MHF1 plays Fanconi anaemia complementation group M protein (FANCM)-dependent and FANCM-independent roles in DNA repair and homologous recombination in plants

Natalie J. Dangel, Alexander Knoll and Holger Puchta*
Botanical Institute II, Karlsruhe Institute of Technology, Hertzstr. 16, Karlsruhe 76187, Germany

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*For correspondence (e-mail holger.puchta@kit.edu).

SUMMARY
Fanconi anaemia complementation group M protein (FANCM), a component of the human Fanconi anemia pathway, acts as DNA translocase that is essential during the repair of DNA interstrand cross-links. The DNA-damage-binding function of FANCM is strongly enhanced by the histone fold-containing FANCM-associated protein MHF1. We identified a single homologue of MHF1 in the genome of Arabidopsis thaliana. Similar to the loss of AtFANCM, the loss of AtMHF1 leads to several meiotic defects, such as chromosome bridges between bivalents and an unequal distribution of chromosomes. Moreover, MHF1, together with FANCM, is involved in interstrand cross-link repair in plants. This phenotype is detectable only in double mutants of the RecQ helicase and BLM homologue RECQ4A, which appears to function in a parallel pathway to the FANCM/MHF1 complex. However, in somatic cells, FANCM has an MHF1-independent function in replicative repair in a parallel pathway to the endonuclease MUS81. Furthermore, MHF1 is required for efficient somatic homologous recombination (HR) – a role antagonistic to FANCM. FANCM and RECQ4A define two parallel pathways of HR suppression in Arabidopsis. Hyperrecombination in the fancm but not the recq4A mutant can be abolished by MHF1 mutations. This finding indicates that MHF1 and FANCM act at different steps of a single, common, HR pathway.

Keywords: MHF1, Fanconi anaemia complementation group M protein, DNA repair, homologous recombination, Arabidopsis thaliana.

INTRODUCTION
Fanconi anaemia (FA) was first described in 1927 by the paediatrician Dr Guido Fanconi (Fanconi, 1927) and is characterised by chromosomal abnormalities, haematopoietic dysfunction and a predisposition to cancer. Although there is a wide range of symptoms, all FA cells possess the same phenotype, which is also used for diagnosis of the disease: They exhibit a characteristic sensitivity to interstrand cross-link (CL)-inducing agents, such as mitomycin C (MMC; Moldovan and D’Andrea, 2009). FA is caused by the mutation of any one of 16 known FANC genes, all of which participate in a common pathway, called the Fanconi anemia pathway, to repair interstrand CLs. Following a lesion search step by the Fanconi anaemia complementation group M protein (FANCM), the orthologue of yeast Mph1 (Scheller et al., 2000), FANCM is able to recognise and bind to the damaged site, leading to the recruitment of the FA core complex formed by seven of the FANC proteins (FANCA, -B, -C, -E, -F, -G and -L). The FA core complex then activates further FA proteins and other repair factors to eliminate the lesion. In addition to the FANC genes, several FA-associated factors, essential for the correct functioning of the pathway, have been described (Kim and D’Andrea, 2012; Kottemann and Smogorzewska, 2013).

The Mph1-associated histone fold-containing proteins MHF1 and MHF2 form a DNA remodelling complex that has been reported to interact with FANCM. Both proteins are involved in the damage-dependent DNA binding of FANCM and in the subsequent recruitment of the FA core complex. In vitro, the MHF heterodimer enhances the DNA translocase (Singh et al., 2010) and replication fork remodelling activities of FANCM (Yan et al., 2010). Both MHF proteins exhibit a typical histone fold domain, a feature of proteins that mediate both protein–protein and protein–DNA interactions (Arents and Moudrianakis, 1993). The crystal structure of the MHF complex shows that MHF1 and MHF2 form a heterotetramer in a manner that resembles the histone H3–H4 heterotetramer, although MHF1 and MHF2 belong to the H2A–H2B family (Tao et al., 2012). Interestingly, MHF1 and
MHF2 also have FANCM-independent functions: they are components of the constitutive centromere-associated network (CCAN) and are also known as CENP-S and CENP-X, respectively (Amano et al., 2009; Nishino et al., 2012). In this respect, MHF1 and MHF2, together with other CENP-proteins, assume important roles in the establishment of kinetochores at the centromeres to ensure proper chromosome segregation. Mutations in MHF1 and MHF2 in chicken DT40 cells and the deletion of MHF2 in HeLa cells result in several defects during mitosis, such as a longer progression time from prophase to anaphase compared with wild-type (WT) cells and anaphase bridging of chromosomes (Amano et al., 2009). In fission yeast, Mhf1 and Mhf2 support the orthologue of FANCM, Fm11, by transforming crossover (COs) into non-crossover (NCO) recombination events (Lorenz et al., 2012), and indicate a role of MHF during meiosis as well. The deletion of the MHF complex in humans leads to reduced FA pathway activation, an increase in chromosomal aberrations and the affected cells exhibit a hypersensitivity to CL-inducing agents and to camptothecin (CPT; Singh et al., 2010). In yeast, both MHF proteins seem to be important in the repair of alkylated bases caused by methyl methanesulfonate (MMS; Yan et al., 2010). Unlike other FANC genes and FA-associated factors, which are only conserved in vertebrates, MHF1, MHF2 and FANCM are widely conserved, with orthologues in yeast and in plants as well (Scheller et al., 2000; Singh et al., 2010; Yan et al., 2010; Knoll et al., 2012), suggesting a functional importance of FANCM-MHF1/2 for DNA repair in all eukaryotes.

Recently, the biological role of a FANC homologue in plants was characterised. It was shown that AtFANC is involved in the suppression of spontaneous HR events in somatic cells (Knoll et al., 2012). Moreover, AtFANC is important for HR during meiosis to ensure proper synopsis and MUS81-dependent CO formation (Crismani et al., 2012; Knoll et al., 2012). Surprisingly, there is no indication of AtFANC involvement in CL repair as its single mutants are not hypersensitive to CL-inducing agents (Knoll et al., 2012). In fact, CL repair differs considerably between plants and animals. Homologues of only a small number of the multiple human FANC genes can be found in plant genomes. Whereas the FA pathway is the major CL repair pathway in mammals, it was shown that the RECC4A and MUS81 proteins are involved in two independent branches of CL repair in Arabidopsis thaliana (Mannuss et al., 2010).

The RecQ family of helicases is highly conserved throughout evolution and is important for ensuring genome stability. Three of the human RecQ helicases are associated with severe genetic diseases due to the fact that the mutations in BLM, WRN or RECC4 lead to Bloom (German et al., 1965; Ellis et al., 1995), Werner (Epstein et al., 1996) or Rothmund–Thomson (Kitao et al., 1999) syndromes, respectively. It was demonstrated previously that the A. thaliana RECC4A is the functional homologue of the human BLM and yeast Sgs1 helicases (Hartung et al., 2007). Furthermore, AtRECC4A is involved in the repair of methylated bases and intrastrand CLs induced by the genotoxins MMS and cisplatin, respectively (Mannuss et al., 2010). As a part of the RTR complex (RecQ helicase, a type IA topoisomerase and the structural protein RM11), AtRECC4A is involved in the suppression of spontaneous HR in somatic cells (Hartung et al., 2007; Bonnet et al., 2013; Schröfer et al., 2013).

Together with its interacting partner, EME1, the endonuclease MUS81 forms a nuclease complex, that resolves DNA intermediates such as stalled replication fork structures, displacement loops and Holliday junctions (Boddy et al., 2001; Chen et al., 2001; Kaliraman et al., 2001). Previously, a homologue of MUS81 in A. thaliana has been identified and it was reported that mus81 mutant lines exhibited a strong sensitivity to cisplatin, MMC and MMS, respectively (Hartung et al., 2006). Additionally, the mutation of the RecQ helicase RECC4A in Atmus81 mutant lines leads to several developmental defects and synthetic lethality, and indicates that MUS81 and RECC4A act in two parallel pathways to resolve stalled replication forks (Mannuss et al., 2010).

Here, we report the identification of a MHF1 homologue in the A. thaliana genome and its relation to FANC, RECC4A and MUS81 in DNA repair and recombination. Similar to FANC, MHF1 is important for the regulation of HR during meiosis. Moreover, we discovered a hidden role of both MHF1 and FANCM in the repair of interstrand CLs in plants. There, both proteins act in a common pathway in parallel to the pathway that involves the RECC4A helicase. MHF1 is also required for the promotion of somatic HR in a step that does not involve FANC. Both proteins seem to act in the same pathway, whereas RECC4A and MHF1 most likely act in different HR pathways.

RESULTS

Identification of a single MHF1 gene in Arabidopsis thaliana

Through BLAST analyses, we identified a single homologue of human MHF1 on chromosome 5 at locus AT5G50930 in A. thaliana. We were surprised to find that alignments of the predicted amino acid (aa) sequence of AtMHF1 with animal MHF1 homologues revealed that the putative plant open reading frame (ORF) had a long N-terminus that was not conserved in animals. The genomic sequence of the AtMHF1 locus and a predicted transcript sequence found in online databases (e.g., the Arabidopsis Information Resource, AT5G50930, accession number 451517436) indicated that the N-terminus contained four putative in-frame start codons, with the most 5′ codon predicted to be the translation start site.
We analysed the 5' end of the AtMHF1 cDNA using 5' rapid amplification of cDNA ends (5' RACE) polymerase chain reaction (PCR) followed by sequencing (Method S1). The result revealed that, in contrast with the predicted mRNA sequence, the 5' end of the cDNA was much shorter and lacked three of the four potential start codons (Figure 1a). Only the proximal ATG was present in the AtMHF1 cDNA and, therefore, the genomic sequence of AtMHF1 consists of four exons and three introns, with a total length of 1600 bp from start to stop codon (Figure 1b) and encodes a protein of 144 aa without a longer N-terminus. In addition, by searching for publicly available expressed sequence tag (EST) sequences available in the Arabidopsis Information Resource TAIR, we could not find any ESTs located upstream of the transcription start found in our RACE experiment. AtMHF1 exhibits an overall protein similarity to HsMHF1 of 25.8%. Similar to HsMHF1 (138 aa) and ScMhf1 (90 aa), the Arabidopsis homologue is predicted to contain a histone fold-like domain named CENP-S [PFAM15630, (Punta et al., 2012)], which shares several conserved amino acids with the CENP-S domain of the human and yeast MHF1 (Figure 1c), respectively.

In humans, MHF1 forms a heterodimer with MHF2, of which we also found a single homologue in Arabidopsis located on chromosome 1 at locus AT1G78790 with an overall protein similarity to HsMHF2 of 44.8%.

To analyse the biological function of MHF1 in A. thaliana, we used two different T-DNA insertion lines named mhf1-1 (SALK_119435) from the SALK collection (Alonso et al., 2003) and mhf1-2 (Wisconsin DsLox507A09) from the Wisconsin DsLox collection (Woody et al., 2007) and characterised their insertion sites and the expression levels of different parts of the ORF [for details see Figure S1(a,b) and Method S2].

According to AtGenExpress microarray experiments, the transcript level of AtMHF1 is only elevated in stamens of flowers stages 12 and 15 and in mature pollen compared with control values (Schmid et al., 2005). In a number of abiotic stress treatments, including genotoxic stress, MHF1 expression was not changed more than two-fold compared with control values.

**AtMHF1 single mutants have no detectable DNA repair defects, but they exhibit a defect in somatic homologous recombination**

In humans it has been shown that the deletion of MHF1, similar to knockouts of other members of the FA pathway, results in an increased sensitivity to CLs, leading to the suggestion that MHF1 is involved in the repair of this type of DNA lesion (Singh et al., 2010; Yan et al., 2010). To analyse whether MHF1 plays a comparable role in A. thaliana, we tested both mhf1 mutant lines for hypersensitivity to the interstrand CL-inducing agent MMC and to cisplatin.
which mostly induces intrastrand CLs (Rink et al., 1996; Boulikas and Vougiouka, 2003). After 1 week of growth under standard conditions, the plantlets were incubated for 2 weeks in different concentrations of the genotoxin, followed by the determination of the fresh weight. To evaluate the results, we compared the fresh weights of the mutant lines with the fresh weights of WT plants that had been treated similarly. Compared with the WT plants, the \textit{mhf1-1} and \textit{mhf1-2} single mutants did not exhibit an elevated sensitivity to the cross-linking agents cisplatin and MMC (Figure 2a,b). Furthermore, neither mutant was hypersensitive to bleomycin, hydroxyurea or MMS, respectively (Figure S2). This result, although different from the phenotype of the animal MHF1 homologue, was not particularly surprising because the mutant of the putative complex partner FANCM in Arabidopsis also did not exhibit increased sensitivity to these agents (Knoll et al., 2012).

It has been shown that the orthologue of MHF1 in \textit{Schizosaccharomyces pombe} is involved in the suppression of CO formation and that it plays an essential role during the promotion of NCO recombination events in somatic cells, in a pathway together with the \textit{S. pombe} orthologue of FANCM, Fm11 (Bhattacharjee et al., 2013). To test the influence of AtMHF1 on HR in somatic cells, we crossed both \textit{mhf1} mutant lines with the reporter line IC9 (Molinier et al., 2004). This reporter line possesses a \textit{β}-glucuronidase (GUS) reporter gene, which is separated into two non-functional parts that share a homologous segment of the gene. Only after interchromosomal homologous recombination (HR), the GUS gene \textit{uidA} can be restored fully and converts the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-GlucA) into a blue stain. Thus, every recombination event results in a quantifiable blue sector on the plant (Puchta and Hohn, 2012). To exclude transcriptional or post-transcriptional repression of the GUS gene, the expression of the gene was tested in both \textit{mhf1} IC9 mutants (Method S2) and was compared with its expression in WT IC9, which revealed no silencing.

\textit{AtMHF1} appears to promote somatic HR because both \textit{mhf1} mutant lines showed a decrease in the frequency of spontaneous recombination compared with WT. The relative HR frequency was reduced to less than half in \textit{mhf1-1} IC9 and \textit{mhf1-2} IC9 compared with WT IC9 plants (Figure 2c). This result was surprising because in \textit{A. thaliana}, FANCM mutants exhibited a hyper-recombination phenotype (Knoll et al., 2012). Thus, \textit{AtMHF1} is involved in the promotion of spontaneous HR events, which is antagonistic to the function of AtFANCM.

**Role of \textit{AtMHF1} during meiosis**

In fission yeast, MHF, together with the FANCM orthologue Fm11, is not only involved in somatic HR but also plays a role in meiotic recombination by processing joint DNA molecules and directing NCO recombination events (Lor-
exhibit meiotic defects similar to those of fancm mutants in A. thaliana. Therefore, we analysed the meiotic stages of DAPI-stained chromosome spreads from mhf1-1 and mhf1-2 pollen mother cells and WT cells that were undergoing meiosis using fluorescence microscopy. In the pachytene stage, the DAPI-positive chromosomes seem to be fully synapsed as in the WT cells. In later stages of meiosis I, such as diplotene, the chromosomes of both mhf1 mutants were arranged as five bivalents linked by chiasmata, as in the WT meiotic cells (Figure 3d–f). Additionally, mhf1-1 and mhf1-2 exhibited connections between bivalents that had been shown before in fancm mutants (Knoll et al., 2012). These connections were also apparent in early anaphase I (Figure 3h) and resulted in defects in chromosome distribution at the end of meiosis II (Figure 3n,o). As our results are reminiscent of the phenotype of the fancm mutant (Knoll et al., 2012), it seems likely that MHF1 indeed controls proper meiotic recombination in a complex with FANCM.

**AtMFH1 has an AtRECQ4A-independent role in interstrand cross-link repair**

In A. thaliana it has been shown previously that CLs are repaired differently than in mammals and that the RecQ family helicase RECO4A, a homologue of the human BLM and yeast Sgs1 proteins, and the endonuclease MUS81 are important during this repair and act in parallel pathways (Mannuss et al., 2010). Although neither mhf1 single mutant was sensitive to MMC or cisplatin, we created and tested different double mutants by crossing mhf1-1 mutants with the mutants of the CL repair genes recq4A-4 and mus81-1, respectively. If MHF1 plays only a minor role, its function might be discernable only in the absence of another important factor in CL repair. As MHF1 interacts with FANCM in humans, we also created a double mutant of Atfancm-1 with Atmhf1-1.

We created the double mutant of mhf1-1 and recq4A-4 by crossing the respective homozygous single mutant lines. The corresponding double mutant, which was homozygous for mhf1-1 and recq4A-4, was identified in the F2 generation through PCR-based genotyping (Method S3). The plants were viable and showed no obvious differences in growth compared with WT plants. To characterise the sensitivity of mhf1-1 recq4A-4, we determined the fresh weights of the double mutant, both single mutants and the WT plants after the induction of interstrand crosslinks by MMC and intrastrand crosslinks by cisplatin. Two different concentrations of each genotoxin were tested.

Both single mutants recq4A-4 and mhf1-1 showed no greater sensitivity to MMC treatment than the WT plants, as the fresh weights of both lines were similar to those of the WT plantlets. Surprisingly, the corresponding double mutant exhibited a strong hypersensitivity to MMC. After 10 μg ml^{-1} MMC treatment, the fresh weight of the double mutant was reduced to a third of the untreated control, whereas both single mutants still had a significantly higher fresh weight compared with the double mutant (Figure 4a). A similar effect was observed after 15 μg ml^{-1} MMC treatment. Thus, although it was not measurable in the single mutants, both AtMhf1 and AtRECO4A play roles in interstrand CL repair and they act in different pathways.

In contrast with the results of MMC treatment, we did not find any evidence of an involvement of AtMhf1 in the repair of intrastrand cross-links induced by cisplatin. The double mutant showed the same level of hypersensitivity as the recq4A-4 single mutant (for details see Figure S3a). Taken together, our results indicated that, in addition to the involvement of RECO4A in intrastrand CLs, both MHF1 and RECQ4A are also involved in independent pathways of interstrand CL repair in A. thaliana.

In A. thaliana, MUS81 acts as an endonuclease and is involved in the resolution of both intra- and interstrand CLs (Hartung et al., 2006). Our discovery of a function of MHF1 in interstrand CL repair due to the analysis of the mhf1-1 recq4A-4 double mutant prompted us to test the sensitivity of the double mutant of mhf1-1 with mus81-1. The plants, which were homozygous for both mhf1-1 and mus81-1, were viable and exhibited no obvious growth defects compared with WT plants. This result was surprising because it has been demonstrated previously that a double mutant of fancm and mus81 showed severe growth defects (see below and Crismani et al. [2012]). Similar to the analysis of mhf1-1 recq4A-4, we also tested the sensitivities of the mhf1-1 mus81-1 double mutant, both single mutants and WT plants to two different concentrations of the cross-linking agents MMC and cisplatin by measuring the fresh weights of the plantlets. However, although mus81-1 single mutants showed a notable sensitivity against both agents, neither treatment revealed a statistically significant enhancement of sensitivity in the double mutant at the various concentrations tested (Figures 4b and S3b,c).

Because the animal MHF1 and FANCM homologues interact, it was interesting to test the double mutant line in Arabidopsis for CL sensitivity. Therefore, we crossed both single mutants and identified the corresponding double mutant line in the F2 generation through PCR-based genotyping (Method S3). The deletion of both genes in A. thaliana did not lead to any differences in growth or viability compared with the WT plants. Similar to the analysis of the other double mutants, we determined the fresh weight of the plantlets after 2 weeks of incubation with MMC or cisplatin, respectively. We did not detect any sensitivity compared with the WT plants (Figures 4c and S3d). These results indicated that if FANCM has a role in interstrand CL repair, it should act in the same pathway as MHF1.
AtFANCM has an AtRECQ4A-independent role in interstrand cross-link repair

Our analysis of double mutants revealed a hidden role for MHF1 in interstrand CL repair. To test whether FANCM is indeed involved in the same pathway, we generated and evaluated the sensitivity to MMC of the corresponding double mutants of \textit{fancm-1} with \textit{recq4A-4} and \textit{mus81-1}.

The \textit{fancm-1 recq4A-4} double mutant was created by crossing both single mutant lines. In the F2 generation, the double mutant was identified through PCR-based genotyping (Method S3) and its seeds were used for the sensitivity analysis. The \textit{fancm-1 recq4A-4} double mutant was viable and had no obvious differences in growth or viability compared with the WT plants. Similar to the analysis of \textit{mhf1-1 recq4A-4}, we tested two concentrations of the interstrand cross-linking agent MMC.

After 10 \(\mu\)g ml\(^{-1}\) MMC induction, the double mutant had a fresh weight of only a fifth of the untreated control and this result was also observed after 15 \(\mu\)g ml\(^{-1}\) MMC treatment, although neither single mutant exhibited this strong hypersensitivity (Figure 4d). Thus, FANCM, similar to MHF1, seems to be involved in interstrand CL repair in a pathway parallel to that of RECQ4A.

Contrary to the results of the \textit{mhf1-1 mus81-1} double mutant, we were unable to produce any plants that were

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**Figure 3.** 4,6-Diamidino-2-phenylindole (DAPI)-stained chromatin spreads of \textit{Arabidopsis thaliana} cells undergoing meiosis of wild-type (WT) (a, d, g, j, m), \textit{mhf1-1} (b, e, h, k, n) and \textit{mhf1-2} (c, f, i, l, o) meioocytes.

In the pachytene stage (a–c), neither \textit{mhf1} mutant exhibited defects compared with WT meioocytes. In the diplotene stage (d–f), connections between the five bivalents in \textit{mhf1-1} and \textit{mhf1-2} were observed (arrows). In early anaphase I (g–i), those bridges were also apparent, resulting in unequal chromosome distributions in both \textit{mhf1} mutants at the end of the second meiotic division in telophase II (m–o, arrowheads). Scale bar = 10 \(\mu\)m.
homozygous for both the fancm-1 and mus81-1 mutations, as the plantlets died shortly after germination. To confirm this observation, we quantified seedling viability of progeny from a line homozygous for mus81-1 and segregating for fancm-1 and revealed that, based on normal Mendelian segregation, the one-quarter of the progeny that were homozygous for both fancm-1 and mus81-1 died very early during vegetative growth (Table S1). This result indicated that FANCM and MUS81 are indispensable in A. thaliana because the plants are unable to survive if both genes are missing. A similar observation has been reported previously, made based on other alleles of fancm-9 and mus81-2. In this case the double mutant showed severe growth defects but was viable (Crismani et al., 2012). As the double mutant phenotype is much more severe than either single mutant phenotype, one must assume that these genes act in separate pathways. Because the mhf1-1 mus81-1 double mutant was viable (see above), FANCM seems to have an MHF1-independent function in the repair of replicative damage that arises in the absence of MUS81.

The AtMHF1-dependent defect in somatic HR can be suppressed by mutations in AtRECQ4A, but not in AtFANCM

Our analysis indicated that MHF1 and FANCM differ in their somatic recombination behaviour. MHF1, similar to MUS81 (Hartung et al., 2006), promotes somatic HR, however, FANCM, similar to RECQ4A, suppresses it (Knoll et al., 2012) and both helicases are involved in independent pathways. It was therefore of interest to define the function of MFH1 in relation to the other factors. For epistasis analysis we tested the recombination frequency of the generated double mutants of mhf1-1 with recq4A-4, mus81-1 and fancm-1, respectively, in the IC9 recombination reporter background. The recombination frequency of the mhf1-1 recq4A-4 double mutant was strongly elevated compared with the recombination frequency of WT IC9 and it was similar to the recombination frequency of recq4A-4 IC9 mutant plants (Figure 5a). Thus, the recombination defect of mhf1-1 mutants can be suppressed by the elimination of the RECQ4A helicase.
In the case of MUS81, the spontaneous recombination frequency of both single mutants in the IC9 background was reduced to less than half in comparison with WT (Figure 5b). Because the double mutant showed a decrease in HR frequency that was almost identical to that of both single mutants, the simplest explanation is that AtMUS81 and AtMHF1 act in a common epistatic pathway to repair spontaneous double-stranded breaks via HR in somatic cells.

As FANCM and MHF1 appear to have opposing functions in HR in *A. thaliana*, it was of special interest to test the double mutant of both genes. Surprisingly, and in contrast with the data obtained with the *RECQ4A* mutant, the loss of *FANCM* did not suppress the recombination defect of the *mhf1*-1 IC9 mutant (Figure 5c).

**DISCUSSION**

Our analyses revealed a quite complex interaction between FANCM and its putative complex partner MHF1. On the one hand, it is obvious that both proteins act together in *Arabidopsis* in certain functions. We supplied strong evidence that this hypothesis is the case for CL repair and, additionally, the meiotic phenotype of the *mhf1* mutants argues for a common complex with FANCM during meiosis. Conversely, we clearly demonstrated that, in somatic cells, these proteins have functions that are independent from each other: MHF1 and FANCM assume opposing roles in HR and only FANCM is essential for replicative repair in the absence of the endonuclease MUS81. A detailed discussion of these findings is presented below.

**AtMHF1 is involved in meiotic HR**

The deletion of AtFANCM is accompanied by a reduction in fertility and several defects during meiotic recombination repair. Similar to FANCM, MHF1 seems to play a role in the regulation of meiotic HR as we observed several defects during meiosis, as well, such as connections between bivalents and an unequal chromosome distribution at the end of meiosis II. The defects observed during meiosis resembled the defects associated with FANCM mutations in *A. thaliana* and indicated the possibility that MHF1 and FANCM act together during meiotic recombination. This hypothesis is supported by observations of the fission yeast orthologues of MHF1 and FANCM, which also function together in meiosis by promoting NCO recombination events (Lorenz et al., 2012; Bhattacharjee et al., 2013).

**AtMHF1 and AtFANCM work in interstrand cross-link repair in a parallel pathway to AtRECQ4A**

The mammalian histone fold protein MHF1, together with its partner MHF2, ensures the correct binding of the DNA translocase FANCM to DNA lesions during the repair of interstrand CLs by the Fanconi anaemia pathway. Mutations in *MHF1*, similar to mutations in other FA genes, lead to a
strong hypersensitivity of the affected cells to interstrand CL-inducing agents (Singh et al., 2010). Although in A. thaliana, single mutants of MHF1 and FANCM do not exhibit a detectable sensitivity phenotype, we revealed here that both proteins do play a role in interstrand CL repair as was demonstrated for their orthologues in humans. The RecQ helicase RECQ4A was described previously as being involved only in the repair of intrastrand CLs induced by cisplatin in A. thaliana (Mannuss et al., 2010). Our analysis of the double mutants of RECQ4A with mhf1-1 and fancm-1, respectively, revealed that AtRECQ4A also plays a role in interstrand CL repair and that both MHF1 and FANCM act in a pathway parallel to that of RECQ4A. Interestingly, these mutants did not exhibit a comparable phenotype for intrastrand CLs induced by the genotoxin cisplatin as the mhf1-1 recq4a-4 double mutant did not show a further reduction in fresh weight compared with recq4a-4. In humans, it has been shown that the RecQ helicase BLM, the functional orthologue of AtRECQ4A, interacts with ubiquitinated FANCD2 and that these proteins colocalise at interstrand CL damage sites to facilitate repair (Pichierri et al., 2004; Hirano et al., 2005). Furthermore, HsFANCM has been shown to interact with the RTR complex (Deans and West, 2009; Hoadley et al., 2012), which consists of the BLM helicase, a type IA topoisomerase and two structural proteins, RMI1 and RMI2. Recently, a similar interaction was shown for FancM in the silkworm Bombyx mori, where FancM interacts with Mfh1 and Rmi1 of the RTR complex, as well (Sugahara et al., 2013). Such an interaction has not been shown for A. thaliana FANCM. As our results indicated, FANCM, MHF1 and RECQ4A are involved in the repair of interstrand CLs but the FA proteins act in a different pathway than does RECQ4A. Thus, the situation in plants seems to differ from that in animals. Similar to the situation in mammals, MHF1 and FANCM seem to act in the same pathway to repair interstrand CLs in A. thaliana, as the sensitivity of the corresponding double mutant did not differ from that of either single mutant or the WT plants. Because AtMHF1, similar to its animal and fungal homologues, contains a histone fold-like domain, one could speculate that Arabidopsis MHF1 is also involved in supporting the binding of FANCM to damaged DNA. Our analysis of the double mutant of mhf1-1 and mus81-1 did not reveal any growth defects compared with WT plants. This result was surprising because it had been shown previously that the deletion of FANCM and MUS81 in A. thaliana was lethal (Crismani et al., 2012), an observation we could confirm by creating the double mutant of fancm-1 and mus81-1. The double mutant of Atmhf1-1 and Atmus81-1 had not been characterised previously but if AtFANCN and AtMHF1, similar to the situation in mammals, always work together, we would have expected that mhf1-1 mus81-1 would also exhibit a lethal phenotype. Because the double mutant mhf1-1 mus81-1 was viable and exhibited a hypersensitivity to interstrand CLs similar to that of the mus81-1 single mutant, it is tempting to speculate that MHF1 and MUS81 function in the same repair pathway. Due to its lethal phenotype, we could not analyse the behaviour of the fancm mus81 double mutant in interstrand CL repair. Nevertheless, taking into account the fact that MHF1 and FANCM, as well as MHF1 and MUS81, seem to act in the same pathway, it is tempting to speculate that all three proteins are members of a single pathway of interstrand CL repair. A respective model is depicted in Figure 6(a).

The deletion of AtMHF1 can suppress the hyper-recombination caused by AtFANCN but not by AtRECQ4A mutation

In addition to its function in interstrand CL repair, AtMHF1 seems to play an important role during somatic HR, as mhf1 mutants exhibited a reduced recombination frequency compared with WT plants. Therefore, AtMHF1 is required to promote a step of HR in somatic plant cells. Interestingly, this phenotype is antagonistic to that of FANCN in A. thaliana (Knoll et al., 2012). Thus, MHF1 and FANCM do have independent functions during somatic HR in Arabidopsis. This result is in contrast with what is known for MHF1 and FANCM in the fission yeast, where Mhf1 and Fm11 act together in the same pathway to promote NCO recombination events (Bhattacharjee et al., 2013). In the Atmhf1-1 fancm-1 double mutant, the HR phenotype of the mhf1-1 single mutant is dominant. Thus, MHF1 is able to suppress the hyper-recombination phenotype caused by a mutation in FANCM. This finding indicates strongly that MHF1 may be involved in the same somatic HR pathway as FANCM, but in a step before FANCN becomes active. If FANCN and MHF1 would act in two different pathways, the HR frequency of the

![Figure 6](https://example.com/figure6.png)

Figure 6. Models of the involvement of MHF1, FANCN, MUS81 and RECQ4A in interstrand CL repair (a), and somatic homologous recombination (HR) (b) in Arabidopsis thaliana. Our analyses indicated that, in both cases, MHF1 acts in the same pathway as MUS81 and in a parallel pathway to RECQ4A. As MHF1 is able to suppress the hyper-recombination phenotype of the fancm mutant, MHF1 seems to act before the helicase in HR. © 2014 The Authors

events in involved in the suppression of spontaneous recombination frequency at the same level of both single mutants. As we could not detect a further reduction in the HR frequency of the double mutant compared with both single mutants, the fact that MHF1 and MUS81 act in two different pathways of spontaneous HR in somatic cells is unlikely. Thus, the most obvious hypothesis is that MHF1 and MUS81 act in the same pathway of somatic HR.

Similar to FANCM, the RecQ helicase RECQ4A is involved in the suppression of spontaneous recombination events in A. thaliana, however it was demonstrated that RECQ4A functions in a parallel pathway to FANCM (Knoll et al., 2012). An investigation of the relationship between AtMHF1 and AtRECQ4A in somatic HR could lead to three different outcomes when comparing the HR frequency of the double mutant with that of each single mutants: the double mutant could show an HR frequency at the level of either the mhf1 or the recq4A single mutant, respectively, if both genes act in a common pathway with one mutation suppressing the second. Alternatively, if the genes act in two parallel pathways one would expect the double mutant to exhibit an intermediate phenotype compared with the single mutant. However, the decrease in HR frequency due to the Mhf1 mutation in relation to the increase observed in recq4A mutants is rather small. Therefore, in the case of two parallel pathways, a small reduction in the high HR frequency of the mhf1 recq4A double mutant might not be apparent as significantly different from the HR frequency of the recq4A single mutant. Because the HR frequency of the mhf1 recq4A double mutant is higher than the WT and similar to the recq4A mutant, we can exclude the possibility that Mhf1 acts before RECQ4A in a single pathway. It is not possible, however, to distinguish between the other two options. AtMHF1 might therefore function in a RECQ4A-dependent pathway at a later step or it might act in a parallel pathway to RECQ4A. Because our data show that FANCM and MHF1 function in a common pathway and that FANCM and RECQ4A are also involved in different pathways of HR suppression (Knoll et al., 2012), we favour the hypothesis that MHF1 and RECQ4A act in two parallel pathways. Figure 6(b) depicts our hypothesis on the role of MHF1 in somatic HR in relation to RECQ4A, FANCM and MUS81.

AtMHF1 and its relation to AtMUS81 and AtRECQ4A

The MUS81 endonuclease and the RECQ4A helicase are key actors in different DNA repair and recombination pathways in Arabidopsis. In the absence of both proteins, Arabidopsis is not viable (Mannuss et al., 2010), this result indicated that they act in different pathways that are essential for DNA replication. It is, therefore, especially interesting to evaluate the outcome of our analysis of MHF1 functions in relation to these two genes. All of our results indicated that MUS81 and MHF1 act in the same pathway in plants. There is no evidence that MHF1 acts independently of MUS81 in interstrand CL repair. In addition, the double mutant exhibited an HR defect similar to that of both single mutants, this result indicated an epistatic relationship between the two genes. The fact that the double mutants are viable, in contrast with the combinations of mhf1 with either fancm or recq4A mutants, indicates that MHF1 is not involved in an essential MUS81-independent pathway of replicative DNA repair. In contrast, we found little evidence that RECQ4A shares pathways with MHF1. On the one hand we demonstrated that MHF1 functions in the repair of interstrand CLs in a different pathway than RECQ4A. Conversely, the suppression of the MHF1 HR defect by the additional mutation of RECQ4A could be explained by the hypothesis that these proteins act in two parallel pathways, as discussed above. Therefore, all the data obtained in this study indicated that AtMHF1 functions in pathways together with MUS81, but parallel to RECQ4A (Figure 6a,b).

Both MHF1 and FANCM are conserved in all eukaryotes and are involved in different important cellular processes. In S. pombe, Mhf1 and Fm11 are involved in the processing of joint DNA molecules and directing NCO recombination events during meiosis (Bhattacharjee et al., 2013). Similar to that finding, our analyses revealed the possibility that, in A. thaliana, MHF1 and FANCM could also work together in terms of meiotic recombination, as both single mutants showed comparable defects.

During HR in somatic cells, the situation seemed to differ between the orthologues of MHF1 and FANCM in yeast and in plants. Contrary to fission yeast, in which Mhf1 and Fm11 act together to promote NCO recombination events (Bhattacharjee et al., 2013), we showed that MHF1 and FANCM have opposing functions during somatic HR in A. thaliana.

Interstrand CLs in humans are processed by the Fanconi anaemia pathway, including FANCM and MHF1 which work together to recognise and bind the damage site. The deletion of FANCM or MHF1 in humans leads to a hypersensitivity to interstrand CL-inducing agents (Singh et al., 2010). Contrary, in yeast, the mutation of Mhf1 or the FANCM orthologue Mhp1 does not lead to a hypersensitivity to MMC but the mutants are sensitive to the genotoxic MMS that leads to replication stress (Scheller et al., 2000; Yan et al., 2010). The situation in plants seems to be similar to that in humans, as we could show that mhf1 and fancm, at least in the double mutants with recq4A, exhibited an increased sensitivity to the interstrand CL-inducing agent MMC, as well.

As homologues of some other FANC genes can be found in the Arabidopsis genome (Patel and Joenje, 2007; Knoll...
and Puchta, 2011), it will be interesting to define the biological functions of these genes in DNA repair and recombination in relation to FANCM and MHF1.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

For the characterisation of MHF1 in Arabidopsis thaliana two mutant lines mhf1-1 (SALK_119435) and mhf1-2 (WiscDsLox507A09) were used (Alonso et al., 2003; Woody et al., 2007). For the generation of double mutants, the mutant lines fancm-1 (SALK_069784), mus81-1 (GABI_113F11) and recq4a-4 (GABI_203C07) were used and which have been described previously (Hartung et al., 2006, 2007; Knoll et al., 2012). For this purpose and for the cytological methods, the plants were grown as described previously (Schröper et al., 2013). For sensitivity and HR assays, an axenic plant culture was used as described recently (Schröper et al., 2013).

Sensitivity assays

The sensitivity assays were performed as described previously (Hartung et al., 2007). The genotoxins tested in this assay were bleomycin (Selleckchem, http://www.selleckchem.com), hydroxyurea (Sigma-Aldrich Chemie), MMC (Duchefa Biochemie, http://www.duchefa-biochemie.com) and MMS (Sigma-Aldrich Chemie).

HR assays

The HR assays were performed as described recently (Hartung et al., 2009; Bonnet et al., 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Characterisation of Atmhf1-1 and Atmhf1-2 mutant lines.

Figure S2. Relative fresh weight of Atmhf1-1 and Atmhf1-2 after the treatment with bleomycin (bleo) (a), hydroxyurea (HU) (b) or methyl methanesulfonate (MMS) (c), respectively.

Figure S3. Relative fresh weights of the generated double mutants after cis-Platin treatment.

Table S1. Segregation analysis of the fancm-1 mus81-1 double mutant.

Methods S1. Rapid amplification of 5’ cDNA ends (RACE).

Methods S2. Quantitative Real-Time Expression Analysis.

Methods S3. Primers used for PCR-based genotyping of T-DNA insertion lines.

REFERENCES


