DNA methylation epigenetically silences crossover hot spots and controls chromosomal domains of meiotic recombination in *Arabidopsis*

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During meiosis, homologous chromosomes undergo crossover recombination, which is typically concentrated in narrow hot spots that are controlled by genetic and epigenetic information. *Arabidopsis* chromosomes are highly DNA methylated in the repetitive centromeres, which are also crossover-suppressed. Here we demonstrate that RNA-directed DNA methylation is sufficient to locally silence *Arabidopsis* euchromatic crossover hot spots and is associated with increased nucleosome density and H3K9me2. However, loss of CG DNA methylation maintenance in *met1* triggers epigenetic crossover remodeling at the chromosome scale, with pericentromeric decreases and euchromatic increases in recombination. We used recombination mutants that alter interfering and noninterfering crossover repair pathways (*fancm* and *zip4*) to demonstrate that remodeling primarily involves redistribution of interfering crossovers. Using whole-genome bisulfite sequencing, we show that crossover remodeling is driven by loss of CG methylation within the centromeric regions. Using cytogenetics, we profiled meiotic DNA double-strand break (DSB) loci in *met1* and found them unchanged relative to wild type. We propose that *met1* chromosome structure is altered, causing centromere-proximal DSBs to be inhibited from maturation into interfering crossovers. These data demonstrate that DNA methylation is sufficient to silence crossover hot spots and plays a key role in establishing domains of meiotic recombination along chromosomes.

**Keywords:** meiosis; crossover; hot spots; epigenetics; DNA methylation; centromeres

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Arabidopsis crossovers are generated by an interfering pathway, which causes events to be more widely distributed than would be expected if they were independent (Copenhaver et al. 2002; Higgins et al. 2004; Mercier et al. 2005). Interfering crossovers are generated by the conserved ZMM pathway (MSH4, MSH5, MMR3, HEI10, ZIP4, SHOCC1, and PTD), which is thought to stabilize strand invasion intermediates and promote dHJ formation (Copenhaver et al. 2002; Higgins et al. 2004, 2008b; Chen et al. 2005; Mercier et al. 2005; Chelysheva et al. 2007, 2010, 2012; Macaisne et al. 2008). The mechanism of crossover interference is unknown but can be modeled as mechanical stress mediated via the meiotic chromosome axis (Zhang et al. 2014). The remaining minority of Arabidopsis noninterfering crossovers is dependent on MUS81 (Berchowitz et al. 2007; Higgins et al. 2008a). The FANCM helicase acts with MHF1 and MHF2 to promote formation of noncrossovers, in the absence of which most strand invasion events enter a noninterfering crossover repair pathway (Crismani et al. 2012; Knoll et al. 2012, Girard et al. 2014). FIDGITTIN, RECQ4A, RECQ4B, over repair pathway (Crismani et al. 2012; Knoll et al. 2010),悸LLUVE, RECQ4B, and TOPOISOMERASE3a have been identified as additional anti-crossover factors in Arabidopsis (Girard et al. 2015; Séguéla-Arnaud et al. 2015).

Meiotic recombination is nonrandomly distributed along chromosomes and tends to occur in narrow hot spots, which are controlled by both genetic and epigenetic information (Lichten and Goldman 1995; Kauppi et al. 2004; de Massy 2013). Plant hot spots are observed at gene transcriptional start sites (TSSs) and transcriptional termination sites (TTSs) and require the histone variant H2A.Z for wild-type activity (Brown and Sundaresan 1991; Fu et al. 2001; Saintenac et al. 2011; Choi et al. 2013; Drouaud et al. 2013; Hellsten et al. 2013; Li et al. 2015). In contrast, crossovers are largely suppressed in repeat-rich heterochromatic regions in plant genomes (Copenhaver et al. 1999; Wei et al. 2009; Mayer et al. 2012; The Tomato Genome Consortium 2012; Yelina et al. 2012; Choulet et al. 2014; Rodgers-Melnick et al. 2015). Plant heterochromatin is densely epigenetically modified with DNA cytosine methylation and histone H3K9me2 methylation, which contribute to suppressed RNA polymerase II [Pol II] transcription, late DNA replication, cytological condensation, and higher-order structural organization (Fransz et al. 2002; Soppe et al. 2002; Zhang et al. 2006; Mathieu et al. 2007; Lister et al. 2008; Lee et al. 2010; Feng et al. 2014). DNA methylation occurs in CG, CHG, and CHH sequence contexts [where H = A, T, or C] in plant genomes (Law and Jacobsen 2010). Using this mechanism, we directly DNA-methylated endogenous Arabidopsis meiotic crossover hot spots located in euchromatin and demonstrate that this is sufficient to epigenetically silence recombination. Using chromatin immunoprecipitation (ChIP), we show that RdDM at hot spots is associated with gain of H3K9me2 and increased nucleosome occupancy. To understand how loss of CG DNA methylation maintenance causes remodeling of crossovers at the chromosome scale, we combined met1 with mutations that alter interfering and noninterfering crossover repair pathways. This demonstrates that crossover remodeling is driven by loss of interfering crossovers from pericentric meric regions and gains in the euchromatic chromosome arms. We performed whole-genome bisulfite sequencing in met1+ heterozygotes and show that crossover remodeling is driven by loss of CG context DNA methylation in the centromeric regions. Despite the extensive changes to met1 chromatin, we show that meiotic chromosomes do not form ectopic DSB foci, indicating downstream changes in crossover maturation. Together, this demonstrates that DNA methylation can potently suppress crossovers at the hot spot scale and impose regional control on recombination patterns at the chromosome scale.

Results

Silencing meiotic crossover hot spots via RdDM

Relatively low numbers of crossover events (~10) occur per meiosis in Arabidopsis (Copenhaver et al. 1998; Giraud et al. 2011; Salomé et al. 2012), and known hot spots have genetic distances of ~0.2–0.5 cM (Table 1; Drouaud and Mèzard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013). Therefore, to measure >100 events at single hot spots, it is necessary to screen hundreds of thousands of meioses. To achieve this, we collected large quantities of pollen (post-meiotic male gametes) from Col/Ler F1 hybrids and extracted genomic DNA. For a given hot spot, this DNA consists of a mixture of crossover and parental molecules, distinguishable by patterns of Col/Ler sequence polymorphisms (Drouaud and Mèzard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013). Therefor, to measure >100 events at single hot spots, it is necessary to screen hundreds of thousands of meioses. To achieve this, we collected large quantities of pollen (post-meiotic male gametes) from Col/Ler F1 hybrids and extracted genomic DNA. For a given hot spot, this DNA consists of a mixture of crossover and parental molecules, distinguishable by patterns of Col/Ler sequence polymorphisms (Drouaud and Mèzard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013). Allele-specific PCR primers that anneal to polymorphic sites can be used to amplify and titrate crossover versus parental DNA molecules (Drouaud and Mèzard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013). Dilutions of template DNA were analyzed until approximately half of the amplifications were negative,
which allowed overall lower error in estimating crossover molecule numbers [Drouaud and Mézard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013]. Single-molecule amplification products could then be genotyped via Sanger sequencing to identify internal crossover locations [Drouaud and Mézard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013].

We previously mapped two hot spots, 3a and 3b, which are located subtelomERICally within the first megabase of chromosome 3 [Fig. 1A; Yelina et al. 2012; Choi et al. 2013]. The 3a and 3b amplicons are 5.8 and 5.7 kb, respectively, and have maximum crossover rates of 83.15 and 78.70 cM/Mb, compared with the male chromosome 3 average of 4.77 cM/Mb [Fig. 1B; Supplemental Tables 1-3; Giraut et al. 2011; Yelina et al. 2012; Choi et al. 2013]. Hot spots of comparable magnitude were detected using linkage disequilibrium-based approaches that measure historical crossover activity in regions overlapping 3a and 3b [Fig. 1C; Choi et al. 2013]. The 3a–3b hot spot region has low DNA methylation [CG, 4.5%; CHG, 0.3%; CHH, 0.4%] compared with the genome average [CG, 27.0%; CHG, 8.9%; CHH, 2.9%], although an intervening gene shows CG body methylation [Fig. 1A]. This is consistent with genome-wide analysis of hot spots in Arabidopsis and maize, which show low levels of DNA methylation [Choi et al. 2013; Wijnker et al. 2013; Rodgers-Melnick et al. 2015].

To directly test the role of DNA methylation on hot spot activity, we used the RdDM pathway [Fig. 1D; Wasseneger et al. 1994; Law and Jacobsen 2010]. Transformation of Arabidopsis with inverted repeat hairpin transgenes produces dsRNAs that are processed into 21- to 24-nucleotide (nt) siRNAs [Fig. 1D; Mette et al. 2000; Zilberman et al. 2004]. These siRNAs are able to direct DNA methylation to homologous sequences in all sequence contexts [CG, CHG, and CHH] via the DRM2 de novo methyltransferase [Cao and Jacobsen 2002; Zilberman et al. 2004]. To methylate the 3a and 3b hot spots, we generated hairpin constructs driven by the ACTIN2 promoter [3a: HP1–HP4, 3b: HP5] [Fig. 1B; Supplemental Table 4]. HAIRPIN [HP] T1 transformants showed accumulation of 21- to 24-nt siRNAs detectable by Northern blotting and hybridization [Fig. 1E]. To assess RdDM at the 3a and 3b target regions in HP transformants, we used McrBC, which digests methylated DNA, followed by PCR amplification. Using McrBC assays, we detected methylation at the boundaries of the HP targeted regions within the 3a and 3b hot spots [Fig. 1F; Supplemental Table 4]. DNA methylation was detectable in HP T1 plants and increased in strength in subsequent T2 and T3 generations [Fig. 1F], consistent with promotion of RdDM during reproductive development [Teixeira et al. 2009]. All McrBC assays detected DNA methylation except in the HP5a region [Fig. 1F; Supplemental Table 4]. We selected HP plants with high levels of DNA methylation at 3a or 3b target regions for further analysis.

To measure 3a and 3b crossover rates after establishment of DNA methylation, we crossed HP1–HP5 Col lines to wild-type Ler plants. The resulting HP/Ler F1 plants were used for pollen collection and genomic DNA extraction alongside untransformed wild-type Col/Ler F1 controls. We used dilution of genomic DNA and amplification to measure the concentration of parental and crossover molecules at the 3a and 3b hot spots in HP lines [Fig. 1G; Table 1]. For the HP1–HP4 transformants that acquired DNA methylation within 3a, we observed that only HP3 and HP4 caused significant reductions in crossover frequency [nonlinear least squares model, HP3, P = 9.95 × 10−05; HP4, P = 0.00136] [Fig. 1G; Table 1]. This is consistent with the HP3 and HP4 hairpins directing DNA methylation toward 3a regions with highest recombination activity in wild type [Fig. 1B].

### Table 1. Silencing of 3a and 3b crossover hot spots using RdDM

| Genotype | Crossovers per milliliter | Parentals per milliliter | 3a | Standard deviation | 3a | 3b | Standard deviation | P |
|----------|---------------------------|--------------------------|----|--------------------|----|----|--------------------|---|
| Col/Ler  | 37.6                      | 18,596                   | 0.202 cM | 0.013 cM | 33.3 cM/Mb | —  |
| HP1/Ler  | 42.1                      | 18,639                   | 0.226 cM | 0.029 cM | 38.8 cM/Mb | 0.590 |
| HP2/Ler  | 24.4                      | 13,813                   | 0.177 cM | 0.025 cM | 30.4 cM/Mb | 0.652 |
| HP3/Ler  | 12.1                      | 17,594                   | 0.069 cM | 0.009 cM | 11.9 cM/Mb | 9.95 × 10−05 |
| HP4/Ler  | 13.0                      | 14,273                   | 0.091 cM | 0.013 cM | 15.6 cM/Mb | 0.00136 |
| HP5/Ler  | 17.6                      | 7784                     | 0.227 cM | 0.041 cM | 39.0 cM/Mb | 0.452 |

Hot spot recombination rates (centimorgans per megabase) were calculated using the ratio of crossover molecules versus parental molecules, measured per microliter of Col/Ler F1 pollen genomic DNA. To test for significant differences in recombination frequency, we compared numbers of crossover molecules in wild-type and hairpin [HP] lines by fitting a nonlinear least squares model [Bates and Watts 1988]. [n.d.] Not done.
Figure 1. RdDM silences Arabidopsis crossover hot spots. (A, top plot) Gene (green) and transposon (blue) density (base pairs [bp] per 100 bp) along Arabidopsis chromosome 3. DNA methylation (percentage; red) is shown for wild-type Col-0. The centromere is indicated by the vertical dotted line, and the 3a–3b hot spots are indicated by the vertical black line. (Bottom plot) DNA methylation frequency (percentage) within the 3a–3b region. (Blue) CG; (green) CHG; (red) CHH. (B) 3a and 3b crossover frequency (centimorgans per megabase) measured by pollen typing. Vertical black lines indicate allele-specific primer-annealing sites. Red X-axis ticks indicate Col/Ler single-nucleotide polymorphisms (SNPs), and black arrows indicate genes. RdDM hairpins (HPs) are indicated by purple and blue bars. The horizontal dotted line indicates chromosome 3 Col/Ler male average recombination (Giraut et al. 2011). (C) Historical crossover frequency within the 3a–3b region estimated from LDhat analysis of SNPs from Eurasian (blue) and Swedish (red) Arabidopsis accessions (Cao et al. 2011; Auton and McVean 2012; Choi et al. 2013; Long et al. 2013). SNP positions are indicated by X-axis ticks. Chromosome average values are indicated by the horizontal dotted lines. (D) Inverted repeat hairpin expression via RNA Pol II from the ACTIN2 promoter produces dsRNA, which is processed by DICER-LIKE (DCL) enzymes into 21- to 24-nucleotide siRNAs, which act in trans to target DNA cytosine methylation (m) to homologous genomic target sequences. (E) Total RNA was blotted and hybridized with radioactively labelled probes complementary to hairpin (HP) sequences to detect siRNAs, and the U6 nuclear RNA was used as a loading control. (F) Total genomic DNA was untreated (−) or digested with McrBC (+) and used for PCR amplification. Methylation of HP target sequences was tested in primary T1 transformants or subsequent T2 or T3 generations. The regions analyzed were HP1a, HP2b, HP3b, HP4b, and HP5b (Supplemental Table 4). The Ta3-LTR retrotransposon and At5g13440 were used as methylated and unmethylated controls, respectively. (G) Recombination rates (centimorgans per megabase) at the 3a and 3b hot spots estimated by pollen typing in wild-type Col/Ler and HP/Ler lines. Asterisks indicate HP/Ler lines with significantly different crossover frequency compared with the Col/Ler control (Table 1).
methylation within 3b directed by HP5 also caused significant suppression of recombination within this hot spot (nonlinear least squares model, \( P = 0.012 \)) [Fig. 1G; Table 1]. In budding yeast, hot spot activity shows compensation when adjacent hot spots are altered over distances of \(
\approx 60 \) kb [Robine et al. 2007]. Due to the physical proximity of 3a and 3b (\(
\approx 10 \) kb), we tested whether their activity was altered when the other hot spot was silenced. However, we did not observe significant differences in 3a when 3b was silenced or vice versa, indicating limited compensatory interactions between these loci [Fig. 1G; Table 1].

**Fine-scale mapping of crossovers and chromatin at silenced hot spots**

We next investigated the fine-scale relationships between DNA methylation and crossover recombination [Fig. 2]. We performed sodium bisulfite sequencing using DNA from closed flower buds of HP/Ler F1 lines that showed

![Figure 2](image-url)

**Figure 2.** Fine-scale patterns of DNA methylation and crossover frequency at 3a and 3b. (A) Recombination rates (centimorgans per mega-base) within the 3a hot spot analyzed by pollen typing. Vertical black lines indicate allele-specific oligonucleotide-annealing sites. Col/Ler SNPs are shown as black X-axis ticks, and genes are indicated by black arrows. Vertical blue lines indicate the HP3 RdDM targeted region. Wild-type Col/Ler recombination rates are shown in red, and those measured in HP3/Ler plants are shown in blue. The chromosome 3 Col/Ler male average recombination rate is shown by the horizontal dotted line (Giraut et al. 2011). Red rectangles indicate regions analyzed for DNA methylation. (B) As in A but for HP4/Ler F1. (C) As in B but for HP5/Ler F1 at the 3b hot spot. (D) Sodium bisulfite sequencing data showing the percentage of DNA methylation for individual cytosines, measured in the regions corresponding to the red rectangles in wild-type Col/Ler or HP3/Ler plots. (Blue) CG; (green) CHG; (red) CHH. (E) As in D but showing bisulfite analysis of HP4/Ler F1. (F) As in D but showing bisulfite analysis of HP5/Ler F1 at the 3b hot spot.
suppressed crossover frequency \(HP3, HP4,\) and \(HP5\) and compared them with untransformed Col/Ler F1 plants [Supplemental Table 4]. \(HP3, HP4,\) and \(HP5\) triggered dense DNA methylation in the targeted regions, with methylation observed in all sequence contexts, consistent with the action of RdDM [Fig. 2D–F; Supplemental Tables 5–14; Cao and Jacobsen 2002; Zilberman et al. 2004; Henderson and Jacobsen 2008]. Low levels of DNA methylation were observed in untransformed plants (Fig. 2D). As levels of CG and CHG methylation exceeded 80% in \(HP5\)/Ler F1 plants, this suggests that both Col and Ler alleles acquired DNA methylation [Fig. 2D–F; Supplemental Tables 5–14].

Limited spreading (<150 base pairs [bp]) of DNA methylation was observed outside of the hairpin targeted regions [Figs. 2D–F], consistent with the known behavior of RdDM [Pelissier et al. 1999; Zilberman et al. 2004]. The \(HP3\) and \(HP4\) hairpins directed dense DNA methylation throughout the \(3a\) targeted region of \(3b\) (Fig. 2F; Supplemental Tables 5–14).

To analyze fine-scale recombination patterns, we Sanger-sequenced \(\sim 100–200\) single-crossover-molecule PCR amplification products from \(HP3/Ler, HP4/Ler,\) and \(HP5/Ler\) F1 lines in addition to untransformed wild-type Col/Ler F1 controls [Fig. 2A–C; Supplemental Tables 1–3]. This approach identifies internal crossover locations to the resolution of individual sequence polymorphisms. The \(HP3\) and \(HP4\) hairpins targeted DNA methylation to adjacent regions within the \(3a\) hot spot, which correlated with shifted patterns of crossover activity [Fig. 2A,B; Supplemental Tables 1–2]. The \(HP5\) hairpin also directed localized crossover suppression within the \(3b\) hot spot [Fig. 2C; Supplemental Table 3]. This demonstrates that de novo DNA methylation causes specific and localized silencing of meiotic recombination in Arabidopsis. Interestingly, in wild type, we saw suppressed crossovers in a region of dense polymorphism \(3’\) to the \(3b\) hot spot and, in \(HP5/Ler\) plants, observed increased crossovers in this region [Fig. 2C; Supplemental Table 3].

To further investigate how chromatin at the \(3a\) and \(3b\) hot spots changes following RdDM, we performed ChIP analysis. As DNA methylation and H3K9me2 are strongly correlated in Arabidopsis (Bernatavichute et al. 2008), we first performed ChIP for this modification within the hot spots. We assayed chromatin from inflorescences of wild-type Col, \(HP3\) \(T3, HP4\) \(T3,\) and \(HP5\) \(T3\) lines that had acquired dense DNA methylation via RdDM [Fig. 3A; Supplemental Table 4].

**Figure 3.** Chromatin analysis of H3K9me2 and nucleosome density at the \(3a\) and \(3b\) hot spots. (A) Plots showing the \(HP\) targeted regions within the \(3a\) \(\text{top}\) and \(3b\) \(\text{bottom}\) hot spots, including the position of amplicons analyzed by ChIP-qPCR [red boxes] [Supplemental Table 4]. Vertical black lines indicate pollen-typing allele-specific primer-binding sites, and vertical blue or purple lines indicate hairpin target regions. (B) ChIP-qPCR analysis of H3K9me2 showing normalized units of PCR enrichment in wild-type Col [light blue], \(HP3\) \(T3\) [orange], \(HP4\) \(T3\) [dark blue], and \(HP5\) \(T3\) [green] lines. Values were normalized against the Ta2 transposon [Johnson et al. 2002]. (C) ChIP-qPCR analysis of unmodified H3 showing normalized units of enrichment in wild type, \(HP3\) \(T3, HP4\) \(T3,\) and \(HP5\) \(T3,\) as in B. Values were normalized against ACTIN7 [At5g09810]. Error bars represent standard deviation of biological replicates.
was performed for regions overlapping DNA methylation and normalized against the Ta2 transposon [Johnson et al. 2002]. We observed strong enrichment of H3K9me2 in HP3, HP4, and HP5 lines, overlapping the DNA methylated regions, but not in Col wild type (Fig. 3B). These increases were specific to the hot spot region targeted for DNA methylation [Fig. 3B]. To monitor nucleosome density, we also performed ChIP using antibodies that recognize unmodified H3, analyzed the same amplicons, and normalized the values against ACTIN7 [At1g09810]. We observed that the HP3, HP4, and HP5 lines showed an increase in nucleosome occupancy in the DNA/H3K9me2 methylated regions [Fig. 3C]. This is consistent with methylated regions showing higher nucleosome occupancy in Arabidopsis [Chodavarapu et al. 2010]. Together, these data demonstrate that establishment of DNA methylation via the RdDM pathway recruits H3K9me2 and causes an increase in nucleosome occupancy. It is likely that combination of these chromatin modifications contributes to suppression of hot spot recombination.

Epigenetic crossover remodeling along met1 chromosome arms

Using RdDM, we demonstrated that establishment of DNA methylation and H3K9me2 is sufficient to epigenetically silence crossover hot spots. However, at the chromosome scale, loss of CG DNA methylation in met1 and ddm1 mutants is associated with remodeling of crossovers, with increases in the euchromatic chromosome arms and decreases in the pericentromeres [Colomé-Tatché et al. 2012; Melamed-Bessudo and Levy 2012; Mirouze et al. 2012; Yelina et al. 2012]. To further characterize epigenetic remodeling along the chromosome arms, we performed genetic mapping. Wild-type or met1-3 Col/Ler F1 was used as male parents and backcrossed into Ler from Col in order to generate the met1 Col/Ler F1 used for mapping [Yelina et al. 2012]. As this causes artefactual crossover calls, chromosome 5 or the south arm alone was excluded from subsequent analysis.

We divided and orientated chromosome arms such that each began at the telomere and ended at the centromere [Fig. 4A–C]. We assigned a proportional value to each crossover along the orientated chromosome arms (excluding the south arm of chromosome 5). Crossover positional values were then binned in windows and normalized by total number of events. We also analyzed mean DNA methylation in CG, CHG, and CHH sequence contexts in 10-kb windows proportionally along the chromosome arms. In wild type, the subtelomeric (0%–0.2%) and pericentromeric (0.7%–0.9%) regions show elevated crossovers, whereas the densely methylated centromeric (>0.9%) regions are crossover-suppressed [Fig. 4A]. In met1+, we observed that CG methylation was depleted along the chromosomes and most strongly in proximity to the centromere, whereas non-CG methylation was less reduced [Fig. 4B]. In met1, the pericentromeric arm (0.7%–0.9%) regions showed a significant decrease in crossovers \(2 \times 2 \chi^2\) test, \(P = 0.01082\), whereas the subtelomeric (0%–0.2%) regions significantly increased crossover frequency \(2 \times 2 \chi^2\) test, \(P = 0.0301\) [Fig. 4B,C]. This genome-wide analysis confirms epigenetic remodeling of crossover frequency in met1 along the telomere–centromere axes of the chromosome arms, with the pericentromeric regions decreasing and the subtelomeric regions increasing recombination.

Crossover remodeling in met1 involves the interfering repair pathway

As total numbers of crossovers are similar between wild type and met1 but regional changes in recombination frequency are observed, we hypothesized that these phenomena are mediated via crossover interference [Yelina et al. 2012]. To test this idea, we combined met1 with meiotic recombination mutants where interfering and noninterfering crossover repair is altered—specifically, the zip4 ZMM mutant, which eliminates interfering crossovers [Chelysheva et al. 2007], and fancm, where noninterfering crossovers are increased at the expense of noncrossovers [Crismani et al. 2012; Knoll et al. 2012]. These mutants were combined with fluorescent FTL lines that measure crossovers within a 1.85-Mb euchromatic interstitial interval on chromosome 1 [I1b] or a 5.40-Mb centromeric/pericentromeric region of chromosome 3 [CEN3] [Fig. 4E,F; Berchwitz and Copenhaver 2008; Yelina et al. 2012]. The CEN3 interval contains a central nonrecombinating region (~12,782,751–14,750,881 bp) [Copenhaver et al. 1998, 1999; Giraut et al. 2011; Salomé et al. 2012; Yelina et al. 2012] in addition to flanking 1.67- and 1.77-Mb regions that show increasing gene density and meiotic recombination [Fig. 4F; Copenhaver et al. 1998, 1999; Giraut et al. 2011; Salomé et al. 2012; Yelina et al. 2012; Choi et al. 2013]. Due to the centromeric region within the CEN3 interval being crossover-suppressed, its overall recombination rate (2.11 cM/Mb) is lower than that of I1b (4.25 cM/Mb). Inbreeding met1 mutants causes...
Figure 4. Epigenetic remodeling along chromosome arms in met1 involves interfering crossovers. (A) Normalized wild-type (Col/Ler) crossovers [black] from a male backcross population are plotted along the proportional length of chromosome arms, orientated from telomere (TEL) to centromere (CEN). Wild-type [Col] percentage of DNA methylation is plotted on the same X-axis. (Red) CG; (green) CHG; (blue) CHH. (B) As in A except that normalized crossovers are from a met1 (Col/Ler) male backcross population, and DNA methylation is from met1-3/+ . (C) Normalized crossovers from wild-type [red] and met1 [blue] backcross populations plotted and overlaid as in A and B. (D) Violin plots showing the distribution of physical distances [in megabases] between double crossover [DCO] events in wild-type and met1 male backcross populations. Observed double crossovers [brown] are compared with distances for an equivalent set of randomly distributed sites [purple]. (E) Gene [green] and transposon [blue] sequence density [base pairs per 100 bp] along Arabidopsis chromosome 3. Wild-type percentage of total DNA methylation [red] calculated for adjacent 10-kb windows. The centromere is indicated by the vertical dotted line and the I1b fluorescent interval is indicated by vertical black lines and colored triangles. (F) As in E but for chromosome 3 and the CEN3 interval. (G) I1b genetic distances [in centimorgans] in the indicated genotypes. Replicate measurements are indicated by black dots, and mean values are indicated by red dots. (H) As in G but for CEN3 genetic distances.
progressive genome demethylation and increasingly severe developmental phenotypes, including decreased fertility [Saze et al. 2003; Mathieu et al. 2007]. We also previously observed that expression of fluorescent FTL transgenes becomes stochastic in met1 homozygotes [Yelina et al. 2012]. However, the effects of met1+/ heterozygosity on recombination are comparable with met1 homozygotes, consistent with demethylation of met1 gametophytes during haploid mitotic divisions (Saze et al. 2003; Yelina et al. 2012). Therefore, to avoid problems associated with analysis of met1 homozygotes, we analyzed crossovers in met1+/ heterozygotes.

Consistent with previous work, we observed that 11b showed a significant increase in crossover frequency in met1+/ [GLM, P < 2.0 × 10^{-16}] [Fig. 4G; Supplemental Table 17; Yelina et al. 2012]. In contrast, the zip4 mutant showed dramatic suppression of crossovers, consistent with the majority of wild-type recombination in this interval being ZMM-dependent [GLM, P < 2.0 × 10^{-16}] [Fig. 4G; Supplemental Table 17; Chelysheva et al. 2007]. Recombination was significantly suppressed in met1+/ zip4 double mutants compared with wild type [GLM, P < 2.0 × 10^{-16}] [Fig. 4G; Supplemental Table 17], which demonstrates that met1+/ 11b recombination increases are dependent on interfering crossovers. In contrast, elevated 11b crossovers observed in fancm are not suppressed in fancm zip4 double mutants due to this increase being dependent on noninterfering crossovers [Fig. 4G; Supplemental Table 17], indicating an additive effect of epigenetic remodeling by met1+/ and increased noninterfering crossovers caused by fancm. Finally, we generated fancm zip4 met1+ mutants and observed a significant reduction in crossovers relative to fancm [GLM, P = 2.77 × 10^{-9}] [Fig. 4G; Supplemental Table 17]. We propose that, due to mutation of multiple recombination and chromatin pathways in the triple mutant, a negative interaction occurs that reduces crossovers below the fancm level.

The CEN3 interval shows significant decreases in recombination in met1+/ compared with wild type, consistent with previous observations [GLM, P < 2.0 × 10^{-16}] [Fig. 4H; Supplemental Table 18; Yelina et al. 2012]. The suppression of CEN3 crossovers observed in met1+/ was not significantly different from that observed in zip4 [GLM, P = 0.141] and remained suppressed in zip4 met1+ double mutants [Fig. 4H]. We observed a significant increase in CEN3 recombination in fancm compared with wild type [GLM, P = 3.20 × 10^{-7}], which was significantly reduced in both fancm zip4 [GLM, P = 3.05 × 10^{-14}] and fancm met1+/ [GLM, P < 2.0 × 10^{-16}] [Fig. 4H]. This is consistent with met1+ causing loss of ZIP4-dependent crossovers in the CEN3 region. CEN3 crossovers were further significantly reduced in fancm zip4 met1+/ compared with fancm met1+/ [GLM, P < 2.0 × 10^{-16}] [Fig. 4H]. As for 11b, this again suggests that a negative interaction is occurring when multiple recombination and chromatin pathways are disrupted. Together, these data support that epigenetic recombination remodelling in met1 primarily reflects changes in the distribution of the interfering, ZMM-dependent crossovers.

Crossover interference is active in met1 mutants

Based on our genetic analyses, we propose that the epigenetic remodeling of crossover frequency observed in met1 is driven by changes in interfering crossovers. Therefore, we next tested whether interference strength was significantly altered in met1 mutants compared with wild type. Arrangement of three linked FTL transgenes expressing different colors of fluorescent protein allows simultaneous measurement of crossovers in adjacent intervals and calculation of interference [Berchowitz and Copenhaver 2008; Yelina et al. 2013]. Therefore, we crossed met1 to two independent three-color intervals on chromosomes 1 ([1bc] and 5 ([5ab]), both of which are located interstitially. Due to MET1 being linked to Isab, we performed experiments with this interval using met1+/ heterozygotes. We observed that all four single genetic intervals tested showed significant crossover frequency increases in met1, consistent with our previous observations at 11b [Table 2; Supplemental Table 19]. For 11bc, we did not observe a significant difference in crossover interference in met1 relative to wild type (P = 0.33) [Table 2; Supplemental Table 19]. However, for Isab, we observed a slight but significant decrease in interference in met1+/ (P = 6.5 × 10^{-6}) [Table 2; Supplemental Table 19].

As an additional measure of crossover interference, we identified 239 and 183 double crossover events in the wild-type and met1 backcross low-coverage sequencing

| Table 2. Analysis of crossover interference using meiotic tetrads in wild type and met1 |
|-----------------|---|---|---|
| 11bc            | Col | met1 | P  |
| 11b             | 7.0 cM | 9.7 cM | 4.50 × 10^{-13} |
| 11c             | 16.7 cM | 20.2 cM | 2.60 × 10^{-9} |
| 11b without adjacent crossover | 8.7 cM | 12.5 cM |
| 11b with adjacent crossover | 3.1 cM | 4.8 cM |
| Total tetrads   | 14,701 | 11,259 |
| Interference    | 0.35 | 0.38 | 0.33 |
| Isab            | Col | met1+/ | P  |
| 15a             | 25.61 cM | 31.16 cM | 1.71 × 10^{-11} |
| 15b             | 15.34 cM | 17.39 cM | 5.83 × 10^{-4} |
| 15a without adjacent crossover | 31.68 cM | 38.30 cM |
| 15a with adjacent crossover | 11.04 cM | 16.53 cM |
| Total (tetrads) | 10,733 | 10,774 |
| Interference    | 0.35 | 0.43 | 6.5 × 10^{-5} |

χ² tests were used to assess significant differences in genetic distances using the number of tetrads with one or two crossover events (1/2PD + 3NPD) [PD] parental diplo; [NPD] nonparental ditype] versus nonrecombinant tetrads. The interference significance tests were performed using Stahl Lab online tools [http://molbio.uoregon.edu/~fstahl].
data [excluding chromosome 5] [Supplemental Table 16]. Physical distances between double crossover events (mean wild type, 11.58 Mb, met1, 12.67 Mb) were greater than an equivalent set of randomly distributed events in both wild type and met1 [mean 7.41 and 6.82 Mb], as expected due to crossover interference [Fig. 4D]. Observed double crossover distances were slightly but significantly greater in met1 compared with wild type [t-test, \( P = 0.0356 \)] [Fig. 4D]. Together, these data demonstrate comparable levels of crossover interference between wild type and met1, which is further consistent with epigenetic crossover remodeling being mediated via interference.

**Loss of centromeric CG methylation drives crossover remodeling in met1**

The met1 mutant shows loss of DNA methylation in centromeric repetitive sequences in addition to dispersed repeats and gene body methylation in euchromatic regions [Cokus et al. 2008; Lister et al. 2008; Stroud et al. 2013]. We therefore sought to investigate which changes in DNA methylation drive epigenetic crossover remodeling in met1. To simultaneously measure euchromatic and heterochromatinic recombination rates, we generated a double 420-CEN3 met1 chromosome 3 reporter line [Melamed-Bessudo et al. 2005, Yelina et al. 2012]. The seed-based 420 and pollen-based CEN3 fluorescent intervals allow euchromatic and pericentromeric regions to be measured for crossovers in the same individuals [Fig. 5, Supplemental Table 20]. We crossed 420-CEN3 to naïve wild-type Col, met1/+ heterozygotes, and met1 homozygotes and measured genetic distances in the resulting F1 progeny [Fig. 5A, Supplemental Tables 20–22]. For crosses to met1/+ , we selected 420-CEN3 met1/+ F1 progeny for analysis. Consistent with previous observations, crossover frequency in the euchromatic 420 interval increased (GLM, met1/+, \( P = 2.72 \times 10^{-4} \); met1, \( P = 9.02 \times 10^{-3} \)) and the pericentromeric CEN3 interval decreased (GLM, met1/+, \( P = 7.91 \times 10^{-15} \); met1, \( P = 4.23 \times 10^{-36} \)) in met1/+ and met1 progeny compared with naïve Col [Fig. 5A, Yelina et al. 2012]. Importantly, we observed a negative correlation between 420 and CEN3 crossovers when measured in the same individuals [Spearman’s, \( r = -0.53, P = 0.0063 \)] [Supplemental Table 20]. This is consistent with compensatory recombination changes between pericentromeric and euchromatic chromosomal domains driven by interference in met1 backgrounds.

Greater variation in the 420 and CEN3 recombination rates was observed within the met1/+ individuals analyzed compared with Col-0 wild-type controls [Fig. 5A]. This is consistent with epigenetic divergence observed during met1/+ inbreeding as a consequence of MET1 function in maintaining CG methylation during the post-miotic gametophytic cell divisions [Saże et al. 2003]. Therefore, we sought to correlate variation in methylation patterns between these met1/+ individuals, as assessed by whole-genome bisulfite sequencing and meiotic recombination rates. We generated bisulfite sequencing data for a 420-CEN3 naïve control, 14 420-CEN3 met1/+ F1 individuals, and a met1/+ heterozygote control that was the progeny of a self-fertilized met1/+ plant [referred to here as met1/+ ] [Supplemental Table 20]. We obtained an average coverage of ∼13.7× for each cytosine, with DNA methylation false positive rates tested using the unmethylated chloroplast genome that were 0.31%, 0.34%, and 0.36% for CG, CHG, and CHH contexts, respectively [Supplemental Tables 23–24]. At the chromosome scale, the 420-CEN3 met1/+ individuals showed decreased CG methylation that was intermediate between the wild-type and met1/+ controls [Fig. 5B]. DNA methylation loss was less pronounced in 420-CEN3 met1/+ F1 individuals compared with the met1/+ control due to one chromosome in 420-CEN3 met1/+ F1 individuals being inherited from the wild-type parent carrying the fluorescent crossover reporters [Fig. 5B]. We observed less reduction in CHG and CHH DNA methylation in 420-CEN3 met1/+ compared with CG methylation, as expected [Fig. 5B; Cokus et al. 2008; Stroud et al. 2013]. A similar pattern was observed for CG methylation within transposable elements and gene bodies, where methylation was depleted in the 420-CEN3 met1/+ F1 individuals to a level intermediate between wild-type and met1/+ controls [Fig. 5C, Stroud et al. 2013]. Therefore, our 420-CEN3 met1/+ individuals represent a range of methylation states intermediate between wild type and met1/+, with a predominant loss of CG methylation.

To investigate the relationship between recombination and DNA methylation, we divided the 14 420-CEN3 met1/+ F1 individuals into five groups based on CEN3 genetic distances [Fig. 5E, Supplemental Table 20]. We then calculated levels of DNA methylation in CG, CHG, and CHH contexts within CEN3 and 420 and correlated these with crossover frequencies in the measured intervals [Supplemental Table 25]. We observed that CEN3 recombination and CEN3 CG DNA methylation were positively correlated [Spearman’s rank, \( r = 0.768, P = 0.0013 \)] [Fig. 5D,E; Supplemental Table 26]. Changes in recombination correlated comparatively strongly with CG methylation in the core versus flanking regions within CEN3 [left flank, \( r = 0.729, P = 0.0029 \); right flank, \( r = 0.745, P = 0.0014 \); core, \( r = 0.786, P = 0.0008 \)] [Supplemental Table 26]. This is consistent with loss of CG methylation within the centromere and surrounding regions driving changes in interfering crossovers in met1/+. No significant correlations were detected between recombination and CG methylation outside of CEN3 or within CEN3 in non-CG sequence contexts [Supplemental Table 26]. For control comparisons, we correlated CEN3 recombination with CG methylation levels in the other chromosome centromeres and with 5 Mb of randomly chosen 1-kb probes throughout the genome and observed no significant correlations [Supplemental Table 26]. We observed a significant negative correlation between 420 recombination and levels of CG methylation within CEN3 [Spearman’s rank, \( r = -0.683, P = 0.005 \)] [Fig. 5D, Supplemental Table 26], which is again consistent with demethylation of the centromeric regions driving met1/+ crossover remodeling.

We correlated 420 centimorgans with 420 DNA methylation in CG, CHG, and CHH contexts, where CG and CHH were not significant, but a positive correlation with CHG was observed \( (r = 0.542, P = 0.037) \). As a control comparison, we correlated...
420 centimorgans to CG methylation levels in two comparably sized subtelomeric regions on the north and south arms of chromosome 1 and did not observe significant relationships [Supplemental Table 26]. Together, this supports that epigenetic remodeling of interfering crossovers observed in met1 is driven by loss of CG context methylation within the centromeric and pericentromeric regions.

DNA DSB foci are unchanged in met1

To investigate the cause of crossover remodeling observed in met1, we considered whether the meiotic DSBs that initiate homologous recombination were altered. Meiotic DSBs can be analyzed cytologically using anther spreads and immunostaining for phosphorylated γ-H2A.X [Fig. 6A–F; Table 3, Supplemental Table 27; Sanchez-Moran...
et al. 2007; Ferdous et al. 2012). γ-H2A.X shows numerous foci on leptotene stage meiotic chromosomes, which are dependent on SPO11 and show numbers comparable with RAD51/DMC1 recombinase foci (Sanchez-Moran et al. 2007; Ferdous et al. 2012). At leptotene stage, meiotic chromosomes are observed to form euchromatic thin fibers connected via the meiotic axis protein ASY1, with clustered heterochromatic chromocenters containing the centromeres (Fransz et al. 2002; Ferdous et al. 2012; Yelina et al. 2012). We therefore immunostained wild-type and met1 nuclei and counted γ-H2A.X foci associated with ASY1 axial structures. In wild type, we observed a mean of 172.3 foci per nucleus with a low number of foci within the DAPI-dense chromocenters (mean = 5.8) (Fig. 6A–C; Table 3; Supplemental Fig. 2). Bars, 10 μm. (G–O) DAPI-stained meiotic chromosome spreads of wild-type (G–I), spo11-1 (J–L), and met1 spo11-1 (M–O) nuclei at late prophase I (G,J,M), metaphase I (H,K,N), and tetrad (I,L,O) stage. Bars, 10 μm.

Figure 6. Meiotic DNA DSB foci are unchanged in met1. (A–F) Localization of ASY1 [green] and γ-H2A.X [red] in wild-type (A–C) and met1-3 (D–F) nuclei at leptotene. DNA was stained with DAPI [blue]. Densely DAPI-staining chromocenters are marked with white arrowheads. The inset boxes show magnifications of the centromeric regions, with yellow arrowheads marking γ-H2A.X foci localized within the DAPI-dense regions (Supplemental Fig. 2). Bars, 10 μm. (G–O) DAPI-stained meiotic chromosome spreads of wild-type (G–I), spo11-1 (J–L), and met1 spo11-1 (M–O) nuclei at late prophase I (G,J,M), metaphase I (H,K,N), and tetrad (I,L,O) stage. Bars, 10 μm.

Loss of DNA methylation in met1 and ddm1 is known to cause transcriptional up-regulation and mobilization of transposable elements (Miura et al. 2001; Zhang et al. 2006; Lister et al. 2008). As transposition can generate DSBs, we considered whether this phenomenon could contribute to met1 meiotic phenotypes. For example, DSBs from exogenous DNA damage agents [e.g., cisplatin] or
X-rays can promote crossovers in Arabidopsis and Caenorhabditis elegans, respectively (Sanchez-Moran et al. 2007; Youds et al. 2010). The spo11-1 mutant is almost completely sterile due to a failure in homologous chromosome pairing and synapsis caused by an absence of meiotic DSBs (Grelon et al. 2001). Therefore, if met1 generates ectopic meiotic DSBs that result in crossovers, then spo11-1 met1 fertility should increase. We compared seed set in spo11-1 and spo11-1 met1 double mutants and did not observe an increase in fertility [Supplemental Table 28]. We also performed DAPI staining on meiotic nuclei from anther spreads (Fig. 6G–O). In wild type, paired homologous chromosomes align along the metaphase I plate as five bivalents connected via chiasmata [Fig. 6H]. In spo11-1 mutants, 10 unpaired univalents are observed at the same stage [Fig. 6K, Grelon et al. 2001]. The spo11-1 met1 double mutant showed patterns of univalent segregation comparable with spo11-1 single mutants [Fig. 6N]. Together, this indicates that any transposition-associated DSBs occurring in met1 are unable to drive meiotic recombination and crossover formation to a significant level.

Discussion

We demonstrated that establishment of DNA methylation via the RdDM pathway is sufficient to potently suppress euchromatic meiotic recombination hot spots in Arabidopsis. This is consistent with low levels of DNA methylation observed at Arabidopsis and maize crossover hot spots (Choi et al. 2013; Wijker et al. 2013; Rodgers-Melnick et al. 2015) and the widespread suppression of recombination in plant heterochromatin (Copenhaver et al. 1999; Fu et al. 2002; Liu et al. 2009; Giraut et al. 2011; Mayer et al. 2012; Salomé et al. 2012; The Tomato Genome Consortium 2012; Bauer et al. 2013; Choulet et al. 2014; Li et al. 2015; Rodgers-Melnick et al. 2015). We show that RdDM is associated with acquisition of H3K9me2 and increased nucleosome occupancy at the targeted hot spots. Therefore, the combination of these chromatin modifications is likely to suppress recombination [Fig. 7A]. As hot spots associate with promoter-associated chromatin marks, including H3K4me3 and H2A.Z, RdDM may also exclude these modifications and thereby reduce crossovers [Liu et al. 2009; Choi et al. 2013; Wijker et al. 2013]. It is also important to consider that hot spots may vary in both their sensitivity to RdDM and the effect of methylation on crossovers, depending on their location in euchromatic versus pericentromeric regions.

As DNA methylation locally inhibits crossover hot spots, this leads to a prediction that loss of CG methylation in met1 centromeric/pericentromeric regions would up-regulate crossovers. However, the opposite is true, with met1 pericentromeric regions showing reduced crossovers, coupled to compensatory increases in the chromosome arms (Colomé-Tatché et al. 2012; Melamed-Besudo and Levy 2012; Mirouze et al. 2012; Yelina et al. 2012). We explain these observations, as the hot spot–RdDM and met1 experiments are not exact reciprocals of one another in terms of epigenetic change. For example, we show that RdDM establishes CG and non-CG DNA methylation, H3K9me2, and increased nucleosome occupancy at the 3a and 3b hot spots, causing suppressed recombination [Fig. 7A]. However, in met1, while CG-context DNA methylation is strongly reduced, substantial non-CG methylation and H3K9me2 remain [Fig. 7B; Cokus et al. 2008; Lister et al. 2008; Deleris et al. 2012; Stroud et al. 2013]. Therefore, to understand these interactions, it will be necessary to obtain further recombination measurements in mutants with different alterations to heterochromatin.

In order to understand meiotic recombination patterns in wild type versus met1, it is also important to consider higher-order chromosome structure. For example, Arabidopsis centromeres physically interact with each other in Arabidopsis somatic nuclei, which is cytologically manifested as the DAPI-dense chromatons from which euchromatin loops emanate [Fransz et al. 2002; Soppe et al. 2002, 2003; Moissiard et al. 2012; Feng et al. 2014; Grob et al. 2014; Wang et al. 2014]. In met1 and ddm1, the chromatons become cytologically decondensed and are observed to interact with euchromatin to a greater extent, as measured by Hi-C [chromosome capture followed by high-throughput sequencing] [Soppe et al. 2002, 2003; Probst et al. 2003; Mathieu et al. 2007; Moissiard et al. 2012; Feng et al. 2014]. It is presently unclear how these changes to higher-order chromosome organization will interact with the chromatin loop–axis arrangement of meiotic chromosomes to influence recombination [Kleckner 2006; Storlazzi et al. 2010; Panizza et al. 2011; Ferdous et al. 2012]. We propose two models to explain crossover remodeling in met1 [Fig. 7B]. First, although the spatial distribution of DSB foci is unchanged in met1, the relative timing of DSB formation between euchromatin and heterochromatin may be altered. For example, it is known that replication occurs later in Arabidopsis heterochromatin [Lee et al. 2010] and that replication is tightly coupled to progression of meiotic recombination in budding yeast [Borde et al. 2000; Murakami and Keeney

Table 3. γ-H2A.X foci counts in wild-type and met1 leptotene nuclei

| Genotype | γ-H2A.X foci (total, mean) | γ-H2A.X foci (total, standard deviation) | γ-H2A.X foci (chromocenters, mean) | γ-H2A.X foci (chromocenters, standard deviation) |
|----------|--------------------------|------------------------------------------|-----------------------------------|-----------------------------------------------|
| Col      | 172.3                    | 27.2                                     | 5.81                              | 4.1                                           |
| met1-3   | 176.5                    | 39.4                                     | 5.25                              | 3.4                                           |

Total foci number are listed in addition to those located within the densely DAPI-staining chromocenters, which contain the centromeres (Yelina et al. 2012).
Therefore, it is possible that *met1* could alter the relative timing of replication between heterochromatin and euchromatin and thereby influence patterns of crossover designation. Second, we propose that loss of CG methylation alters *met1* chromosome structure, including its interaction with the meiotic axis, such that interhomolog invasion sites closer to the centromere are inhibited from crossover designation. As total crossovers are similar in number in *met1* and as interference is active, crossovers are favored in the gene-rich arms (Fig. 7B).

Together, our results highlight the importance of epigenetic information for determining distributions of meiotic recombination along eukaryotic chromosomes. We demonstrate that analyzing the roles of chromatin marks at both the whole chromosome and hot spot scales is important. Although DNA methylation is widely conserved, its distribution and function between eukaryotes vary, and it has been lost from several model species [Feng et al. 2010; Zemach et al. 2010]. For example, within fungi, DNA methylation strongly suppresses crossovers in *Ascobolus immersus* [Maloisel and Rossignol 1998], whereas this modification is absent in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. However, in fission yeast, other heterochromatic marks, including H3K9me2, mediate a repressive effect on meiotic crossovers [Ellermeier et al. 2010]. Therefore, it will be important to study the interactions of DNA methylation and additional markers of heterochromatin in multiple species to more completely understand control of meiotic recombination by epigenetic information.
Materials and methods

\textbf{Plant material}

\textit{Arabidopsis} lines used in this study were Col-0, Ler-0, \textit{met1-3} (Saze et al. 2003), \textit{fancm-1 zip4-2} (Crismani et al. 2012), and \textit{spo11-1-3} (SALK_146172) (Stacey et al. 2006). Plants were grown at 20°C and 60% humidity with a long day photoperiod (16 h light) and light intensity of 150 \textmu mol. Genotyping of \textit{met1-3} was performed by PCR amplification using \textit{met1-3-F} and \textit{met1-3-R} oligonucleotides for the T-DNA alleles. Genotyping for \textit{fancm-1} was carried out by PCR amplification using fancm-F and fancm-R oligonucleotides followed by MboII restriction endonuclease digestion. PCR fragments corresponding to \textit{RdDM} as described (Stacey et al. 2006) for the \textit{spo11-1-3} alleles. Genotyping for \textit{spo11-1-3} was performed as described (Stacey et al. 2006).

\textbf{Northern blot and hybridization analysis of small RNAs}

Five to seven inflorescences were collected per genotype, and total RNA was extracted using TRI reagent (Sigma). Ten micrograms of total RNA was separated on 15\% polyacrylamide/8 M urea gels, blotted on Zeta probe membrane (Bio-Rad), and hybridized with \textsuperscript{32}P-labeled probes synthesized using Rediprime II kits (Amersham Biosciences). Hybridization was carried out overnight at 37°C in PerfectHyb Plus hybridization buffer (Sigma). Templates for \textsuperscript{32}P-labeled probes were obtained by PCR amplification of \textit{Arabidopsis} genomic DNA.

\textbf{DNA methylation analysis}

DNA isolated from \textit{Arabidopsis} closed flower buds was used for McrBC analysis. One-hundred nanograms of DNA was digested with 10 U of McrBC (New England Biolabs) for 6 h at 37°C. The enzyme was heat-inactivated for 20 min at 65°C. Two nanograms of McrBC-digested DNA was used as a template for PCR amplification. For Sanger sequencing analysis, DNA sodium bisulfite conversion was carried out using an EZ DNA methylation gold kit (Zymo Research). Prior to conversion, DNA was digested at the restriction sites introduced into the hairpin arms in order to enrich for endogenous target sequences. Following conversion, DNA was PCR-amplified using oligonucleotides designed as described (Henderson et al. 2010). PCR products were cloned into pGem-T Easy (Promega), and 15–26 individual clones were Sanger-sequenced to determine the percentage of DNA methylation for cistogenes in the amplified regions (Gruntman et al. 2008). For genome-wide bisulfite sequencing, DNA was isolated from leaves of 3- to 5-wk-old \textit{Arabidopsis} plants. One microgram of genomic DNA was sheared to yield fragments with an average size of 340 bp using a Covaris E220 instrument and purified using Agencourt AMPure XP beads (ratio 1.8×; Beckman Coulter). DNA was end-repaired and A-tailed using T4 DNA polymerase and Klenow fragment (New England Biolabs) and purified again using XP beads (ratio 1.8×). Finally, double-stranded paired-end adapters were ligated using quick-stick ligase (Bioline), and DNA was purified using XP beads (ratio 1.8×). Four-hundred-fifty nanograms of DNA was used for sodium bisulfite conversion using an EZ DNA methylation gold kit. DNA was quantified with a Bioanalyzer 2100 using the high-sensitivity DNA kit (Agilent Technologies) and size-selected to yield fragments with the average size of 400 bp using XP beads (ratio 0.8×). DNA was barcoded using PCR amplification with KAPA HiFi HotStart uracil+ ready mix (Kapabiosystems) with PE1.0 and one of the iPCRtag 1–17 oligonucleotides and then purified using XP beads (ratio 1×), and libraries were quantified on a Bioanalyzer 2100. Final libraries were mixed in equal molar ratios to a final concentration of 10 nM and used for 150-bp paired-end sequencing on a HiSeq 2000 instrument. FastQ files were analyzed and DNA methylation was identified using Bismark (Krueger and Andrews 2011) and SeqMonk (http://www.bioinformatics.bbsrc.ac.uk/projects/ seqmonk).

\textbf{Pollen typing}

Pollen typing was performed as previously described (Drouaud and Mézard 2011; Yelina et al. 2012; Choi et al. 2013; Drouaud et al. 2013). Genomic DNA was isolated from Col/Ler F\textsubscript{1} or HP/Ler F\textsubscript{1} pollen and used for nested PCR amplifications using parental or crossover configurations of allele-specific oligonucleotide primers (Yelina et al. 2012; Choi et al. 2013). To test for significant differences in recombination frequency, we compared numbers of crossover molecules in wild-type and hairpin lines by fitting a nonlinear least squares model [Bates and Watts 1998]. The model fit is given by $\text{log}(X_{ijk}) = \text{parental} + \text{crossover} \times \text{strain effect} - \text{HP} + E_{ijk}$, where $X_{ijk}$ is the log of the number of estimated molecules for the $i$th observation, with the index $j$ identifying whether the estimation is for the parental or crossover molecules, and the index $k$ indicating whether the estimation is for wild-type or HP strains. The model thus describes for each observation a strain effect ($S_k$). If the observation is for the parental molecules, a parental effect ($P$) is added, if the observation is for crossover molecules, the parental effect is modified by a crossover factor ($Z \sim z \times \text{mutant}[k]$, where $z$ represents the change in the crossover factor attributed to the HP line. The key result in the model fit for evaluating whether the number of crossovers is influenced by the hairpin is thus to evaluate whether $z$ significantly deviates from 0.

\textbf{H3 and H3K9me2 ChIP}

ChIP was performed as described (Johnson et al. 2002). Five micrograms of antibodies ab1220 (Abcam) and ab1791 (Abcam) was used to ChIP H3K9me2 and unmodified H3, respectively. Primer sequences used for ChiP-qPCR are provided in Supplemental Table 29. Two biological replicates were performed for each experiment, and each was analyzed using two technical replicates.

\textbf{Mapping crossovers via low-coverage sequencing}

We applied a method to identify crossovers from low-coverage (<0.5×–2×) sequencing, using 96 barcoded genomic DNA libraries generated from recombinant individuals (Rowan et al. 2015) with the following modifications. DNA was digested with 0.3 U of dsDNA Shearase (Zymo Research) in a final volume of 15 μL.
The resulting DNA fragments were end- repaired with 3 U of T4 DNA polymerase [New England Biolabs], 10 U of T4 polynucleotide kinase [Thermo Fisher Scientific], and 1.25 U of Klenow fragment [New England Biolabs] in the presence of 0.4 mM dNTPs in a reaction volume of 50 µL for 30 min at 20°C. DNA fragments were cleaned as described (Rowan et al. 2015), and the protocol was followed until the DNA fragment size selection step. To size-select, following barcoded Illumina adaptor ligation, 30 µL of a mixture of eight concentrated DNA libraries was combined in a tube containing 48 µL of a 1:1 mix of AMPure XP magnetic SPRI beads [Beckman-Coulter; referred to as SPRI beads] in water. After 5 min of incubation at room temperature, the samples were placed on a magnetic rack and allowed to clear before supernatant was transferred to a fresh tube and mixed with 0.12 vol of undiluted SPRI beads. After 5 min of incubation at room temperature, the tubes were placed on a magnetic rack and allowed to clear. The supernatants were discarded, and the beads were washed twice with 80% ethanol. DNA was eluted in 20 µL of 10 mM Tris [pH 8.0]. Twelve microliters of the eluate was used for PCR amplification in a reaction volume of 50 µL using KAPA HiFi Hot-Start ReadyMix PCR kit [Kapabiosystems] and the reported DNA oligonucleotides [Rowan et al. 2015]. Twelve cycles of amplification were performed, and PCR products were then purified on SPRI beads [1× ratio] and quantified with a Bioanalyzer. The resulting libraries were subjected to paired-end 150-bp sequencing on an Illumina NextSeq instrument.

We pooled data from 94 wild-type libraries and aligned reads to the TAIR10 Col genome assembly using Bowtie 2 (Langmead and Salzberg 2012). Variant sites were called using SAMTools and BCFtools (Li et al. 2009). Sites were filtered to remove those with qualities <100 and those with >2.5× mean coverage and repeat-masked as described previously (Choi et al. 2013). Sites were also intersected with an independent set of high-quality Ler SNP calls provided by K. Schneeberger, yielding a final set of 153,842 SNPs. For each barcoded library, the number of reads supporting reference versus variant bases at these sites was obtained and used as input for the TIGER pipeline, and the crossovers identified were used for subsequent analysis (Rowan et al. 2015). To test for significant differences in crossovers between wild type and met1, we used a GLM assuming a negative binomial distribution. To analyze chromosomal distributions, chromosomes were split and orientated such that they began at the telomere and ended at the centromere. Each crossover was assigned a proportional distance value along the chromosome arms.

To test whether recombinant and nonrecombinant counts were significantly different between replicate groups, we used a GLM. We assumed the count data are binomially distributed—\( Y_i \sim \text{Bin}(n_i, p_i) \), where \( Y_i \) represents the recombinant counts, and \( n_i \) is the total counts—and modeled the proportions \( \hat{Y}_i/n_i \). Then, \( E[Y_i/n_i = p_i] \) and

\[
\text{var}(Y_i/n_i) = \frac{p_i(1 - p_i)}{n_i}.
\]

Thus, our variance function is \( V[\mu_i] = \mu_i(1 - \mu_i) \), and our link function must map from \([0, 1] \rightarrow [-\infty, \infty]\). We used a logistic link function, which is

\[
g(\mu_i) = \logit(\mu_i) = \log \frac{\mu_i}{1 - \mu_i} = \beta X + e_i,
\]

where \( e_i \sim \mathcal{N}(0, \sigma^2) \). Both replicates and genotypes were treated as independent variables \( \{x\} \) in our model. We considered \( P \)-values <0.05 as significant.

Cytological analysis

Chromosome spreads of Arabidopsis pollen mother cells [PMCs] were immunostained as described (Ferdous et al. 2012). The following antibodies were used for immunostaining: a-ASY1 [rat, 1/500 dilution] [Armstrong et al. 2002] and a-YH2A.X [Ser 139, rabbit, 1/100 dilution, Upstate Biotechnology, catalog no. 07-164]. Microscopy was conducted using a DeltaVision Personal DV microscope [Applied Precision/GE Healthcare] equipped with a CCD Coolsnap HQ2 camera [Photometrics]. Image capture, image analysis, and processing were performed using SoftWoRx software version 5.5 [Applied Precision/GE Healthcare]. The images were deconvolved using the function “Mexican hat” of SoftWoRx software version 5.5. Deconvolution improved the contrast and resolution of images, allowing better visualization of the signal.

Accession numbers

Accession numbers for the next-generation sequencing data in this study have been uploaded to EMBL European Bioinformatics Institute Annotare [https://www.ebi.ac.uk/fg/annotare] and are as follows: for whole-genome bisulphite sequencing of met1-3/+, ArrayExpress accession E-MTAB-3927; for mapping meiotic crossovers in Arabidopsis using low-coverage whole-genome sequencing, ArrayExpress accession E-MTAB-3926.

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