Arabidopsis thaliana FANCD2 Promotes Meiotic Crossover Formation[OPEN]

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Fanconi anemia (FA) is a human autosomal recessive disorder characterized by chromosomal instability, developmental pathologies, predisposition to cancer, and reduced fertility. So far, 19 genes have been implicated in FA, most of them involved in DNA repair. Some are conserved across higher eukaryotes, including plants. The *Arabidopsis thaliana* genome encodes a homolog of the *Fanconi anemia D2* gene (*FANCD2*) whose function in DNA repair is not yet fully understood. Here, we provide evidence that *At*FANCD2 is required for meiotic homologous recombination. Meiosis is a specialized cell division that ensures reduction of genomic content by half and DNA exchange between homologous chromosomes via crossovers (COs) prior to gamete formation. In plants, a mutation in *AtFANCD2* results in a 14% reduction of CO numbers. Genetic analysis demonstrated that *AtFANCD2* acts in parallel to both *MUTS HOMOLOG4* (*AtMSH4*), known for its role in promoting interfering COs and *MMS AND UV SENSITIVE81* (*AtMUS81*), known for its role in the formation of noninterfering COs. *At*FANCD2 promotes noninterfering COs in a MUS81-independent manner and is therefore part of an uncharted meiotic CO-promoting mechanism, in addition to those described previously.

INTRODUCTION

Fanconi anemia (FA) is a human autosomal recessive disorder characterized by chromosomal instability, bone marrow failure, congenital skeletal abnormalities, a predisposition for cancer, reduced fertility, hypogonadism, testicular failure, and cellular sensitivity to DNA cross-linking agents (Fanconi, 1967; Kee and D'Andrea, 2012). So far, mutations in 19 genes responsible for these clinical manifestations have been identified (Fanconi anemia complementation group D2, -I, -A, -B, -C, D1, -E, -F, -G, -J, -L, -M, -N, -O, -P, -Q, -R, -S, and -T; reviewed in Ceccaldi et al., 2016; Michl et al., 2016; Supplemental Data Set 1). Many interacting factors, not directly implicated in FA pathologies, have been identified, including Bloom syndrome RecQ helicase-like, DNA ligase 4, DNA-dependent protein kinase, both subunits of the KU heterodimer (KU70 and KU80), and FANCD2/FANCI-associated nuclease 1 (FAN1). All of these factors have a direct role in DNA repair and/or in DNA repair pathway choice (Nakanishi et al., 2005; Kottemann and Smogorzewska, 2013).

The mammalian FA DNA repair pathway is activated by the protein kinase ATR (ATM and Rad3-related; Andreassen et al., 2004). The FA core ubiquitin ligase complex, comprising FANCA, B, C, E, F, G, L, and M then modifies FANCD2 with a single ubiquitin moiety. Modified FANCD2 localizes to nuclear foci at DNA lesion sites together with its heterodimeric partner FANCI and other protein factors like RAD51 (FANCR), BRCA1 (FANCS), and BRCA2 (FANCD1) (reviewed in Jacquemont and Taniguchi, 2007). In addition, FANCD2 is phosphorylated by the kinase ATM (ataxia telangiectasia mutated) in response to DNA damage, eliciting a cell cycle checkpoint signal (Wang and D'Andrea, 2004).

FANCD2 is essential for DNA repair by homologous recombination (HR) in response to DNA interstrand cross-linking, but its molecular functions are still not fully understood. Despite the lack of a defined nuclease domain, it has been reported that the FANCD2 protein has a 3′-5′ directed DNA exonuclease activity and modifies DNA ends so that they are less favorable substrates for error-prone repair pathways like nonhomologous end joining (NHEJ) and more likely repaired via the faithful HR pathway (Pace et al., 2010). Furthermore, in its ubiquitinated form, FANCD2 attracts the FAN1 nuclease to DNA interstrand crosslinks (ICLs) that block the progression of replication forks. FAN1 is endowed with endo- and 5′-3′ exonuclease activities and is also essential for HR DNA repair and reestablishment of collapsed replication forks

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(Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Klein Douwel et al., 2014; Pizzolato et al., 2015; Lachaud et al., 2016). Additionally, FANCD2 may serve as a scaffold for several other nucleases like XPF-ERCC1 and MUS81-EME1 via recruitment of SLX4 (Castor et al., 2013; Boisvert and Howlett, 2014; Klein Douwel et al., 2014). Yet, the precise orchestration of the different nucleases at sites of DNA lesions is still unknown. FANCD2 also attracts CtIP to DNA lesion sites and cooperates with it in the repair of ICLs (Murina et al., 2014). This latter finding is especially intriguing since CtIP, together with the MRE11-complex, is known to process DNA ends at DNA lesion sites promoting HR DNA repair (Liu and Huang, 2016).

Considering the importance of FANCD2 for human health, understanding the underlying molecular mechanisms is of crucial importance and motivated studies of FANCD2 homologs in different model organisms. The *fancd2*$^{-/-}$ knockout mice display unpaired and mispaired chromosome regions in male meiotic cells but checkpoint activation followed by apoptosis impedes further analysis (Houghtaling et al., 2003). *Caenorhabditis elegans* encodes homologs of several FA factors (reviewed in Youds et al., 2009). It is the only animal model organism where FANCD2's (FCD-2) function in meiotic DNA repair has been extensively analyzed. The *C. elegans* FA pathway responds to double-strand breaks (DSBs) in replicating cells and *fcd-2* deletion mutants show hypersensitivity to ICL agents during somatic development (Collis et al., 2006; reviewed in Youds et al., 2009). During *C. elegans* meiosis, when crossover (CO) pathways are compromised, FCD-2 channels a subset of DNA repair intermediates into HR pathways, thereby preventing them from being processed by error-prone NHEJ pathways (Adamo et al., 2010).

Meiosis ensures the reduction of the parental genome prior to the formation of generative cells and allows the exchange of genetic information between maternal and paternal chromosomes, leading to novel combinations of genetic traits in the following generation. The molecular basis of this process is recombination between homologous chromosomes and depends on the formation of DSBs, introduced at nonrandom sites throughout the genome. DSBs are formed by an evolutionarily conserved protein complex, with the SPO11 transesterase being its catalytically active subunit (Keeney et al., 1997; Robert et al., 2016; Vrielynck et al., 2016). Following DNA cleavage, SPO11 is covalently bound to the 5′ ends of DSBs and has to be removed prior to DNA end processing. Processing creates 3′ single-stranded DNA overhangs, which are coated by the recombinases RAD51 and DMC1 to form nucleoprotein filaments that find and invade preferentially non-sister chromatids. Specialized meiotic DNA repair proteins, together with other DNA repair factors, mediate capture of the second DSB end, ligation, DNA synthesis, and resolution to generate COs (reviewed in Hunter, 2007; Muyt et al., 2009; de Massy, 2013).

Two classes of COs exist in most organisms. Class I COs are sensitive to interference (one CO inhibits formation of other COs nearby) and depend on the ZMM proteins: Zip1, Zip2, Zip3, Zip4/Spo22, Msh4/Msh5, and Mer3 in yeast (reviewed in Lynn et al., 2007). Class II COs are interference insensitive and depend on the resolvase Mus81 (Hollingsworth and Brill, 2004). Meiotic DSBs can also be repaired by mechanisms that result in non-crossovers, such as the synthesis-dependent strand annealing pathway (McMahill et al., 2007). In most organisms, at least one CO per homologous chromosome pair is essential for correct chromosome segregation (obligatory CO), but the vast majority of DSBs are repaired by non-CO pathways or by sister chromatid interactions (reviewed in Muyt et al., 2009; Phadnis et al., 2011; Wang et al., 2015).

Meiotic processes and many protein factors are widely conserved among eukaryotes, including the model plant *Arabidopsis thaliana.* Homologs of several FA pathway members have been identified in Arabidopsis and implicated in DNA repair and, in the case of the helicase FANCM and its interaction partners MHF1 and 2, also in meiotic CO formation (Crismani et al., 2012; Knoll et al., 2012; Dangel et al. 2014; Girard et al., 2014). Here, we present the identification and characterization of the Arabidopsis *FANCD2* homolog, *AtFANCD2*, and demonstrate its importance for meiotic recombination. Fertility is reduced in *Atfancd2* mutants and 14% fewer meiotic COs are formed. Strikingly, *At*FANCD2 acts in parallel to both *At*MSH4, known for its role in promoting interfering COs (class I), and *At*MUS81, known for its role in promoting noninterfering COs (class II). Taken together, our findings suggest that *At*FANCD2 processes a subset of meiotic DNA repair intermediates into noninterfering COs. Previously, disrupting class I and II CO pathways, by mutating *MSH4* and *MUS81*, did not entirely eliminate COs, suggesting the existence of additional, as yet uncharacterized, CO-forming mechanisms. Our results suggest that *At*FANCD2 is part of such an additional mechanism contributing to class II CO formation.

RESULTS

Characterization of the *AtFANCD2* Gene and Mutant Alleles

We identified an Arabidopsis gene encoding a protein with 25% identity (and 44% similarity) to human FANCD2 by homology search and named it *AtFANCD2* (At4g14970). Cloning and sequencing of the cDNA revealed that the gene comprises 31 exons and its open reading frame is 4431 base pairs in length (GenBank accession number MF327383). RT-PCR detected transcripts in all tested plant organs but especially in proliferating tissue and rapidly dividing cell suspension culture cells (Figure 1A).

To study *At*FANCD2's function, two mutant lines carrying T-DNA insertions in *AtFANCD2* exons were identified in the Salk Institute Genomic Analysis Laboratory T-DNA (Alonso et al., 2003) and GABI-KAT (Kleinboelting et al., 2012) collections. The mutant lines were named *fancd2-1* (SALK_113293) and *fancd2-2* (GABI_209C01) and analyzed (Figures 1B to 1F). Sequencing the T-DNA insertion sites revealed that *fancd2-1* has two "left borders" and that a 9-bp fragment of the 6th exon of the *AtFANCD2* sequence is deleted in the mutants. The T-DNA insertion of *fancd2-2* plants has one "left" and one "right border" and 35 bp of exon 19 and 19 bp of the adjacent intron are deleted (Figure 1C). Both mutant alleles do not generate a continuous mRNA across the T-DNA insertion sites, indicating that no functional *At*FANCD2 protein can be produced (Figure 1D). Homozygous mutant plants develop normally and are mildly but significantly affected in fertility, with seed per silique counts of 50.5 in *fancd2-1* ($\pm$5.4; n = 150 siliques) compared with 53.4 in the wild type ($\pm$8.1; n = 235 siliques; $P < 0.0001$). Pollen viability, as assessed by

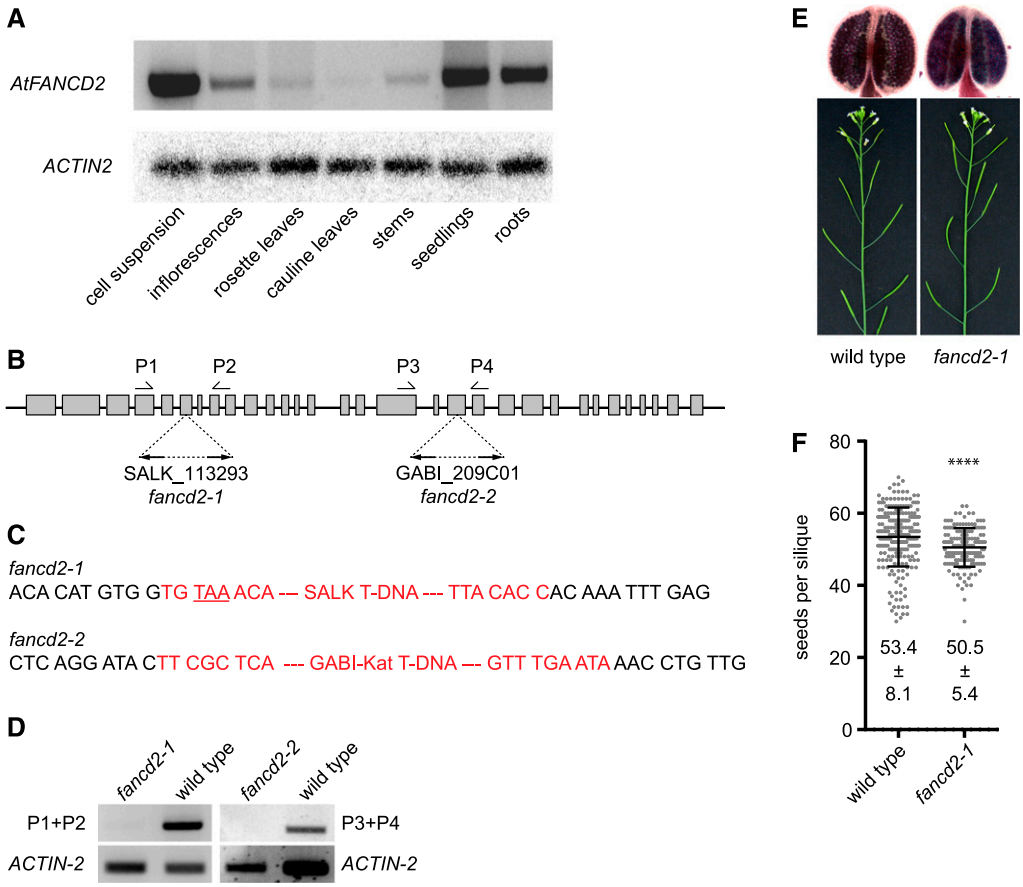

Figure 1. Characterization of the *AtFANCD2* Gene (At4g14970) and Two Corresponding T-DNA Insertion Mutants.

(A) The *AtFANCD2* gene is expressed in all tested plant organs.
(B) The exon and intron structure of the *AtFANCD2* gene has been defined by cloning and sequencing of the cDNA (GenBank accession number MF327383). The positions of the T-DNA insertion sites of the mutant lines SALK_113293 and GABI_209C01 are indicated.
(C) Sequences of regions flanking the T-DNA insertion sites are shown in black letters, with sequences corresponding to the T-DNA sequences in red letters. The first in-frame stop codon in mutant line *fancd2-1* is underlined, and the first in-frame stop codon in mutant line *fancd2-2* is found 72 bp further downstream within the T-DNA sequence.
(D) No *AtFANCD2* mRNA is produced across the T-DNA insertion sites in *fancd2-1* and *fancd2-2* mutant lines. Primer positions relative to the T-DNA insertions are indicated in **(B)**.
(E) Somatic growth, silique length, and pollen formation appear unaffected in *fancd2-1* mutants.
(F) *At*FANCD2 is required for full fertility. Average and SD of seeds per silique are indicated (****P ≤ 0.0001).

Alexander staining, appears similar to the wild type (Figures 1E and 1F) and *fancd2-2* mutants were phenotypically indistinguishable from *fancd2-1* mutant plants.

*At*FANCD2 Is Involved in Meiotic DSB Repair

To analyze FANCD2's role in meiosis, spreads of pollen mother cells (PMCs) were prepared and chromosomes were visualized by light microscopy after 4′,6-diamidino-2-phenylindole (DAPI) staining (Ross et al., 1996). Meiosis appeared to be normal in *fancd2-1* until the diplotene stage but in 12.6% of diakinesis and metaphase I cells unpaired chromosomes (four bivalents and one pair of univalents) were observed (n = 190 cells; Figure 2A).

Data from other organisms show that the FA pathway is activated by ATR and interacts with ATM, so we assessed the epistatic relationship between *AtFANCD2*, *AtATM*, and *AtATR*. *atm-2* mutant plants have meiotic defects and only generate 6.8 (±7.2; n = 364 siliques) seeds per silique (Figure 3; Garcia et al., 2003). However, in the *atm-2 fancd2-1* double mutant, fertility was further reduced to 0.9 (±1.9; n = 317 siliques) seeds per silique. In *atm-2* mutant PMCs, pachytene nuclei appeared normal, whereas metaphase I chromosomes sometimes showed interconnections and chromosome fragmentation in later stages (Figure 3; Garcia et al., 2003). These phenotypes were aggravated in the *atm-2 fancd2-1* double mutant and could be recapitulated in *atm-2 mus81-2* mutants (Figure 3). Conversely, *fancd2-1 atr-2* double mutants had no aberrant meiotic chromosome segregation or structural integrity phenotypes (Supplemental Figure 1). Chromosome fragmentation was not observed in *atm-2 fancd2-1 spo11-2-3* triple mutants, demonstrating that the observed DSB

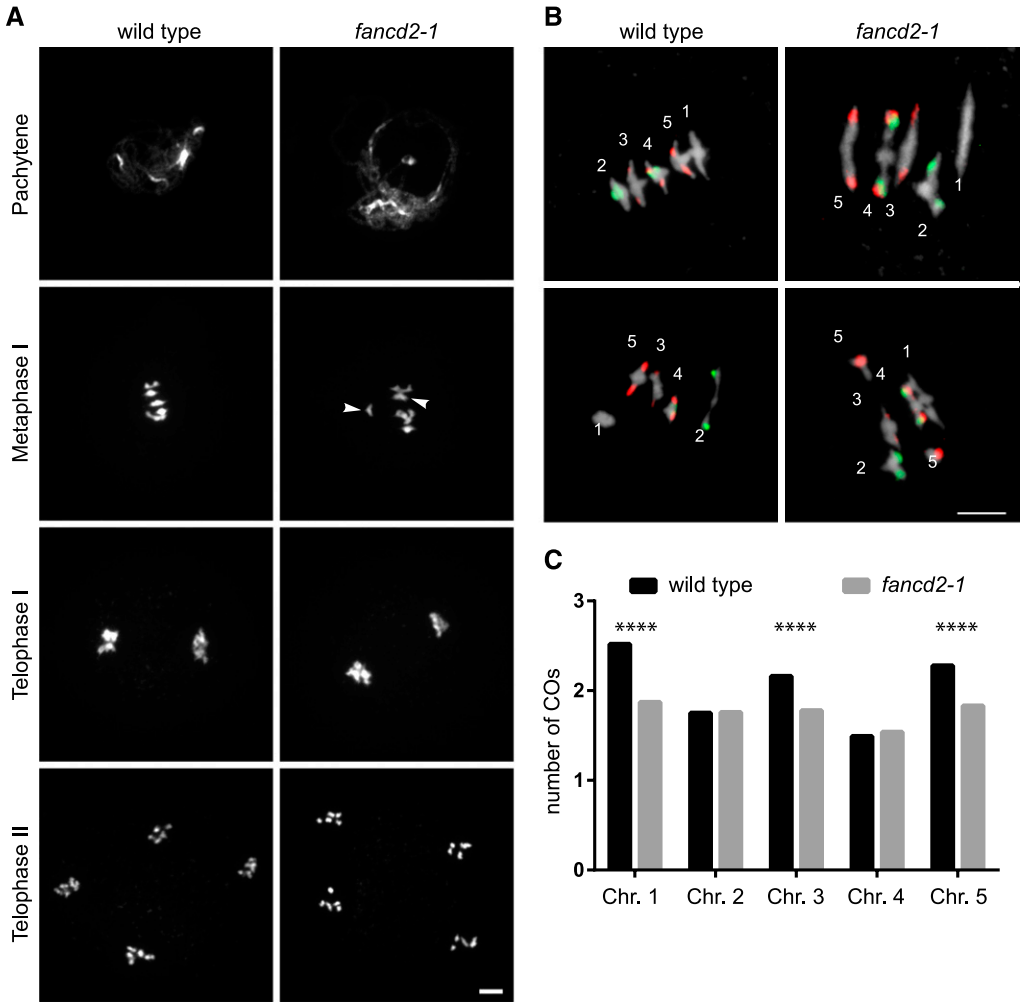

Figure 2. AtFANCD2 Promotes Meiotic Chiasma Formation.

(A) Chromosome spreads of wild-type and *fancd2-1* meiocytes with indicated stages. Arrowheads point to univalents. Bar = 5 μm.
(B) Chromosome spreads of wild-type and *fancd2-1* metaphase I cells. FISH probes against 45S (green) and 5S (red) rDNA were used to identify chromosomes, allowing for chiasma counting depending on chromosome morphology. Chromosome identities are indicated. Bar = 5 μm.
(C) Quantification of chiasmata per chromosome in wild-type (black bars) and *fancd2-1* mutant plants (gray bars) (****P ≤ 0.0001)

repair defects occur downstream of meiotic DSB formation (Supplemental Figure 2). Taken together, these results indicate that *At*FANCD2 and *At*ATM function in separate meiotic pathways following DSB formation. Alternatively, and also discussed in more detailed below, meiocytes without functional *At*ATM may produce more DNA repair intermediates that depend on *At*FANCD2 for correct processing, while these are not formed in cells devoid of *At*ATR.

*At*FANCD2 Is Required for Normal Levels of Meiotic Crossovers

To analyze CO formation in *fancd2-1* mutants, we used DAPI staining and fluorescent in situ hybridization (FISH) of metaphase I chromosome spreads to count chiasmata on individual chromosome pairs (Sanchez-Moran et al., 2002; Armstrong, 2013). While 10.2 chiasmata (±0.14) are formed on average per wild-type meiocyte (n = 69), only 8.78 chiasmata (±0.13) were found in *fancd2-1* cells (n = 111), representing a statistically significant reduction of 14% of chiasma frequency ($P < 0.001$) (Figures 2B and 2C; Supplemental Table 1). Chiasmata were specifically reduced on the long chromosomes 1 ($P < 0.0001$), 3 ($P < 0.0001$), and 5 ($P < 0.0001$), while chiasma frequency was almost unaffected on the short, NOR-bearing, chromosomes 2 ($P = 0.974$) and 4 ($P = 0.644$).

To analyze meiotic recombination frequency more closely and in a defined chromosomal region, we used the fluorescent tagged lines (FTLs) system (Francis et al., 2007; Berchowitz and Copenhaver, 2008). Transgenes encoding fluorescent marker proteins, inserted at defined positions on the chromosomes, are driven by the pollen-specific, postmeiotic *LAT52* promoter. In addition, mutation of the *QUARTET1* gene enables tetrad analysis because the four pollen grains originating from each meiotic division remain physically attached and do not separate during development (Preuss et al., 1994; Francis et al., 2006). Assaying the marker

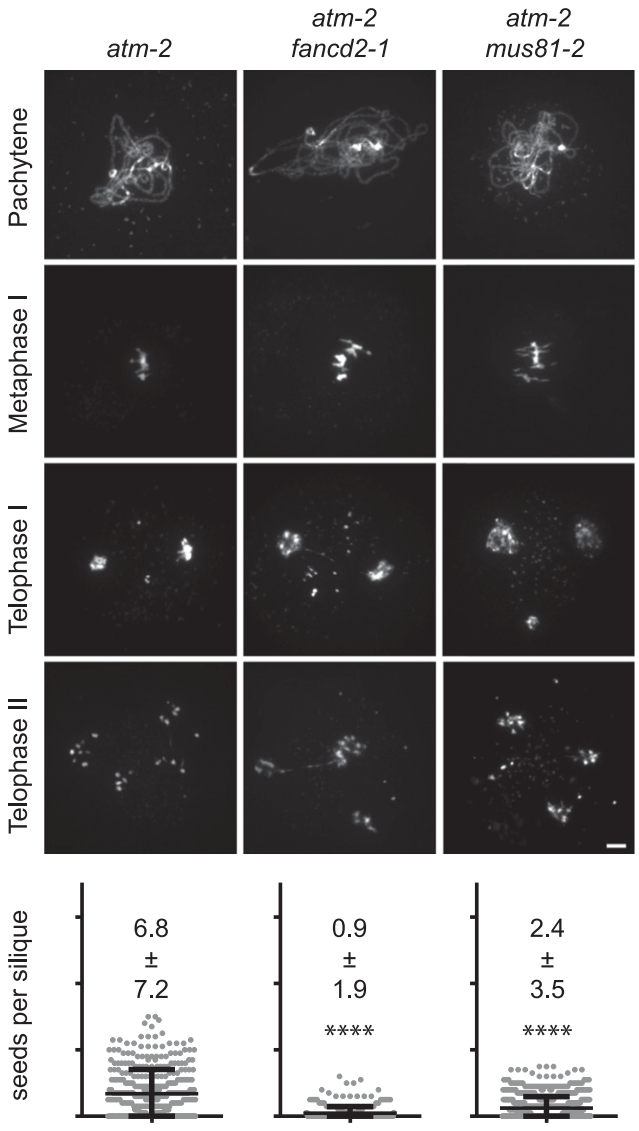

Figure 3. Meiotic Progression and Seed Formation in *atm-2* and Indicated Double Mutant Plants.

The stages of meiotic progression are indicated. *fancd2-1 atm-2* and *mus81-2 atm-2* double mutants are similarly affected with significantly reduced fertility compared with *atm-2* single mutants. Plots represent seed formation; average and SD are indicated (****P ≤ 0.0001). Bar = 5 μm.

distribution in the four meiotically related pollen grains allows for the calculation of genetic distances within and interference between selected intervals. We determined CO frequency in two neighboring intervals, I5c and I5d, on chromosome 5 by counting fluorescent pollen tetrads from wild-type and *fancd2-1* mutant plants (Figure 4A). I5c (1.4 Mb) spans 5.9 cM and I5d (1.7 Mb) 6.2 cM in wild-type plants (n = 4682 pollen tetrads from five plants). No difference in recombination frequency was observed for interval I5c (5.9 cM) in *fancd2-1* mutants, but recombination was significantly reduced to 5.2 cM in I5d (n = 4789 pollen tetrads from six plants; P = 0.0022). We also analyzed two additional intervals, I2a and I2b, on chromosome 2. No significant difference in recombination frequency was observed for I2b (1.5Mb; 4.7 to 4.6 cM) between the wild type and *fancd2-1* mutants, but recombination was significantly reduced in the I2a interval (0.5 Mb), from 3 cM in the wild type to 2.5 cM in *fancd2-1* mutants (P = 0.046; wild type, n = 4542 pollen tetrads from five plants; *fancd2-1*, n = 4544 pollen tetrads from 10 plants). Interestingly, interference was in place in all analyzed regions and genotypes (interference ratios I2ab: wild type, 0.19; *fancd2-1*, 0.27; I5cd wild type, 0.36; *fancd2-1*, 0.28) (Figure 4A; Supplemental Table 2). Further evidence that the interference-sensitive CO I pathway remains unaffected in the *fancd2-1* mutant background is provided by the analysis of HEI10 foci numbers. HEI10 is an E3 ubiquitin ligase known to promote interference-sensitive COs (Ward et al., 2007; Chelysheva et al., 2012). The same numbers of HEI10 foci are observed in wild-type (10.4 ± 1.5; n = 24) and *fancd2-1* mutant (10.6 ± 1.5; n = 21) meiocytes at pachytene stage (P = 0.8581; Figure 4B).

DMC1 but Not RAD51 Foci Numbers Are Altered in *fancd2-1*

Placing *At*FANCD2 functions downstream of DSB formation but upstream of (or during) CO formation, we sought to define its role in meiotic recombination more precisely. To this end, we quantified the number of foci of the recombinase RAD51 in zygotene stages in PMCs using immunofluorescence. We observed an average of 210 RAD51 foci (±40; n = 22 cells) in *fancd2-1* cells and 233 (±45; n = 14 cells) foci in wild-type cells, representing a nonsignificant difference (P = 0.0966) (Figure 5A). We also stained for the meiosis-specific recombinase DMC1 and found a mild but significant increase in DMC1 foci (P = 0.0001) in the absence of *At*FANCD2. In *fancd2-1* zygotene cells, 250 (±32; n = 10 cells) DMC1 foci were observed, while only 188 (±41; n = 34 cells) foci are formed in wild-type cells (Figure 5B). These numbers indicate that the absence of *At*FANCD2 does not affect meiotic DSB formation, that DNA ends at meiotic DSB sites are processed, and that they acquire recombinase proteins. In case *At*FANCD2 affected meiotic DSB formation (e.g., as part of a negative feedback regulatory loop), it would be expected that RAD51 and DMC1 foci numbers were equally altered. As only DMC1 foci are increased in the absence of *At*FANCD2, this rather suggests that DNA repair events involving DMC1 are slowed (Duroc et al., 2014; Thacker et al., 2014) but not blocked, since complete synapsis is established in *fancd2-1* pachytene meiocytes (Figure 6).

*At*FANCD2 Promotes CO Formation in Parallel to Both *At*MSH4 and *At*MUS81

The indication that interhomolog DNA repair events may be affected in *Atfancd2-1* mutants together with the observed 14% reduction of chiasma numbers prompted us to analyze the epistasis of *AtFANCD2* and genes encoding known CO-promoting factors. *fancd2-1* mutants were crossed to *msh4* mutant plants, defective in class I interference-sensitive COs, and *mus81-2* mutant plants, defective in class II, interference-insensitive COs (Higgins et al., 2004, 2008a; Hartung et al., 2006; Berchowitz et al., 2007). CO reduction and specifically loss of the so-called obligatory CO results in chromosome missegregation and the occurrence of univalent chromosomes instead of bivalent pairs. Using DAPI staining on

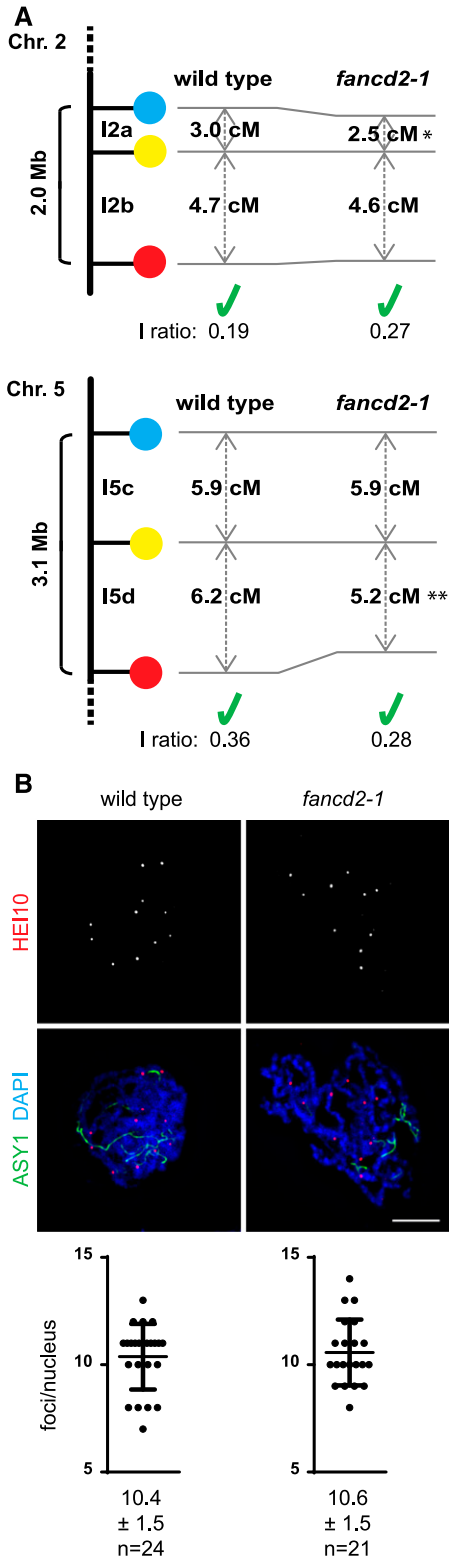

Figure 4. *At*FANCD2 Is Required for Normal Levels of Meiotic Interference-Insensitive Crossovers.

chromosomes of PMC spreads, we observed no univalents in wild-type cells ($n > 190$), whereas we found univalents in 12.6% of all analyzed *fancd2-1* cells ($n = 190$, $P < 0.0001$ compared with the wild type). We found univalents in 86.5% of *msh4* cells ($n = 37$), with many cells displaying only one, two, or three bivalents, consistent with published data (Higgins et al., 2004). In *msh4 fancd2-1* double mutants, all observed cells ($n = 36$) contained univalents and have a clear shift to less bivalents per meiocyte, compared with *msh4* single mutants ($P = 0.0006$; Figure 7).

mus81-2 mutant plants have so far not been reported to display univalents, despite a ~10% reduction in meiotic CO frequency (Berchowitz et al., 2007; Higgins et al., 2008a). We observed two cells with univalents out of 46 *mus81-2* cells scored (Supplemental Figure 3). Crossing *fancd2-1* to *mus81-2*, the percentage of cells with univalents increased from 4.3% in *mus81-2* to 26.3% in the double mutant ($n = 38$, $P = 0.0038$ compared with *mus81-2*), indicating that *At*MUS81 and *At*FANCD2 are involved in formation of obligatory COs. We also observed cells with more than one chromosome pair affected in the double mutant (Figure 7).

Thus, *fancd2-1* exhibits an additive effect in combination with both *msh4* and *mus81-2*. It has been shown before that *msh4* and *mus81* mutant combinations have an additive effect on CO number reduction in various organisms (de los Santos et al., 2003; Argueso et al., 2004; Whitby, 2005; Berchowitz et al., 2007; Higgins et al., 2008a; Holloway et al., 2008) and the corresponding proteins have been placed in either the interfering CO (class I) or the noninterfering CO (class II) pathway (Copenhaver et al., 2002). Eliminating both pathways does not result in a complete absence of COs, which lead to the speculation that one or more additional CO-promoting mechanisms may exist (de los Santos et al., 2003; Argueso et al., 2004; Whitby, 2005; Berchowitz et al., 2007; Higgins et al., 2008a). *At*FANCD2 appears to be involved in a pathway that acts in parallel to both *At*MSH4 and *At*MUS81.

To investigate this further, we generated a triple mutant line combining the *msh4*, *mus81-2*, and *fancd2-1* mutant alleles. The *msh4 mus81-2 fancd2-1* triple mutant ($n = 34$) has fewer bivalents than the *msh4 mus81-2* double mutant ($n = 41$) ($P < 0.0001$), presumably due to fewer COs; 67.6% of the *msh4 mus81-2 fancd2-1* triple mutant meiocytes contain no bivalents (2.4% in the double mutant), 26.5% of the cells display one, and only 5.9% have two bivalents (compared with 31.7% and 46.3%, respectively, in the *msh4 mus81-2* double mutants). Nonetheless, CO formation does not appear to be completely abrogated, since

(A) CO frequencies in two neighboring intervals on chromosomes 2 and 5 were analyzed. Recombination is significantly reduced in two of the four intervals in *fancd2-1* mutants, while interference is still in place (see text for details). Interval I2a (0.5 Mb): FTL1506 (eCFP) and FTL1524 (eYFP). Interval I2b (1.5 Mb): FTL1524 and FTL965 (DsRed2). Interval I5c (1.4 Mb): FTL 1963 (eCFP) and FTL 1143 (eYFP). Interval I5d (1.7 Mb): FTL1143 and FTL2450 (DsRed2). *$P \leq 0.05$ and **$P \leq 0.01$.

(B) Spreads of pachytene cells stained for the axial element protein ASY1 (green) and the interference-sensitive CO marker HEI10 (red). Dot plots show foci numbers per nucleus. Average and SD are indicated. Foci numbers are not significantly different between wild-type and *fancd2-1* nuclei ($P = 0.8607$). Bar = 5 μm.

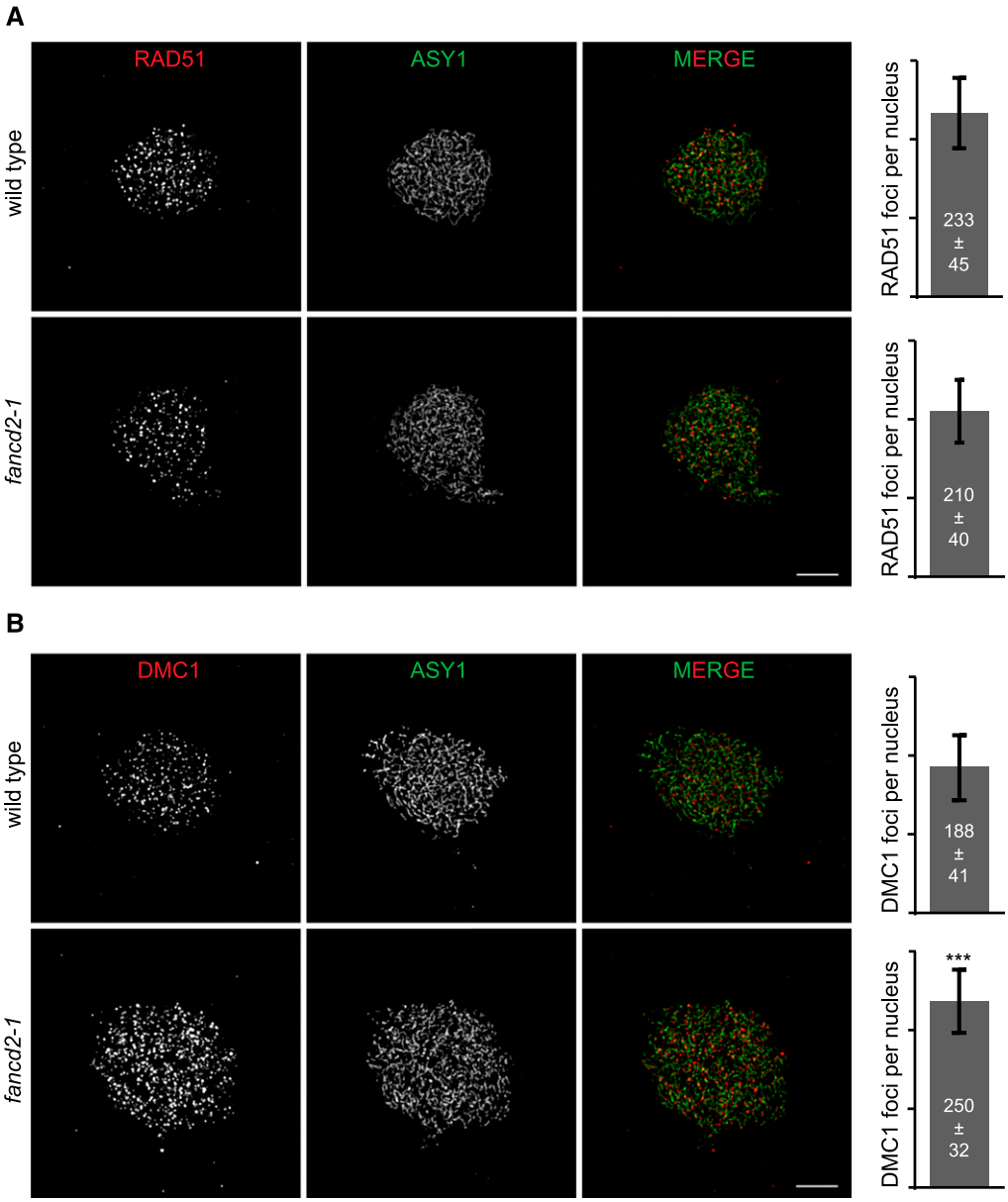

Figure 5. Immunolocalization of Recombinases RAD51 and DMC1 in Wild-Type and *fancd2-1* Meiocytes.

(A) Spreads of zygotene cells stained for the axial element protein ASY1 (green) and the recombinase RAD51 (red). RAD51 foci numbers are not significantly different (P = 0.0966) between wild-type (n = 14) and *fancd2-1* mutant plants (n = 22).

(B) Spreads of zygotene cells stained for ASY1 (green) and DMC1 (red). DMC1 foci numbers are significantly different (P = 0.0001) between wild-type (n = 34) and *fancd2-1* mutant plants (n = 10). Bar graphs show average foci numbers per nucleus; SD is indicated. Bars = 5 μm.

some bivalents are still observed. The data also demonstrate that in the *msh4 mus81-2* background *At*FANCD2 is needed for ~80% of the remaining COs, while in wild-type meiocytes, it is required for only 14% of COs (Figure 7).

Mutation of *AtFAN1* Compromises Meiotic Progression

Reports from other organisms show that FANCD2 interacts with the FAN1 nuclease that is also essential for HR DNA repair and reestablishment of replication forks (Liu et al., 2010; Smogorzewska et al., 2010). Inspired by these findings, we used homology searching to identify the Arabidopsis gene At1g48360 as a nonambiguous *FAN1* homolog, encoding a protein with 23% identity and 36% similarity to its human counterpart. An insertion mutant was identified in the Saskatoon Arabidopsis T-DNA collection (Robinson et al., 2009) and named *fan1-2* (Figure 8). Puchta and colleagues also independently identified *AtFAN1* and analyzed *fan1-1* mutants (Herrmann et al., 2015). They found that *At*FAN1 is involved in

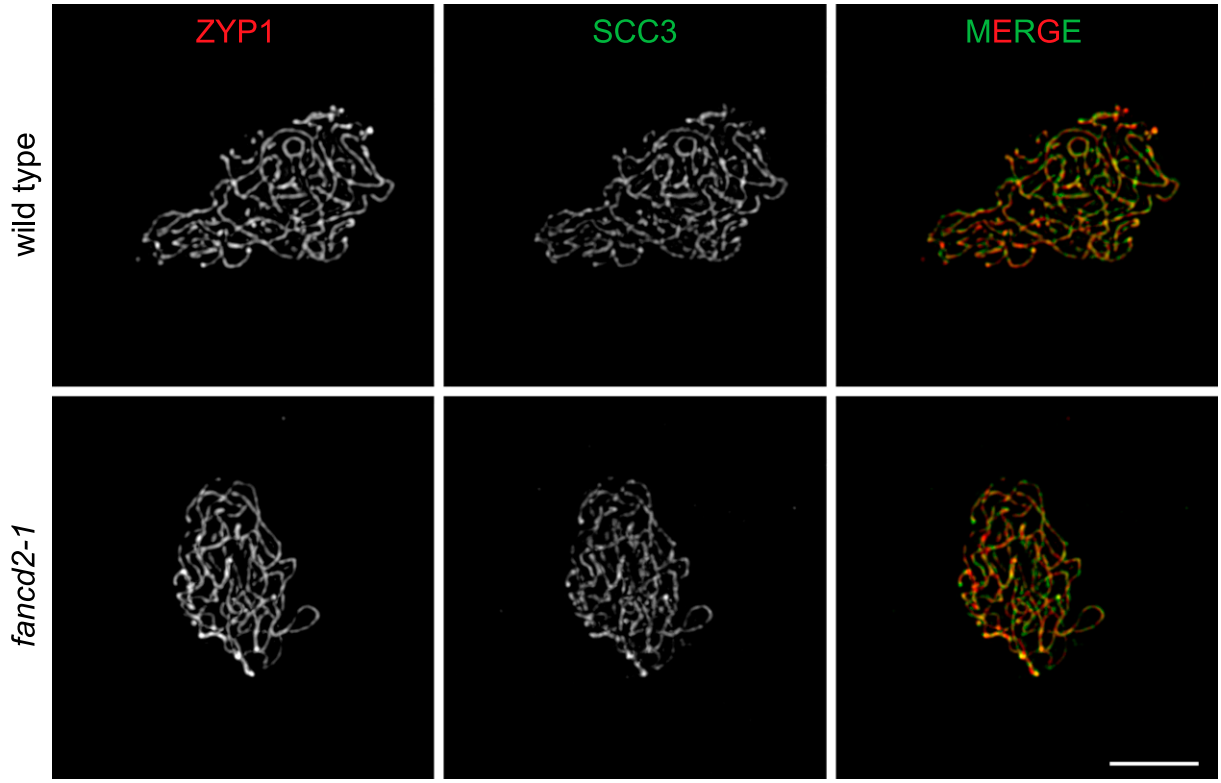

Figure 6. Regular Pachytene and SC Formation in the Absence of *At*FANCD2.

Immunolocalization of the synaptonemal complex protein ZYP1 (red) and the cohesin protein SCC3 (green) in wild-type and *fancd2-1* meiocytes. Continuous ZYP1 staining along all chromosomes in spread pachytene cells confirms that synapsis is complete in the absence of *At*FANCD2. Bar = 5 μm

somatic DNA crosslink repair acting independently of *At*MUS81. Our analysis showed that *fan1-2* mutant plants displayed normal growth and vegetative development and that seed set was similar to the wild type (55.9 ± 4.4, n = 49 siliques, P = 0.0926). Analysis of DAPI-stained *fan1-2* meiotic chromosomes revealed no obvious meiotic phenotype and meiotic progression seemed unaffected (Figure 8). In contrast to *fancd2-1*, none of the *fan1-2* meiocytes showed univalents (n = 58), but importantly meiocytes from *fan1-2 atm-2* double mutants displayed severe chromosome fragmentation and a drastic reduction of fertility (1.9 ± 2.8; n = 123 siliques; Figure 8), compared with *atm-2* single mutants (6.8 ± 7.2; n = 364 siliques; P < 0.0001; Figure 3). In this sense, the *fancd2-1* and *fan1-2* mutants behave very similarly. Based on these observations, we propose that in *atm-2* meiocytes more DNA repair intermediates are generated, and these extra intermediates depend on *At*FANCD2 and *At*FAN1 for correct processing. Assuming that *At*FAN1 is recruited by *At*FANCD2, it should work in parallel to *At*MUS81. This is consistent with published data on somatic DNA repair in plants (Herrmann et al., 2015).

DISCUSSION

Here, we report that the Arabidopsis homolog of FANCD2 is required for meiotic recombination. Fertility is mildly reduced in *fancd2* mutants and we observed occasional univalents in male meiocytes, with ~13% of all cells displaying at least one chromosome pair without the obligatory CO. Even though these will lead to a small number of chromosomally unbalanced male gametes, the majority of developing pollen grains in anthers will have the correct number of chromosomes and therefore not substantially compromise fertility.

We also observed an overall reduction of COs using both cytological and genetic assays. The latter experiment had further implications: First, we found that recombination is not homogenously affected, with "hotter" regions showing larger changes and second, that CO interference is still intact in *fancd2-1* plants, implying that *At*FANCD2 promotes noninterfering COs. Chiasma formation on chromosomes 2 and 4 was not affected in *fancd2-1*. We attribute this to the fact that interference-insensitive crossovers are already generally reduced on these short, NOR-bearing chromosomes (Lam et al., 2005).

*At*FANCD2 has also been previously tested in a large survey for class I CO formation defect suppressors but was shown to not be involved. A decrease of bivalent formation was observed, corroborating our results (Girard et al., 2014).

The reduced recombination frequency phenotype motivated us to further investigate *At*FANCD2's involvement in CO formation. We crossed *fancd2-1* mutants to *msh4* and *mus81-2* mutant lines. MSH4, together with its heterodimeric partner MSH5, is thought to form a protein ring around DNA repair intermediates that will mature into interfering COs (Higgins et al., 2004, 2008b; reviewed in Manhart and Alani, 2016). MUS81, together with its partner EME1, has been shown to be a structure specific nuclease, to

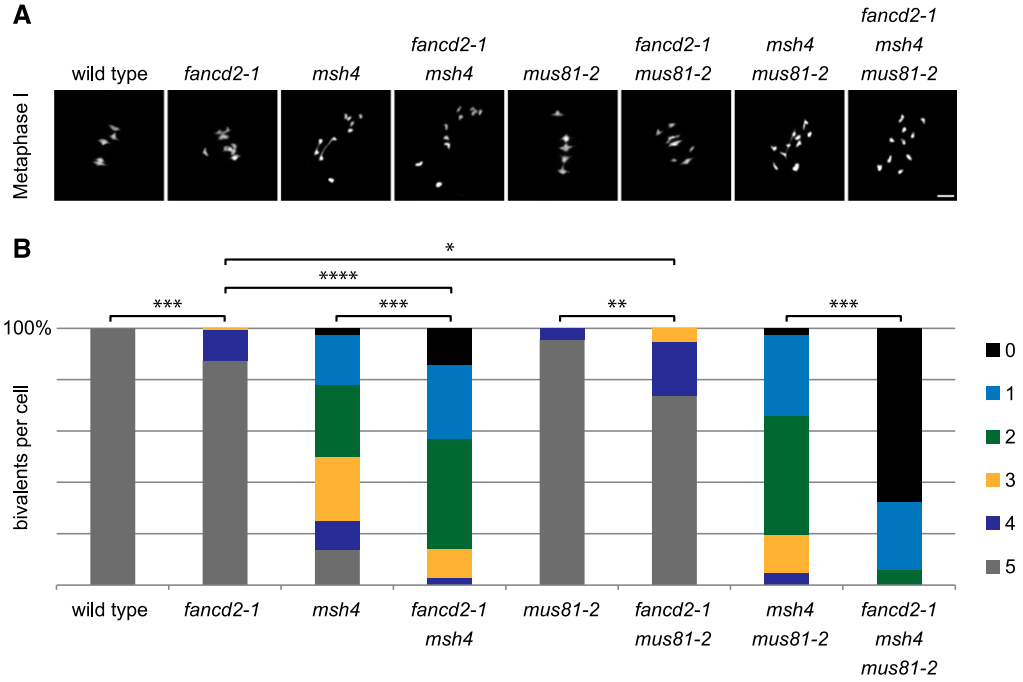

Figure 7. *At*FANCD2 Promotes CO Formation Independently of *At*MSH4 and *At*MUS81.

(A) Representative images of metaphase I cells are shown.

(B) Bivalents, as a readout for CO formation, were assessed in chromosome spreads of the indicated mutant lines. The colors indicate bivalents per cell (gray indicates that all chromosomes found their respective partners, and black indicates that no bivalent has been formed) in percent. Mutation of *AtFANCD2* leads to a significant decrease (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001) of bivalent formation in all tested mutant backgrounds. Bar = 5 μm.

process nicked and mature Holliday junctions (Hartung et al., 2006; Berchowitz et al., 2007; Amangyeld et al., 2014; reviewed in Ciccia et al., 2008; Wyatt and West, 2014; Blanco and Matos, 2015), yielding noninterfering COs or non-crossovers (Oke et al., 2014). In plants and other organisms, the MSH4-dependent and MUS81-dependent COs are generated in parallel. Interestingly, Arabidopsis *msh4 mus81* double mutants (and further double mutants that affect either one or the other mechanism) still form residual COs (Berchowitz et al., 2007; Higgins et al., 2008a), which has led to the suggestion that additional proteins and pathways may exist that contribute to CO formation. This CO pathway or mechanism was thought to exclusively generate noninterfering COs, since all mutations that affect the interfering COs are not additive and are believed to act in a single pathway.

*At*FANCD2 is essential for 14% of all chiasmata and the remaining COs in the corresponding mutant plants show proficient interference. Furthermore, *At*FANCD2 acts in parallel to both, *At*MSH4 and *At*MUS81, as the corresponding double mutants are more severely affected in terms of CO formation than the respective single mutants. Importantly, the remaining COs in the *msh4 mus81-2* double mutant plants depend to a large extent on *At*FANCD2. Therefore, we suggest that *At*FANCD2 is part of a previously uncharacterized mechanism, yielding class II COs in plants, with at least equal importance as *At*MUS81. *At*FANCD2 serves in parallel to the well-characterized interference-sensitive (class I) CO pathway and represents an additional player for the production of interference-insensitive (class II) COs, independent of *At*MUS81. It appears that meiotic DNA repair intermediates are processed by a range of different proteins that have affinity for them, rather than depending on a series of precisely orchestrated events for repair. *At*FANCD2 appears to be used for a subset of these intermediates in wild-type meiocytes. When the "class I" pathway is compromised or the structure specific resolvase *At*MUS81 is missing, more intermediates are channeled into CO repair via *At*FANCD2. This is consistent with our observations that in the wild type, only 14% of the COs depend on *At*FANCD2, but in *msh4 mus81-2* double mutants, most of the remaining COs depend on *At*FANCD2 (bivalent formation is reduced by 80% in the triple compared with the double mutant). Since CO formation is not fully abrogated in the triple mutants, further factors implicated in (most likely) class II CO formation remain to be identified.

Further support for the interpretations above comes from the analysis of the *atm-2 fancd2-1* double mutant. ATM has been established as a negative regulator of meiotic DSB formation in various organisms (Joyce et al., 2011; Lange et al., 2011; Zhang et al., 2011; Carballo et al., 2013; Garcia et al., 2015; Mohibullah and Keeney, 2017). We propose that the aggravation of the fertility and chromosome fragmentation defects observed in the double mutant compared with the *atm-2* single mutant is a result of supernumerary DSBs formed in the absence of ATM and that the additional DSBs may result in more DNA repair intermediates that depend on *At*FANCD2 and further class II CO factors. This is in line with our findings that the reduction of fertility is similar in *atm-2 mus81* and *atm-2 fan1-1* double mutants (Figures 3 and 8).

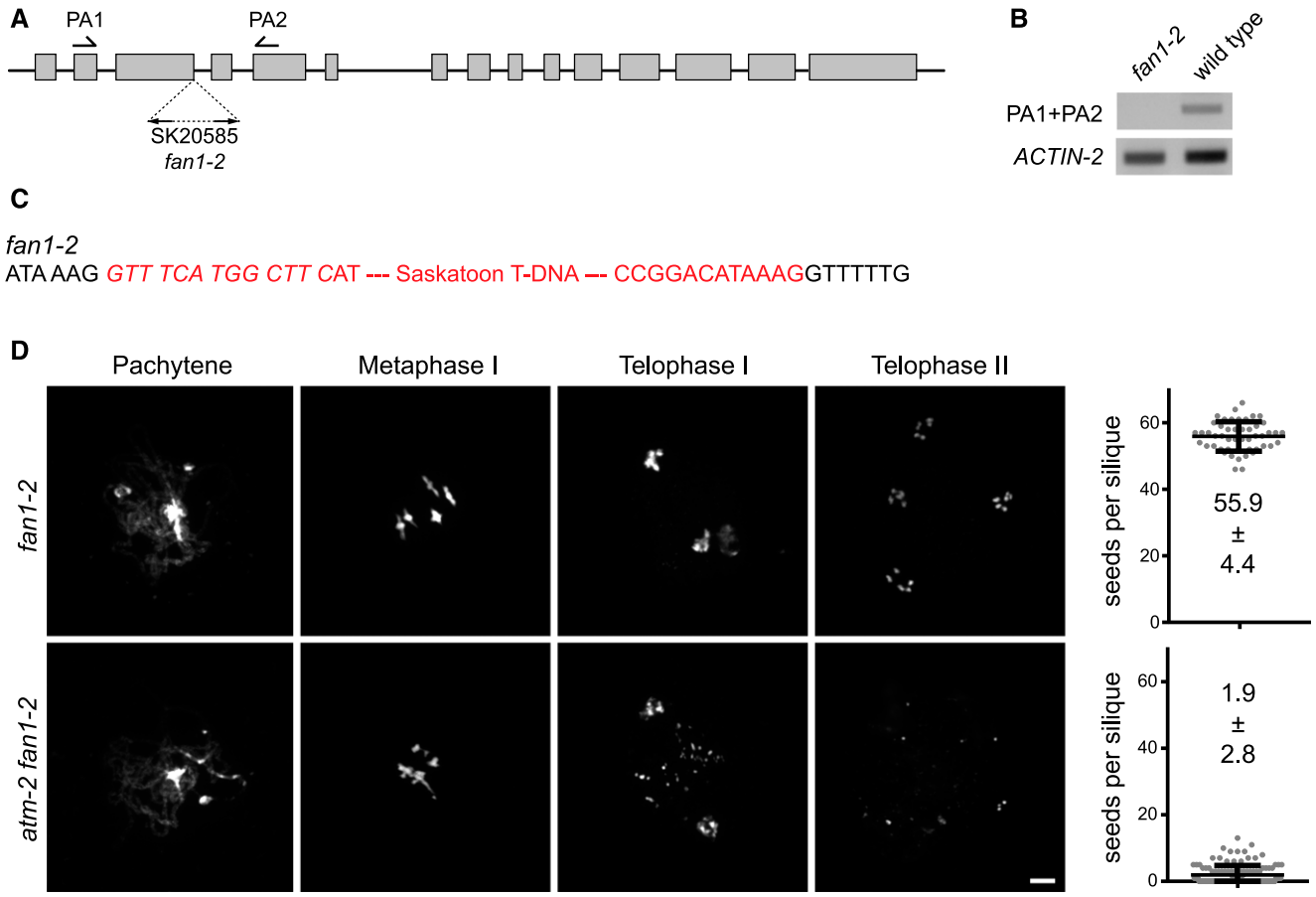

Figure 8. Characterization of the *Atfan1-2* T-DNA Insertion Mutant.

(A) Exons and introns of the Arabidopsis *FAN1* gene have been defined before (Herrmann et al. 2015). The positions of the T-DNA insertion site in the mutant line are indicated. Primer binding positions for expression analysis are given.
(B) Using primers spanning the insertion site, no expression of *AtFAN1* could be detected in *fan1-2* mutants.
(C) Sequences of genomic regions flanking the T-DNA insertion site are shown in black letters, and sequences corresponding to the T-DNA are in red letters. Inserted bases are given in italics. The first in-frame stop codon in the mutant line is found 66 bp downstream of the insertion site within the T-DNA sequence.
(D) Meiotic progression and seed formation in *fan1-2* and *atm-2 fan1-2* mutant plants. Meiotic stages are indicated.

Unexpectedly, our data also shed new light on CO assurance, a mechanism that ensures that each pair of homologs is connected by at least one CO (Jones and Franklin, 2006; Martini et al., 2006). It appears that both class I and II COs contribute to CO assurance.

In summary, we present data that establish *At*FANCD2 as a CO-promoting factor in plants. Considering the evolutionary conservation of FANCD2 and its related proteins, we speculate that this function will also be conserved. A previous study implicated the *C. elegans* FANCD2 protein (FCD-2) in meiotic DNA repair, yet the predominant *fcd-2* phenotype was aberrant DNA repair via NHEJ in the *msh-4* mutant background. Univalents in the single *fcd-2* mutant have not been observed. It should be noted that *C. elegans* lacks DMC1 (Rinaldo et al., 2002) and has very rigorous CO interference control, with typically only one CO per bivalent (Hillers and Villeneuve, 2003). In Arabidopsis, it appears that especially the interhomolog, DMC1-dependent HR events are affected in the absence of *At*FANCD2. This may be interpreted that DNA repair machineries in *C. elegans* are less redundant and upon loss of MSH-4 and FANCD-2, NHEJ proteins can acquire access to unrepaired DNA breaks or DNA repair intermediates. Future studies are needed to understand the precise molecular role of FANCD2 during meiosis in different organisms. We advocate the idea that FANCD2, likely together with its conserved partner FANCI, serves as an adaptor for nucleases and other DNA repair factors, as it has been shown for somatic DNA repair (Garner and Smogorzewska, 2011; Yamamoto et al., 2011; Chaudhury et al., 2013; Boisvert and Howlett, 2014): First, it serves to recognize specific DNA repair intermediates (Park et al., 2005; Sobeck et al., 2007; Sato et al., 2012; Liang et al., 2016) that, at least in the case of Arabidopsis, are formed in ~14% of all interhomolog meiotic DNA repair events, and, second, to recruit (meiotic) DNA repair proteins that then cleave and process the DNA (Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Boisvert and Howlett, 2014; Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014). We furthermore present a plant candidate for such a recruited protein, the Arabidopsis relative of the nuclease FAN1, which recapitulates

the aggravation of DNA damage and loss of fertility in the *atm-2* mutant background. Future experiments will show which factors, possibly including *At*FAN1, are recruited to meiotic chromatin by *At*FANCD2.

METHODS

Plant Growth Conditions

Plants were grown under long-day conditions (16 h light, 8 h dark, 21°C; 60% humidity, 15,550 lux, T5 Tube illumination). All lines were *Arabidopsis thaliana*, ecotype Columbia.

Mutant Plant Lines

The following mutant plant lines were used: *fancd2-1* (SALK_113293), *fancd2-2* (GABI_209C01), *fan1-1* (GABI_815C08; Herrmann et al., 2015), *fan1-2* (SK20585), *atm-2* (SALK_006953; Garcia et al., 2003), *atr-2* (SALK_032841; Culligan et al., 2004), *msh4* (SALK_136296; Higgins et al., 2004), and *mus81-2* (SALK_107515; Hartung et al., 2006). Confirmation of genotyping was performed by PCR using the following primers: *fancd2-1*: FancUbup, P1, and SALK LBa1; *fancd2-2*: FD2-2L, FD2-2R, and GABItest1; *fan1-1*: F1-1_LP, F1-1_RP, and GABItest1; *fan1-2*: PA1, FAN1_RP, and GABItest1; *atm-2*: ATM-F1, ATM-R1, and SALK LBc1; *atr-2*: atr2_1, atr2_3, and SALK LBa1; *msh4*: MSH4_F1, MSH4EXP_R1, and SALK LBa1; *mus81-2*: Mus81_LP, Mus81_RP, and SALK LBa1 (Supplemental Table 3).

Cloning of the *AtFANCD2* cDNA

The cDNA has been cloned using primers based on the gene predictions of TAIR 9. Various primer combinations have been used to obtain exonic parts that were not correctly predicted. Ultimately, the full-length cDNA of *AtFANCD2* was obtained ligating six PCR generated fragments by conventional cloning techniques. Fragments were constructed using the following primer pairs: F1CK and Mfe_up, Mfe_dn and F3CK, 3-no and Sac_up, Sac_dn and Aha_up, Aha_dn and Eco_up, and Eco_dn and Sma_up (Supplemental Table 3). The cDNA was submitted to GenBank (accession number MF327383).

Expression Analysis of *AtFANCD2*

RNA was isolated from various tissues using the TRI reagent solution (Sigma-Aldrich) according to the manufacturer's instructions and remaining DNA removed by a subsequent DNase treatment. Two micrograms of RNA was reverse-transcribed using RevertAid H minus M-MuLV reverse transcriptase (Fermentas) according to the manufacturer's instructions. A 20-cycle PCR was performed using primers Actin2.1 and Actin2.2 and *Taq* polymerase (Fermentas). PCR products were separated on an agarose gel and blotted on a nylon membrane. The *ACTIN7* sequence (At5g09810) was detected using a probe constructed by PCR using primers Actin2.1 and Actin2.2 and cDNA obtained from buds as template. The intensity of the 1631-bp signal was measured using Quantity One (Bio-Rad) and normalized to the band with lowest expression. The cDNA was subsequently diluted according to the normalized expression and the same amount of cDNA was used in a subsequent PCR amplifying the *AtFANCD2* cDNA using primers Aha_dn and Sma_up. Amplification products were analyzed on an agarose gel.

Sequencing of T-DNA Junctions

The upstream T-DNA junction of *fancd2-1* was amplified using primers LBc1 and FancUbup. The downstream border was amplified using primers LBc1 and P2. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

The upstream T-DNA junction of *fancd2-2* was amplified using primers GABItest1 and P3. The downstream border was amplified using primers LBc1 and P2. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

The upstream T-DNA junction of *fan1-2* was amplified using primers PA1 and o8409. The downstream border was amplified using primers pAC106RB and FAN1_RP. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

Seed per Silique Counts

Mature but still green siliques originating from the fifth to the thirtieth flower per stem were harvested into 96% ethanol and left at room temperature for 1 to 3 days for destaining. Ethanol was exchanged several times until the tissue was destained and seeds inside siliques were counted manually under a dissection microscope.

Cytology

Spreads of PMCs for DAPI staining were prepared as described (Vignard et al., 2007). Prophase spreads for cytological detection of proteins were prepared as described before (Kurzbauer et al., 2012). The following primary antibodies were used as described previously: anti-HEI10 raised in rabbit (Chelysheva et al., 2012), anti-ASY1 raised in rabbit (Armstrong et al., 2002) or rat (Higgins et al., 2004), anti-RAD51 raised in rat (Kurzbauer et al., 2012), anti-DMC1 raised in rabbit (Chelysheva et al., 2007), anti-SCC3 raised in rabbit (Chelysheva et al., 2005), and anti-ZYP1 raised in rat (Higgins et al., 2005). The secondary antibodies used were as follows: Goat-anti-rabbit conjugated to FITC (1:300; Sigma-Aldrich; F9887), goat-anti-rat conjugated to Cy3 (1:300; Chemicon; AP183C), goat-anti rabbit conjugated to Alexa Fluor 568 (1:500; Invitrogen; A11036), and donkey-anti-rat conjugated to Alexa Fluor 488 (1:500; Invitrogen; A21208).

Genome-Wide Chiasma Counting

Fixation, spread preparation of PMCs, and FISH were performed as previously described (Sanchez Moran et al., 2001; López et al., 2012). FISH using 45S and 5S rDNA probes, combined with chromosome morphology, allowed the unambiguous identification of each chromosome and chromosome arm (Sanchez-Moran et al., 2002).

Determination of Genetic Distances and Interference Ratios

Genetic distances and interference were determined as described before (Berchowitz and Copenhaver, 2008). The following lines were used: FTL1506, FTL1524, and FTL965 for analysis of chromosome II and FTL1963, FTL1143, and FTL2450 for analysis of chromosome V. Analyzed plants were all grown at the same time and tetrads from at least five individual plants per genotype were evaluated.

Statistical Analyses

Chiasma data were analyzed using the SPSS statistical package. Due to the binary nature of the results (recombination or no recombination), differences in genetic distances were analyzed by binary logistic regression. All other statistical analyses were performed using the GraphPad Prism software version 6.07. Unpaired, two-tailed Mann-Whitney tests were performed, since D'Agostino Pearson omnibus K2 normality testing revealed that most data were not sampled from a Gaussian population and nonparametric tests were therefore required. Error bars indicate standard deviations.

Accession Numbers

Sequence data from this article can be found in the GenBank database under accession number MF327383.

Supplemental Data

Supplemental Figure 1. Meiotic DNA repair is unaffected in *fancd2-1 atr-2* double mutants.

Supplemental Figure 2. Meiotic DNA repair defects in *atm-2 fancd2-1* double mutants depend on presence of *At*SPO11-2.

Supplemental Figure 3. Univalents are formed in *mus81-2* meiocytes.

Supplemental Table 1. Mean chiasma frequencies per cell, per bivalent, and per bivalent arm.

Supplemental Table 2. Tetrad analysis

Supplemental Table 3. Oligonucleotides used in this study.

Supplemental Data Set 1. Fanconi anemia genes and their functions in mammals and plants.

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AUTHOR CONTRIBUTIONS

M.-T.K., M.P., C.K., J.S., R.L., C.O., M.P.J., and M.M. performed the experiments shown. M.-T.K., D.S., G.P.C., and P.S. conceived the experiments. M.-T.K., M.P., and P.S. analyzed the data. M.-T.K. and P.S. wrote the manuscript with substantial contribution from G.P.C.

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