Chapter 4

AtMND1 is Indispensable for Homologous Chromosome Pairing and Recombination
4.1 Introduction

Homologous chromosomes must match, pair and exchange DNA segments during prophase I to ensure the successful completion of meiosis. Defects in any of the above mentioned processes can lead to the production of aberrant gametes, which is deleterious to the long term success of a species (Anuradha and Muniyappa, 2005). Stepwise segregation of homologous chromosomes at meiosis I, and sister chromatids at meiosis II requires the prior pairing of homologues. Homologous recombination machinery plays a very crucial role in bringing the two homologous chromosomes to close proximity and in ensuring physical linkage between them. The formation of at least one crossover between pairs of homologous chromosomes is essential for their correct segregation at meiosis I (Gerton and Hawley, 2005). In most species, the cellular machinery makes sure that at least one crossover is made between a pair of homologues. In budding yeast, it has been shown that the lack of crossover triggers the spindle check point, followed by metaphase arrest (Rudner and Murray, 1996). However, plants seem unique in this aspect, where meiocytes never exhibit meiotic arrest in any of the mutants described so far. Instead, all of them complete meiosis and produce aberrant gametes with altered chromosome number or damaged DNA. This unique aspect of plants let researchers to study the effects of a meiotic gene mutation beyond its site of action, which is otherwise not possible. The various pairing interactions that take place at the DNA level later culminate in the formation of the synaptonemal complex (SC) – a tripartite proteinaceous structure. The dissolution of the SC at the end of pachytene marks the completion of recombination and crossing over, leaving the homologues connected by cohesins distal to chiasmata. Two kinds of crossover (CO) pathways co-exist in Arabidopsis, class I COs and class II COs, where class I CO accounts for 85% of the chiasmata and class II COs generates the remaining 15% of the chiasmata (Ross-Macdonald and Roeder, 1994; Zalevsky et al., 1999). Class I COs show interference and are controlled by
bacterial MutS homologues MSH4 and MSH5 (Wang et al., 1999). Whereas class II COs are interference insensitive and are mediated by MUS81/MMS4 (de los Santos et al., 2003; Hollingsworth and Brill, 2004). The extents at which these pathways operate in different organisms vary widely. In fission yeast it is known that all COs arise by Class II pathway (Osman et al., 2003). While in Caenorhabditis elegans, all COs exhibit interference (Villeneuve and Hillers, 2001).

Recombination at the DNA level in yeast and in other organisms is initiated by double strand breaks (DSBs) made by a topoisomerase II-like transesterase enzyme Spo11 (Bergerat et al., 1997; Keeney et al., 1997). Interaction between DSBs and a homologous intact chromosome can lead to crossover and noncrossover recombination products which are formed by two different pathways (Allers and Lichten, 2001). Processing of DSBs by 5' end resection yields 3' single-stranded ends that asymmetrically invade a homologous chromosome and lead to the formation of a double-Holliday junction intermediate which has been proposed to account for the majority of crossovers (Schwacha and Kleckner, 1997; Hunter and Kleckner, 2001). Interaction between homologous chromosomes at the sites of DSBs is promoted by the action of the RecA-like strand exchange proteins Rad51 and Dmc1 (Sung and Robberson, 1995; Hong et al., 2001b). Several lines of evidence suggest that Rad51 and Dmc1 have different but overlapping functions (Rockmill et al., 1995; Shinohara et al., 1997) and interact with distinct sets of proteins in promoting recombination (Dresser et al., 1997; Sugawara et al., 2003; Tsubouchi and Roeder, 2004). Rad51 acts in mitosis and in meiosis (Shinohara et al., 1992) whereas Dmc1 is specific to meiosis (Bishop et al., 1992).

Mechanisms involved in homologous pairing and recombination remain poorly understood in plants. Much of the information comes from functional
analysis of meiotic mutants, and the majority of the genes so far characterized are functional homologues of yeast genes (Hamant et al., 2006). Mutants such as *Atdmc1* and *Atspo11* show no bivalent formation during male and female meiosis (Couteau et al., 1999; Grelon et al., 2001). These mutants are also defective in recombination and crossing over. Characterization of *AtSP011* has demonstrated the importance of DSB generation in plant meiosis. Similarities in sequence and function between yeast and plant meiotic genes such as *AtDMC1*, *AtSPO11*, *AHP2*, *ASY1*, *AESP* and *SYN1/DIF1* (Couteau et al., 1999; Grelon et al., 2001; Armstrong et al., 2002; Cai et al., 2003; Schommer et al., 2003; Liu and Makaroff, 2006) strongly suggest that the mechanism of meiotic recombination in yeast could be conserved in plants. It was believed that synapsis is an essential prerequisite for meiotic recombination to take place. However, studies in budding yeast mutants *zip1* and *red1* shows that recombination can happen even in the absence of SC formation and synapsis (Rockmill and Roeder, 1990; Sym et al., 1993; Rockmill et al., 1995). In addition, none of the meiotic mutants identified so far has been shown to assemble the SC in the absence of DSBs. Therefore it is likely that processing of DSBs could be the main mechanism that promotes synapsis and recombination during meiosis.

In this study, I describe the role of *AtMND1* in *Arabidopsis* meiosis and I provide evidence for its function during meiotic recombination. Genetic and cytological experiments carried out on *Atmnd1* establish that this gene is indispensable for homologous chromosome pairing and repair of DSBs in *Arabidopsis*.

4.2 Results

4.2.1 *AtMND1* is required for proper execution of meiosis

The expression pattern of *AtMND1* and the phenotype of the *AtMND1* mutant discussed in the third chapter are together suggestive of a defect in
Figure 4.1. Meiotic prophase defects in the *Atmnd1* mutant

(A-E) Wild type, (F-J) *Atmnd1*. (A-J) Male meiosis. (A,F) Unsynapsed elongated strands of chromosomes at late leptotene radiating from densely stained synizetic knot (arrow). The NOR is indicated by arrowhead. (B) Zygotene stage with partially synapsed chromosomes. (C) Synapsis is complete at pachytene and chromosomes have a shorter and thicker appearance. (D) Late diplotene stage, where bivalents have undergone partial decondensation of the arms but not at centromeric regions. Arrow indicates the NOR. (E) Diakinesis with brightly stained bivalents. (G) Desynapsed chromosomes at stage corresponding to zygotene with the chromosomes remaining as univalents. (H) Pachytene equivalent stage showing irregular and unsynapsed univalent chromosomes. (I) Diplotene stage with patchy and fragmented chromosomes. (J) Occurrence of more than ten brightly stained spots indicates univalent chromosomes and their fragmented form at diakinesis.

Scale Bars: 10\(\mu\)m
meiosis. Therefore meiotic prophase stages from the mutant and wild type were compared using spread preparations of meiotic chromosomes following the method of Ross et al., (Ross et al., 1997). The initial stages of meiotic prophase corresponding to early leptotene were seen to occur in the Atmnd1 mutant and thread-like chromosomes were apparent (Fig. 4.1G). Association of the nucleolar heterochromatin present on chromosomes 2 and 4 as well as the synizetic knot, which is formed during late leptotene concomitant with pairing and the start of synapsis (Ross et al., 1996) could also be observed (Fig. 4.1F). However, abnormalities could be detected starting at the zygotene stage, both with respect to the appearance of the chromosomes and the organization of pericentromeric heterochromatin. Chromosomes in the mutant appeared less compact when compared to wild type and pericentromeric heterochromatin regions were more extended and unpaired than at the corresponding stage in wild type. During the course of zygotene, the differences became more pronounced, and synapsis was defective (Fig. 4.1H). The thickening of chromosomes along segments of their length representing synapsed regions that characteristically appears during zygotene and is complete by pachytene, was largely missing in the mutant. Chromosome fragments formed during early prophase I remained unrepaired throughout meiosis. Later on, these fragments segregated disproportionately at anaphase I and II thereby giving rise to aberrant spores at the completion of meiosis.

4.2.2 In Atmnd1 chromosomes undergo severe fragmentation.

The most predominant feature of the mutant revealed by meiotic chromosome preparation was chromosome fragmentation (Fig. 4.1H, I). Chromosome fragmentation could be detected as early as pachytene in the mutant. Chromosomes appeared disorganized as indicated by pronounced differences in chromosome compaction. At diplotene the chromosomes appeared as a dispersed and fragmented mass with 10-12 separate spots of
Figure 4.2. Chromosomes undergo severe fragmentation in *Atmnd1*
Wildtype (A-E, K), *Atmnd1* (F-J, L, M), male meiosis (A-J), female meiosis (K-M). (A) Five separating univalents at early anaphase I. Residual chiasma can be seen in one of the separating bivalents (arrowhead). (B) Late anaphase I. (C) Metaphase II. (D) Anaphase II. (E) Telophase II. (F) Anaphase I with numerous chromosome fragments migrating to either pole. (G) Abnormal segregation of fragmented chromosomes at late anaphase I. (H) Fragmented chromosomes aligned at the metaphase II plate. (I) Anaphase II, showing scattered chromosome fragments and bridges probably representing sister chromatid cross over. (J) Telophase II with polyads. (K) Female meiocytes at pachytene with fully synapsed chromosomes. (L) Corresponding stage as in U with chromosomes appearing fuzzy, remaining as univalents. (M) Female meiocyte at late prophase I showing extensive chromosome fragmentation. Scale Bars: 10μm.
condensed pericentromeric heterochromatin (Fig. 4.1I). At diakinesis, separated chromosomes and fragments could be clearly distinguished (Fig. 4.1J). The segregation of the chromosome fragments at anaphase I and II was at random and in several cases bridges could be observed (Fig. 4.2F, I). In most cases polyads that contained a variable number of fragmented chromosomes were formed (Fig. 4.2J). These gave rise to defective spores that did not form viable pollen. Female meiosis in the mutant was also defective and chromosome fragmentation was observed (Fig. 4.2L, M). These data suggested the possibility that the mutant is defective in homologous pairing and synapsis and the accumulation of fragments may arise from defects in repair of DSBs that are formed during leptotene.

4.2.3 *Atmnd1* show higher number of anaphase II bridges.

One of the most prominent phenotype seen among the mutant meiocytes is chromosome bridges (Fig. 4.2F, I; Fig. 4.3A). In *Atmnd1* the chromosomes undergo fragmentation during prophase I itself; however, a small proportion of meiocytes seems to progress beyond this stage with less chromosome fragmentation, and produces one or two viable pollen grains/anther. Presently, it is not clear how this progression happens in a null mutant background. I examined the progression of meiosis in 425 meiocytes, among which 158 nuclei (~37%) showed at least one or more bridges during anaphase I, while the remaining displayed chromosome bridges during anaphase II (Fig. 4.4B). Since chromosome fragmentation is comparatively less in these meiocytes, I assume that a large proportion of the chromosome bridges would have arisen from unresolved recombination intermediates. Increase in the anaphase II bridges could be an indication that nonhomologous recombination is taking place in the mutant meiocytes, as reported in the Mnd1 mutant of budding yeast (Zierhut et al., 2004).
Figure 4.3. *Atmnd1* meiocytes exhibit chromosome bridges. (A) Anaphase I and Anaphase II bridges in *Atmnd1*. Cartoon of chromosome represents the possible chromosome configuration in each case. (B) Bar diagram showing percentage of bridges observed in different anaphase stages.
4.2.4 *Atspo11-1* mutation is sufficient to remove the chromosome fragmentation in *Atmnd1*

The chromosome fragmentation phenotype together with the absence of homologous pairing suggested that the *Atmnd1* mutant was defective in recombination possibly due to defects in the repair of meiotic DSBs. *AtSPO11-1* is one of three *SPO11* homologues in *Arabidopsis* and is specifically required for recombination and synapsis during meiosis (Grelon et al., 2001; Yin et al., 2002). To test this hypothesis I generated an *Atmnd1 Atspo11* double mutant. F1 plants that carried both insertions were identified in the F1 and homozygous *Atmnd1 Atspo11-1* double mutants were obtained in the F2. Chromosomes in the double mutant did not undergo fragmentation after diplotene (Fig. 4.4C, H) and remained as intact univalents that segregated randomly at the first meiotic division (Fig. 4.4C, D, H, I). Analysis of male meiotic chromosome spreads of the double mutant and comparison to the *Atspo11-1* single mutant indicated that the chromosome fragmentation phenotype of *Atmnd1* was suppressed by *Atspo11-1* (Fig. 4.4). In addition, the double mutant phenotype resembled that of the *Atspo11-1* mutant indicating that *AtSPO11-1* is epistatic to *AtMND1*.

4.2.5 *AtMND1* is indispensable for homologous chromosome pairing

To examine homologous pairing we carried out FISH experiments (Fig. 4.5) using a telomere repeat based oligonucleotide probe that hybridizes strongly to the centromere of chromosome 1 but not to centromeres of the remaining chromosomes (Li et al., 2005). In wild type, two well separated signals were observed at leptotene indicating that chromosomes were unpaired. A single signal was observed at late zygotene (46/46 nuclei) and pachytene (23/23 nuclei) indicative of pairing and synapsis having taken place. At diplotene, twin signals close to each other (6/6 nuclei) were seen indicative of centromere regions having desynapsed. These twin signals again merged and at metaphase I only a single signal was seen. In the *Atmnd1* mutant, nuclei showed two widely
Fig 4.4. Suppression of chromosome fragmentation in Atmnd1 by Atspo11-1. (A-E) Atspo11-1 single mutant. (F-J) Atmnd1 Atspo11-1 double mutant. (A,F) Pachytene equivalent stage with unpaired chromosomes. (B,G) Diplotene stage showing ten condensed univalents; four nucleolus organizing chromosomes are attached together at the NOR. (C,H) Diakinesis with ten brightly stained univalent chromosomes. (D,I) Anaphase I showing 7:3 and 6:4 unequal segregation of chromosomes respectively. (E,J) Polyad. Scale Bar: 10 μm.
separated signals starting from leptotene and throughout all subsequent stages (58/61 nuclei for zygotene and 42/43 for pachytene) indicating that homologous pairing as well as synapsis was defective (Fig. 4.5I).

4.3 Discussion

Pairing and recombination between homologous chromosomes at meiosis relies on search for homology using resected ends that are created at the sites of DSBs. This search is mediated by the action of RecA-like strand exchange proteins Rad51 and Dmc1 which bind to single stranded DNA and promote the formation of joint molecules (Sung and Robberson, 1995; Hong et al., 2001a). The strand exchange activity of Dmc1 and Rad51 is stimulated by Hop2 and Mnd1 which cooperate together as a complex (Tsubouchi and Roeder, 2002; Chen et al., 2004). Both Hop2 and Mnd1 are required in yeast for homologous pairing and meiotic DSB repair (Leu et al., 1998a; Rabitsch et al., 2001; Gerton and DeRisi, 2002; Tsubouchi and Roeder, 2002). In this study I establish that the Arabidopsis orthologue of MND1, AtMND1 is required for homologous pairing during meiosis in Arabidopsis.

Occurrence of DNA fragmentation in Atmnd1 meiocytes raises two possibilities regarding the function of AtMND1. One possibility is that in the absence of AtMND1, mutant meiocytes are unable to repair DSBs, which would be visible as chromosome fragments upon DNA condensation. Another possibility is DNA damage induced apoptosis taking place in Atmnd1 meiocytes as reported in white-cap mutant of Coprinus cinereus and cep-1 mutant of mice (Derry et al., 2001; Lu et al., 2003). Yeast proteins Mnd1 and Hop2 act synergistically to promote meiotic recombination (Chen et al., 2004; Petukhova et al., 2005; Enomoto et al., 2006). However, neither of them is required for DSBs in yeast or mammals. Therefore it is likely that fragmentation seen in Atmnd1 could
Early Leptotene

Leptotene-Zygotene

Late Zygotene

Pachytene

Diplotene

WT

Atmnd1

Figure 4.5. FISH analysis of homologous pairing in wild type and Atmnd1 meiocytes. (A-E) Wild type, (F-J) Atmnd1. (A,F) Early leptotene with two green signals indicating that homologous chromosomes are unpaired. (B,G) Two signals observed at leptotene-zygotene transition. (C) Late zygotene stage with only one signal indicating that the chromosomes have synapsed. (D) Pachytene stage with one signal. (E) Desynapsis occurring at centromeres during diplotene as evident by two closely appearing signals. (H) Late zygotene stage, with two signals placed far apart indicating unpaired state of homologous chromosomes. (I) Pachytene equivalent stage, where two signals are evident (J) Diplotene with two widely separated signals. Scale Bars: 10 μm.
be a result of unrepaired DSBs. In Arabidopsis, DSBs are made early during meiosis by AtSPO11-1; mutation in AtSPO11-1 causes formation of univalents. Using immunolocalization experiments Vignard et al., shown that AtDMC1 foci accumulates on meiotic DNA in Atmnd1 mutant, which is an indication of unsuccessful meiotic strand invasion process (Vignard et al., 2007). Suppression of DNA fragmentation seen in Atspo11-1 Atmnd1 double mutant clearly establishes that fragments observed in Atmnd1 was due to unrepaired DSBs made by AtSPO11-1. This also proves that AtMND1 acts in the same pathway as that of AtSPO11-1 to promote DSB repair and meiotic recombination.

One of the features of Atmnd1 meiocytes was that chromosomes remained connected during anaphase I and anaphase II and formed chromosome bridges. It has been proposed that such bridges can possibly arise by non-homologous end joining (NHEJ) (Siaud et al., 2004). Chromosome bridges at anaphase I can arise by defective crossover or exchange of DNA strands between homologous chromosomes or due to NHEJ or can be a combination of all the above. While, presence of chromosome bridges at anaphase II is suggestive of DNA exchange between sister chromatids and/or carryover NHEJ molecules from meiosis I. Studies on budding yeast MND1 gene have demonstrated that it is vital for promoting DSB repair from homologues (Zierhut et al., 2004). Similarly in Arabidopsis, absence of AtMND1 may have lead to removal of strand bias thus allowing the default sister chromatid-mediated repair pathway to operate.

The early defects in the Atmnd1 mutant with respect to overall appearance of chromosomes during meiosis were a lack of thickening during zygotene and absence of subsequent synapsis at pachytene. Fragmentation of chromosomes became apparent at diplotene and isolated univalents and fragments were first visible at diakinesis. Earlier in this section I have given evidence for absence of
DSB repair in the \textit{Atmnd1} mutant. Studies from yeast mutants such as \textit{zip1} and \textit{red1} show that pairing is an essential prerequisite for the repair of DSBs (Sym et al., 1993; Smith and Roeder, 1997). From the meiotic analysis carried out, it was observed that 5-densely stained heterochromatin regions, which is an indication of homologous pairing in wild type, was absent in \textit{Atmnd1} (Fig. 4.1H). Therefore we hypothesized that defects in DSB repair in \textit{Atmnd1} could be due to lack of homologous pairing. FISH analysis using a centromere 1 specific probe indicated that homologous pairing did not take place in the mutant during zygotene and homologous chromosomes remained apart throughout meiotic prophase and meiosis I. Synapsis involves polymerization of SC proteins along arms of homologous chromosomes, mutation in SC protein such as Hop1, Zip1 and Red1 are known to cause asynapsis. Similar defects are reported in the \textit{Atmnd1} mutant, where localization of one of the synaptonemal complex protein AtZYP1 is severely compromised (Vignard et al., 2007), which supports the defective pairing observed in \textit{Atmnd1}. A similar phenotype was seen other aynaptic mutants like \textit{Atdmc1} and \textit{Atrad51} (Vignard et al., 2007). At present it is not very clear how AtMND1 affects the localization AtZYP1. Since AtMND1 is necessary for the chromosomal localization of AtRAD51 and AtDMC1, the requirement of AtRAD51 and AtDMC1 function for AtZYP1 localization cannot be excluded.

The meiotic phenotype of \textit{Atmnd1} is similar to that caused by a mutation in \textit{AHP2} which encodes the \textit{Arabidopsis} orthologue of \textit{HOP2}. In both cases there is a failure to repair double strand breaks and a defect in homologous pairing. The failure to synapse and the appearance of fragmented chromosomes late in meiotic prophase I is a feature of several \textit{Arabidopsis} mutants that are affected in processing and repair of DSBs (Yin et al., 2002; Schommer et al., 2003) and supports the interpretation that \textit{Atmnd1} is also defective in meiotic DSB repair. A major difference between the meiotic phenotype of yeast \textit{mnd1} and that for \textit{Atmnd1} in \textit{Arabidopsis} is the absence of meiotic arrest in
Arabidopsis whereas mnd1 shows prophase arrest which is alleviated by a mutation in MEC1, a major regulator of DNA damage induced checkpoints (Zierhut et al., 2004). A lack of arrest is also seen in the case of ahp2 whereas hop2 shows prophase arrest (Leu et al., 1998b). The failure to arrest in the case of Arabidopsis is likely to be due to the absence or leakiness of meiotic DNA damage checkpoints and has also been observed for other Arabidopsis meiotic mutants for which the yeast counterparts show prophase arrest (Caryl et al., 2003).

So far there are no reports that explain the actual mechanism by which Mnd1 promotes strand exchange activity and inter-homologue bias in recombination. Using in vitro biochemical assays Petukhova et al., have shown that Mnd/Hop2 complex can stimulate strand invasion activity of DMC1 (Petukhova et al., 2005). Similarly, in Arabidopsis, AtMND1 and AHP2 has been shown interact each other in yeast two-hybrid assays indicating that the complex is conserved in plants as well (Kerzendorfer et al., 2006). AtMND1 localizes throughout chromatin during prophase I, and this localization requires AHP2 (Vignard et al., 2007). However, AtMND1 does not co-localize either with AtDMC1 or AtRAD51; indicating that most likely AtMND1 influences the activity of AtDMC1 and AtRAD51 indirectly. As per the proposed mechanism, binding of Mnd1 leads to alterations in superhelicity of the DNA thereby making it more accessible to Dmc1/Rad51 complex (Chen et al., 2004).

In most species, number of crossovers formed is about 1 to 3 per homologous pair; nevertheless number of DSBs created initially at prophase I is 10-40 fold higher than that of actual crossovers (Bishop, 1994). It has been proposed that majority of these breaks are repaired from the sister chromatids by AtRAD51 and AtXRCC3 (Vignard et al., 2007). However, a subset of the DSBs goes through the crossover pathway catalyzed by AtDMC1/AtRAD51 complex.
An insight into the above was gained by analyzing *Atrad51* and *Atdmc1* mutants. In *Atrad51* mutant, chromosomes displayed severe fragmentation during meiosis (Li et al., 2005); surprisingly, *Atdmc1* mutant did not show any chromosome fragmentation, instead chromosomes remained as univalents throughout prophase I (Klimyuk and Jones, 1997). In light of above observation it is suggestive that AtDMC1 in itself is insufficient to repair DSBs whereas AtRAD51 can repair most of the DSBs even in the absence of AtDMC1. Previous studies have demonstrated the vital role of ASY1 in promoting interhomologue recombination by stabilizing AtDMC1 loading at the site of DSBs (Sanchez-Moran et al., 2007). Absence of AtDMC1 has been shown to abolish the crossover pathway leading to deficiency in pairing and formation of univalents. Moreover, data presented here show that *Atmnd1* phenotype is cytologically similar to that of *Atdmc1* but is different from that of *Atrad51* regarding the formation of univalents. Therefore, it is possible that AtMND1 is required for the activity of AtDMC1/AtRAD51 complex as well as AtRAD51 alone in meiosis. Collectively, these observations propose that in *Atmnd1* mutant, both AtRAD51 and AtDMC1 functions are largely compromised and uncoupled thereby severely impairing the recombination repair machinery.

In budding yeast, *mnd1* mutant meiocytes arrest at prophase I due to activation of Mec1 mediated DNA damage checkpoint. However, a mutation either in *RED1* or *HOP1*, which disrupts the SC formation, could suppress the fragmentation seen in *mnd1* mutant (Zierhut et al., 2004). This observation is not only suggestive of a suppression of Rad51-mediated DSB repairs in *mnd1* mutant but also indicative of strong interhomologue bias exerted by SC proteins during recombination. Using in vitro binding assays Vignard *et al.*, have demonstrated that AtMND1 functionally interacts with AtRAD51 (Vignard et al., 2007). Since AtMND1 and AtRAD51 can functionally interact it is likely that AtMND1 is essential for the activity of AtRAD51. Another possibility is one in
which AtRAD51 function is independent of AtMND1 and the fragmentation occurred in *Atmnd1* could be due to suppression of AtRAD51 activity by interhomologue bias imposed by SC proteins such as ASY1. Therefore it is worth testing if mechanisms seen in yeast exist in plants as well. Further studies by combining *Atmnd1* mutation with *asy1* may provide some deeper insights into the function of *AtMND1* in plants.