MCM8 Is Required for a Pathway of Meiotic Double-Strand Break Repair Independent of DMC1 in Arabidopsis thaliana

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Abstract

Mini-chromosome maintenance (MCM) 2–9 proteins are related helicases. The first six, MCM2–7, are essential for DNA replication in all eukaryotes. In contrast, MCM8 is not always conserved in eukaryotes but is present in Arabidopsis thaliana. MCM8 is required for 95% of meiotic crossovers (COs) in Drosophila and is essential for meiosis completion in mouse, prompting us to study this gene in Arabidopsis meiosis. Three allelic Atmcm8 mutants showed a limited level of chromosome fragmentation at meiosis. This defect was dependent on programmed meiotic double-strand break (DSB) formation, revealing a role for AtMCM8 in meiotic DSB repair. In contrast, CO formation was not affected, as shown both genetically and cytologically. The Atmcm8 DSB repair defect was greatly amplified in the absence of the DMC1 recombinase or in mutants affected in DMC1 dynamics (sds, asy1). The Atmcm8 fragmentation defect was also amplified in plants heterozygous for a mutation in either recombinase, DMC1 or RAD51. Finally, in the context of absence of homologous chromosomes (i.e. haploid), mutation of AtMCM8 also provoked a low level of chromosome fragmentation. This fragmentation was amplified by the absence of DMC1 showing that both MCM8 and DMC1 can promote repair on the sister chromatid in Arabidopsis haploids. Altogether, this establishes a role for AtMCM8 in meiotic DSB repair, in parallel to DMC1. We propose that MCM8 is involved with RAD51 in a backup pathway that repairs meiotic DSB without giving CO when the major pathway, which relies on DMC1, fails.


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Introduction

Meiosis is a process that occurs in the germlines of sexually reproducing organisms. Two successive rounds of chromosome segregation (meiosis I and II) follow a single round of DNA replication (S phase). The resulting four cells each contain half the genetic content of the pre-meiotic mother cell. The genetic complement of these gametes is a mosaic of the paternal and maternal DNA due to meiotic recombination that occurs between S phase and the first meiotic division [1].

Meiotic recombination begins with programmed DSBs that are dependent on SPO11 and multiple cofactors, including PRD1 in plants [2,3]. DSBs are subsequently resected to yield 3’ overhangs that invade the homologous chromosome. At this step, two recombinases co-operate to achieve efficient strand exchange with the homolog, RAD51 and DMC1 [4]. RAD51 is a recombinase involved both at mitosis and meiosis while DMC1 is specific to meiosis. Importantly, it has been recently shown in S. cerevisiae that only the strand exchange activity of DMC1, and not of RAD51, is required for meiotic crossover formation [5]. RAD51 appears thus to be an accessory factor for DMC1 for meiotic homologous crossover formation, but may also serve as a backup to repair breaks when DMC1 fails [5]. In Arabidopsis thaliana, RAD51 is indispensable for repair of meiotic DSBs as shown by the extensive meiotic chromosome fragmentation which occurs at meiosis in Atrad51 mutants [6,7]. AtDMC1 is required for CO formation but not meiotic DSB repair. Indeed, in Atdmc1 mutant, meiotic DSBs are repaired in a AtRAD51-dependent manner which does not promote chromosome pairing and does not yield COs between homologs, likely using the sister chromatid as a template [7,8]. In addition, consistent with a role of RAD51 in helping DMC1 in wild type, the number of DMC1 foci is severely decreased in a Atrad51 mutant [7,9], while RAD51 foci are unaffected in Atdmc1 [9]. Thus two meiotic functions of RAD51 emerge, helping DMC1 to promote COs and promoting DSB repair on the sister without DMC1.

Two other Arabidopsis mutants, sds and asy1, have phenotypes reminiscent of Atdmc1, repairing breaks using AtRAD51 but exhibiting major homologous chromosome pairing defects and making no or few COs [10–12]. Both sds and asy1 show localization defects of AtDMC1 but not of AtRAD51, suggesting that they work with DMC1 to promote interhomolog recombination [12,13]. Based on its amino acid sequence, SDS is a cyclin-like protein and ASY1 is a HORMA domain protein making it the likely functional homologue of S. cerevisiae Hop1.
DSB repair events form intermediates that are resolved as either crossovers (COs) or non-crossovers (NCOs) (gene conversion). COs are required for accurate segregation of chromosomes during meiosis I and can arise from at least two independent pathways known as class I and class II COs. These two pathways coexist in budding yeast, mammals and Arabidopsis [1,14–17]. Class I COs are subject to a phenomenon known as interference, whereby the occurrence of a CO significantly reduces the probability of a CO occurring at an adjacent locus, in a distance dependent manner. This pathway is dependent on the ZMM proteins (defined as ZIP1, ZIP2/SHOC1, ZIP3, ZIP4, MSH4, MSH5, MER3) and, in most eukaryotes, is responsible for the majority of COs during meiosis. Class II COs, that do not display interference, require MUS81 [1,14–17].

Here we addressed the meiotic function of MCM8. MCM8 is a member of the eight MCM family proteins (MCM2–9), that all share a well conserved helicase domain. Together MCM2–7, as a hexamer, form a well characterized DNA helicase, which is essential for replication in all eukaryotes [18]. In contrast, MCM8–9 is not present in all eukaryotes [19], being notably missing in S. cerevisiae, S. pombe and C. elegans, but existing in vertebrates and plants. A study in Xenopus showed that MCM8 functions during DNA replication at the elongation stage but it is not required for replication licensing. The Xenopus MCM8 protein is the only MCM8 representative for which helicase activity has been demonstrated in vitro [20]. MCM8 is also involved in, but not essential for the assembly of the pre-replicative complex in human [21]. Very recently, MCM8 and MCM9 has been shown to be involved in homologous recombination-mediated DNA repair in mouse and chicken somatic cells [22,23]. MCM8 has also been shown to be involved in meiosis. In the fruit fly (Drosophila melanogaster), in which MCM9 has not been identified, MCM8 (also known as REC) is required for 95% of meiotic COs. In contrast to COs, the frequency of NCOs increases in the absence of Dmrec [24]. Finally, a very recent study pointed out a role for MCM8, but not MCM9, in meiotic recombination in mouse [22]. Indeed meioocytes in the mouse mcm8 mutant accumulate DMC1 foci, display synapsis defects and go into apoptosis, consistent with a defect in meiotic DSB repair. The meiotic function of MCM8 has been analyzed only in Drosophila and mouse, with contrasting conclusions. This raises the question of the conservation of this function in eukaryotes. The aim of the present study was to further explore the meiotic function of MCM8 by deciphering its role in the model plant Arabidopsis.

### Results

**Identification of the AtMCM8 gene and Atmcm8 mutations**

Phylogenetic analyses of the MCM family [19,24], showed that the Arabidopsis genome contains one clear homolog for each MCM2–9. At3g09660 being the MCM8 homolog. We sequenced the At3g09660 CDS using RT-PCR on mRNA from Arabidopsis inflorescences. Because of some differences in splicing sites, the At3g09660 CDS slightly differed from the predicted sequence found in the genebank (NM_111800), measured 2,406 bp and contained 17 exons (Figure 1) (genebank BankIt1577803 MCM8 KI09786). We nonetheless confirmed by reciprocal BLAST
analysis and multiple protein alignment that At3g09660 encodes the Arabidopsis MCM8 homolog (Figure S1 and [24]).

We identified three T-DNA insertions from the public collections within the AtMCM8 gene: Atmcm8-1, Atmcm8-2 and Atmcm8-3 (Figure 1). Plants homozygous for the insertions showed normal vegetative growth but reduced fertility as shown by Alexander staining of pollen (Figure 2). This phenotype (and others described below) was detected only in homozygotes of each mutant. Moreover seed counts showed that Atmcm8-1 has significantly less seeds than wild type (44.8 ± 5.2 (n = 41) compared to 52.4 ± 5.8 (n = 77), $z$ test $p < 10^{-11}$). Allelism tests showed that the meiotic defects observed (see below) were due to the insertions in Atmcm8.

**Atmcm8 chromosomes fragment during meiosis**

To investigate if this reduction in fertility was linked to a meiotic defect, we analyzed meiotic progression by DAPI (4',6-diamidino-2-phenylindole) staining of meiotic chromosome spreads in all three mutant alleles. In wild type meiosis (Figure 3A–3E), chromosomes condense at leptotene. Then, synapsis is initiated at zygotene until its completion in pachytene when the two homologous chromosomes are connected along their entire length by a proteinous structure called the synaptonemal complex [25] (Figure 3A and Figure 4A). Desynapsis occurs at diplotene and further condensation of the chromosomes occurs. Five bivalents continue to condense and become visible at diakinesis. At metaphase I, the five bivalents align on the metaphase I plate (Figure 3B). At anaphase I homologous chromosomes segregate to opposite poles (Figure 3C). At telophase I the two groups of five recombinant chromosomes begin to decondense. At prometaphase II chromosomes recondense and align on the two metaphase II plates (Figure 3D). At anaphase II each of the ten chromosomes segregate their two sister chromatids to opposite poles resulting in four balanced groups of five chromatids (Figure 3E).
In all three Atmcm8 alleles, meiosis appeared to progress normally from leptotene through to pachytene (Figure 3F) where chromosomes condensed, aligned and fully synapsed like wild type. The completion of synapsis in Atmcm8 was confirmed by immunolabeling meiotic chromosomes with antibodies against ASY1 and AtZYP1 (Figure 4B), that are components of the axial elements and of the transverse filament of the synaptonemal complex, respectively [26,27]. Chromosomes desynapsed normally during diplotene and we observed five bivalents as condensation progressed during diakinesis, revealing the presence of chiasmata (the cytological manifestation of CO). At metaphase I, five bivalents were systematically observed in all mutant alleles, showing that at least one CO is formed per pair of homologous chromosomes (Figure 3G). Anaphase I proceeded, however chromosome fragmentation was observed in all three Atmcm8 alleles (Figure 3H–3K), with 1 to 10 chromosome fragments detected in 60 to 80% of the cells (Figure 5). Chromosomes aligned on the metaphase II plate, with fragments dispersed throughout the cell (Figure 3L). Anaphase II proceeded but additional chromosome fragments appeared (Figure 3M–3O). This fragmentation persists at telophase II. We also observed fragmentation in female meiosis showing that Atmcm8 mutation also affects female meiosis (data not shown).

Chromosome fragmentation in Atmcm8 is dependent on meiotic DSB formation

In Atspo11-2 and Atprd1, no meiotic DSBs are formed and therefore recombination does not occur [3,28]. Thus at metaphase I, ten univalents are observed and segregate randomly (Figure 6A–6B and 6E–6F). To test whether the chromosome fragmentation seen in Atmcm8 mutants are dependent on DSB formation or not, we introduced the Atspo11-2 and Atprd1 mutations independently into Atmcm8. At meiosis, we observed ten univalents at metaphase I in the Atmcm8/Atspo11-2 or Atmcm8/Atprd1 and, importantly, the chromosome fragmentation was abolished (Figure 6C–6D and 6G–6H, Figure 5). Therefore, the fragmentation defect of Atmcm8 is dependent on AtSPO11-2 and AtPRD1. Thus, AtMCM8 is required for efficient repair of the DSBs that initiate meiotic recombination.

Atmcm8 does not affect CO frequency

We then tested if the Atmcm8 fragmentation phenotype is dependent on the presence of any of the known pathways of CO formation, using epistasis tests. We used Atmsh4 and Atzip4 that are both required for class I CO formation and Atmus81 that is required for class II CO formation. In the Atmcm8/Atmsh4, Atmcm8/Atzip4, Atmcm8/Atmus81 double mutants and the Atmcm8/Atmsh4/Atmus81 triple mutant, we still observed a chromosome fragmentation defect as in the Atmcm8 single mutant (Figure 5 and Figure 7, data not shown for Atmcm8/Atzip4). Thus the Atmcm8 fragmentation phenotype is independent of MSH4, ZIP4 and MUS81.

In Atmcm8 and Atmcm8/Atmus81 we invariably observed five bivalents at metaphase I, suggesting that the formation of class I COs, which account for most of the CO in wild type, is not grossly
affected by the \textit{Atmcm8} mutation. This was further supported by counts of \textit{AtMLH1} foci, a marker of class I COs at late prophase of meiosis I \cite{29,30} (Figure S2), that revealed no significant differences between wild type (10.1 ± 1.4 per cell; \(n = 81\)) and the \textit{Atmcm8} mutant (10.3 ± 1.9; \(n = 86\) (\(Z_p = 0.55\))). In \textit{Atmcm8/Atmsh4} (Figure 6), the residual number of bivalents at metaphase I was unchanged compared to the single \textit{Atmsh4} mutant (1.5 ± 1.0; \(n = 86\); vs 1.3 ± 1.1; \(n = 91\) (\(Z_p = 0.94\))), strongly suggesting that class II CO formation is not affected neither by \textit{Atmcm8} mutation. We then measured recombination frequency and crossover interference genetically in \textit{Atmcm8}. This was achieved using tetrad analysis (Fluorescent-Tagged Lines, FTL) which is a visual pollen assay allowing the measurement of multiple COs simultaneously with access to all four chromatids from the same meiosis \cite{31}. Two different sets of adjacent intervals on chromosome 5 have been analyzed, \{I5aI5b and I5cI5d\}, representing four intervals in total. We did not detect any difference in recombination frequency between the \textit{Atmcm8} and wild type for any of these intervals (Table 1, Genetic Distance), consistent with the cytological data. Also, interference, that affects the distribution of crossovers, was unchanged compared to wild type for both sets of adjacent

**Figure 6. Epistasis tests between \textit{Atmcm8} and two mutants affected in DSB formation.** Meiotic spreads with (A–B) \textit{Atspo11-2}, (C–D) \textit{Atmcm8/Atspo11-2}, (E–F) \textit{prd1}, (G–H) \textit{Atmcm8/Atspo11-2} using DAPI staining at anaphase I and anaphase II. Bar, 10 μm. doi:10.1371/journal.pgen.1003165.g006

**Figure 7. Epistasis tests between \textit{Atmcm8} and mutants affected in crossover formation.** Meiotic spreads with (A–C) \textit{Atmsh4}, (D–F) \textit{Atmcm8/Atmsh4}, (G–I) \textit{Atmus81}, (J–K) \textit{Atmcm8/Atmus81}, (L–O) \textit{Atmsh4/Atmus81}, (P–R) \textit{Atmcm8/Atmsh4/Atmus81} using DAPI staining at metaphase I, anaphase I and anaphase II. Bar, 10 μm. doi:10.1371/journal.pgen.1003165.g007
intervals (Table 1, Interference Ratio). Taken together these data suggest that dMCM8 is not involved in CO formation. This contrasts from the observation that the absence of MCM8 reduces COs frequency by 95% in Drosophila [24].

Meo9/Rad1 is another gene required for the formation of more than 90% of the COs in Drosophila [32]. Given the major difference in MCM8 function between Arabidopsis and Drosophila, we tested the role of AtRAD1 [33–35] in crossover formation in Arabidopsis. Cytological analysis showed that the single Atrad1 mutant has no obvious defect in CO formation. We then analyzed if AtRAD1 has a minor effect. To achieve this, we constructed a sho1/Atrad1 double mutant and a Atmcm8/sho1/Atrad1 triple mutant to be able to detect a weak reduction in CO formation, in a sensitive context where there are no class I and class II COs. However, this triple mutant was not different from Atmcm8/sho1 (0.99±0.84 (n = 74) versus 1.15±1.28 (n = 75), \( \chi^2 \) p = 0.36) and neither was sho1/Atrad1 different from sho1 (1.47±1.07 (n = 51) versus 1.56±0.86 (n = 32), \( \chi^2 \) p = 0.67). These genes, MCM8 and MEJ9/RAD1, are essential for CO formation in Drosophila but not in Arabidopsis showing divergent functions. However, contrary to RAD1, MCM8 has conserved a meiotic function in Arabidopsis.

The Atmcm8 DSB repair defect is amplified by DMC1 mutation

DMC1 is involved in the strand invasion stage of meiotic recombination and AtMCM8 mutants fail to synapse and to make COs (Figure 8A–8B, 8G–8H). However, DSBs are repaired in Atmcm8, in an AtRAD51-dependent manner, without CO formation, suggesting that the DSBs are repaired on sister chromatids in these mutants [8,12]. In the Atmcm8/Atmcm8 double mutant, from metaphase I to the end of the meiosis, we observed extensive chromosome fragmentation in all cells, which was much more intense than in the single Atmcm8 mutant (compare Figure 8C–8D to Figure 3I–3K and see quantification in Figure 5). Consistently, the Atmcm8/Atmcm8 double mutant was completely sterile whereas Atmcm8 has moderate fertility reduction and Atmcm8 produce some residual seeds [8,12] (Table 2). Mutating SPO11-2 in this Atmcm8/Atmcm8 double mutant abolished the chromosome fragmentation (Figure 8E–8F, Figure 3), demonstrating that MCM8 and DMC1 act in parallel pathways of meiotic DSB repair.

Furthermore in the Atmcm8 mutant context, we observed a more drastic meiotic chromosome fragmentation in plants heterozygous for DMC1 (Atmcm8/−/−ΔDMC1+/−) than wild type for DMC1 (Atmcm8/−/−ΔDMC1+/+) (compare Figure 8I–8J to Figure 3I–3K, quantification on Figure 5), accompanied by a strong reduction of fertility (Table 2). However, the fragmentation observed in Atmcm8/−/−ΔDMC1+/− was less dramatic than in the double mutant (Atmcm8/−/−ΔMcm8+/−) (Figure 5), which is also supported by the fertility levels (Table 2). This is despite the ΔDMC1 mutation being recessive (in an ΔMCM8+/− or ΔMCM8−/− context). Thus, in the absence of Atmcm8, the mutation of one of the two copies of DMC1 was enough to enhance fragmentation, which is even more drastic when both DMC1 alleles are disrupted.

The Atmcm8 DSB repair defect is amplified by mutation of ASY1, SDS, or one copy of RAD51

Therefore we tested the relationship of AtMCM8 with ASY1 and SDS, two proteins that are required for normal DMC1 localization [3,13]. In the sds and asy1 single mutants, COs are greatly reduced (Figure 9E–9F) [10,11]. In the Atmcm8/asy1 and Atmcm8/sds double mutant, we observed chromosome fragmentation from anaphase I onwards, which was much greater than that seen in the Atmcm8/−/− single mutant (compare Figure 9G–9H with Figure 3I–3K, quantification on Figure 5). Thus, mutation of SDS or ASY1 amplified the fragmentation phenotype of Atmcm8. Finally, both the single Atrad51 mutant and the double Atmcm8/Atrad51 mutant show intense chromosome fragmentation (Figure 10). Interestingly, while ΔRAD51/−/+ does not show chromosome fragmentation, Atmcm8/−/ΔRAD51+/− showed more chromosome fragmentation that Atmcm8 (Figure 10, Figure 5). Thus, in the absence of Atmcm8, the mutation of one of the two copies of RAD51 was enough to enhance fragmentation.

AtDMC1 foci number is unaffected in Atmcm8

Given the relationship between DMC1 functional gene copy number and the degree of Atmcm8-dependent fragmentation, we looked at DMC1 behavior in Atmcm8. No significant difference in DMC1 foci shape or number was observed in Atmcm8−/− compared to wild type (Table 2). Similarly, we did not detect any differences in number or shape of DMC1 foci in Atmcm8−/−ΔDMC1+/− or Atmcm8−/−ΔMcm8+/− compared to either wild type or Atmcm8−/− (Figure S3, Table 2). In the Atmcm8 Atrad51 double mutant, we observed a marked decrease of DMC1 foci number, which was however similar to what was previously observed in a single Atrad51 mutant [7] (Table 2). It is intriguing that Atmcm8−/−ΔDMC1+/− and Atmcm8−/−ΔRAD51+/− exhibit a more drastic meiotic defect than Atmcm8−/−ΔMcm8+/−, while DMC1 foci number and shape appear similar. However, it is

Table 1. Genetic distances and interference in Atmcm8 using FTLs.

<table>
<thead>
<tr>
<th>Genetic distance (cM)</th>
<th>Interference ratio**</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td>Atmcm8</td>
<td><strong>Wild type</strong></td>
</tr>
<tr>
<td>I5a</td>
<td>24.2±0.8</td>
<td>22.3±1</td>
</tr>
<tr>
<td>I5b</td>
<td>14.4±0.6</td>
<td>16.1±0.9</td>
</tr>
<tr>
<td>I5c</td>
<td>5.9±0.4</td>
<td>7.5±0.8</td>
</tr>
<tr>
<td>I5d</td>
<td>5.7±0.4</td>
<td>6.3±0.7</td>
</tr>
</tbody>
</table>

Values are means ± Standard Error. Number of tetrads: Wild type I5a15b n = 1986, Atmcm8 I5a15b n = 1022, wild type I5c15d n = 1860, Atmcm8 I5c15d n = 646. "Z-test between wild type and Atmcm8.

**The interference ratio is defined as the ratio of genetic distance of I5a with a CO in I5b by the genetic distance of I5a without a CO in I5b. The same was done for the interference ratio between I5c and I5d. Absence of interference would give a ratio of 1 that would tend to 0 with increased interference [57]. The chi square test shows a deviation from 1, and thus the presence of interference [31].

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possible that immunolocalization fails to detect subtle differences in DMC1 protein quantity or dynamics.

In the absence of homologous chromosomes, DSBs fail to be repaired in the absence of both MCM8 and DMC1

Next we explored the functional relationship between MCM8 and DMC1, in haploid plants, where homologous chromosomes are not present. Thus, the only template available for meiotic DSB repair is the sister chromatid. Meiotic chromosome spreads, in a wild-type haploid, showed that the five chromosomes were intact and segregated randomly at anaphase I [36] (Figure 11A–11B), suggesting that DSBs are efficiently repaired. The haploid Atmcm8 mutant had a limited fragmentation defect (Figure 11C–11D), similar to the defect in the diploid Atmcm8 mutant (Figure 5 for quantification). The Atdm1 haploid had no fragmentation (Figure 11C–11F). In clear contrast, in the double Atmcm8/Atdm1 haploid, we observed extensive meiotic chromosome fragmentation (Figure 11G–11H, see Figure 5 for quantification). This shows that in a haploid context, DSB repair is efficient in wild type and Atdm1, only slightly affected in Atmcm8, but ineffective in the Atmcm8/Atdm1 double mutant. This suggests that in the absence of a homologous template, AtMCM8 and AtDMC1 catalyze DSB repair on the sister chromatid in a redundant manner.

Discussion

Here AtMCM8 was shown to be involved in meiotic DSB repair but not CO formation. This study thus revealed a pathway for DNA DSB repair that does not yield COs. This pathway depends on AtMCM8 and acts in parallel to the AtDMC1 pathway from which COs originate.

AtMCM8 is required for efficient meiotic DSB repair but not for CO formation

Arabidopsis MCM8 is required for effective meiotic DSB repair as all Atmcm8 mutant alleles had a clear, albeit limited, chromosome fragmentation defect at meiosis. The fragmentation is dependent on meiotic DSB formation as it disappears when AtSPO11-2 or AtPRD1 is absent. However, in contrast to Drosophila re(mcm8) mutants, genetic and cytological data strongly support that CO formation is not affected by AtMCM8 mutation: (1) In the absence of AtMSH4 or AtZIP4 (class I COs) or AtMUS81 (class II COs) fragmentation still occurred and the number of bivalents was unchanged. (2) MLH1 foci numbers, a marker of class I COs, were unchanged in Atmcm8. (3) The genetic analysis using FTLs revealed no difference in terms of genetic distance and the strength of interference. These data showed that AtMCM8 acts in a pathway which repairs a subset of meiotic DSB and does not lead to CO formation.

Two pathways for DSB repair: one dependent on AtMCM8 and one on AtDMC1

A striking finding was that AtMCM8 becomes crucial when the DMC1 pathway was affected. Indeed, we observed a drastic amplification of the Atmcm8 mutant chromosome fragmentation defect when one of the two allelic copies of DMC1 was mutated, which was even more drastic when both DMC1 copies were mutated. This extensive fragmentation defect reflects a failure of DSB repair, as it is abolished by SPO11-2 mutation. Further, this extensive fragmentation was consistently confirmed in the absence of AtMCM8 and SDS, or AtMCM8 and AtS1. SDS and ASY1 are essential for AtDMC1 loading/stability [12,37]. Extensive fragmentation was also observed when one copy of RAD51 was

Figure 8. Epistasis tests between Atmcm8 and Atdm1. Meiotic spreads with (A–B) Atdm1+/−, (C–D) Atmcm8+/−/Atdm1+/−, (E–F) Atmcm8+/−/Atdm1+/−/Atspo11−/−, (G–H) Atmcm8+/−/AtDMC1+/−, (I–J) Atmcm8+/−/AtDMC1+/−/Atspo11−/−, using DAPI staining at anaphase I and anaphase II. Bar, 10 μm. doi:10.1371/journal.pgen.1003165.g008
mutated in the Atmcm8 mutant. A function of RAD51 as a cofactor of DMC1 has been recently identified in yeast [5], and consistently DMC1 foci number is drastically reduced in the Arabidopsis rad51 mutant [7,9]. We thus propose that two pathways of DSB repair coexist, one dependent on AtMCM8 and the other one on AtDMC1. In the absence of AtDMC1, efficient DSB repair occurs without CO formation. This repair depends on AtRAD51 [7,8,12] and on AtMCM8 (this study). Such RAD51-mediated, DMC1-independent repair also exists in S. cerevisiae but is normally inhibited by RAD51 regulators [38–42]. Consequently, we suggest that, in the Admcl context, AtMCM8 and AtRAD51 can cooperate to repair DSBs using the sister as a template. In addition to this function, AtRAD51 is required for the Admcl-dependent pathway (possibly as an accessory factor for the DMC1 strand-exchange activity as shown in yeast [5]) as repair is completely defective in the single Atrad51 mutant [6], like in the double Atmcm8/Atrad51 mutant.

The fact that the fragmentation defect is limited in the single Atmcm8 mutant, suggests that the AtMCM8/AtRAD51 pathway would be essential for a limited number of events in wild type, when DMC1 fails. The repair events promoted by AtMCM8 are likely not intended to become a CO, as CO formation was not affected in Atmcm8, leaving sister chromatid repair or NCOs as the only other known possibilities. The absence of synopsis in Admcl [7,8], in which the AtMCM8/AtRAD51 pathway must be active, favors the hypothesis of sister chromatid repair. In contrast, the DMC1 pathway promotes CO formation. However, DMC1 foci in wild type, outnumber COs by approximately 25 to 1 [7,43]. This suggests that repair of many DSBs catalyzed by DMC1 do not become CO, but NCO (that involve the homologous chromosome) or sister chromatid exchange (SCE). In Arabidopsis, the genome-wide frequency of NCOs and SCEs is currently unknown. We favor the hypothesis that DMC1 promotes NCOs, as DMC1 promotes synopsis. However, it should be noted that DMC1 is also able to promote SCE, notably in the haploid mcm8 context. Indeed, only the simultaneous mutation of AtDMC1 and AtMCM8 in haploids led to extensive chromosome fragmentation. The capacity of DMC1 to promote inter-sister repair was previously shown in other mutant background in both Arabidopsis [9] and yeast [44].

In summary we suggest that two pathways of DSB repair exist in wild type meiosis: The first pathway relies on the strand exchange activity of DMC1, and is also promoted by ASY1, SDS and RAD51 as a co-factor of DMC1 [5]. This pathway generates the COs, but also NCOs and SCEs in a ratio that remains to be determined. The second pathway of the model, which may be viewed as a backup pathway in case of failure of DMC1, relies on the strand exchange activity of RAD51 and the helicase activity of MCM8, and uses the sister chromatid as a template.

MCM8 function varies among eukaryotes

The function of MCM8 appears to differ markedly in Arabidopsis and in Drosophila. Interestingly, DMC1 and MCM8 appear to be partially redundant in Arabidopsis while the Drosophila genome seems devoid of a DMC1 homolog [43]. Thus CO formation in Drosophila appears to rely on a RAD51/MCM8 pathway, which has only a minor role in wild type meiotic DSB repair in Arabidopsis. The CO pathways appear to differ considerably in the two species, mainly using ZMMs in Arabidopsis but not RAD1, and

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### Table 2. Seed per fruit and fragmentation levels in different combinations of double mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seeds per fruit</th>
<th>Bivalent</th>
<th>Fragmentation</th>
<th>Number of DMC1 foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>46.5±1.8 (46)</td>
<td>yes</td>
<td>none</td>
<td>221.6±10.8 (25)</td>
</tr>
<tr>
<td>MCM8&lt;sup&gt;+/-&lt;/sup&gt;DMC1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>47.8±2.2 (51)</td>
<td>yes</td>
<td>none</td>
<td>187.3±10.6 (20)</td>
</tr>
<tr>
<td>MCM8&lt;sup&gt;-/-&lt;/sup&gt;DMC1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>49.0±1.8 (44)</td>
<td>yes</td>
<td>none</td>
<td>ND</td>
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<tr>
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<tr>
<td>mcm8&lt;sup&gt;+/+&lt;/sup&gt;DMC1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>30.5±2.2 (69)</td>
<td>yes</td>
<td>+</td>
<td>227.8±10.9 (50)</td>
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<td>7.0±1.2 (85)</td>
<td>yes</td>
<td>++</td>
<td>212.9±4.8 (87)</td>
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<tr>
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<td>1.9±0.2 (76)</td>
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<td>none</td>
<td>ND</td>
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<tr>
<td>mcm8&lt;sup&gt;-/-&lt;/sup&gt;dmc1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1.3±0.2 (65)</td>
<td>no</td>
<td>none</td>
<td>ND</td>
</tr>
<tr>
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<td>0.0±0.0 (50)</td>
<td>no</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
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<td>few</td>
<td>++</td>
<td>ND</td>
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<tr>
<th>Genotype</th>
<th>Seeds per fruit</th>
<th>Bivalent</th>
<th>Fragmentation</th>
<th>Number of DMC1 foci</th>
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<tr>
<td>wt</td>
<td>58.4±1.1 (48)</td>
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<td>none</td>
<td>221.6±10.8 (25)</td>
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<tr>
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<td>56.8±1.6 (52)</td>
<td>yes</td>
<td>none</td>
<td>202.7±6.3 (43)</td>
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<tr>
<td>MCM8&lt;sup&gt;+/-&lt;/sup&gt;RAD51&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>57.4±1.3 (50)</td>
<td>yes</td>
<td>none</td>
<td>ND</td>
</tr>
<tr>
<td>MCM8&lt;sup&gt;-/-&lt;/sup&gt;RAD51&lt;sup&gt;+/+&lt;/sup&gt;</td>
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<td>none</td>
<td>ND</td>
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<tr>
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<td>+</td>
<td>ND</td>
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<tr>
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<td>yes</td>
<td>++</td>
<td>182.5±8.0 (29)</td>
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<tr>
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<td>no</td>
<td>+++</td>
<td>50.1±11 (21)</td>
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<td>no</td>
<td>+++</td>
<td>ND</td>
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<tr>
<td>mcm8&lt;sup&gt;-/-&lt;/sup&gt;rad51&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>0.0±0.0 (50)</td>
<td>no</td>
<td>+++</td>
<td>512.2±7.0 (8)</td>
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</table>

Values are means ± Standard Error. The number of fruit or cells counted is indicated in brackets. ND: not determined, a–e: indicates significant differences among groups (Newman Keuls test, p<0.05). Number of crosses indicates fragmentation levels, based on Figure 5.

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the reverse in Drosophila, i.e. RAD1 but not ZMMs (that are absent from the Drosophila genome). Drosophila appears to be unique, as in distant species like S. cerevisiae, mammals and C. elegans CO formation depends mainly on ZMM. Adding to the complexity, MCM8 exists in mammals but not in S. cerevisiae and C. elegans [19,24]. In mouse, MCM8 mutation leads to a meiotic arrest, with defects in homologous synapsis and over-accumulation of DMC1 foci before apoptosis, suggestive of defects in DSB repair [22]. We would like to suggest that these defects may compatible with MCM8 being required for a backup pathway in the case of failure of DMC1 to repair breaks, like in Arabidopsis. The lack of the backup pathway may lead to the accumulation of DMC1 foci and a failure to repair a subset of breaks, triggering apoptosis (it is noteworthy that DSB repair defects do not trigger meiotic arrest or apoptosis in Arabidopsis). This illustrates the variety of mechanisms that arose in the course of evolution to fulfill the conserved outcome of meiotic DSB repair and CO formation.

In conclusion, our data reveals the meiotic function of MCM8 in Arabidopsis. Cytological and genetic analyses showed that ΔMCM8 is involved in DSB repair but it is not a determinant
Materials and Methods

Plant material

A. thaliana accession Columbia (Col-0) was the wild type reference. Atmcm8-1 (Salk_023274, N532764), Atmcm8-2 (Salk_104007, N604007) Atmcm8-3 (Salk_099327, N599327) were obtained from the collection of T-DNA mutants at the Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) [46] via NASC (http://nasc.nott.ac.uk/). Other mutants used in this study were Atspo1-2 (Gabrielle_749C12, N359272) [47], Atprd1 (Salk_024703, N524703) [3], Atmcm8-1 (Sail_170_F06, N871769) [48], Atmcm8-1 (Atmcm8/1) [6], atyl-1 (Salk_026722, N532764), atyl-2 (Sail_129_F09, N906294) [12], Atspo1-2 (Salk_068052, N568052) [43], AtDmc1-4 (Salk_136296, N636296) [49], mus81-2 (Salk_107515, N607515), mus81-3 (Salk_002761, N502761) [50,51], and sho1-1 (Salk_057589, N557589), rad1-1 (wkl-1) has a EMS (ethyl methanesulfonate) mutation [33,34] and was provided by C. White.

Growth conditions

Plants were cultivated in greenhouse or growth chamber with a 16 h/day and 8 h/night photoperiod, at 20°C and 70% humidity.

Genetic analysis

Allelism tests were performed by crossing Atmcm8-1/- with Atmcm8-2/- and selecting F1 plants hemizygous for both alleles and likewise for Atmcm8-2/- with Atmcm8-3/- Double mutants were obtained by crossing heterozygous plants for each mutation and selling the double heterozygous F1 plants. Atmcm8/Atmus81/Atmus81 triple mutant was identified by crossing Atmcm8/Atmus81 double heterozygous with Atmus81 single mutant. As Atmus81 and Atmus81 are linked, a plant heterozygous for Atmcm8/Atmus81 was self-fertilized and homozygous for Atmus81 to identify the triple mutant in the offspring. Haploid Atmcm8 and Atmcm8/AtDmc1 were obtained by crossing a heterozygous plant for Atmcm8 or Atmcm8/AtDmc1 mutations as male and the GEM line as female [36,52]. In F1, haploid plants of the desired genotype were selected.

Oligonucleotides for PCR genotyping

Plants of interest were selected by PCR genotyping using diagnostic primer sets. The three AtMCM8 insertions were genotyped by PCR using following primer combinations to amplify genomic DNA flanking the T-DNA insertions. Atmcm8-1: left borders (LB) with LSBalk2/5'-GGCTTTCTTCCCTTCTCTTCTCTC-3' and with LSBalk2/N359272U 5'-GGCAGCTTCACTTCTTGACAGT-3'. Wild type allele with N359272U/N359272L. Atmcm8-2: left borders (LB) with LSBalk2/N604007L 5'-TCACACAGTGACGAAAGCT-3', right borders (RB) with RBsalk1 5'-ATGACGATAGACTATGATCCGC-3' and likewise for Atmcm8-3. Atmcm8-3: left borders (LB) with LSBalk2/N349327L 5'-ATGATATGATCGACGCAAGCTGAG-3' and with RBsalk2/N349327L 5'-ATGATATGATCGACGCAAGCTGAG-3'. Wild type allele with N349327U/N349327L. T-DNA right borders were amplified using primers N524703U (5'-AGCGAGGAAAATTCAATGGAAATATGTCACGATTCTCTCTG-3') and N532764L (5'-CGCTTTCTTCCCTTCTCTTCTCTC-3'). Wild type allele with N532764U/N532764L. Atmcm8-1 triple mutant was identified by crossing Atmcm8/Atmus81 double heterozygous with Atmus81 single mutant. As Atmus81 and Atmus81 are linked, a plant heterozygous for Atmcm8/Atmus81 was self-fertilized and homozygous for Atmus81 to identify the triple mutant in the offspring. Haploid Atmcm8 and Atmcm8/AtDmc1 were obtained by crossing a heterozygous plant for Atmcm8 or Atmcm8/AtDmc1 mutations as male and the GEM line as female [36,52]. In F1, haploid plants of the desired genotype were selected.
GACGTAAGTGGAGAC-3'). We amplified SDS wild type allele using primers N060294U (5'-CTTGCCCTGTAAC-3') and N060294L (5'-CTTAAACCATGCGAAGGCA-3') and mutant allele using N060294U and LbSail. AtSDS wild type allele was amplified using primers N060296U (5'-CTTTTCTTGAGCCGTGTTTG-3') and N060296L (5'-GCCACGCTTATTTTTGTTGC-3') and mutant allele using N060296L and LbSalk2. AtMUS81A wild type allele was amplified for Salk_107515 using primers N060715U (5'-CATGTCGACGTTGAGGTC-3') and N060715L (5'-CCTCAGCTTCTCCAAAT-3') and mutant allele using N060715L and LbSalk2. AtMUS81A wild type allele was amplified for Salk_002176 using primers N502176U (5'-TACGTTTTTGGTTCCC-3') and N502176L (5'-AGTGTCAAGTCTGGTTTCC-3'). AtZIP4 wild type allele was amplified using primers N568052U (5'-TCTTCCACAGGACCTGACC-3') and N568052L (5'-GACTGGTGAGAGAAACT-3') and mutant allele using N568052L and LbSalk2. ASY1 wild type allele was amplified using primers N546272U (5'-TCTATGGTTGTAGCCGTTAATCAG-3') and N546272L (5'-AGTGGTGCTGGTCTAAGC-3') and mutant allele using N546272L and LbSalk2. SHOC1 wild type allele was amplified for Salk_002176 using primers N502176U (5'-TACGTTTTTGGTTCCC-3') and N502176L (5'-AGTGTCAAGTCTGGTTTCC-3'). AtRAD1 was amplified using primers o629 (5'-CTTGTTGAAGACATTTGCTCAG-3') and o630 (5'-CTCTATGTTTGTTGGTTGTC-3') and mutant allele using N607515L and LbSalk2. AtDde1 digestion.

Polymorphism between wild type and mutant alleles was revealed with Dde1 digestion. Fluorescent tagged lines

FTL lines were obtained from G.P. Copenhaver. For this study, we used two couple of adjacent intervals: I5aI5b and I5cI5d [31]. The protocol described by [55] was used to fluorescent tagged lines and the immunolocalization of DMC1 immunolocalization. DNA (DAPI, blue) and Figure S3

change in the overlay of both signals (merge) at diplotene in (A) wild type and in (B) Atmcm8 mutant. Bar, 10 μm.

Figure S2

Coimmunolocalization of ASY1 and AtMLH1. ASY1 (red), AtMLH1 (green) are shown as well as the overlay of both signals (merge) at zygotene in (A) wild type and in (B) Atmcm8 mutant. Bar, 10 μm.

Figure S3

DMC1 immunolocalization. DNA (DAPI, blue) and AtMCM8 (green) are shown as well as the overlay of both signals (merge) at zygotene in (A) wild type and in (B) Atmcm8 mutant. Bar, 10 μm.

Acknowledgments

We thank Christine Mézard, Mathilde Grelon, and Arnaud Demuyt for critical reading of the manuscript and helpful discussions. We thank Gregory P. Copenhaver and Charles White for generously providing the fluorescent tagged lines and the Abad1 mutant, respectively.

Author Contributions

Conceived and designed the experiments: WC VP RM. Performed the experiments: WC VP NF LC CH NV RM. Analyzed the data: WC VP RM. Wrote the paper: WC VP RM.

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