild type and 2147 *ahp2* mutant *Arabidopsis* meiocytes were readily identified by chromosome morphology/organization and substage association analysis as described in section 2.8.1. Meiosis I meiocytes were found in wild type buds that ranged in size from 0.39 mm to 0.50 mm, +/- 0.01 mm. Wild type buds in this size range were processed (n = 59). The distribution was as follows (with the number of buds given in parentheses): 0.39 mm (6), 0.40 mm (5), 0.41 mm (5), 0.42 mm (4), 0.43 mm (4), 0.44 mm (5), 0.45 mm (6), 0.46 mm (4), 0.47 mm (5), 0.48 mm (4), 0.49 mm (6), and 0.50 mm (5). Meiosis I meiocytes were found in *ahp2* buds that ranged in size from 0.35 mm to 0.45 mm, +/- 0.01 mm; buds in this size range were processed (n = 47). The distribution was as follows: 0.35 mm (4), 0.36 mm (5), 0.37 mm (4), 0.38 mm (5), 0.39 mm (3), 0.40 mm (4), 0.41 mm (5), 0.42 mm (3), 0.43 mm (5), 0.44 mm (4), and 0.45 mm (5). The percentage of meiotic cells at each stage of meiosis for wild type and *ahp2* meiocytes are shown in Figure 24a.

Using the calculated durations of meiosis, 23 hours for wild type and 30 hours for *ahp2*, we used the percentage of cells at each stage to estimate the durations of each stage as described in section 3.2.2.3; see Figure 24b. An F distribution analysis demonstrated that the only duration difference between wild type and the *ahp2* mutant that was statistically significant was the early-mid zygotene interval (p = 0.05, df = 5 and 4 respectively). From this analysis it is clear that the extent of delay in progression through zygotene can account for the extended meiosis seen in *ahp2* mutants. These findings strongly suggest that the lack of AHP2 protein function disrupts and delays the events of zygotene in *Arabidopsis*. 

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Fig. 23 The *ahp2* mutant has an extended meiosis. The data from the BrdU experiments is presented as follows: a) a schematic summarizing both the average times for BrdU label to appear in tetrads and the average duration of meiosis is given for wild type and *ahp2* meiocytes. L leptotene; TII telophase II (tetrads). The bracketed times apply to the *ahp2* meiocytes. The one hour time until BrdU appearance in G2 meiocytes and the 7 hr (G2 duration) measurements were previously determined by Armstrong et al. (2003). b) a table of the average times and the range of times measured from the mid-point of the BrdU pulse until peak BrdU signal was observed in the tetrads from the three wild type and three *ahp2* time course experiments performed. c) a table of the raw data from the three wild type BrdU experiments is shown. d) a table of the raw data from the three *ahp2* mutant BrdU experiments is given. NTF = no tetrads found, NA = not assayed
BrdU time course results for wild type Arabidopsis and ahp2 mutant meiocytes

<table>
<thead>
<tr>
<th>Meiocyte Type</th>
<th>Avg. time from the midpoint of the 2 hr BrdU pulse label to peak BrdU signal in the tetrads (hrs)</th>
<th>Range of times from the midpoint of the 2 hr BrdU pulse label to peak BrdU signal in the tetrads (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>31</td>
<td>30.5-32.5</td>
</tr>
<tr>
<td>ahp2</td>
<td>38</td>
<td>37.5-39.5</td>
</tr>
</tbody>
</table>

BrdU time course - raw data for wild type Arabidopsis meiocytes

<table>
<thead>
<tr>
<th>Time elapsed after the mid-point of the two hour pulse (h) for wild type</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative control #1 (no BrdU)</td>
<td>0/8 (0)</td>
<td>0/5 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>neg con #2 (30 min after pulse end)</td>
<td>0/3 (0)</td>
<td>0/5 (0)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>27-28</td>
<td>2/28 (7)</td>
<td>NTF</td>
<td>0/22 (0)</td>
</tr>
<tr>
<td>29-30</td>
<td>0/1 (0)</td>
<td>NTF</td>
<td>19/40 (47)</td>
</tr>
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<td>30-31</td>
<td>13/17 (76)</td>
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<tr>
<td>32-33</td>
<td>9/12 (75)</td>
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<td>17/37 (46)</td>
</tr>
<tr>
<td>34-35</td>
<td>0/9 (0)</td>
<td>NTF</td>
<td>0/27 (0)</td>
</tr>
</tbody>
</table>

BrdU time course – raw data for Arabidopsis ahp2 mutant meiocytes

<table>
<thead>
<tr>
<th>Time elapsed after the mid-point of the two hour pulse (h) for ahp2</th>
<th>Exp #1</th>
<th>Exp #2</th>
<th>Exp #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative control #1 (no BrdU)</td>
<td>0/7 (0)</td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>neg con #2 (30 min after pulse end)</td>
<td>NTF</td>
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<td>0/6 (0)</td>
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<tr>
<td>31-32</td>
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<tr>
<td>40-41</td>
<td>0/3 (0)</td>
<td>10/17 (59)</td>
<td>0/2 (0)</td>
</tr>
</tbody>
</table>
Fig. 24  Early to mid-zygotene is prolonged in the *ahp2* mutant. A comparative distribution analysis of meiosis I substages for *Arabidopsis* wild type (wt) and *ahp2* male meiocytes is shown: (a) A histogram showing the percentages of meiocytes observed for each stage in both wild type and the *ahp2* mutant, b) approximate durations of the meiotic stages in both wild type and the *ahp2* mutant [calculated from percentages shown in (a) and the estimated length of meiosis for wild type and *ahp2* respectively]. The red asterisk indicates that the only statistically significant difference in substage duration, between wild type and the *ahp2* mutant, was seen in early to mid-zygotene meiocytes.
3.3.2.4 AHP2 protein localization to chromosomes is punctate

The immuno-localization pattern of AHP2 protein in Arabidopsis was examined using a rabbit polyclonal antibody that was raised against a domain of the AHP2 protein (corresponding to the 3rd exon of the AHP2 gene). In western blots the AHP2 antibody recognized the exon 3 polypeptide antigen (3 ng) but did not recognize any proteins from wild type or ahp2 Arabidopsis bud protein extracts (data not shown here but given in Fig. 16).

AHP2 labeling was punctate throughout prophase I from leptotene to diakinesis (Fig. 25a-e). For wild type meiocytes the AHP2 signal was sparsely punctate in early leptotene (Fig. 25a) and became densely punctate in zygotene (Fig. 25b). AHP2 signal intensity and pattern was maintained in pachytene (Fig. 25c) and then became reduced during diplotene (Fig. 25d). AHP2 labeling of diakinesis spreads once again was sparsely punctate (Fig. 25e) and the AHP2 signal disappeared entirely by metaphase I (Fig. 25f). Negative controls included pre-immune serum done with wild type spreads and AHP2 antibody done with ahp2 spreads; neither negative control gave any signal; examples are shown in Figure 25g and h.

3.3.2.5 AHP2 and MND1 protein’s localization to chromosomes is temporally synchronous

The temporal and spatial co-localization of HOP2 and MND1 proteins was reported in yeast (Tsubouchi and Roeder 2002). In our work with Arabidopsis we are presenting the first cytological examination of the co-localization of AHP2 and MND1 proteins in a higher eukaryote. Co-immunostaining in wild type meiocytes revealed a similar temporal pattern for AHP2 and MND1 signal; both signals appeared during leptotene and persisted through zygotene and pachytene (Fig. 26a, b, c). Both AHP2 and MND1 signal diminished during diplotene and diakinesis and disappeared at metaphase I (data not shown).

The spatial distribution of AHP2 and MND1 signal overlapped but was not identical. AHP2 signal was entirely foci-based, whereas MND1 had diffuse signal all along the chromosome in addition to foci. The difference between AHP2 and MND1 localization was particularly apparent in leptotene chromosome spreads (Fig. 26a) but was also seen in other substages (Fig. 26b, c). MND1 antibody also stained the nucleolus (Fig. 26b) as was previously reported by Vignard et al. (2007) but the nucleolus was not stained with the AHP2 antibody.

There was no AHP2 immuno-staining of mnd1 chromosome spreads (Fig. 26d). This clearly demonstrates AHP2 protein’s dependence on the MND1 protein for proper chromosomal localization. Vignard et al. (2007) reported that chromosomes of the ahp2 mutant were not immuno-labeled by MND1 antibody. In contrast, while punctate foci-based staining was lacking, we observed diffuse MND1 staining of chromosomes in 69% of ahp2 nuclei examined (n = 39). In most of these ahp2
Fig. 25 AHP2 signal initially peaks in zygotene meiocytes but remains punctate from leptotene to diakinesis. Fluorescent immuno-localization of AHP2 protein (green) to wild type Arabidopsis chromosome spreads are illustrated: (a-f) were counterstained with DAPI (white): (a) mid-leptotene, (b) mid-zygotene, (c) mid-pachytene, (d) mid-diplotene (e) mid-diakinesis and (f) metaphase I. No AHP2 signal was seen associated with the ahp2 chromosome spreads used as a negative control; an example spread is shown in image (g). Additional AHP2 negative control images (h) Scale Bar 10 μm
**Fig. 26 AHP2 and MND1 protein localization to chromosomes is temporally synchronous but not spatially identical.** Fluorescent co-immuno-localization of the meiotic proteins AHP2 (green) and MND1 (red) to wild type *Arabidopsis* chromosome spreads are shown in (a-c), a *mnd1* spread (d) and an *ahp2* spread (e). All spreads were counterstained with DAPI (white). The prophase I substages are as follows: (a) mid-leptotene, (b, d, e) mid-zygotene, c) mid-pachytene. The white arrow in (b) indicates the MND1 stained nucleolus. Note that AHP2 antibody does not label the nucleolus. The insets in (c) provide further magnification of a portion of this pachytene spread. The inset in (e) merges DAPI and MND1 images and clearly shows MND1 staining of the *ahp2* nucleolus (marked with white arrow). Note the absence of both AHP2 and MND1 related signal in the *mnd1* spread (d) and the presence of MND1 signal but not AHP2 signal in the *ahp2* chromosome spread (e). *Scale Bar* 10 μm
Fig.27 MND1 protein can localize to ahp2 chromosomes. Additional images are given demonstrating MND1 labeling of Arabidopsis ahp2 chromosome spreads and the nucleolus, if present. MND1 antibody did not label mnd1 chromosomes. Note: All experimental parameters were kept constant for the ahp2 and mnd1 mutants; this included primary and secondary antibody dilutions and incubation conditions. Scale Bar 10 um
spreads the nucleolus was visible and also was stained with MND1 antibody (Fig. 26e). See Figure 27 for additional images of MND1 labeled ahp2 chromosome spreads.

### 3.3.2.6 Chromosome axes appear normal in the ahp2 mutant

We observed that chromosomes appeared normal in DAPI-stained ahp2 leptotene chromosome spreads. We wanted to examine this more closely. The deposition of ASY1 protein along the chromosomal axis prepares homologous chromosomes for subsequent synapsis (Armstrong et al. 2002). We used the ASY1 antibody (Armstrong et al. 2002) to determine ASY1 protein localization in ahp2 meiocytes and found it to be identical to wild type throughout prophase I, from loading in leptotene (Fig. 28a, e) to disassociation from chromosomes during diplotene (Fig. 28d, h). ASY1 antibody labeling was not observed in either wild type or ahp2 diakinesis meiocytes (data not shown). TEM analysis of ahp2 meiocyte sections revealed the formation of normal looking chromosomal axes in the ahp2 mutant (Fig. 28j). Negative control images for immunocytochemistry are shown in Figure 28k.

### 3.3.2.7 TEM analysis revealed substantial unstabilized, close alignment of ahp2 chromosomes

Our LM analysis of DAPI-stained ahp2 chromosome spreads revealed a drastic reduction in stabilized chromosome alignment – most chromosome regions were unpaired. We wished to investigate whether close alignment of chromosomes occurred in Arabidopsis ahp2 meiocytes but was not stable enough to survive the spreading protocol. Such alignment might be preserved in intact, fixed meiocytes that could be visualized once nuclei were examined using TEM. Serial sections were collected and nuclei reconstructed. Axial elements running in parallel that were separated by approximately 200-400 nm (with no visible central element) were considered to be closely aligned. The average amount of close alignment (as a percentage of total chromosome length) found in late zygotene/early pachytene ahp2 nuclei was 44%; the data is shown in Figure 29. Thus a significant amount of close alignment occurs in ahp2 mutants but most is not stabilized sufficiently to withstand the spreading forces that occur in typical LM analysis.

### 3.3.2.8 The short arms of ahp2 chromosomes 2 and 4 exhibit stabilized homologous pairing

In DAPI-stained preparations, stabilized alignment in the ahp2 mutant was seen to extend from the NORs along the short arms of chromosomes 2 and 4 to their respective centromeres; examples are provided in Figure 30. Using FISH analysis, we confirmed that this was indeed stabilized homologous pairing specific for these short arms. A chromosome specific multi-BAC probe targeting the sub-terminal NOR-associated region of the short arm of chromosome 2 resulted in a single probe signal in 92% of the labeled ahp2 pachytene
Fig.28  Chromosome axes appear normal in the \textit{ahp2} mutant.  
Fluorescent immuno-localization of ASY1 protein to \textit{Arabidopsis} chromosome spreads are provided for: (a-d) wild type, (e-h) \textit{ahp2} meiocytes. The images shown are the result of merging the image of ASY1 protein (green) localization signal and the DAPI counterstained (white) image of the same chromosome spread. The prophase I substages are as follows: (a) early leptotene (e) late leptotene, (b, f) zygotene, (c,g) pachytene and (d, h) diplotene. \textit{Scale Bar} 10 \textmu m   TEM images of wild type (i) and \textit{ahp2} (j) meiocyte sections provide representative examples of unpaired chromosomal axis (black arrows). Note: TEM images were provided by Nazia Pathan \textit{Scale Bar} 1 \textmu m   k) negative control image; ASY1 pre-immune serum did not label wild type spreads. \textit{Scale Bar} 10 \textmu m
Fig. 29  Significant amounts of chromosome close alignment are observed in *ahp2* nuclei. Representative transmission electron microscopy images of chromosome close alignment and SC presence are given in (a) and (b) respectively. *Scale Bar* 1 um A histogram showing the average amounts of close alignment and SC formation as a % of total chromosome length for five *Arabidopsis* meiotic nuclei is given in (c). The mean amount for three *ahp2* late zygotene/early pachytene nuclei and for two wild type late zygotene/early pachytene nuclei is also given.
Fig. 30 Homologous pairing occurs along the short arms of *ahp2* chromosomes 2 and 4. DAPI-stained, pachytene, *ahp2* chromosome spread images. Arrows indicate the region of pairing that extends from the centromere to the NOR. *Scale Bar* 10 µm
spreads examined (n = 26) (Fig. 31b). A specific multi-BAC probe targeting the sub-terminal NOR-associated region of the short arm of chromosome 4 also resulted in a single probe signal in 85% of the labeled ahp2 pachytene spreads examined (n = 48) (Fig. 31d). A second chromosome 4 multi-BAC probe, specific for a region of the short arm just distal to the NOR, also exhibited a single probe signal in 100% of the labeled ahp2 chromosome spreads examined (n = 25) (data not shown). In the 8% and 15% of nuclei that yielded two distinct probe signals, these signals were still within close proximity to one another. In sharp contrast an interstitial chromosome 1 specific FISH probe (Fig. 31f) localized as two distinct probe signals in all labeled ahp2 pachytene chromosome spreads examined (n = 20). A sub-terminal, chromosome 1 multi-BAC probe localized as a single signal in 53% of the labeled ahp2 pachytene spreads examined (n = 39; data not shown). The pairing seen with this sub-telomeric probe likely reflects the earlier leptotene pairing of ahp2 telomeres (the ahp2 bouquet appeared normal) that occurs in the mutant. Thus NOR-associated regions have substantially more stabilized pairing than other non-NOR associated regions, that were tested, even the sub-telomeric region of chromosome 1.

3.3.2.9 Two prominent linear tracts of SC are observed in most ahp2 nuclei
ZYP1 protein localizes to the central element of the synaptonemal complex (reviewed by Osman et al. 2006). We examined SC formation using antibodies to ZYP1 protein. We observed some ZYP1 foci associated with both our wild type and ahp2 mutant leptotene chromosome spreads (Fig. 32a, c).

Arabidopsis ahp2 mid-zygotene to mid-pachytene chromosome spreads had only short linear tracts of ZYP1 signal as compared to the much longer tracts seen in wild type. A representative example of the short linear ZYP1 tracts observed in an ahp2 pachytene chromosome spread is shown in Figure 32d.

In 87% of the ahp2 mid-zygotene to mid-pachytene spreads examined (n = 129) there were 2 predominant ZYP1 signal tracts and a variable number of ZYP1 foci, some of which were slightly extended. These two predominant ZYP1 tracts ranged from 2 µm to 9 µm in length. The average total length of ZYP1 tract per ahp2 meiotic nuclei was 8.9 µm. Fransz et al. (1998) estimated that the average total bivalent length in DAPI-stained wild type Arabidopsis pachytene chromosome spreads was approximately 330 µm. Therefore the average length of ZYP1 stained regions measured per ahp2 meiocyte nuclei represents 2.7% of wild type. Evidence of a small amount of normal looking SC also was obtained from the TEM analysis (Fig. 32f). The average amount of SC measured in serially reconstructed ahp2 meiocyte nuclei (n=3) was approximately 5.1% of the total chromosome length observed (Fig. 29). Negative control images for the ZYP1 immuno-cytochemistry as shown in Fig. 32g.
Fig. 31 Paired BAC signals are evidence of the homologous pairing of the short arms of chromosomes 2 and 4 in the ahp2 mutant. Fluorescent *in situ* hybridization analysis of wild type (a, c, e) and ahp2 (b, d, f) was done of *Arabidopsis* pachytene chromosomes using chromosome specific multi-BAC probes (red); chromosomes have been counterstained with DAPI (white). In (a, b) a single sub-terminal chromosome 2 multi-BAC signal is associated with both the wild type and the ahp2 pachytene spreads indicating homologous pairing in this sub-terminal region of the short arm of chromosome 2. In (c, d) a single sub-terminal chromosome 4 multi-BAC signal is associated with both wild type and ahp2 pachytene chromosome spreads indicating homologous pairing in this sub-terminal region of the short arm of chromosome 4. In the wild type pachytene spread (e) there is one interstitial chromosome 1 multi-BAC signal and in the ahp2 pachytene spread (f) there are two interstitial chromosome 1 multi-BAC signals indicative of non-pairing. Note: The chromosome specific multi-BAC probes used in these FISH experiments are shown pictorially to the right of the images in which they were used and the probes are also described in the methods section. *Scale Bar* 10 µm
Fig. 32 Two predominant linear tracts of SC are observed in most ahp2 meiocytes. Synaptonemal complex (SC) presence in Arabidopsis chromosome spreads was detected with ZYP1 immuno-staining for wild type (a, b) and ahp2 chromosome spreads (c, d). The merged images are ZYP1 protein (green) on chromosome spreads counterstained with DAPI (white). The prophase I substages are as follows: (a, c) leptotene and (b, d) pachytene. Arrows indicate linear tracts of ZYP1 staining in pachytene ahp2 chromosome spreads. Scale Bar is 10 μm for (a-d). Electron micrographs of wild type (e) and ahp2 meiocyte nuclei (f) are shown of ultra thin sections (the insets provide additional magnification of the synaptonemal complex). The very limited SC seen in the ahp2 null mutant has a normal appearance. Arrowheads indicate synaptonemal complex formation. TEM images were provided by Nazia Pathan. The scale bar is 1 μm. A representative negative control image is shown in (g); wild type spreads were not labeled with ZYP1 pre-immune serum. Scale Bar 10μm
3.3.2.10 Homologous synapsis of the short arms of ahp2 chromosomes 2 and 4
Given that we observed stabilized pairing for the short arms of 2 and 4 we thought it likely that the two longest ZYP1 tracts, seen in ahp2 chromosome spreads, represented these arms. We confirmed this with a combination of ZYP1 immuno-staining of ahp2 chromosome spreads followed by FISH. In each of the ZYP1 stained ahp2 chromosome spreads where chromosome 4 probe localized, a single probe signal was associated with one of the predominant linear ZYP1 tracts thus identifying the tract as belonging to the short arm of chromosome 4 (n = 14); an example is shown in Figure 33b (1-4).

FISH also was performed using a chromosome 2 short arm specific probe on previously ZYP1 stained chromosome spreads. In each of the ZYP1 stained ahp2 chromosome spreads where chromosome 2 probe localized, a single probe signal was associated with one of the predominant linear ZYP1 tracts, thus identifying the tract as belonging to the short arm of chromosome 2  (n = 6); an example is shown in Figure 33c (1-4).

3.3.2.11 The cohesin complex is not involved in the ahp2 ‘sticky’ chromosome phenotype
The plant meiotic cohesin complex plays a role in both meiosis I and meiosis II chromosome segregation events (Lam et al. 2005). Therefore it was possible that problematic cohesin release between sister chromatids could contribute to the sticky chromosome phenotype observed in the ahp2 mutant during both reductional and equational meiotic divisions.

We determined the distribution of SYN1 (REC8) and SMC3 cohesin proteins in the ahp2 mutant (Fig. 34) and found the immuno-staining pattern of antibodies to these proteins to be normal throughout meiosis I. The SYN1 protein began to disassociate from ahp2 chromosomes during diplotene (Fig. 34i) as seen in wild type (Fig. 34d) which is indicative of its proper removal from chromosome arms of sister chromatids prior to anaphase I. SMC3 labeling of both wild type and ahp2 diakinesis (Fig.34n, s) chromosomes is observed as localized foci. These persistent SMC3 foci may represent the cohesin complex that remains in centromeric regions to help keep sister chromatids tethered until after anaphase I. These foci appear more distinct and of uniform size in wild type meiocytes as compared to ahp2 meiocytes which most likely reflects the abnormal morphology of ahp2 diakinesis chromosomes. After anaphase I completion neither SYN1 nor SMC3 labeling was apparent on either wild type or ahp2 mutant chromosome spreads (Fig.34e, o and j, t respectively). Thus there is no evidence that the sticky phenotype is due to abnormal loading or unloading of cohesins.
Fig. 33 The short arms of NOR-bearing chromosomes 2 and 4 undergo homologous synapsis in *ahp2* nuclei. Fluorescent immuno-localization is shown of ZYP1 protein on *Arabidopsis* wild type and *ahp2* chromosome spreads and subsequent identification of chromosome 4 or 2 short arm with FISH using sub-terminal multi-BAC probes specific to these regions: a (1–4) wild type and b, c (1–4) *ahp2* meiocytes. In (a, b, c): (1) DAPI (white), (2) ZYP1- FITC signal (green), (3) either chromosome 4 or chromosome 2 specific multi-BAC probe- rhodamine signal (red) and (4) DAPI/ZYP1/chromosome probe merged image. (d) Schematics of both of the chromosome 2 and chromosome 4 multi-BAC probes that were used in (a, b, c) is shown. Arrows indicate linear tracts of ZYP1 staining. *Scale Bar* 10 µm
**Fig. 34 The appearance and disappearance of the cohesins SYN1 and SMC3 are normal in ahp2 meiocytes.** Fluorescent immuno-localization is shown for the meiotic cohesin proteins SYN1 (a-j) and SMC3 (k-t) on *Arabidopsis* wild type and *ahp2* chromosome spreads counterstained with DAPI (white). The SYN1 antibody (green) was localized to both wild type (a-e) and *ahp2* nuclei (f-j) of the following substages: (a, f) leptotene, (b, g) mid-zygotene, (c, h) mid-pachytene, (d, i) diplotene and (e, j) post diakinesis. The micrographs shown are merged DAPI and SYN1 images. The SMC3 antibody (green) was localized to both wild type (k-o) and *ahp2* nuclei (p-t) of the following substages: (k, p) leptotene, (l, q) mid-zygotene, (m, r) mid-pachytene, (n, s) diakinesis and (o, t) post diakinesis. The micrographs shown are merged DAPI and SMC3 images. Negative control images for SYN1 and SMC3 are shown in (u, v respectively); there is no labeling of wild type spreads with either SYN1 or SMC3 pre-immune sera.

*Scale Bar 10 um*
Results (Part IV)  Bioinformatic comparison of the AHP2 and MND1 genes/proteins

The work in this section was performed by Patti Stronghill
3.4 Results (Part 4)-Bioinformatic comparison of the *Arabidopsis* AHP2 and MND1 genes/proteins

3.4.1 Overview
Because of differences found in the immuno-labeling of *Arabidopsis* AHP2 and MND1 proteins a bioinformatic analysis of these two proteins was undertaken. This bioinformatic analysis included a comparison of global gene expression patterns and relative amounts of transcript, algorithm-based predictions of protein localization, DNA binding motifs and potential kinase phosphorylation sites.

3.4.2 Results

3.4.2.1 AHP2 and MND1 gene expression comparison in *Arabidopsis*
Unlike their orthologs in yeast, the AHP2 and MND1 genes are expressed in both vegetative and reproductive tissue, in *Arabidopsis*, as determined by microarray data (Schmid et al 2005). Using this data, Winter et al. (2007) created a fluorescent pictogram to illustrate the expression patterns for the AHP2 and MND1 genes (Fig. 35). AHP2 and MND1 gene expression occurs primarily in apical vegetative and reproductive meristematic tissue and young buds that develop from the latter. The *Arabidopsis* microarray data also indicates that the relative level of MND1 expression is at least twice that of the AHP2 gene (in apex and flower tissue) but expression levels for both are typically a hundred fold less than seen for histone H1 transcript (Fig. 36). Finally the AHP2 gene has the E2Fα-DPα transcription factor target site but this site is not seen in the MND1 gene; there could be a different transcription factor that allows for pre-meiotic transcription of the MND1 gene and different responses.

3.4.2.2 AHP2 and MND1 predicted protein localization
There were some differences in the predictions for cellular/nuclear localization of the AHP2 and MND1 proteins (Fig. 37). The AHP2 protein was predicted to be found almost exclusively in the nucleus whereas MND1 protein might be found in both the nucleus and the mitochondria using protein localization algorithms (www.bar.utoronto.ca). Fluorescent microscopy of meiotic cells confirmed the presence of AHP2 and MND1 proteins in the nucleus; MND1 localized to the nucleolus and chromosomes whereas AHP2 localized to chromosomes only (Fig. 26). No immuno-localization studies were done for the mitochondria.

3.4.2.3 The DNA binding properties of the AHP2 protein
Bioinformatic analysis of the AHP2 protein’s secondary structure confirmed the presence of the helix-turn-helix (HtH) motif (residues 28-80) in the N-terminal region of the protein. This putative DNA binding motif is known to bind the major groove of the DNA double helix (Luscombe et al. 2000).
Fig. 35 Gene expression patterns for *AHP2* and *MND1* are very similar. The relative gene expression pattern for the *Arabidopsis* *AHP2* and *MND1* genes, shown here, is based on microarray data (Schmid et al. 2005); www.bar.utoronto.ca. The color red indicates a relatively high level of expression, orange indicates medium expression and yellow indicates relatively low expression levels. Note: *AHP2* and *MND1* genes are expressed in both vegetative and reproductive tissue that includes: 1) shoot apex inflorescence, 2) shoot apex transition meristem, 3) shoot apex vegetative meristem and 4) stage 9 flower buds.
Fig. 36 *MND1* expression is approximately twice that observed for *AHP2* in all tissues examined. Graphical representation is given of the relative gene expression of *Histone H1* (blue), *MND1* (green) and *AHP2* (red) genes in various tissue types in *Arabidopsis* from the same microarray data as shown in Figure 35 (Schmid et al. 2005); [http://www.weigelworld.org/resources/microarray/AtGenExpress](http://www.weigelworld.org/resources/microarray/AtGenExpress). Numbers mark samples containing vegetative, pre-meiotic and meiotic tissue: 1) shoot apex vegetative meristem, 2) shoot apex intermediate meristem, 3) shoot apex reproductive meristem, 4) shoot apex reproductive meristem, 5) flower – stage 9 (includes stamens whose anthers contain meiocytes).
**Fig. 37** AHP2 and MND1 proteins are predicted to localize to the nucleus but MND1 may also localize to mitochondria. Cellular localization of the AHP2 and MND1 proteins based on predictions made by Wolfsport and Mitpro algorithms [www.bar.utoronto.ca](http://www.bar.utoronto.ca) High confidence predictions are colored red, medium are colored orange and low confidence predictions are indicated by yellow coloring. Confidence for AHP2 and MND1 localization is based on the number of different algorithms that place them in a particular cell compartment.
A GenBank search and secondary structure analysis revealed that this motif is conserved for all AHP2 (HOP2) protein orthologs in several plant and animal species and *S. pombe* (fission yeast) (Fig. 38a).

We detected a second DNA binding motif in the AHP2 protein. This positively charged motif ‘WRKRKR(M/I/L)’ is located in the C-terminal portion of the AHP2 protein (residues 168-174). A GenBank search revealed that the WRKRKR(M/I/L) motif was highly conserved in all of the AHP2 (HOP2) protein orthologs examined (except in budding yeast) despite, in most instances, a low degree of overall AHP2 (HOP2) protein sequence homology (Fig. 38a). There appears to be a truncated version of the motif in the C-terminus of the *S. pombe* AHP2 ortholog (MEU13) but the ‘RKK’ sequence found may be this protein’s nuclear localization signal. The other (HOP2) AHP2 orthologs have both the 7-residue motif and a nuclear localization motif located at the C-terminus of the protein.

The primary amino acid sequence of budding yeast HOP2 ortholog is shown separately (Fig. 38b) as it failed to align with the other proteins using the alignment tool. Presumably its sequence divergence exceeded that allowed by the default parameters of the clustalW alignment algorithm. It can be seen from Figure 38b that the 7-residue motif is entirely absent from the budding yeast HOP2 protein.

### 3.4.2.4 The DNA binding properties of the MND1 protein

The MND1 protein does not have any obvious DNA minor groove binding domain. The MND1 protein has a different DNA major groove binding motif from the AHP2 protein [Dr. L. Aravind (personal communication)]. This MND1 DNA binding motif is located in the C-terminal portion of the protein (residues 149-164) and is comprised of a 16 residue helical domain containing three aromatic residues, one of which is phenylalanine [phenylalanine is thought to facilitate DNA binding via intercalation with nucleotide bases (Kim et al. 1993)]. A GenBank search and a secondary structure analysis demonstrated that this DNA binding motif is conserved in both plant and animal MND1 proteins (Fig. 39). An altered version of this motif may be present in both fission and budding yeast. The presence of a DNA binding motif in the MND1 protein gives it the ability to bind DNA in the absence of AHP2 protein.

### 3.4.2.5 Potential phosphorylation control of *Arabidopsis* AHP2 and MND1 protein activity

The NetphosK algorithm predicts specific phosphorylation target sites in proteins. The potential kinase phosphorylation target sites in AHP2 and MND1, are shown in Figure 40. The algorithm identified 16 possible kinase phosphorylation sites in AHP2 and 27 sites in MND1. In the MND1 protein 6 of the 26 possible phosphorylation sites were potential targets for cell cycle kinases whereas only one potential cell cycle kinase (ATM) target site was found in AHP2. Ataxia Telangiectasia Mutated and Cell Division Cycle (both cell cycle kinases) each have three potential phosphorylation target sites in the MND1 protein.
Fig. 38  A highly conserved ‘WRKRRK(M/I/L)’ motif was found in the C-terminal region of all animal and plant AHP2 orthologs examined.  a) The clustal W alignment of AHP2 (HOP2) orthologous proteins is presented. The clustal W alignment was created using the tool at: http://www.ebi.ac.uk/Tools/clustalw. The N-terminal region contains a helix turn helix DNA binding motif (h = helix and t= turn); the conserved residues within this motif have been highlighted in orange; the HTH motif was identified using the protein secondary structure algorithm at: http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html. A highly conserved 7-residue sequence motif (that has the potential to target this protein to the minor groove of AT-rich DNA) in the C-terminal region of the AHP2 orthologs is highlighted in red; fission yeast demonstrated a possible truncated version of this motif.  b) Budding yeast HOP2 is shown separately as it failed to align with the other orthologs using the alignment tool; note the total absence of the 7-residue positive motif. The green highlighted residues are probable nuclear localization signals that likely target this protein to the nucleus.
human =MSKGRBABAAAAGAAGLRLYLQEQNRPYSQDVFGNLOQREHLGKAVVVTTELEQLAQCCQ
rat =MSKSRABAAGAGPILRLYLQENRPSQDFVGNLOQREHGLGKAVVVKALDQLAQCCQ
mouse =MSKRSRAAGAGSILRLYLQENRPSQDFVGNLOQREHGLGKAVVVKALDQLAQCCQ
zebrafish =--=MSKMEPASSAASIIILYLDNQRPSTQDFVSNLORDHLGKTVAKMELQACQ
frog =----=MKEKKEEASSAASIIILYLDNQRPSTQDFVSNLORDHLGKTVAKMELQACQ
flatworm =--=MTKGSKDAAIIVKVELYQGNYPSYIDINIFNNLHKEYGKTNQAVDLVVEECK
arabidopsis =-----=MAPKDNTEAALIVNENQKNPKLQTQNAADDLQFNKLK-TAVQCDALDLSAQG
grape =----=MAPKDSVEGLIVNENQPKNPLQNQVADSLQFNKLK-KSSQVCDALDLSGR
poplar =-----=MAPKDSSEAALIVNENQKNPLQNQVADSLQFNKLK-KRAGKCDALDLSAQG
rice =FAGGGGGGAARLCLNLADKMRKLQFQVADSLQFNKLK-TAVQCDALDLSAQG
maize =------------------------=QQPRLNPLQVWADLALQICGK-AQAVQVMDTLAANG
fission yeast =KEVKAPFICKEEAKLELYQKRTNPSRTYDVSAANK--=NVPSKQVQAKALELQDGTQ

a

human =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
rat =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
mouse =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
zebrafish =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
frog =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
flatworm =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
arabidopsis =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
grape =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
poplar =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
rice =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS
maize =------------------------=QKPRLNPLQVWADLALQICGK-AQAVQVMDTLAANG
fission yeast =IEKVKGYKQKCYFADQDOFDVMSDLQVLQDQIVALTAKQSLQSSCRDEMEAKELS

human =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
rat =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
mouse =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
zebrafish =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
frog =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
flatworm =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
arabidopsis =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
grape =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
poplar =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
rice =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
maize =------------------------=QKPRLNPLQVWADLALQICGK-AQAVQVMDTLAANG
fission yeast =ALKTVFQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR

human =ALTFEMQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
rat =ALTFEMQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
mouse =ALTFEMQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
zebrafish =ALTFEMQKEQKEKCECQERLKNK=AATNHSVPEKEQKVRKLEKKYKECKWRKR
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b

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**Fig. 39** MND1 animal and plant orthologs have a conserved C-terminal region motif that can potentially bind the major groove of DNA. The clustal W alignment of MND1 orthologous proteins was created using the alignment tool [http://www.ebi.ac.uk/Tools/clustalw](http://www.ebi.ac.uk/Tools/clustalw). The orange high-lighted residues represent a conserved motif consisting of an alpha helical region (h) containing one or more of the following aromatic residues: F=phenylalanine, W= tryptophan, Y=tyrosine (colored dark blue). The helical region in the C-terminus of MND1 orthologs was identified using the protein secondary structure algorithm at: [http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html](http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html). This is the region of the MND1 protein that most likely interacts with the major groove of DNA (Dr. Aravind, personal communication). b) fission yeast and c) budding yeast may have an altered versions of this motif. The green highlighted residues, in the MND1 orthologs, are nuclear localization signals that likely target the MND1 protein to the nucleus.
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### b)

| ATM  | Ataxia Telangiectasia Mutated kinase |
| CDC  | Cell Division Cycle kinase          |
| CK   | Casein Kinase                       |
| PKA  | Protein Kinase A                    |
| PKC  | Protein Kinase C                    |
| PKG  | Protein Kinase G                    |
| DNAPK| DNA-dependent Protein Kinase         |
| SRC  | Sarcoma Kinase                      |
| EGFR | Epidermal Growth Factor Receptor kinase |
| INSR | INSLulin Receptor kinase            |

**Fig. 40** MND1 has several potential target sites for ATM and CDC kinases that are involved in cell cycle control. a) The tables show the potential kinase phosphorylation sites, predicted by the algorithm NetphosK at [http://www.cbs.dtu.dk/services/NetphosK](http://www.cbs.dtu.dk/services/NetphosK), for the AHP2 and MND1 proteins in *Arabidopsis*. Phosphorylation target sites of cell cycle-associated kinases are highlighted in red. Higher scores correlate with increased confidence in the phosphorylation site prediction, b) The full names of the kinases, whose acronyms are seen in part (a) of this figure, are provided.
4.0 Discussion

4.1 The importance of detailed cytological analysis, accurate meiocyte staging and estimation of individual prophase I substage durations in the evaluation of meiotic mutant phenotypes

A detailed ‘pictorial’ documentation of chromosome movement and the changes in chromosome morphology, which occur during wild type meiosis I are crucial to the comprehensive analysis of meiotic mutants. A wild type pictorial can be used to identify subtle deviations in chromosome movement and morphology within the nuclei of meiotic mutants. For example the presence or absence of the bouquet in meiotic mutants is relatively easy to observe but the detection of subtle changes in bouquet formation or structure require detailed cytological knowledge of wild type bouquets and techniques that preserve it when present. Detailed images of the bouquet and other meiotic chromosomal features (eg the synizetic knot in zygotene or the diffuse stage in diplotene) will be useful in the identification of meiotic mutants that exhibit subtle prophase I abnormalities.

The largest challenge to accurate meiotic staging is in the differentiation of zygotene and diplotene. This is because in both of these prophase I substages a combination of paired and unpaired chromosomes are observed. The accurate differentiation of zygotene from diplotene is critical to the correct determination of function for many meiotic proteins. Proteins localized to zygotene chromosome spreads could potentially be involved in pairing and synapsis of homologous chromosomes unlike proteins that localize to post-synaptic diplotene spreads.

Meiotic substage durations are often affected in meiotic mutants. Knowledge of the individual durations of each of the prophase I substages in wild type Arabidopsis allows for a more precise determination of substage duration changes in meiotic mutants; providing another means to determine when specific meiotic proteins function. The caveat is that a change in a meiotic substage duration may reflect a problem in that substage or an earlier one; further experiments are required to pinpoint the substage in which a specific meiotic protein functions. Various subsets of proteins become involved with chromosomes as they progress through meiosis. Care must be taken not to draw conclusions from one line of evidence. Knowing when a meiotic protein functions is helpful in determining its function.

In recent years cytological analysis has relied largely on chromosome spreads. Such analysis allows the investigator to assess whether or not chromosome alignment has been achieved and if used in conjunction with ZYP1 immuno-localization or electron microscopy can determine if SC formation occurs. However such techniques do not allow a determination of which earlier steps may have failed – bouquet formation, rough alignment, close alignment, or close alignment stabilization. The cytological techniques developed in part of this thesis: substage identification, improved light microscopy (LM)
spreading techniques that retain telomere and centromere associations from leptotene to diplotene, duration of prophase I substages in normal and aberrant cells, as well as techniques to readily gather accurately staged meiotic cells for subsequent transmission electron microscopy (TEM) analysis provide new perspectives on the events of the highly important meiotic substage of prophase I relative to the processes that lead up to reciprocal genetic exchange. Our comparison of LM analysis of ahp2 chromosome spreads with TEM analysis of ahp2 nuclei sections detected a lack of stabilized pairing in mutant meiocytes. These techniques were used to further our understanding of the role of the AHP2 protein as studied in wild type and mutant Arabidopsis microsporocytes.

4.2 The Arabidopsis AHP2/MND1 protein complex has a role on the chromosomal axis but MND1 alone is also found on non-axial portions of the chromosomes

The DNA binding motifs found in AHP2 and MND1 proteins are quite different and this may explain the differences observed in their nuclear localization pattern. The most striking difference is the conserved positively charged 7-residue DNA binding motif in the C-terminal region of the AHP2 protein that is not present in the MND1 protein. This type of positively charged motif is well suited to interact with the DNA within the particularly narrow minor groove of AT-rich DNA (M/SARs) found intermittently along the chromosomal axis (Roque et al. 2004; Ottaviani et al. 2008). This may explain why AHP2 protein’s localization pattern is primarily foci-based. AHP2 did not localize to chromosome spreads of mnd1 mutants suggesting that it only binds DNA as a complex with the MND1 protein. Perhaps the binding of the AHP2 protein to MND1 protein ‘in vivo’ induces a conformational change in the AHP2 protein that exposes its DNA binding motifs and allows it to correctly distribute along chromosomes. MND1-dependent AHP2 localization to chromosomes is important as AHP2 protein has intrinsic recombinogenic ability that is inhibited by its association with the MND1 protein until the functional interaction with DMC1 occurs (Petukhova et al. 2005). AHP2 appears to function, in meiosis, only as a complex with MND1; this complex has been reported to have dual roles both in stabilizing DMC1-ssDNA and RAD51-ssDNA nucleoprotein filaments and facilitating duplex DNA capture in the presence of DMC1/RAD51 protein complex (Pezza et al. 2007; Chi et al. 2007).

AHP2 and MND1 signal overlaps significantly but the MND1 protein localizes at other locations, in a diffuse pattern away from the chromosome axis. This binding may depend on MND1 protein’s C-terminal DNA major groove binding motif. Vignard et al. (2007) reported that MND1 appeared to bind both chromosomal axis and loop regions. The diffuse nature of MND1 staining in the ahp2 mutant may reflect its localization to loop regions only; whereas in wild type meiocytes, as part of the AHP2/MND1 complex, MND1 protein can also bind to the chromosomal axis. Our AHP2-MND1 coimmuno-
localization results suggest that a proportion of MND1 protein does not bind AHP2 protein and non-AHP2 complexed MND1 protein appears to localize to chromosomes in a different manner than AHP2/MND1 complex.

MND1 protein (but not AHP2 protein) also localizes to the nucleolus. The nucleolar localization may reflect a requirement for MND1 protein accumulation or a need for this protein to be sequestered to regulate its function apart from the AHP2/MND1 complex. Although primarily known for ribosome biogenesis the nucleolus has been associated with numerous other regulatory functions (Boisvert et al. 2007).

Our finding of MND1 presence on ahp2 chromosomes conflicts with earlier reports of a lack of MND1 immuno-labeling of ahp2 spreads by Vignard et al. (2007). A possible explanation may involve non-complexed MND1 protein’s low DNA binding affinity and differences in fixation protocols. Chi et al. (2007) found the ‘in vitro’ DNA binding affinity of non-complexed MND1 to be considerably less than found for the AHP2/MND1 complex. Consequently non-complexed MND1 protein may be more prone to disassociation from chromosomes during the spreading process. Our ‘two stage’ PFA fixation protocol (see methods) may have prevented the loss of MND1 protein from ahp2 spreads.

The Atmnd1 dmc1 double null mutant examined by Vignard et al. (2007) provides further evidence of an additional role for the MND1 protein that does not utilize the DMC1-mediated DSB pathway. The double mutant exhibited an intermediate phenotype with respect to chromosome segregation during metaphase/anaphase I and II (as compared to dmc1 and mnd1 single mutants). The mnd1 dmc1 double null mutant had a dmc1-like anaphase I and a mnd1-like anaphase II. An intermediate phenotype normally indicates that more than one pathway is involved.

4.3 A prolonged ahp2 zygogene may relate to problems in the stabilization of chromosome close alignment

The formation of the chromosomal axis appeared normal in both DAPI stained spread preparations and in meiocyte ultra-thin sections examined with TEM. The deposition of ASY1 along chromosomes also appeared normal. But examination of ahp2 mutant dispersed zygogene/pachytene chromosome spread preparations revealed very limited amounts of paired chromosomes. In contrast TEM analysis of serially sectioned ahp2 late zygogene/early pachytene meiocytes revealed significant amounts of closely associated/paired (∼200 nm separation) chromosomal axis (lateral elements). The apparent chromosome alignment/pairing disparity likely occurs because the ahp2 mutant can accomplish chromosome alignment/pairing but fails to stabilize it; unstabilized chromosome alignments do not survive the rigors of the LM spreading protocol.
In most wild type meiocytes extensive homologous alignment and pairing stabilization occurs during early to mid-zygotene (Zickler and Kleckner 1999). In our work early/mid-zygotene was determined to be substantially prolonged in the ahp2 mutant. This is most likely a consequence of problems in chromosome pairing stabilization based on the amount of unstabilized chromosome pairing seen in ahp2 meiocyte spread preparations. Slowed meiotic progression and problems in chromosome pairing stabilization have also been reported in the S. pombe meu13 (ahp2-cognate) null mutant (Nabeshima et al. 2001).

In a broad range of organisms closely aligned chromosomes appear to be connected at discrete sites known as axial associations. Because these axial associations immediately precede SC formation and are obscured by the SC once it forms they can only be observed in TEM preparations when SC fails to occur (Rockmill 1995; Zickler 2006; Tsubouchi 2008). In the yeast zip1 mutant (that fails to form SC), axial associations are clearly visible in TEM spread preparations, but absent in zip1 hop2 double mutants. This evidence suggests that HOP2 (AHP2) protein function is required for axial association formation and pairing stabilization (Leu et al. 1998).

The evidence from our study suggests that the AHP2 (HOP2) protein has a similar function in Arabidopsis. This conclusion is based on the fact that most aligned homologous chromosome regions are not stabilized (not associated) in our ahp2 spread chromosome preparations, but are seen to be closely aligned in our TEM sections (that have not been subjected to spreading forces).

4.4 Arabidopsis NORs are observed to operate as “Cis acting” pairing sites in the absence of AHP2 function

Overall, the Arabidopsis ahp2 mutant has greatly reduced levels of stabilized alignment; thus, in light-microscope examined spreads, there is a widespread lack of chromosome pairing. Nonetheless, it is remarkable that the short arms of NOR-bearing chromosomes 2 and 4 accomplish stable homologous pairing in prophase I and undergo synapsis. The amount of SC formation estimated from ZYP1 staining was comparable to the amount of SC observed in the TEM analysis of ahp2 meiocyte sections. Vignard et al. (2007) also reported the presence of short linear stretches of ZYP1 staining in mnd1, rad51, xrcc3, and dmc1 mutant meiocytes. Through FISH, with chromosome-specific probes, we have confirmed that the sites of significant ZYP1 tracts are the short arms of ahp2 chromosomes 2 and 4. These NOR-associated regions do not require the AHP2-related functions of the DSB repair pathway to stabilize alignment and promote SC formation. The NOR ability observed in Arabidopsis is similar to the situation in Drosophila melanogaster where XY pairing is promoted by both rDNA and the
intergenic spacer DNA situated between the rRNA genes (McKee and Karpen 1990; McKee et al. 1992). In *Drosophila*, the NOR-related capacity for pairing may involve DNA sequence homology and/or rDNA transcriptional activity (McKee 1996). More recently, Sherizen et al. (2005) have suggested that chromosome continuity between specialized sites on each *Drosophila* chromosome is responsible for pairing and SC formation in female *Drosophila*. In *C. elegans*, the DSB repair pathway is not required for stabilized pairing but instead requires the presence of functional pairing centers, located at one end of each chromosome (Dernburg et al. 1998; MacQueen et al. 2005). *C. elegans* pairing sites appear to function in a different manner from those observed in *Drosophila*. In *C. elegans*, specific proteins are involved that uniquely recognize and interact with a particular pairing site. The HIM8 protein has been shown to interact with the pairing site on the X chromosome (Phillips et al. 2005). It has since been shown that there is a family of four related C2H2 zinc-finger proteins (ZIM-1, ZIM-2, ZIM-3, ZIM-4) that play a central role in pairing site-mediated homologous pairing (Phillips and Dernburg 2006). The NOR ability observed in *Arabidopsis* coupled with the DSB-independent synopsis seen in *D. melanogaster* and *C. elegans* points to the fact that stabilized pairing may be the obligate precursor of homologous SC formation but this stabilized pairing need not involve DSBs. Further investigation of the *Arabidopsis* NORs as pairing sites will reveal whether NOR-associated proteins, homology between repetitive rDNA sequences, or transcriptional activity (or a combination thereof) underlies NOR pairing ability in *Arabidopsis*.

### 4.5 Meiosis and plant reproductive development

Meiotic surveillance likely is involved in the coordination of plant reproductive development with meiotic progression. In our work with *Arabidopsis*, the short stamen phenotype was observed in both *ahp2* and *mnd1* mutant plants and was determined to be the result of problematic cell elongation. Several other meiotic mutants in *Arabidopsis* also exhibit the short stamen phenotype; these include [*rad51* (a recombinase) (Li et al. 2004); *xrec3* (a *rad51* paralog) (Bleuyard and White 2004); *cdc45* (a DNA replication initiator) (Stevens et al. 2004); *aesp* (a separase) (Liu and Makaroff 2006); and *ask1* (an E3 ubiquitin ligase) (Zhao et al. 2003)]. One chromosome phenotype common to all of these mutants is tangled and/or bridged chromosomes at anaphase I. Detection of illegitimate connections between chromosomes at anaphase I may trigger cell-to-cell signals that result in reduced epidermal cell elongation of the stamen. In contrast, normal stamen length was observed in nearly all of the *Arabidopsis* mutants reported to have univalents present in both anaphases I and II meiocytes [*e.g., *dmc1* (Couteau et al. 1999); *sds* (Azumi et al. 2002); *mer3* (Chen et al. 2005); *ptd* (Wijeratne et al. 2006); *prd1* (Grelon personal communication 2009); *rpa1* (Osman personal communication 2009);
Thus, failure to form bivalents alone does not produce short stamens; unresolved DSB intermediates are required.

### 4.6 Anaphases I and II: problematic segregation events in the Arabidopsis ahp2 mutant

The repair of DSBs can potentially involve sister chromatids, complementary DNA of non-homologous chromosomes, or homologous chromosomes as DNA templates during meiosis. It is important that a homolog repair bias be present during meiosis to ensure an adequate number of crossovers for the proper segregation of chromosomes and prevent non-homologous exchanges. Homolog bias during meiotic recombination, in most organisms, likely arises from mechanisms that suppress non-homologous and intersister events e.g. via proper axis formation and mechanisms that promote homologous recombination, e.g. DMC1/RAD51 complex-mediated DSB repair pathway (Schwacha and Kleckner 1997). The correct localization of the AHP2/MND1 complex along chromosomes could influence homolog bias. The AHP2/MND1 complex appears on chromosomes in early leptotene prior to the continuous distribution of ASY1 protein along chromosomes (Vignard et al. 2007). The early localization of the AHP2/MND1 complex could potentially effect proper axis formation as it pertains to the homolog bias, through its participation in the DMC1/RAD51 complex-mediated DSB repair pathway.

The extreme chromosome “stickiness” phenotype, observed at metaphase I, may be the result of numerous unresolved DSB intermediates between homologous chromosomes. At anaphase I, these unresolved DSB intermediates could lead to the observed chromosome fragments. However, such connections would be expected to be broken prior to meiosis II. The involvement of the cohesins in the “sticky” anaphase II chromosome phenotype is unlikely as their appearance/disappearance and localization, on ahp2 chromosomes, was normal. Therefore the observation of residual stickiness and bridges in anaphase II suggests that either there are unresolved DSBs occurring between sister chromatids or illegitimate exchanges have occurred between non-homologous chromosome regions in a manner that has produces dicentric chromosomes.

### 4.7 AHP2 protein ortholog comparisons

From previous studies it has been noted that there appears to be only modest primary amino acid sequence conservation between orthologous proteins whose function is of a non-enzymatic nature. An example would be the synaptonemal complex protein ZYP1 in Arabidopsis whose orthologs in yeast (ZIP1) and mouse (SCP1) vary considerably in primary amino acid sequence. In contrast orthologous proteins that function in an enzymatic manner exhibit much more primary sequence conservation as
very small changes in conformation could have profound effects on function; examples include DMC1 and RAD51 proteins. The significant divergence in primary amino acid sequence observed for AHP2 (HOP2) protein orthologs, except for the 7-residue motif in the C-terminal region, may be indicative of a structural or non-enzymatic role for this protein.

Both mouse and Arabidopsis HOP2 (AHP2) protein have a WRKRRK motif. Deletion analysis of the mouse HOP2 (AHP2) protein demonstrated the importance of this conserved 7-residue motif to both DNA binding and in vitro DNA strand invasion. In the mouse HOP2 protein analysis the (residues 179-216) deletion did not affect DNA binding or protein function whereas the (residues 163-216) deletion, which included the entire 7-residue motif (residues 173-179) severely affected DNA binding and the strand invasion capabilities of the protein (Enomoto et al. 2004). Bioinformatic analysis clearly shows that the Arabidopsis AHP2 (HOP2) gene/protein, aside from other plants, is most similar to mouse for the parameters that were examined. This finding is congruent with the observation that both Arabidopsis ahp2 and mouse hop2 mutants have very little SC formation (in contrast to budding yeast).

4.8 Comparison of AHP2 and MND1 gene expression and regulation of their protein function

Microarray data and quantitative RT-PCR assays comparing the expression of AHP2 and MND1 genes from mouse spo11 null mutants found that HOP2 (AHP2) gene expression was down regulated 2.5 fold whereas MND1 expression was up regulated 5 fold as compared to wild type (Smirnova et al. 2006). These results demonstrate that even though these two gene products work together as part of the same complex during prophase I their expression is controlled differently in mice. In Arabidopsis differential regulation of AHP2 and MND1 genes is suggested by the presence of an E2Fα-DPα transcription target site in the AHP2 gene’s promoter but not in MND1.

Potential phosphorylation sites in Arabidopsis AHP2 and MND1 proteins also reveal the capacity for differential regulation. The ATM kinase protein is known to bind at the sites of unrepaired DNA double strand breaks and to be involved in regulation of the cell cycle during Arabidopsis meiosis (Garcia et al. 2003; Kurz et al. 2004). The CDC2 kinase protein is a cell cycle regulatory subunit that stably associates with mitotic cyclins and functions during both G1/S and G2/M transitions (Iwakawa et al. 2006). Only one of the potential AHP2 phosphorylation sites is a target of kinases known to be involved in the regulation of cell cycle. If AHP2/MND1 complex and/or uncomplexed MND1 function is coordinated with the progression of meiosis then this, most likely, is executed via phosphorylation of serine-8, 28, and/or threonine 109 of the MND1 protein by ATM. Whereas the phosphorylation of serine 57, 68, 75, 201 of the MND1 protein by CDC2 kinase may coordinate MND1 protein function with mitotic progression in the somatic/vegetative cells in which the MND1 protein is expressed.
The MND1/AHP2 protein complex functions, in wild type *Arabidopsis*, in the meiotic DMC1-mediated DSB repair pathway that uses homologous chromosomes as DNA repair templates (Vignard et al. 2007). There is some evidence that suggests that MND1 can also function, with RAD51 and XRCC3 proteins to repair DSBs using sister chromatids as templates (Vignard et al. 2007). Phosphorylation of specific amino acids in the MND1 protein likely controls how MND1 functions via subtle changes in its conformation. Changes in MND1 conformation, in wild type *Arabidopsis*, are probably coordinated with the progression of meiosis. The MND1 protein may also undergo conformational change in meiotic mutants that fail to repair their DSBs in a timely fashion; in this case, despite a failed meiosis, potentially harmful DSBs would ultimately be repaired using a sister chromatid’s DNA as a template.

Bioinformatic comparison of AHP2 and MND1 proteins supports the theory put forth by Vignard et al. (2007) that suggests the MND1 may have two distinct roles. Which role MND1 plays may be controlled by the ATM kinase through its ability to sense unrepaired DSBs and its potential to alter MND1 protein conformation to one that favors interaction with the RAD51/XRCC3 complex. As well, our immuno-localization of MND1 suggests that the second function of MND1 does not require AHP2 and likely occurs in the non-axial regions of meiotic chromosomes.

### 4.9 *Arabidopsis* AHP2/MND1 protein complex - a hypothesis for the mechanism of function

I suggest the following as a possible mechanism for the functioning of *Arabidopsis* AHP2, as part of the AHP2/MND1 complex. AHP2 needs to form a complex with MND1 to be able to bind DNA. After forming a complex with MND1 the AHP2 protein likely undergoes a conformational change that exposes its DNA binding motifs. The ‘WRKRKRM’ motif of AHP2 could target the AHP2/MND1 complex to the minor groove of AT-rich DNA; this type of DNA is known to exist at the base of chromatin loops (Ottaviani et al. 2008). In meiocytes chromatin is organized into a linear array of loops whose bases are elaborated with specific proteins thus forming the chromosomal axis (Kleckner et al. 2004).

The minor groove of AT-rich DNA is particularly narrow (Roque et al. 2004). The AHP2/MND1 complex is ideally suited for binding the minor groove of AT-rich DNA as both AHP2 and MND1 are relatively small (26 and 28 kDa respectively) and both have an abundance of positively charged residues. The *Arabidopsis* AHP2 protein has an especially large percentage of positively charged residues and in this regard bears some resemblance to the histone proteins, known to interact with the DNA double helix minor groove (Zhao et al. 1999).
The positioning of the positively charged AHP2/MND1 complex at the base of chromatin loops may induce a DNA conformational change that allows DMC1 preferentially to bind at axis-associated DSB sites during mid to late leptotene. Sanchez-Moran et al. (2007) have shown that DMC1 localizes to chromosomes during mid-leptotene (after AHP2/MND1 complex localization). DMC1 binds to the 3’ DNA overhang that exists at sites of resected DSBs (Pezza et al. 2007); these DSB sites can potentially exist in both chromosome axis and loop-associated regions of DNA. The recruitment of DMC1 (by AHP2/MND1) to axial regions could contribute to DNA strand invasion into homologous duplex DNA and facilitate DMC1’s ability to promote homologous chromosome, non-sister chromatid exchange.

Petukhova et al. (2005) have shown, ‘in vitro’, that the AHP2/MND1 complex enhances single stranded DNA invasion into homologous duplex DNA via a functional interaction with the RAD51/DMC1 complex. It thus appears that the AHP2/MND1 complex may have two functions: the recruitment of DMC1 to axis-associated DSBs (during leptotene) and the enhancement of DNA strand invasion (during zygotene). Both of these functions contribute to the inter-homolog repair of DSBs.

My work has demonstrated that the duration of meiosis was increased in Arabidopsis ahp2 meiocytes, largely due to a significantly prolonged early to mid-zygotene. This finding strongly suggests that AHP2 functions mainly during zygotene; it is during this meiotic substage that strand invasion of homologous duplex DNA largely occurs (Zickler and Kleckner 1998). In my work, the immuno-labeling of Arabidopsis AHP2 showed that its presence initially peaks during zygotene, further supporting a role for AHP2 during this prophase I substage.

Strand invasion into homologous duplex DNA leads to the formation of axial associations that stabilizes homologous chromosome close alignment and is followed by synaptonemal complex formation; these events are required for successful RGE (Wilson et al. 2005). In yeast, axial associations are not observed in hop2 meiocytes (Leu et al. 1998); lack of stabilized alignment (except in NOR regions), in the Arabidopsis ahp2 mutant, suggests that axial associations also are not formed.

It is likely that both roles proposed for the AHP2/MND1 complex are non-enzymatic; this would explain the overall poor conservation in both AHP2 and MND1 amino acid sequences (excluding the 7 residue motif found in the C-terminal region of AHP2 orthologs).

**4.10 Arabidopsis AHP2 function - a unifying model**

Figure 41 illustrates a unifying model that attempts to show the relationships between Arabidopsis chromosome organization, the molecular steps in reciprocal genetic exchange, the initial localization of meiotic proteins (relevant to my work) and the timing of their function with respect to successive meiotic stages. The proposed timing of AHP2’s function, as a complex with MND1, is placed in relationship to these chromosomal and DNA events.
The progression of chromosomes from axis formation to homolog rough alignment to close alignment to that of alignment stabilization (axial association formation) and finally to SC formation, has been reported in *Arabidopsis* meiosis (Wilson et al. 2005). The molecular steps (DNA events) involved in reciprocal genetic exchange have been documented in yeast (Bishop and Zickler 2004) and are applicable to plants (Ma 2006). The timing of chromosomal and DNA events, with respect to the meiotic stages, are estimated in the model. Individual chromosome regions proceed through successive chromosomal and DNA events, but these events do not occur at the same time for all chromosome regions.

Shown in my model are the approximate timing of ASY1, ZYP1, SPO11-1, SPO11-2, MND1, DMC1, RAD51, XRCC3 and AHP2 initial localization onto *Arabidopsis* chromosomes (Armstrong et al. 2002; Higgins et al. 2005; Sanchez-Moran et al. 2007; Vignard et al. 2007; Stronghill et al. 2010). SPO11-1 and SPO11-2 localization is pre-meiotic and occurs just prior to DSB formation. ASY1 localizes pre-meiotically to chromatin as foci but this localization becomes linear during early leptotene. Sanchez-Moran et al. (2007) suggest that ASY1 signal linearization is commensurate with axis formation and DSB formation. The AHP2/MND1 complex is shown to localize initially to early leptotene chromosomes. MND1 (not complexed to AHP2) also separately localizes to chromosomes during early leptotene. XRCC3, DMC1 and RAD51 localize, during mid-leptotene, to the 3’ single stranded DNA overhangs that are present at the sites of resected DSBs.

The model shows that the AHP2/MND1 complex functionally interacts with the RAD51/DMC1 complex and this function has been placed in temporal alignment with the 3’ DNA single strand invasion (SSI) and chromosome alignment stabilization events that occur during zygotene. It is known from *in vitro* work in mice that the AHP2/MND1 complex functionally interacts with the RAD51/DMC1 complex and strongly enhances DNA strand invasion into homologous duplex DNA (Petakhova et al. 2005). I have experimentally shown that the AHP2/MND1 complex functions in early to mid-zygotene to stabilize chromosome close alignment (section 3.3.2.3).

Vignard et al. (2007) have suggested that MND1 (not associated with AHP2) may be required for the RAD51/XRCC3 complex to function efficiently during the same stage of meiosis (DNA strand invasion) to repair DSBs using sister chromatids as repair templates. This separate role for MND1 is consistent with our finding of MND1 at locations that were distinct from the AHP2/MND1 complex. How could the DSB repair activity of these two separate functional complexes, which both include MND1 and RAD51, be coordinated in wild type *Arabidopsis* meiocytes? MND1 and RAD51 affinity-changes may occur as DSB repair proceeds through zygotene. I suggest that in early to mid-zygotene,
Fig. 41 The functional interaction of the AHP2/MND1 complex with the DMC1/RAD51 complex is likely coordinated with MND1/RAD51/XRRC3 function during zygotene; this coordination may involve ATM kinase. The unifying model presented here illustrates the temporal relationships between wild type *Arabidopsis* chromosome organization (A), the molecular steps in reciprocal genetic exchange (B), meiotic protein initial localization (C) and time of function (D) in successive meiotic stages from leptotene to metaphase I (MI). The proposed function of AHP2, as a complex with MND1, is placed in the context of chromosomal and DNA events. Row A of the model shows the meiotic stages from leptotene to metaphase I. Row B gives the major chromosomal-based events that occur from early leptotene to late diakinesis. Red arrows show the progression of chromosomes from rough alignment to synaptonemal complex formation. Blue arrows indicate that changes in chromosome organization, within a meiocyte, occur progressively for individual chromosome regions but do not occur at the same time for all chromosome regions. The green arrow shows that chromosomes progressively condense (shorten) throughout meiosis I except for a brief period in late diplotene called the ‘diffuse stage’ when there is a temporary reduction in the overall level of chromosome condensation (centromeres excepted); NOR symbolizes the Nucleolar Organizer Region. Row B gives the major DNA-based events that are required for the repair of double strand breaks and for the completion of reciprocal genetic exchange (RGE); these DNA events occur from early leptotene to late pachytene. Red arrows show the progressive molecular steps that result in reciprocal genetic exchange from double strand break formation to double holiday junction resolution. Blue arrows indicate that these steps in RGE occur progressively for individual chromosome regions but do not occur at the same time for all chromosome regions; symbols used are: DSB = Double Strand Break, SSI = Single Strand Invasion, DHJ = Double Holiday Junction. Row C provides the approximate timing, for some meiotic proteins, of initial localization to chromosomes with respect to meiotic substage. Circled proteins represent a protein complex. Row D gives the approximate meiotic substage in which various proteins and protein complexes are hypothesized to function. Functionally interactions between complexes or a complex and a protein are represented by black bars. Question marks denote a speculative estimate of time of function. For example both ZYP1 and AHP2/MND1 complex bind to early leptotene chromosomes but whether they function during leptotene is not yet proven. The blue triangle represents the decreasing axis-associated DSB repair activity of the AHP2/MND1/RAD51/DMC1 functional complex; the red triangle represents the increasing loop-associated DSB repair activity of the MND1/RAD51/XRCC3 functional complex. The transition in complex activities is hypothesized to be controlled by the *Arabidopsis* ATM kinase.
MND1’s conformation favors AHP2/MND1 complex formation and RAD51’s conformation favors RAD51/DMC1 complex formation.

I speculate that the DSB repair in wild type meiosis, during early to mid-zygotene, primarily involves the functional interaction of the AHP2/MND1 complex with the RAD51/DMC1 complex (producing chiasmata) but toward the later part of zygotene there is a transition to the repair of remaining DSBs (possibly more distal to the axis). These latter, non-axial repairs involve MND1’s functional interaction with RAD51/XRCC3 complex using sister chromatids as repair templates (with no chiasma formation). The change in MND1 and RAD51 conformations to those favoring MND1’s functional interaction with RAD51/XRCC3 complex could be achieved via phosphorylation of MND1 and RAD51 by ATM kinase. The *Arabidopsis* MND1 and RAD51 both have three possible ATM kinase phosphorylation sites.

*Arabidopsis* ATM is known to bind at the sites of DSBs and as DSBs are repaired ATM is released to the nucleoplasm (Culligan et al. 2008). In addition *Arabidopsis* ATM has the ability to self-phosphorylate (Waterworth et al. 2007). I suggest that in wild type meiocytes the activity of the AHP2/MND1- RAD51/DMC1 complex repairing DSBs causes the release of a threshold amount of ATM into the nucleoplasm that stimulates ATM self-phosphorylation. ATM self-phosphorylation could lead to ATM phosphorylation of MND1, allowing MND1 to functionally interact with the RAD51/XRCC3 complex. ATM activation also could result in the phosphorylation of cell cycle proteins required for meiosis progression. This theory provides one plausible explanation for *ahp2*’s extended zygotene; ATM may not be released from most DSB sites due to the lack of AHP2 function.

### 4.11 *ahp2*’s prolonged zygotene and ‘sticky’ chromosome phenotype – a possible explanation

The increased duration of zygotene that is seen in *Arabidopsis* *ahp2* meiocytes likely results from a failure of the AHP2/MND1/DMC1/RAD51 complex to properly process DSB intermediates and stabilize pairing via axial associations. The *Arabidopsis* ATM gene is expressed during meiosis and may be a key regulatory factor that senses accumulation of unrepaired DSBs, regulates DSB repair complexes and has the potential to control the progression of meiosis via the phosphorylation of cell cycle genes. ATM is known to have a meiotic function, in *Arabidopsis*, as *atm* mutants are partially sterile (Garcia et al. 2003).

Meiotic surveillance in wild type *Arabidopsis* probably controls meiosis progression and allows time for DSB repair. In wild type the majority of axis-associated DSBs are repaired by the DMC1-mediated pathway due to the inter-homolog repair bias. In the *ahp2* mutant axis-associated DSB cannot be repaired by the DMC1 pathway. However some loop-associated DSBs could still be repaired by the MND1/RAD51/XRCC3 complex from sister chromatid DNA templates. Without the DMC1-mediated
pathway there would be a slower than normal release of ATM to the nucleoplasm. The activation of ATM (via self phosphorylation) would be delayed but would eventually lead to the phosphorylation of cell cycle proteins necessary for meiosis progression. Delayed ATM activation would explain the *ahp2* mutant’s prolonged early-mid zygotene.

In the *ahp2* mutant DMC1 might not preferentially bind to axis-associated DSB sites. I suggest that in the absence of the AHP2/MND1 complex there might be significant DMC1 binding to loop-associated DSB sites (that does not occur in wild type). DMC1 in loop regions might promote illegitimate (non-homologous), hard to resolve, non-sister DSB intermediates, hindering the repair of loop-associated DSBs via sister chromatid exchange despite the presence of functioning MND1/RAD51/XRCC3 complexes. Indeed there appears to be a delayed loss of DMC1 foci from meiotic chromosomes in *mnd1* mutants (Vignard et al. 2007). I propose that both the impairment of legitimate axial and loop-associated DSB repair and enhancement of illegitimate associations in the loop regions contribute to abnormal connections between chromosomes that results in the ‘sticky’ chromosome phenotype observed in *ahp2* meiocytes.

5.0 Summary and future research on *Arabidopsis* meiosis

Our cytological investigation included the first comprehensive characterization of prophase I for AHP2 in plants using an *Arabidopsis* *ahp2* mutant, a modified spreading protocol and a new multi-criteria technique that we developed for detailed prophase I substage identification. This combined approach should be useful in studying other mutants. For example another meiotic protein, DMC1, has not been thoroughly examined in plants for its prophase I characteristics; if the *Arabidopsis dmc1* mutant was comprehensively examined using the techniques we have developed here, new information could be generated about this protein’s function.

Ours was the first report of the duration of individual prophase I substages in wild type *Arabidopsis*. We combined BrdU-determination of meiosis duration and the relative durations of each substage; this allowed us to calculate the duration of each prophase I substage in wild type nuclei and revealed that early to mid-zygotene was prolonged in the *ahp2* mutant. This finding coupled with AHP2 immunolabeling of zygotene chromosomes is consistent with our proposed role for AHP2/MND1 in stabilizing homologous chromosome close alignment. Various meiotic mutants have been reported to have a prolonged prophase I. Our method of calculating individual substage durations will allow for a more exact determination of which substage of prophase I is affected by specific mutations.

*Arabidopsis* AHP2 functions during zygotene in wild type plants by stabilizing the close alignment of homologous chromosomes that is necessary for successful RGE and plant fertility. The main
evidence for our conclusion on AHP2 function comes from our finding of substantial chromosome close alignment in ultrathin sections of *ahp2* nuclei that was not observed in counterpart chromosome spreads; spreads do not maintain unstable associations. We found that stabilization of close alignment of homologous chromosomes, involving the AHP2/MND1 complex, is required for SC formation in non-NOR regions of *Arabidopsis* chromosomes. Our comparison of TEM analysis of meiotic nuclei ultrathin sections with LM analysis of chromosome spreads could be extended to other meiotic mutants to ascertain whether a lack of close alignment is due to an alignment problem or an inability to stabilize chromosome close alignment.

Our immuno-localization of AHP2 was the first done in a higher eukaryote. We found that AHP2 required the presence of MND1 to localize to chromosomes, but MND1 could bind to the chromosomes in the absence of AHP2, at non-axial locations. We found independent diffuse staining of MND1 in the *ahp2* mutant that likely indicates a role for MND1 along the loop-regions of chromosomes that does not involve AHP2. This is consistent with the work of Vignard et al. (2007) who observed an intermediate phenotype for *mnd1dmc1* metaphase I meiocytes that suggested MND1 may function in two different pathways. My work has generated further evidence in support of this theory through both cytological observations and bioinformatic analysis. In silico analysis showed that MND1 function is most likely controlled by the kinase ATM that is known to be involved in the surveillance of DSB repair in meiosis.

In wild type meiosis it is possible that most DSBs that are repaired early use homologs as repair templates, but as meiosis progresses sister chromatid-based repair becomes more prevalent. If there is a transition, during wild type prophase I, in the DSB repair bias from homolog-based repair to sister chromatid-based repair then it appears that MND1 and RAD51 may be two of the proteins involved in the later repair. RAD51, like MND1 also has the potential to be under ATM control as it contains SQ/TQ motifs.

In several organisms cis-acting DNA sequence can facilitate the stabilization of homologous chromosome close alignment. Nuclear organizer regions can perform as cis-acting homologous chromosome pairing sites in *Drosophila*; I also have found this to be true for NORs in *Arabidopsis*. This is the first report of NOR-induced homologous chromosome pairing in plants, and is based on our finding of localized synaptonemal complex formation along the short arms of NOR-bearing chromosome 2 and 4 in the *ahp2* mutant that otherwise demonstrates a drastic reduction in synopsis in non-NOR regions. The examination of *Arabidopsis spo11* chromosome spreads could determine whether NOR-derived synopsis is dependent on DSB formation. In addition antibodies that mark the sites of crossovers could be used to ascertain whether successful RGE occurs on the short arms of NOR bearing *ahp2* chromosomes; this would provide evidence that the SC that occurs in these regions is functionally normal.
The ‘sticky’ chromosome phenotype observed in the Arabidopsis ahp2 and mnd1 meiocytes is the result of irregular connections between chromosomes. Chromosome bridging and fragmentation in both ahp2 and mnd1 anaphase II meiocytes indicate these two mutants appear to experience problems in the separation of chromatids and or non-homologous chromosomes; our investigation showed that the cohesin complex is likely not involved in this ahp2 mutant phenotype. Vignard et al. (2007) found that the localization of SCC3, a cohesin protein, was normal in the mnd1 mutant. While the AHP2/MND1 complex works with DMC1 in wild type meiosis, the Arabidopsis dmc1 mutant does not display the sticky phenotype; it displays mainly univalents at metaphase I. The presence of univalents in dmc1 mutants (instead of entangled sticky chromosomes) suggests the absence of unresolved DSBs and a lack of illegitimate exchanges. Thus dmc1 mutants appear to repair DSBs using sister chromatids as repair templates (instead of normal inter-homolog repair). It would be interesting to follow DSB repair in the Arabidopsis ahp2, mnd1 and dmc1 mutants with γH2AX immuno-cytochemistry; this histone 2A variant marks the sites of DSBs. We know that DSBs in hop2, mnd1 and dmc1 mutants are left mainly unrepaired in budding yeast, but what is the situation in plants for these mutants? Vignard et al. (2007) observed an interesting intermediate phenotype for mnd1dmc1 metaphase 1 meiocytes. It would be interesting to determine how DSB repair might be affected in this mutant and in ahp2dmc1 and ahp2mnd1 double mutants.

The function of the Arabidopsis AHP2 appears to more similar to its counterpart in mouse than in budding yeast. This comparison is based on both bioinformatic analysis and cytological observations that I and others have reported; this may indicate that the AHP2’s meiotic function, in plants, is most similar to HOP2’s function in mice.

Our investigation of the Arabidopsis AHP2/MND1 complex has advanced our understanding of its role in plant meiosis. We also have provided evidence for an additional role for the MND1 protein function apart from the DMC1-mediated DSB repair pathway. Also our work has shown that ‘cis-acting’ DNA such as NORs can stabilize chromosome pairing; thus extending this type of stabilization beyond the animal kingdom. The knowledge we gain in meiotic research will impact both agriculture and medicine.
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