HIGH Crossover RATE1 encodes PROTEIN PHOSPHATASE X1 and restricts meiotic crossovers in Arabidopsis

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Meiotic crossovers are tightly restricted in most eukaryotes, despite an excess of initiating DNA double-strand breaks. The majority of plant crossovers are dependent on class I interfering repair, with a minority formed via the class II pathway. Class II repair is limited by anti-recombination pathways; however, similar pathways repressing class I crossovers have not been identified. Here, we performed a forward genetic screen in Arabidopsis using fluorescent crossover reporters to identify mutants with increased or decreased recombination frequency. We identified HIGH CROSSOVER RATE1 (HCR1) as repressing crossovers and encoding PROTEIN PHOSPHATASE X1. Genome-wide analysis showed that hcr1 crossovers are increased in the distal chromosome arms. MLH1 foci significantly increase in hcr1 and crossover interference decreases, demonstrating an effect on class I repair. Consistently, yeast two-hybrid and in planta assays show interaction between HCR1 and class I proteins, including HEI10, PTD, MSH5 and MLH1. We propose that HCR1 plays a major role in opposition to pro-recombination kinases to restrict crossovers in Arabidopsis.

Meiosis is a specialized type of cell division occurring in eukaryotes, in which a single round of DNA replication is coupled to two rounds of chromosome segregation, generating haploid cells that can undergo sexual fusion. During meiotic prophase I, homologous chromosomes pair and undergo programmed recombination, which can produce reciprocal crossovers between chromosomes. Meiotic recombination and chromosome segregation cause the haploid gametes to be genetically mosaic. As a consequence, sex has a profound effect on genetic variation and adaptation.

Meiotic recombination initiates with the formation of DNA double-strand breaks (DSBs), via the conserved topoisomerase-related protein SPO11. In plants, SPO11-1 and SPO11-2 form a heterotetramer with MEIOTIC TOPOISOMERASE VIB-LIKE (MTOPVIB) to generate meiotic DSBs. Mutation of spo11-1, spo11-2 or mtopyvib prevents homologue pairing, causing univalent segregation at metaphase I and aneuploid gametes in Arabidopsis. Meiotic DSBs are resected to generate single-stranded DNA (ssDNA) that is bound by the RecA-related proteins DMC1 and RAD51. DMC1–RAD51 nucleofilaments mediate interhomologue strand invasion to form displacement loops. In wild-type Arabidopsis, around 150–250 DSB-associated foci are evident along the meiotic chromosome axis when DMC1, RAD51, RPA1a and yH2A.X are immunostained during early meiotic prophase. In wild-type Arabidopsis, only around ten of these DSBs are ultimately repaired as interhomologous crossovers. The remaining strand invasion events are disassembled by non-crossover pathways, which include FANCM, RECQ4A, RECQ4B and FIGLI. Meiotic DSBs may also be repaired using the sister chromatid.

In plants, the major pathway generating crossovers is termed class I (also known as the ZMM pathway). Class I crossovers show interference, meaning they are more widely spaced than expected by chance. In plants, around 80–85% of crossovers are dependent on the class I pathway, which includes MSH4, MSH5, ZIP4, SHOC1, PTD, HEI10, HEIP1, MERA3, MLH1 and MLH3. The class I pathway functions to stabilize interhomologue joint molecules and promotes crossover resolution via double Holliday junctions. Within this pathway; MSH4 and MSH5 form the MutSy heterodimer that associates with meiotic chromosomes and stabilizes interhomologue joint molecules. SHOC1 and PTD form a catalytically inactive XPF–ERCC1 endonuclease-related complex that has affinity for joint molecules. A HEI10 orthologue, has been shown to be a target of Mec1/Tel1 kinase activity.

In anti-recombination pathway mutants, for example recq4a recq4b, there are large increases in the numbers of class II crossovers. Progression of the meiotic cell cycle and recombination are regulated by multiple protein kinase pathways, whose targets include DSB proteins, the class I pathway and the chromosome axis.

In mammals and budding yeast, the ATM/ATR (also known as Mec1/Tel1) DNA-damage kinases are activated by meiotic DSBs, and mediate feedback signalling on recombination in cis and trans. Zip3, a HEI10 orthologue, has been shown to be a target of Mec1/Tel1 in budding yeast; it is antagonized by the PPH3–PP4 protein.

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A forward genetic screen for mutants with altered meiotic crossover frequency. To isolate factors controlling meiotic crossover frequency we performed a forward genetic screen in Arabidopsis thaliana (Fig. 1a). We made use of fluorescent reporters of crossover frequency, which consist of linked fluorescent tagged lines (FTL)/Col traffic lines (CTL) T-DNA insertions expressing fluorescent proteins of different colours in the seed (under control of the NapA promoter) or pollen (under the LAT52 promoter) (Fig. 1b). When FTLs are hemizygous, inheritance of fluorescence can be used to score crossover frequency within the interval defined by the T-DNAS (Fig. 1b). We selected the 420 FTL for mutagenesis, which defines a 5.1 megabase interval located in the left sub-telomeric region of chromosome 3 (Fig. 1a,b). 420 was selected for mutagenesis, as crossover frequency in this region is known to be sensitive to multiple recombination and chromatin pathways.

We generated approximately 10,000 420/++ hemizygous seeds by crossing, and used them for ethyl methanesulfonate (EMS) mutagenesis (Fig. 1a). From these seeds, about 7,000 M1 plants were grown and M2 seeds were collected (Fig. 1a). The seeds from 12 independent M1 plants were combined to generate about 600 M2 pools (Fig. 1a), and seeds within these pools were pre-selected to be red–green fluorescent (420/++) (Fig. 1b). Approximately 150 pre-selected seeds were grown from each M2 pool and allowed to self-fertilize (Fig. 1a). Seeds from individual M3 plants were used to score crossover frequency within 420 (Fig. 1a,b). In our growth conditions, 420 in self-fertilized wild-type Col/Col inbred plants shows a mean crossover frequency of 20.19 cM (s.d. 1.43 cM) (Fig. 1c and Supplementary Table 1). In total, 2,883 M3 individuals were screened and the majority (81.4%) showed 420 crossover frequency within the range of 18–22 cM (Extended Data Fig. 1). Nineteen putative high or low crossover-frequency mutants were self-fertilized and M1 progeny were tested for 420 crossover frequency, of which 5 were confirmed to show a heritable recombination phenotype in the next generation (Fig. 1a).
HIGHER CROSSTYPE RATES1 encodes PROTEIN PHOSPHATASE X1. We used PCR with reverse transcription (RT–PCR) to amplify and sequence PPX1 mRNA from hcr1 plants, revealing retention of intron 3, causing a premature stop codon (Fig. 2c and Extended Data Fig. 3). The stop codon is predicted to truncate PPX1 (retaining 143 of 305 residues) and remove conserved metal-binding histidine residues in the C-terminal region (Fig. 2c and Extended Data Fig. 4a). However, the truncated protein has the potential to encode three of the four conserved PPX1 catalytic motifs (GDXHG, GDXVDRG and GNHE) in the N-terminal region (Fig. 2c and Extended Data Fig. 4a). PPX1 is the catalytic subunit of the hetero-multimeric PP4 serine/threonine protein phosphatase complex, which includes two additional regulatory subunits (PP4R2 and PP4R3) (Fig. 2d). PP4 complexes have multiple roles in mitotic and meiotic DNA recombination and repair in diverse eukaryotes (26–28).

To demonstrate whether the splice acceptor mutation in PPX1 causes the hcr1 420 crossover phenotype, we performed a complementation test (Fig. 2f and Supplementary Table 5). A 4.515-bp genomic fragment containing the PPX1 gene was amplified by PCR from wild type (Col) and inserted into an Agrobacterium binary vector and used to transform hcr1 420/++ plants. We observed that the hcr1 plants transformed with PPX1, but not those transformed with empty vector, showed 420 crossover frequency not significantly different to the wild type (Welch’s t-test, P = 0.357) (Fig. 2f and Supplementary Table 5). We obtained a second T-DNA insertion (GK_651B07) mutation in PPX1, located in the 5′-untranslated region (UTR), which we term hcr1-2, and term the EMS allele hcr1-1 (Fig. 2c,g and Supplementary Table 6). We measured 420 crossover frequency in hcr1-2 homozygotes and observed a significant increase compared to wild type (Welch’s t-test, P = 5.43 × 10⁻⁶) (Fig. 2g and Supplementary Table 6), although the phenotype was weaker than that of hcr1-1 mutants. We crossed hcr1-1 with hcr1-2 to generate hcr1-1/hcr1-2 F1 hybrids, which showed significantly higher 420 crossovers compared with wild type, demonstrating allelism (Welch’s t-test, P = 4.91 × 10⁻¹⁰) (Fig. 2g and Supplementary Table 6). Together, these genetic data identify PPX1 as HCR1.

The Arabidopsis genome encodes a second PP4C catalytic subunit gene, PPX2 (At5g55260), which shows 93.8% amino acid sequence identity to PPX1 (Fig. 2d and Extended Data Fig. 4). Functional redundancy between Arabidopsis PPX1 and PPX2 has been observed (29). We obtained a T-DNA insertion in PPX2 (GK_488H09), which disrupts mRNA expression, but did not observe a significant effect on 420 crossovers, compared to wild type (Welch’s t-test, P = 0.119) (Fig. 2h, Extended Data Fig. 3a,b and Supplementary Table 7). However, hcr1-2 ppx2-1 double mutants showed a significant increase in 420 crossovers compared with hcr1-2 (Welch’s t-test, P = 1.42 × 10⁻⁴) (Fig. 2h and Supplementary Table 7). We also crossed hcr1-1 with a second ppx2 T-DNA insertion allele (ppx2-2) but did not obtain any hcr1-1 ppx2-2 double mutants in the F2 generation. As the siliques of hcr1-1/++ ppx2-2/++ plants contained aborted seeds that were not seen in wild-type controls, these data suggest that the double mutant is embryo or seedling lethal (Extended Data Fig. 3d–f). Arabidopsis encodes a single gene for the PP4R2 regulatory subunit (At1g17070), and we obtained a T-DNA insertion that disrupts mRNA expression of this gene (Extended Data Fig. 3a,b). We observed that pp4r2 shows a significant increase in 420 crossover frequency, compared to wild type (Welch’s t-test, P = 4.24 × 10⁻⁴), with a similar phenotypic strength to hcr1-2 (Fig. 2h and Supplementary Table 7). As pp4r2 mutants are viable, this indicates that the T-DNA insertion is likely to be hypomorphic. Together, these results are consistent with HCR1/PPX1 and PPX2 acting in PP4 complexes with PP4R2 to repress meiotic crossovers in Arabidopsis. We also note that recent mass spectroscopy data from Arabidopsis has confirmed the presence of HCR1/PPX1, PPX2, PP4R2L and PP4R3A complexes in vivo (30).

Meiosis-specific knockdown of HCR1/PPX1 and PPX2 using meiMIGS. Our genetic analysis indicates functional redundancy between PPX1 and PPX2 (Fig. 2h). This is consistent with null ppx1 ppx2 double mutants causing severe developmental phenotypes that are not observed in the single mutants (31). Therefore, we sought to silence both PPX1 and PPX2 specifically during meiosis. For this purpose, we adapted miRNA-induced gene silencing (MIGS) for use during meiosis (32). In MIGS constructs, a microRNA173 (miR173) target site is inserted upstream of target transcript sequences (33). Transcription cleavage of the fusion RNA by endogenous miR173 is an efficient trigger of 22-nucleotide trans-acting siRNAs, which act to silence endogenous gene transcripts that share sequence homology in trans (34). To drive MIGS specifically during meiosis (meiMIGS), we expressed miRNA173-targeting PPX1 and PPX2 gene fusions from the DMC1 promoter (35, 36). We measured...
PPX1 and PPX2 transcript levels from meiotic stage floral buds in meiMIGS-transformed plants and observed a significant reduction of both genes in all tested lines, compared to wild type (Welch’s t-test, all P < 1.51 × 10⁻⁹) (Extended Data Fig. 5). Cross-silencing of PPX1 and PPX2 by the meiMIGS constructs is expected, as these genes share 86.6% nucleotide identity. The constructs were transformed into 420/++ plants and we observed a significant increase in crossover frequency compared to wild type (Welch’s t-test, all
P < 1.01 × 10⁻⁴ (Fig. 3b and Supplementary Table 8). We correlated relative expression of PPX1 and PPX2 in these backgrounds with 420 crossover frequency and observed a significant negative correlation in both cases (PPX1 r = -0.76, P = 6.73 × 10⁻³; PPX2 r = -0.64, P = 1.81 × 10⁻⁴) (Fig. 3b,c and Extended Data Fig. 5). Together, these data demonstrate quantitative increases in crossover frequency that correlate with the degree of PPX1 and PPX2 silencing.

Euchromatic crossovers increase and the strength of interference decreases in hcr1 and meiMIGS-PPX1-PPX2. To investigate the effect of hcr1 and meiMIGS-PPX1-PPX2 on crossover frequency in other genomic regions, we crossed these lines with additional FTL/CTL recombination reporters30–31 (Fig. 3d) expressing fluorescent proteins using either seed (Fig. 3e,f), or pollen promoters (Fig. 3g). Plants carrying seed-based CTL reporters were self-fertilized and measure both male and female meiosis (Fig. 3e,f). We observed that distal FTL intervals CTL1.17, CTL1.26, CTL3.15 and CTL5.4 showed significantly higher crossover frequency in hcr1-1, compared to wild type (Welch’s t-test, all P < 1.08 × 10⁻⁴) (Fig. 3e and Supplementary Table 9). By contrast, the centromere-spanning interval CTL5.11 was not significantly different in hcr1-1 (Fig. 3e and Supplementary Table 9). The same patterns were confirmed using meiMIGS-PPX1-PPX2, which showed significant crossover increases in the distal and interstitial FTL intervals CTL1.13, CTL1.22, CTL2.2, CTL2.7, CTL4.7, CTL5.1 and CTL5.13, compared with wild type (Welch’s t-test, all P < 1.71 × 10⁻⁴), whereas the centromeric interval CTL5.5 were not significantly different (Fig. 3f and Supplementary Table 10).

We crossed meiMIGS-PPX1-PPX2 with pollen-based FTL intervals, which are combined with the quartet mutation1 (Fig. 3d,g,h and Supplementary Tables 11 and 12). This assay measures crossover frequency and interference specifically in male meiosis1. For analysis, we used a deep learning pipeline DeepTetrad, which enables high-throughput analysis of fluorescent tetrads4. We tested four three-colour FTL intervals located in distal chromosome regions: I1bc, I1fg, I3bc and I5ab. All intervals, except the relatively narrow I1fg, showed significant crossover increases in meiMIGS-PPX1-PPX2 compared with wild type (Welch’s t-test, all P < 7.28 × 10⁻³) (Fig. 3g, Extended Data Fig. 6 and Supplementary Table 11). We also tested the centromere-spanning FTL CEN3, which significantly decreased in meiMIGS-PPX1-PPX2 (Welch’s t-test, P = 5.05 × 10⁻⁴) (Fig. 3g and Supplementary Table 12). Across all FTL data, we correlated the proximity of each interval midpoint to the centromere, with the change in crossover frequency that occurred in hcr1-1 or meiMIGS-PPX1-PPX2 relative to wild type (Fig. 3i and Supplementary Tables 9–12). This analysis revealed a significant negative correlation (r = -0.709, P = 1.48 × 10⁻⁴) between the crossover increase and proximity to the centromere (Fig. 3i). These results show that the distal chromosome regions significantly increase crossovers in hcr1 and meiMIGS-PPX1-PPX2 when measured in male meiosis alone, or in both male and female meiosis. To specifically compare male and female recombination, we backcrossed wild type, hcr1 and meiMIGS-PPX1-PPX2 plants that were 420/++ hemizygous, as either male or female parents. The 420 interval is heterochromatic and shows significantly higher crossover frequency in males (24.23 cM), compared with females (10.98 cM) (Welch’s t-test P = 2.92 × 10⁻⁴) (Fig. 3j and Supplementary Table 13). We observed that both hcr1 and meiMIGS-PPX1-PPX2 showed significant crossover increases in male (Welch’s t-test P = 6.25 × 10⁻⁴ and 2.15 × 10⁻⁴) and female (Welch’s t-test P = 2.81 × 10⁻⁴ and 1.75 × 10⁻⁴) meiosis, compared with wild type (Fig. 3j and Supplementary Table 13).

For three-colour, pollen-based FTL intervals we are able to measure crossovers in adjacent regions and thereby measure interference6,54 (Fig. 3h, Extended Data Fig. 6b and Supplementary Table 14). Crossover interference ratios (IFRs) are calculated using the genetic map distance in the test interval, with and without a crossover occurring in the adjacent interval. An IFR of 1 indicates an absence of interference6,54. We observed that meiMIGS-PPX1-PPX2 causes an increase in crossover frequency, but a decrease in the strength of interference in FTLs I1bc, I1fg, I3bc and I5ab (Welch’s t-test, all P < 3.05 × 10⁻⁴) (Fig. 3g,h and Supplementary Table 14). Therefore, a higher incidence of double crossovers in adjacent intervals occurs in meiMIGS-PPX1-PPX2, compared with wild type (Extended Data Fig. 6d). We repeated three-colour analysis using FTL intervals I1bc and I3bc in hcr1-1 and again observed significantly increased crossover frequency and decreased crossover interference (higher IFR) (Welch’s t-tests, P = 2.7 × 10⁻⁴ and P = 8.1 × 10⁻⁴, respectively) (Extended Data Fig. 6a-c and Supplementary Table 14).

Genome-wide mapping of crossovers in meiMIGS-PPX1-PPX2. Our FTL data indicate that the euchromatic chromosome arms undergo an increase in crossover frequency in hcr1 and meiMIGS-PPX1-PPX2. Notably, these FTL experiments were performed in a Col/Col inbred background. Therefore, we sought to test the effect of meiMIGS-PPX1-PPX2 on crossovers in a hybrid background (Fig. 4a). We crossed wild type (Col), or a meiMIGS-PPX1-PPX2 transgenic line on the Col background carrying the 420 FTL to Ler and generated Col/Ler F1, hybrids (Fig. 4a and Supplementary Table 15). We measured 420 crossover frequency in wild type and meiMIGS-PPX1-PPX2 Col/Ler F1 hybrids and observed a significant increase in meiMIGS-PPX1-PPX2 on the Col background (Welch’s t-test, P = 6.55 × 10⁻⁴) (Fig. 4a and Supplementary Table 15). This demonstrates that PPX1 and PPX2 repress crossovers in both inbred and hybrid backgrounds.

We self-fertilized wild-type and meiMIGS-PPX1-PPX2 Col/Ler F1 plants and generated 144 wild-type and 192 meiMIGS-PPX1-PPX2 F2 plants, from which we extracted genomic DNA. This DNA was sequenced and data were analysed using the TIGER pipeline6,54 to identify crossover locations in each wild-type and meiMIGS-PPX1-PPX2 F2 individual (Fig. 4a,c–f). Crossovers
were mapped to an average of 962 bp and 936 bp in wild-type and \textit{meiMIGS-PPX1-PPX2} F\textsubscript{2} populations, respectively (Supplementary Table 15). We observed a significant increase in crossovers per F\textsubscript{2} plant, from 7.86 in wild type, to 8.57 in \textit{meiMIGS-PPX1-PPX2} (Welch’s \( t \)-test, \( P = 7.7 \times 10^{-3} \)) (Fig. 4c), increased crossover numbers on each chromosome in \textit{meiMIGS-PPX1-PPX2} compared with wild type (Fig. 4d), and a positive correlation between crossover number and chromosome length (wild type \( r = 0.986 \), \textit{meiMIGS-PPX1-PPX2} \( r = 0.983 \)) (Fig. 4d and Supplementary Table 16).

We analysed the crossover landscape in wild type and \textit{meiMIGS-PPX1-PPX2} (Fig. 4e,f). We averaged all chromosome arms along their telomere–centromere axes and plotted crossover frequency per F\textsubscript{2} in wild-type and \textit{meiMIGS-PPX1-PPX2} plants (Fig. 4e,f). Wild type and \textit{meiMIGS-PPX1-PPX2} show a U-shaped distribution of crossover frequency along the chromosomes, with high recombination in the distal sub-telomeres and pericentromeres (Fig. 4e,f). We observed that the first 60–70% of the chromosome arms from the telomeres showed increased crossovers.
in *meiMIGS-PPX1-PPX2* compared with wild type, whereas the pericentromeres and centromeres showed a similar level of recombination (Fig. 4e,f), consistent with our previous FTL analysis (Fig. 3d–g,i). DNA methylation is highest in the centromeric region\(^{33}\), where recombination is suppressed in both wild type and *meiMIGS-PPX1-PPX2* (Fig. 4e,f). We compared crossover frequency to Col/Ler single nucleotide polymorphism (SNP) frequency, which follows an ascending gradient from the telomeres to the centromeres (Fig. 4e,f). The distal regions of the chromosomes with lowest SNP density and lowest DNA methylation underwent the greatest crossover increase in *meiMIGS-PPX1-PPX2* compared with wild type (Fig. 4e,f). We analysed nucleosome occupancy (MNase-seq) and SPO11-1-oligonucleotides (a marker of meiotic DSBs) around crossover locations in wild type and *meiMIGS-PPX1-PPX2*, compared with the same number of randomly chosen locations\(^{46,57}\). We observed that crossovers in both

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**Fig. 4 | Genome-wide mapping of crossovers in meiMIGS-PPX1-PPX2.** a, Schematic showing crossing of *meiMIGS-PPX1-PPX2* Col-420 (black) and wild-type Col-420 (black), to Ler (red) to generate F\(_2\) populations for genotyping by sequencing. Green and red triangles indicate 420 T-DNAs on chromosome 3. b, 420 crossover frequency (cM) in wild type and *meiMIGS-PPX1-PPX2* Col/Ler F\(_1\) hybrids. c, Histogram of crossover number per F\(_2\) individual in wild-type (blue) Col/Ler and *meiMIGS-PPX1-PPX2* (red) populations. Vertical dashed lines indicate mean values. d, Crossovers per chromosome per F\(_2\) compared with chromosome length in wild type (blue) and *meiMIGS-PPX1-PPX2* (red). e, Normalized crossover frequency plotted along chromosome arms orientated from telomere to centromere in wild-type (blue) and *meiMIGS-PPX1-PPX2* (red) F\(_2\) populations. Mean values are indicated by horizontal dashed lines. Also plotted is Col/Ler SNP frequency (green, top) and DNA methylation (pink, bottom). f, As e, but without telomere-centromere scaling. Vertical solid lines indicate telomeres and vertical dashed lines indicate centromeres.
genotypes showed a similar depletion of nucleosome occupancy and enrichment of SPO11-1-oligonucleotides, compared to random positions (Extended Data Fig. 7). This indicates that while distal regions increase crossovers in meiMIGS-PPX1-PPX2, recombination retains a local bias for accessible DNA that experiences higher levels of DSB.

Fig. 5 | Meiotic MLH1 foci are elevated in hcr1 whereas RAD51, ASY1 and ZYP1 immunostaining are unchanged. a, Representative images of male meiocytes spread and stained with DAPI in wild type (Col) and hcr1-1, at the indicated stages of meiosis. Scale bars, 10 μm. b, Representative images of ASY1 (green) and ZYP1 (red) immunostaining of wild type (Col-0) and hcr1-1 male meiocytes at pachytene. Nuclei spreads were also stained with DAPI. Scale bars, 10 μm. c, Quantification of RAD51 foci number per cell in wild type and hcr1-1. e, Representative images of MLH1 immunostaining of male meiocytes at diakinesis stage in wild type, hcr1-1 and meiMIGS-PPX1-PPX2. Cells were also DNA stained with DAPI. Arrows represent MLH1 foci at distal locations on the chromosomes. Scale bars, 10 μm. f, Quantification of MLH1 foci number per cell scored at diakinesis stage in wild type (blue), hcr1-1 (red) and meiMIGS-PPX1-PPX2 (red). All cytological experiments represent data collected from at least two biological replicates.
**hcr1** and **meiMIGS-PPX1-PPX2** show elevated class I MLH1 foci at diakinesis stage. We used cytological analysis to analyse meiosis in **hcr1-1** compared with wild type. We spread wild-type and **hcr1-1** male meiocytes and stained chromosomes using 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 5a). We observed normal chromosome morphology during prophase I (leptotene and pachytene) in **hcr1-1**, normal bivalent morphology at metaphase I and chromosome segregation during anaphase I and meiosis II (Fig. 5a). This is consistent with **hcr1-1** showing no difference in fertility compared with wild type (Supplementary Table 16). To investigate formation of the chromosome axis and homologue synopsis, we immunostained wild-type and **hcr1-1** meiocytes for the HORMA domain protein ASY1 and the synaptonemal complex protein ZYP1 during prophase I (Fig. 5b). Wild type and **hcr1-1** showed normal homologue synopsis and immunostaining of ASY1 and ZYP1 (Fig. 5b).

We immunostained meiocytes in early prophase I for ASY1 and the DSB marker RAD51 and observed no significant difference in RAD51 foci number between wild type and **hcr1-1** (Fig. 5c,d and Supplementary Table 18) (Wilcoxon t-test, \( P = 0.32 \)). This is consistent with normal levels of meiotic DSBs forming in **hcr1** relative to wild type. Finally, we immunostained for the MLH1 class I protein at diakinesis stage on DAPI-stained male meiocyte spreads (Fig. 5e,f and Supplementary Table 19). Quantification of MLH1 foci per nucleus showed a significant increase in **hcr1-1** (mean = 12.1 foci), compared with wild type (mean = 10.4 foci) (Wilcoxon test, \( P = 5.3 \times 10^{-7} \)) (Fig. 5e,f and Supplementary Table 19). We also measured MLH1 foci in wild type (Col) and **meiMIGS-PPX1-PPX2**, using the same transgenic line as for genotyping by sequencing. We observed that **meiMIGS-PPX1-PPX2** showed significantly higher numbers of MLH1 foci (mean = 12.8 foci), compared with wild type (mean = 10.7 foci) (Wilcoxon test, \( P = 2.5 \times 10^{-6} \)) (Supplementary Table 20). Together, this is consistent with the increases in crossovers observed in **hcr1** and **meiMIGS-PPX1-PPX2** being mediated mainly via the class I repair pathway.

**HCR1 interacts with the class I crossover pathway proteins HEI10, PTD, MSH5 and MLH1.** Since we observed elevated MLH1 foci in **hcr1** and **meiMIGS-PPX1-PPX2** (Fig. 5e,f), we sought to investigate genetic interactions with the class I and class II repair pathways. Class I pathway mutants, for example zip4, have low fertility due to reduced crossovers, unbalanced chromosome segregation and aneuploid gametes\(^{14}\) (Fig. 5a). Fertility of class I mutants can be restored by mutations that block non-crossover formation and increase class II crossovers, for example **fancm**\(^{-}\). We generated **zip4 hcr1** double mutants and observed that fertility was not restored (Fig. 6a). We performed meiotic chromosome spreads and counted chiasma, bivalents and univalents in wild type (Col), **zip4** and **zip4 hcr1** (Supplementary Table 21). We observed that **zip4** and **zip4 hcr1** showed strongly reduced bivalents (**zip4** mean = 0.8, **zip4 hcr1** mean = 1.3), compared with wild type (mean = 5) (Wilcoxon test, **Col vs zip4** \( P = 5.22 \times 10^{-12} \), **Col vs zip4 hcr1** \( P = 1.43 \times 10^{-11} \)). The bivalent counts for **zip4** and **zip4 hcr1** were not significantly different from one another (Wilcoxon test \( P = 0.11 \)).

This is also consistent with a major effect of **hcr1** on the class I pathway. We also generated **hcr1 fancm** double mutants carrying the 420 FTL interval, and observed an additive increase in genetic distance in the double mutant compared to **hcr1** and **fancm** single mutants (Welch’s t-tests, \( P = 2.7 \times 10^{-11} \) and \( P = 6.77 \times 10^{-4} \), respectively) (Fig. 6b and Supplementary Table 20). The **hcr1 fancm zip4** triple mutant exhibited a 420 crossover frequency lower than that of **hcr1 fancm**, but higher than that of **fancm zip4** (Welch’s t-test, \( P = 5.60 \times 10^{-4} \) and \( P = 9.93 \times 10^{-4} \), respectively) (Fig. 6b and Supplementary Table 22). This suggests that **hcr1** may also increase the number of class II crossovers, at least in a **fancm zip4** mutant background (Fig. 6b and Supplementary Table 22).

We investigated whether **HCR1** physically interacts with known components of the meiotic recombination pathways. We cloned **HCR1/PPX1** into Y2H AD and BD vectors and tested interactions with class I proteins, as well as the PP4 regulatory subunits PP4R2L and PP4R3A (Fig. 6c,d, Extended Data Fig. 8 and Supplementary Table 23). As expected\(^5\), **HCR1** interacted strongly with the PP4 regulatory subunits PP4R2L and PP4R3A (Fig. 6c and Supplementary Table 23). Of the tested class I combinations, we observed strong **Y2H** interactions between **HCR1** and HEI10, MSH5 and PTD (Fig. 6c,d). We also detected weaker interactions between **HCR1** and the class I pathway proteins MER3, ZIP4, SHOC1 and MLH1 (Extended Data Fig. 8d and Supplementary Table 22). Within the class I pathway we observed strong interactions between HEI10, HEI11 and MSH5, and between SHOC1 and PTD (Extended Data Fig. 8a,b), consistent with data in rice and Arabidopsis\(^{6,7}\). We additionally tested a wider set of 13 meiotic proteins that included the synaptonemal complex protein ZYP1a, DNA repair factors (DMC1, RAD51 and RPA1A), DSB proteins (PRD1, PRD2, PRD3, SPO11-1 and MTOPVIB) and meiotic chromosome axis proteins (ASY1, ASY3, SWI1 and REC8). Using serial dilutions, we observed that **HCR1** shows strong interactions with REC8, SPO11-1, PRD1, RPA1A, MTOPVIB and PRD2 and weaker interactions with ASY1, RAD51, DMC1, ZYP1a and CDKA;1 (Fig. 6c,d and Extended Data Fig. 8a,b). Thus, although **HCR1** represses the class I crossover pathway, it may have a more widespread role regulating protein phosphorylation during Arabidopsis meiosis.

The human PP4 complex targets multiple proteins by recognizing a short motif (FxxP) via the PP4R3 Eev/Vasp homology 1 (EVH1) domain\(^6\). To explore whether a similar mechanism is relevant in Arabidopsis we performed Y2H experiments using the Arabidopsis **PP4R3A** (At3g06670) EVH1 domain (residues 1–166) (Extended Data Fig. 9). The **PP4R3A** EVH1 domain interacts with 14 of 15 proteins observed as **HCR1** interactors (Extended Data Fig. 9). Additionally, **PP4R3A** showed **Y2H** interactions with PRD3 and SWI1 (Extended Data Fig. 9). These data are consistent with **HCR1/PPX1** and **PP4R3A** subunits interacting with a diverse set of proteins that regulate meiotic chromosomes and recombination, including class I factors.

We sought to further test protein–protein interactions between **HCR1** and class I proteins in planta using transient transfection and co-localization studies in Arabidopsis protoplasts (Fig. 6c). As reported\(^6\), expression of a **HCR1**–cyan fluorescent protein (CFP)
fusion protein showed nuclear localization (Fig. 6c). We co-expressed PPX1–CFP with PTD–yellow fluorescent protein (YFP), HEI10–YFP, MSH5–YFP and MLH1–YFP fusion proteins and observed nuclear co-localization in all cases (Fig. 6c). We confirmed physical association of PPX1 using co-immunoprecipitation following transient expression in Arabidopsis protoplasts of PPX1–Myc,
undergoes dephosphorylation by at least one FXXP motif (Extended Data Figs. 9 and 10). Interestingly, 15 out of 18 PPX1 interactors, and 12 out of 16 meiocyte-specific expression34,60 (Arabidopsis and recombination in meiosis (Extended Data Fig. 10g). Together, these interactors are also significantly enriched in Gene Ontology (GO) categories according to the criteria of: (1) FXXP motifs (n = 13,803), (2) predicted nuclear location (n = 10,595) and (3) meiocyte-specific expression46 (n = 4,528). This search identified 1,367 candidate targets for the PP4 complex during meiosis (Extended Data Fig. 10d). Of these proteins, 1,315 (96.2%) have at least one phosphorylation consensus site (Extended Data Fig. 10e). Furthermore, 15 out of 18 PPX1 Y2H interactors, 12 out of 16 PP4R3A EVH1 Y2H interactors, 49 out of 84 known meiotic interactors also possess multiple consensus sites used by CDK, DDK and ATM/ATR kinases (Extended Data Fig. 10c and Supplementary Table 20). We searched genome-wide for potential meiotic substrates according to the criteria: (1) FXXP motifs (n = 1,000 random sets of 1,367 proteins, P-values were then randomly generated using 1,000 random sets of 1,367 proteins, P-values were then randomly generated using -test expectation (compared with numbers of phosphorylation sites one phosphorylation site is significantly higher than the random expectation (compared with numbers of phosphorylation sites in 1,000 random sets of 1,367 proteins, P-test = 7.02 × 10−31) (Extended Data Fig. 10f). The 1,367 predicted meiotic PP4 substrates are also significantly enriched in Gene Ontology (GO) terms for DNA repair, DNA recombination, chromatin organization and meiosis I cell cycle (Extended Data Fig. 10g). Together, these data indicate the wide potential for PP4 regulation of meiosis and recombination in Arabidopsis.

Discussion

We identified the HCR1/PPX1 phosphatase as a repressor of crossover frequency in Arabidopsis. We provide genetic, cytological and protein–protein-interaction data that a major target of HCR1/PPX1 is the class I crossover pathway, and that it has a minor role repressing class II crossovers (Fig. 7). Our protein-interaction data indicate that HEI10, PTD, MSH5 and MLH1 are probably direct targets for HCR1/PPX1–PP4 phosphatase activity within the class I pathway. However, we also observed that HCR1/PPX1 and PP4R3A interact in a Y2H assay with components of the chromosome axis (ASY1, ASY3, REC8 and SWI1), DSβ proteins (SPO11-1, MTOPVIB, PRD1 and PRD2) and recombinases (RPA1A, RAD51 and DMC1), consistent with a broader regulatory role during meiosis.

In the absence of HCR1/PPX1, we propose that the action of pro-recombination kinases on the class I pathway promotes stabilization of interhomologue strand invasion and crossover formation (Fig. 7). The crossovers increase observed in hcr1 and meiMIGS–PPX1–PPX2 were most pronounced in the distal chromosome ends. Notably, distal crossover increases are characteristic of situations with elevated class I activity in Arabidopsis, including male meiosis, HEI10 and CDKA;16,61, although distal increases are also observed in mutants that increase class II crossovers (for example, rec4a rec4b)64,65. The causes of distal biases in crossover formation in these backgrounds remain incompletely understood. Chromatin may be an important influence, as meiotic DSβs are elevated in gene-associated nucleosome-free regions, and there are positive associations with euchromatic chromatin marks, including H3K4me3 and H2AZ66,67. By contrast, heterochromatic modifications including H3K9me2 and dense DNA methylation are associated with crossover suppression68,69. Additionally, class I crossovers are subject to interference, which inhibits formation of adjacent crossovers in a distance-dependent manner70. A complete understanding of the crossover landscape in hcr1 will require further investigation of how chromatin, chromosome structure and interference co-operate spatially and temporally during meiosis.

Within the class I pathway, HEI10 belongs to a family of conserved ubiquitin or SUMO E3 ligases that promote interfering crossover formation in diverse eukaryotes11,23. In Arabidopsis, HEI10 is a dosage-sensitive promoter of class I crossover repair6,68,71. HEI10 shows a dynamic localization pattern along plant meiotic chromosomes, initially showing numerous foci along the axis, which become restricted to a small number of foci that overlap MLH1 foci during late prophase I66,67. In budding yeast, the HEI10 orthologue Zip3 is phosphorylated in a DSβ-dependent manner by Mec1 (ATR), which is antagonized by PH311. This is of particular interest as PH3 is a HCR1/PPX1 orthologue, indicating that repression of the class I pathway by PP4 phosphatases may be conserved between plants and fungi.

In mice, orthologues of HEI10 (for example, RNF212) act to regulate association of the MutSy Msh4–Msh5 heterodimer with meiotic chromosomes68,69. Msh4–Msh5 heterodimers are capable of forming sliding clamps on DNA in vitro and associate with recombination foci along meiotic chromosomes in vivo70,71. MutSy is proposed to bind nascent joint molecules and protect them from dissolution by anti-recombinases, including Sgs1–Top3–Rmi1 in budding yeast70,72,73. MutSy can also directly or indirectly recruit the MutLy (Mlh1–Mlh3) endonuclease heterodimer to promote crossover resolution74,75. Budding yeast Msh4 was recently identified as an intrinsically unstable protein that is degraded by the proteasome via an N-terminal degron23. Phosphorylation of the degron by the cell cycle kinase Cdc7–Dbf4 (DDB) inhibits Msh4 degradation and thereby promotes crossover repair23. As Arabidopsis HCR1/PPX1 physically interacts with MSH5 and MLH1 this may promote MutSy and MutLy dephosphorylation and thereby repress class I crossover repair.

We observed physical interaction between HCR1/PPX1 and PTD, which is the partner protein of SHOC1, which together form...
a XPF–ERCC1-related complex. Orthologues of the SHOC1–PTD complex include budding yeast Zip2–Spo16, which binds branched DNA molecules in vitro, lacks endonucleolytic activity and acts with Zip4 to promote crossover formation. However, phosphorylation of Zip2–Spo16–Zip4 has not been reported in budding yeast or other organisms. Since Arabidopsis PTD interacts with HCR1 and PP4R3A EVH1 and contains consensus phosphorylation sites, it is possible that plant SHOC1–PTD–ZIP4 complexes may be regulated by phosphorylation.

It is also possible that HCR1/PPX1 may regulate phosphorylation of the DSB machinery, or components of the meiotic chromosome axis, as observed in Caenorhabditis elegans. Furthermore, orthologues of ASY1 (Hop1), REC8 (Rec8) and ZYP1 (Zip1) proteins in budding yeast are known to be regulated via phosphorylation. Hence, it is possible that Arabidopsis ASY1, REC8 and ZYP1 may be dephosphorylated by PP4. However, we did not observe significant changes to RAD51 foci or ASY1 and ZYP1 immunostaining during meiosis in hcr1 at the cytological level.

We consider three pro-recombination kinase pathways as candidates for HCR1/PPX1–PP4 antagonism (Fig. 7). First, CDK–cyclin complexes are drivers of cell cycle progression, including during meiosis and are known to regulate recombination. Second, Dbf4-dependent kinase (DDK) (Cdc7–Ddf4) has a prominent role in the initiation of DNA replication, and also in regulation of recombination and kinetochore behaviour during meiosis. Third, the ATM/ATR phosphatidylinositol 3-kinase related kinases are activated by DSBs and regulate meiotic DSB number and distribution in yeast and mammals. Together these kinase pathways play complex and interacting roles in the promotion of crossovers during meiosis.

In Arabidopsis, CDKA;1 (the homologue of human Cdk1 and Cdk2) has a role in promoting class I crossovers. Hence, HCR1/PPX1 may remove phosphorylation from CDKA;1 targets within the class I pathway and thereby limit crossovers (Fig. 7). Interestingly, mutation of CDK consensus motifs (S/T-P) in budding yeast Zip3 had no effect on phosphorylation, whereas mutation of Tel1/Mec1 sites (S/T-Q) did. As noted earlier, Zip3 phosphorylation has been shown to be regulated by PP4, meaning that HCR1 may regulate HEI10 phosphorylation in an analogous manner in Arabidopsis.

Indeed, it has been shown that many targets of Mec1 phosphorylation, including Zip1, are also PP4 substrates in budding yeast. In Arabidopsis, ATM and ATR are redundantly required for DSB repair. The atm single mutant is partially sterile with increased meiotic DSBs, chromosomal fragmentation and moderately increased class I crossovers. In budding yeast, DDK is responsible for Msh4 degron phosphorylation and stabilization. Thus, it is possible that HCR1 could dephosphorylate MutSβ and thereby promote its destabilization and repress crossovers (Fig. 7). However, the meiotic function of DDK kinases in plants is currently unknown.

Studies in diverse systems and contexts have identified PP4 phosphatase complexes as key regulators of DNA repair and recombination. For example, the DNA-damage response involves kinase regulation, which is balanced with antagonizing phosphatases. Defined roles for PP4 complexes include: (1) dephosphorylation of γ-H2AX during recovery from DNA-damage checkpoints in Dro sophila, budding yeast and human; (2) prevention of Rad53 hyperphosphorylation during DSB repair and promoting DNA end resection in budding yeast; (3) dephosphorylating RPA2 to promote DNA repair via homologous recombination; (4) promoting non-homologous end joining-mediated DSB repair, which occurs partially via KRAB-associated protein1 (KAPI); (5) regulation of Mec1 during DSB repair and at sites of replication fork collapse; and (6) regulating Zip1 phosphorylation during meiosis to control homology-independent centromere pairing. Our work identifies PPX1–PP4 phosphatase complexes as repressing the class I crossover pathway during Arabidopsis meiosis. We propose that PP4 complexes may generally act in opposition to pro-recombination kinases to regulate meiotic crossovers in eukaryotes.

Methods

Plant materials. Arabidopsis thaliana plants were grown under controlled conditions of 22°C, 50–60% humidity and 16:8 light-dark cycles. Seeds were incubated at 4°C in the dark for 3–4 d to stratify germination. Seed-expressed FTL/CTL and pollen-expressed FTL lines were used. T-DNA insertion lines in ppx1 (GK_651B07), ppx2 (GK_488H09), pp4r2 (SALK_093051), zip4-2 (SALK_68052) and the fancm-1 EMS mutant were provided by Nottingham Arabidopsis Stock Centre. Genotyping of hcr1-1 was performed by PCR amplification using oligonucleotides ppx1-F and ppx1-R for wild type, and ppx1-F and GABI_LB for the T-DNA allele. Genotyping of hcr1-2 was carried out by PCR amplification using primers ppx2-F and ppx2-R for wild type, and ppx2-R and GABI_LB for the T-DNA allele. Genotyping of pp4r2 was performed by PCR amplification using oligonucleotides pp4r2-F and pp4r2-R for wild type, and pp4r2-L and GABI_LB for the T-DNA allele. Genotyping of hcr1-1 was performed by PCR amplification using hcr1-1 and hcr1-R DACPS markers, followed by FokI restriction endonuclease digestion. zip4-2 and fancm-1 genotyping was performed as previously described. Genotyping oligonucleotide sequences can be found in Supplementary Table 24.

Ethyl-methyl sulfonate mutagenesis of A. thaliana seed. Approximately 10,000 seeds from 420 GR/++ hemizygote plants were obtained by crossing 420 GR/GR homozygote to wild type (Col-0). These seeds were soaked in 40 ml of 100 mM phosphate buffer (pH 7.5) in a 50 ml tube for 1 h. Seeds were washed with fresh 100 mM phosphate buffer and then treated with 0.3% (v/v) ethyl-methyl sulfonate and incubated for 12 h at room temperature. Seeds treated with ethyl-methyl sulfonate were washed ten times with distilled water and immediately sown on soil. From these seeds, ~7,000 M1 plants were germinated and grown. The seeds from 12 independent M1 plants were combined to generate ~600 M2 pools. From each M1 pool, ~150 seeds were pre-selected as 420/++ hemizygotes on the basis of red and green fluorescence, grown and self-fertilized. The resulting seeds were analysed for 420 crossover frequency.

Measurement of crossover frequency and interference using fluorescent seed and pollen. Crossover frequency was measured by analysing counts of fluorescent and non-fluorescent seeds from FTL/++ hemizygote plants using a CellProfiler image analysis pipeline. CellProfiler enables the quantification of the numbers of green-only fluorescent seeds (Nmg), red-only fluorescent seeds (Nrg), and total seeds (Ntotal). Crossover frequency (cM) is calculated using the formula:

$$cM = 0.5T + 3NPD \times \frac{1}{PD + T + NPD} \times 100$$

where PD is the parental ditype frequency, T is the tetra type frequency, and NPD is the non-parental ditype frequency. Crossover frequency was calculated using the Perlkin’s equation:

$$\chi^2 = \frac{T^2 - NTPD}{NPD(T + PD)}$$

Three-colour FTL intervals (11bc, 11fg, 12fg, 13bc and 15ab) produce 12 tetrad classes: no recombination (A), single crossover interval 1 (B; SCo-1), single crossover interval 2 (C; SCo-2), two-strand double crossover (D; 2stDCO), three-strand double crossover a (E; 3stDCoa), three-strand double crossover b (F; 3stDCOb), four-strand double crossover (G; 4stDCO), non-parental ditype interval 1, non-crossover interval 2 (H; NPD-1 NCO-2), non-crossover interval 1, non-parental ditype interval 2 (I; NPD-2 NCO-1), single crossover interval 2 (J; NPD-1 SCo-2), single crossover interval 1, non-parental ditype interval 2 (K; SCo-1 NPD-2) and non-parental ditype interval 1, single crossover interval 2 (L; NPD-1 NPD-2). Fluorescent tetrad states were identified using DeepTetrad, and cM was calculated using the Perlkin’s equation.
Identification of candidate hcr1-1 mutations using DNA sequencing and SHOREmap. Sixty hcr1 BC1F1 individuals with high (>27 cm) ±20 crossover frequency were identified and 5 mg of seeds from each BC1F1 individual were pooled. Pooled seeds were germinated on MS agar plates with 7-day-old seedlings were collected. About 3 g of pooled seedlings was ground in liquid N. using a mortar and pestle. The leaf powder was transferred into a pre-chilled mortar with 40 ml of fresh nuclear isolation buffer (25 mM Tris-HCl, pH 7.5, 0.44 M sucrose, 10 mM MgCl2, 0.5% Triton X-100, 2 mM β-mercaptoethanol, and triaze (free protease inhibitor cocktail) and the contents were homogenized. The tissue lysate was kept on ice and incubated for 30 min with rocking. The filtered contents were centrifuged at 4 °C at 3000g for 25 min. The supernatant was removed and the pellet was subjected to DNA extraction using cetyl trimethylammonium bromide (CTAB). CTAB-extracted and purified DNA was sheared to a size range 200–500 bp using a BioRuptor sonicator. One microgram of input DNA was diluted in 150 μl of TE buffer and was sonicated for 22 min using high voltage with 30 s ON/OFF cycles. The sonicated DNA was concentrated in a 60 μl volume and DNA in the size range ~300–400 bp from a 2% agarose gel stained with 1x SYBR gold using a UV transilluminator. Fifty nanograms of purified DNA in 60 μl was used as input for library construction using an Illumina Truseq Nano DNA LT library prep kit. The hcr1 BC1F1 library was sequenced using an Illumina Genome Analyzer (100 bp paired) Hiseq 2000 instrument.

SHOREmap (v.3.0) was applied to align paired-end reads to the TAIR10 reference genome using the GenomeMapper tool. Raw reads were trimmed according to quality and included a cut-off of 50% reads or a quality score (Q score) of 10 in SHORE import. SHORE function consensus was used to detect sequence variation between the hcr1 BC1F1 and the TAIR10 reference assembly. SNPs with high-quality marker scores (>40), supported by at least 10 unique reads, were applied using SHOREmap backcross for analysis of allele frequency. Using SHOREmap annotate, we compared the TAIR10 gene annotation and obtained a list of EMS-derived mutations that included predicted effects on gene expression and function. Mutations were screened for those with (1) greater than 80% allele frequency, and (2) non-synonymous, splice site or premature stop codon changes predicted in genes. Additionally, candidate mutations were examined on the basis of their location within genes with predicted or known functions relevant to meiosis, protein location in the nucleus and known molecular functions provided in the The Arabidopsis Information Resource (TAIR) database.

Genetic complementation of hcr1-1 by PPX1. A 4.5-kb genomic DNA fragment containing HCR1/PPX1 was amplified by PCR using primers PPX1-F and PPX1-R (Supplementary Table 24). The PCR product was digested by PstI and Smal restriction enzymes and cloned into the binary vector pGREEN0029. The pGREEN0029-PPX1 and empty vector constructs were electroporated into Agrobacterium strain GV3101-SPSOUP and transformed into Arabidopsis plants by floral dipping. T. plants were selected for kanamycin resistance and genotyped using primers designed from left and right borders of the HCR1/PPX1 transgene (Supplementary Table 24).

Construction of PPX1/PPX4 phylogenetic tree. The neighbour-joining method was used to construct a PPX1/PPX4 phylogenetic tree. Amino acid sequences of AtPPX1 (NP_1944002.1, AIPPX2 (NP_2003371.1), OsPPX (XP_015612628), DmPPX (NP_001022898), HsPPP4C (NP_001290432), Cepph-4.1 (NP_499603), Cepph-19C (NP_194402.1), AtPPX2 (NP_200337.1), OsPPX (XP_015612628), 10 units of T4 DNA polymerase (Thermo Fisher Scientific), 1.25 units of Klenow fragment (New England Biolabs) and 0.44 M sucrose, 10 mM MgCl2, 0.5% Triton X-100, 2 mM β-mercaptoethanol, and triaze (free protease inhibitor cocktail) and the contents were homogenized. The tissue lysate was kept on ice and incubated for 30 min with rocking. The filtered contents were centrifuged at 4 °C at 3000g for 25 min. The supernatant was removed and the pellet was subjected to DNA extraction using cetyl trimethylammonium bromide (CTAB). CTAB-extracted and purified DNA was sheared to a size range 200–500 bp using a BioRuptor sonicator. One microgram of input DNA was diluted in 150 μl of TE buffer and was sonicated for 22 min using high voltage with 30 s ON/OFF cycles. The sonicated DNA was concentrated in a 60 μl volume and DNA in the size range ~300–400 bp from a 2% agarose gel stained with 1x SYBR gold using a UV transilluminator. Fifty nanograms of purified DNA in 60 μl was used as input for library construction using an Illumina Truseq Nano DNA LT library prep kit. The hcr1 BC1F1 library was sequenced using an Illumina Genome Analyzer (100 bp paired) Hiseq 2000 instrument.

Immunocytological analysis of wild-type and hcr1 meiocytes. Chromosome spreads of Arabidopsis pollen mother cells were prepared using fixed buds and stained with DAPI (1:200 dilution), propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) at 1:4000 dilution. ASY1 and ZYP1, and diakinesis cells were immunostained for MLH1, using fixed buds, as described94. Leptotene-stage meiocytes were immunostained for ASY1 and RAD51 using fresh buds, as described101. The antibodies were used: ASY1 (rabbit, 1:200 dilution), ZYP1 (rabbit, 1:200 dilution), RLH1 (rabbit, 1:200 dilution) and RAD51 (rabbit, 1:300 dilution)101,102. Microscopy was performed with a DeltaVision microscope (Applied Precision/GE Healthcare). DNA was visualized using a CCD CoolSnap HQ2 camera (Photometrics). Image capture was carried out using SoftWoRx software version 5.5 (Applied precision/GE Healthcare). For ASY1 and RAD51 co-immunostaining of leptotene-stage nuclei, individual cell images were acquired as 2-z-stacks of 10 optical sections of 0.2 μm each, and the maximum intensity projection for each cell was used to calculate Number of MLH1 foci per meiotic cell and RAD51 foci per cell associated with the axis protein ASY1 were manually scored. Wuxiong tests were used to assess significant differences between wild type and hcr1-1 MLH1 and RAD51 foci counts.

Y2H assays. For Y2H assays, the open reading frames of Arabidopsis genes were cloned into pGBK7 TD and pGAD77 TD vectors (Clontech, 630490) using BamHI and Stul sites, using a Gibson assembly cloning system (NEB E2621L). Information on all oligonucleotides used for Y2H assays is presented in Supplementary Table 24. BD and AD vectors were co-transformed into S. cerevisiae strain AH109 and selected on synthetic dropout medium lacking leucine (L) and tryptophan (−T). The colonies of yeast transformant cells were streaked onto both (−L) and (−LTH) (histidine) A (adenine) synthetic media and grown for 3–5 d at 30°C. The cells grown in synthetic medium (−L) were grown until OD<sub>600</sub> = 1 and diluted 10-, 100- and 1,000-fold in water and spotted on synthetic medium (−LTHA) for 3–7 d.

Transient expression of fusion proteins in Arabidopsis protoplasts for co-localization and co-immunoprecipitation analysis. Transient expression vectors in protoplasts were constructed using Golden Gate cloning. The full-length coding regions of PPX1/HCRI and meiotic genes were amplified by PCR from cDNA and cloned into LV0 universal vector (pICH1331). For epitope and fluorescent protein tagging, LV0 vectors with coding regions lacking a stop codon were assembled in the LV1 transient expression vector (pICH74742), using the 35S promoter vector (pICE51266), C-terminal vectors (YFP, CFP, Myc tag/pcISL50010 and HA tag/pcISL50009) and NOS terminator vector (pICH14121). Information on all oligonucleotides for protoplast transient expression is provided in Supplementary Table 24.

Plasmid DNA and mesophyll protoplasts were prepared as described104. Twenty-thousand protoplasts were transfected with 20 μg of total plasmid DNA and incubated for 6–12 h at room temperature. To detect co-localization of PPX1–CFP and meiotic protein–YFP, 20 μg of total plasmid DNA (a mixture of PPX1–CFP with YFP fusion constructs HCR1–YFP, PTD–YFP or MSFP–TFP) were co-transfected into 20 x 10<sup>4</sup> protoplasts and incubated at room temperature for 12 h. As a negative control, PPX1–CFP alone or YFP fusion plasmid alone were transfected. The fluorescence of transfected mesophyll protoplasts was detected using a confocal microscope (LSM 800, Zeiss).

Co-immunoprecipitation analysis. 40 μg of PPX1-Myc tag and meiotic gene-HA tag DNA plasmids were co-transfected into protoplasts, or individually transfected as a negative control. Total protein was extracted using extraction buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, protease inhibitor cocktail (Roche) and 1% Triton X-100). The extracted proteins were separated by SDS–PAGE using 8% polyacrylamide gels, transferred to a

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Prediction of PP4 complex target proteins in Arabidopsis. To predict PP4 target proteins during meiosis, proteins containing the FXXP motif were identified by searching protein sequences from TAIR. Nuclear proteins were obtained from Arabidopsis thaliana using the protocol described by Allen et al. (2016). The catalytically active tyrosine residues of both SPO11-1 and FANCM were identified by searching for the consensus sites of CDK, DDK and ATM/ATR, predicted using GPS 5.0 (Hunter, 2006). The predicted phosphosite overlaps were compared with the random using a Z-test.

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The authors declare no competing interests.

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Extended Data Fig. 1 | 420 crossover frequency in wild type and M₂ plants derived from the EMS population. Box and whisker plot showing 420 crossover frequency (cM) for wild type (Col/Col) 420/++ plants (n=75) and EMS-treated M₂ 420/++ plants (n=1,217). Black dots indicate 420 crossover frequency in individual plants. Horizontal lines of black (wild type, Col) and red (EMS M₂) box plots represent maximum, 3rd quartile, median, 1st quartile and minimum in 420 cM, respectively. In this study, wild type plants show a mean value of 19.5 cM (standard deviation=1.5) within 420, and the majority (81.4%, 991/1,217) of M₂ plants display 420 crossover frequency within the range of 18-22 cM (Mean=21.4 cM, SD=1.5). 420 crossover frequency in M₂ plants was significantly increased compared to wild type (one-sided Welch’s t-test P=2.2×10⁻¹⁶), which may have been caused by heterozygous EMS polymorphisms.
Extended Data Fig. 2 | EMS mutations identified in FANCM (hcrl4) and TAF4B (lcr1). a, FANCM gene structure is shown, including the EMS mutation site in hcrl4/fancl-11. The red arrow indicates the G to A substitution within exon 15, which causes a G to S amino acid substitution. Exons are shown as boxes (black=CDs, grey=UTRs). Scale bar=0.5 kb. b, Multiple sequence alignment of the DEHDc (blue line) and HELICC (green line) domains of FANCM in different species. The mutation positions of the fancl-1 to fancl-10 alleles that were previously identified, and fancl-11 (hcrl4), are shown. The fancl-11 mutation is located in a conserved motif within the SF2 helicase domain (bold arrow). c, Gene structure of TAF4B is shown with the location of the lcr1 (tof4b-3) mutation indicated in exon 3 (red arrow), which causes a premature stop codon.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | T-DNA insertions in Arabidopsis PP4/PPX complex genes. **a**, The gene structures of PPX1 (At4g26720), PPX2 (At5g55260) and PP4R2 (At5g17070) are shown. Exons are shown as boxes (black=CDS, grey=UTR). Scale bar=0.5 kb. The EMS induced hcr1-1 mutation is located at the splice donor site of the 3rd intron, shown by the asterisk. The red arrows indicate the location of primers for RT-qPCR in PPX1 and PPX2. The hcr1-2 T-DNA (GK_651B07) insertion position in the 5’-UTR is indicated. The position of the ppx2-1 (GK_488H09), ppx2-2 (SALK_049725), and pp4r2 (SALK_093051) T-DNA insertions are shown, which are located in the 4th intron, 8th exon and 7th intron, respectively. The arrows spanning the ppx2 and pp4r2 T-DNA insertions indicate primer positions used for RT-PCR. **b**, RT-PCR amplification and quantification for PPX1, PPX2 and PP4R2 mRNA expression in wild type Col, hcr1-1, ppx1-2, ppx2-1 and pp4r2. Floral cDNA from two biological replicates were evaluated by RT-PCR amplification for PPX1, PPX2, PP4R2 (shown in a) and GAPC expression. RT-PCR amplicon sizes for wild type, hcr1-1, ppx1-2, ppx2-1, pp4r2 cDNAs and wild type genomic DNA (positive/negative control) are shown. **c**, Plot showing RT-qPCR enrichment of PPX1 and PPX2 in hcr1-1 and ppx2-1. Relative transcript levels of PPX1 and PPX2 were measured in wild type, hcr1-1, and ppx2-1 using qRT-PCR. TUB2 was used for normalization. The y axis indicates fold-enrichment of PPX1 and PPX2 transcript levels, compared to PPX1 and PPX2 in wild type. RT-qPCR reactions of two technical replicates for each of four biological samples were shown as dots. Mean values are indicated by horizontal lines. Significance between wild type and mutants was assessed by one-sided Welch’s t-tests. The P values between Col and hcr1-1 for PPX1 was 0.186, for PPX2 was 3.49×10^{-5}; between Col and ppx2-1 for PPX1 was 1.77×10^{-5} and for PPX2 was 3.64×10^{-9}. Asterisks indicate P<0.001. **d**, Photograph showing developmental phenotypes of wild type, hcr1-1, ppx2-1, hcr1-1, ppx2-1 and hcr1-1 ppx2-1 grown alongside one another. **e**, Photograph showing seeds of wild type and hcr1-1/+ ppx2-2/+ plants. Asterisks indicate defective seeds. **f**, Photograph showing F2 seedlings grown from self-fertilization of F1 hcr1-1/+ ppx2-2/+ plants, with asterisks indicating developmentally delayed seedlings.
Extended Data Fig. 1 | Alignment of PP4 homolog protein sequences from diverse eukaryotes. a, Amino acid sequence alignment of AtPPX1, the predicted hcr1-1 truncated protein, AtPPX2 and PP4 homologs from different eukaryotic species. The predicted hcr1-1 truncated protein consisting of 143 residues is shown. The underlined region indicates amino acids generated due to the retention of the 3rd intron. Hash symbols indicate the locations of conserved PP4 catalytic motifs (GDXHG, GDXVDRG and GNHE) and the histidine (H) residues required for metal binding in C-terminal region. b, As for a, but showing percent identity of amino acid sequence between PP4 homologs.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Meiosis-specific knockdown of PPX1 and PPX2 in meiMIGS transgenic plants. a, qRT-PCR analysis of PPX1/HCR1 and PPX2 transcripts in floral buds of wild type and meiMIGS-PPX1, meiMIGS-PPX2 and meiMIGS-PPX1-PPX2 T2 transgenic lines. The y axis indicates fold-enrichment of PPX1 and PPX2 transcripts, compared to PPX1 in wild type. DMC1 was used as a meiotic gene for normalization. Replicate measurements are shown as dots and mean values shown by horizontal lines. b, Correlation between PPX1 and PPX2 transcript levels in wild type, meiMIGS-PPX1, meiMIGS-PPX2, and meiMIGS-PPX1-PPX2 lines. The x and y axis indicate relative PPX1 and PPX2 transcript levels in meiMIGS-PPX1 (blue), meiMIGS-PPX2 (red), and meiMIGS-PPX1-PPX2 (green) lines respectively, compared to PPX1 and PPX2 expressions in wild type Col plant. The correlation coefficient between PPX1 and PPX2 expression values across these samples was \( r = 0.80 \), which was significantly different than expected if there were no relationship (\( P \) value=1.21\( \times 10^{-5} \)).
Extended Data Fig. 6 | Crossover frequency and interference measured in wild type and hcr1-1 using fluorescent pollen. a, Crossover frequency measured using the pollen FTLs I1bc and I3bc from wild type and hcr1-1. Crossover frequency in each interval of the three-color FTLs was measured using the DeepT etrad pipeline (Supplementary Table 20). b, Crossover interference ratio measured using FTL pollen tetrads in wild type and hcr1-1. Crossover interference ratio (IFR) were calculated using the DeepT etrad pipeline. c, Plots showing the % of tetrads containing double crossovers, using data from the three-color FTL intervals in wild type and hcr1-1. d, As for c, but showing FTL data from the I1bc, I1fg, I3bc and I5ab intervals in wild type and meiMIGS-PPX1-PPX2. Tetrads were classified into 12 fluorescence classes (A-L) by DeepT etrad. Mean values are indicated by horizontal lines.
**Extended Data Fig. 7 |** SPO11-1-oligonucleotides and nucleosome occupancy around wild type and *meiMIGS-PPX1-PPX2* crossovers. 10 kb windows surrounding crossover midpoints identified from wild type or *meiMIGS-PPX1-PPX2* plants, or the same number of randomly selected positions, were analysed for SPO11-1-oligos (log₂(SPO11-1-oligos/gDNA), red) or nucleosome occupancy (log₂(MNase-seq/gDNA), blue).
Extended Data Fig. 8 | Yeast two hybrid assays showing interactions of HCR1/PPX1 with meiotic proteins. a, Yeast two hybrid assays testing interaction between HCR1/PPX1 and Class I (ZMM) proteins. The yeast co-transformants were grown until OD$_{600}$ = 1 and spotted on synthetic dropout media (SD) lacking leucine/tryptophan (-LT) and leucine/tryptophan/histidine/adenine (-LTHA) for 3, 5 or 7 days. b, Yeast two hybrid assays of HCR1/PPX1 and meiotic proteins involved in axis formation, DSB formation and DNA repair. The yeast transformants were grown until OD$_{600}$ = 1, then diluted 10-, 100- and 1,000-fold in water, and spotted on SD (-LT) and SD (-LTHA) plates to examine growth in 3, 5, or 7 days (Supplementary Table 23).
Extended Data Fig. 9 | The EVH1 domain of Arabidopsis PP4R3A interacts with meiotic proteins. **a**, Yeast two-hybrid assays testing interaction between the PP4R3A EVH1 domain and meiotic proteins. PP4R3A-N indicates the PP4R3A N-terminal region (1-166 aa) containing the EVH1 domain. The yeast co-transformants were grown until OD$_{600}$ = 1 and spotted on synthetic dropout media (SD) lacking leucine/tryptophan (-LT) and leucine/trypotphan/histidine/adenine (-LTHA) for 3 and 5 days. The yeast transformants were grown until OD$_{600}$ = 1, then diluted 10-, 100- and 1,000-fold in water, and spotted on SD (-LT) and SD (-LTHA) plates to examine growth. **b**, Venn diagram summarizing yeast two hybrid assays of meiotic proteins that interact with HCR1/PPX1 and the PP4R3A EVH1 domain. **c**, A schematic model of Arabidopsis PP4 holoenzyme complex that recognizes target protein HEI10 for dephosphorylation via the PP4R3A EVH1 domain and PPX1.
Extended Data Fig. 10 | Genome-wide prediction of PP4 complex target proteins during meiosis. a, Protein domain (green) structure of Arabidopsis PP4 subunits PPX1, PPX2, PP4R2L and PP4R3. b, Amino acid alignment of the PP4R3A homolog EVH1 domain (red box). Hash symbols (#) indicate conserved tyrosine (Y) and tryptophan (W) residues. c, As for a, but showing the positions of FxxP motifs and phosphorylation consensus sites in PTD, HEI10, MSH5 and MLH1. d, Venn diagram showing overlap of meiotically expressed, nuclear proteins with FxxP motifs. e, Venn diagram showing overlap of candidate PP4 target proteins with CDK, DDK or ATM/ATR kinase consensus motifs, predicted using GPS 5.0. The location of HCR1 Y2H interactors are indicated within the Venn diagram. f, Histogram showing a significant enrichment of proteins containing phosphorylation sites in the predicted 1,367 PP4 targets, compared to 1,000 sets of randomly chosen genes (n=1,367). The vertical red line indicates observed predicted PP4 target proteins containing phosphorylation sites, compared to the random sets (black lines). g, Gene ontology (GO) enrichment analysis of the predicted PP4 targets, using PANTHER (http://pantherdb.org/). Benjamini-Hochberg False Discovery Rate (FDR) correction was used for enrichment test.
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| Data exclusions | No data were excluded from analysis. |
| Replication | In response to the reviewers concerns about growth conditions the GBS experiment has been replicated entirely in South Korea. We also provide data collected in Cambridge and South Korea to rule out significant differences in growth conditions influencing recombination. |
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Antibodies

Antibodies used

ASY1, RAD51, ZYP1 and MLH1 antibodies were provided as gifts by Prof Chris Frankin and Prof Mathilde Grelon and appropriate references cited. For immunoprecipitation experiments the following antibodies were used: anti-HA (Roche 12013819001) or anti-Myc (Santa Cruz sc-9E10).

Validation

The ASY1, RAD51, ZYP1 and MLH1 antibodies have been extensively published and validated in the literature, the relevant papers have been cited in the manuscript. The HA and Myc antibodies were used according to manufacturer's instructions and were validated via appropriate negative controls.