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Research

The RecQ-like helicase HRQ1 is involved in DNA crosslink repair in Arabidopsis in a common pathway with the Fanconi anemia-associated nuclease FAN1 and the postreplicative repair ATPase RAD5A

Sarah Röhrig, Annika Dorn, Janina Enderle, Angelina Schindele, Natalie J. Herrmann, Alexander Knoll and Holger Puchta

Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe BW 76131, Germany

Author for correspondence:
Holger Puchta
Tel: +49 721 60848894
Email: Holger.Puchta@kit.edu

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Summary

- RecQ helicases are important caretakers of genome stability and occur in varying copy numbers in different eukaryotes. Subsets of RecQ paralogs are involved in DNA crosslink (CL) repair. The orthologs of AtRECQ2, AtRECQ3 and AtHRQ1, HsWRN, DmRECQ5 and ScHRQ1 participate in CL repair in their respective organisms, and we aimed to define the function of these helicases for plants.
- We obtained Arabidopsis mutants of the three RecQ helicases and determined their sensitivity against CL agents in single- and double-mutant analyses.
- Only At*hrq1*, but not At*recq2* and At*recq3*, mutants proved to be sensitive to intra- and interstrand crosslinking agents. AtHRQ1 is specifically involved in the repair of replicative damage induced by CL agents. It shares pathways with the Fanconi anemia-related endonuclease FAN1 but not with the endonuclease MUS81. Most surprisingly, AtHRQ1 is epistatic to the ATPase RAD5A for intra- as well as interstrand CL repair.
- We conclude that, as in fungi, AtHRQ1 has a conserved function in DNA excision repair. Additionally, HRQ1 not only shares pathways with the Fanconi anemia repair factors, but in contrast to fungi also seems to act in a common pathway with postreplicative DNA repair.

Introduction

RecQ helicases are important caretakers of genome stability and thereby involved in manifold processes such as DNA replication, repair and recombination. These enzymes are conserved in all domains of life and are represented by at least one helicase coding gene in each organism. While there is one RecQ helicase in *Escherichia coli* and yeast, various numbers of orthologs are found in multicellular eukaryotes.

Mutations in three of the five human *RecQ* helicase genes (Hs*BLM*, Hs*WRN* and Hs*RECQ4*) are associated with different heritable diseases that share a predisposition for cancer (Ellis *et al.*, 1995; Yu *et al.*, 1996; Kitao *et al.*, 1999). Interestingly, three distinct diseases alone are linked to the mutation of Hs*RECQ4*, Rothmund–Thomson syndrome (RTS), Baller–Gerold syndrome and RAPADILINO syndrome (Siitonen *et al.*, 2009). Functional analyses of HsRECQ4 were shown to be complex, as the N-terminal part of the protein exhibits homologies towards the replicative factor ScSld2, while an additional helicase domain is present. This indicates a role for HsRECQ4 in both DNA repair and replication initiation (Xu & Liu, 2009; Liu, 2010).

So far, seven *RecQ* genes have been identified in the plant model organism *Arabidopsis thaliana*: RECQ1, RECQ2, RECQ3, the duplicated gene pair RECQ4A and RECQ4B, RECQ5 and RECQsim (Hartung *et al.*, 2000). There is still only little known about most of these plant RecQ helicases, the exception being RECQ4A and RECQ4B. RECQ2 together with the small exonuclease containing protein WRNexo was postulated to form the functional homolog of human WRN helicase (Knoll & Puchta, 2011). Yeast two-hybrid assays confirmed an interaction between WRNexo and RECQ2, and further biochemical analyses highlighted similar characteristics of RECQ2 and HsWRN (Hartung *et al.*, 2000). *In vitro* analyses of RECQ2 and RECQ3 demonstrated 3′-5′ helicase activity for both of them, while only RECQ2 showed Holliday junction branch migration activity (Kobbe *et al.*, 2008, 2009). Recent single-molecule analyses underlined their different functionalization by uncovering a highly repetitive DNA unwinding activity for RECQ2, while RECQ3 preferably rewinds DNA forks (Klaue *et al.*, 2013). However, neither protein has been characterized *in vivo* as yet.

As part of *in silico* analyses, a search for HsRECQ4 homologs in plants and fungi was performed, whereby HRQ1 (homologous to RecQ helicase 1) was identified as an additional RecQ helicase

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in several fungal and plant species (Barea *et al.*, 2008). First characterizations in yeast demonstrated important tasks for Hrq1 in genome stability and DNA repair. In conformity with other RecQ helicases, an ATP-dependent 3′-5′ helicase activity was shown for Hrq1 (Groocock *et al.*, 2012; Kwon *et al.*, 2012). Epistasis analyses revealed a role for Hrq1 in the repair of cisplatin-induced intrastrand crosslinks (CLs) via nucleotide excision repair (NER), in parallel to postreplicative repair (PRR) and homologous recombination (HR) (Groocock *et al.*, 2012; Choi *et al.*, 2014). Epistasis analyses with the other RecQ helicase in baker's yeast, Sgs1, revealed that Hrq1 is the main helicase in response to mitomycin C (MMC)-induced interstrand CLs, while Sgs1 possesses only a backup function (Bochman *et al.*, 2014). Recent *in vitro* analyses reinforce Hrq1 as the true homolog of HsRECQ4 with comparative biochemical attributes and similar structural features (Rogers *et al.*, 2017). While HRQ1 homologs from plants were identified almost 10 years ago, a further characterization was still missing (Barea *et al.*, 2008).

With the discovery of Hrq1, the second RecQ helicase in yeast involved in the repair of DNA crosslinks was identified, with this appearing to be a conserved function for multiple RecQ orthologs. As intrastrand CLs affect only one DNA strand, the other can serve as a repair template. Therefore, repair can be accomplished using DNA damage tolerance mechanisms or NER. In contrast to this, the repair of interstrand CLs is more complex, as both DNA strands are affected. Repair is performed sequentially for each strand with specific endonucleases mediating the initial unhooking of the CL, followed by mechanisms including NER, translesion synthesis and HR (Clauson *et al.*, 2013). In mammals, the Fanconi anemia (FA) pathway is involved in interstrand CL recognition and repair. It consists of a multitude of proteins that interact in CL repair with mutations in one of the 17 characterized FA genes leading to the hereditary disease FA (Kim & D'Andrea, 2012). At present, the FA pathway does not seem to be conserved in plants. Only seven homologs of the 17 known *FANC* genes were identified in Arabidopsis, with FANCM currently being the only protein linked to CL repair (Knoll *et al.*, 2012). Interestingly, two FA-associated factors, AtFAN1 and AtMHF1, were recently characterized in Arabidopsis, highlighting their involvement in CL repair (Dangel *et al.*, 2014; Herrmann *et al.*, 2015). A model with two independent repair pathways for CL repair, defined by the nucleases AtFAN1 and AtMUS81, was postulated. The nuclease HsFAN1 is an essential factor additional to the *FANC* genes in the FA pathway (Kratz *et al.*, 2010; Liu *et al.*, 2010; MacKay *et al.*, 2010). As HsFAN1 exhibits 5′flap activity, an involvement of the protein in the unhooking step of CL repair was postulated. For the endonuclease complexes, XPF-ERCC1 and MUS81-EME1, processing of the opposing end of the CL was described (Ciccia *et al.*, 2008). Mutations in both plant FA factors, AtFANCM and AtMHF1, do not lead to hypersensitivity against crosslinkers. Nevertheless, an additional mutation in the RecQ helicase AtRECQ4A revealed functions in CL repair for both proteins (Knoll *et al.*, 2012; Dangel *et al.*, 2014). As double mutants of AtRECQ4A with the endonuclease AtMUS81 exhibit a synthetic lethal phenotype, parallel involvement of both proteins in the repair of blocked replication forks was proposed (Mannuss *et al.*, 2010). The central factor in the third branch of CL repair is the ATPase, AtRAD5A. AtRAD5A is the functional homolog of yeast Rad5 and human HLTF, which both show functions in the error-free pathway of DNA damage tolerance (Gangavarapu *et al.*, 2006; Blastyák *et al.*, 2007, 2010; Unk *et al.*, 2008). Mutants of At*RAD5A* exhibit hypersensitivity against CL agents and base methylations (Chen *et al.*, 2008). Recently, it was shown that AtRAD5A acts in a pathway independent of the main DNA repair pathways, NER, single-strand break repair, as well as microhomology-mediated double-strand break repair (Klemm *et al.*, 2017). As AtFAN1 was found to genetically interact with both AtRECQ4A and AtRAD5A, it was classified into the CL repair pathway above both factors, uniting them into a FAN1-dependent branch independently of AtMUS81 (Herrmann *et al.*, 2015). Another nuclease involved in DNA repair in Arabidopsis, AtRAD1, forms the functional homolog of HsXPF, a classical factor of NER (de Laat *et al.*, 1999; Fidantsef *et al.*, 2000; Gallego *et al.*, 2000). The human HsXPF-HsERCC1 complex was shown to act in NER in endonucleolytic cutting next to the DNA lesion, thereby cutting the affected single strand 5′ and 3′ from the damage (Staresincic *et al.*, 2009; Fagbemi *et al.*, 2011).

The aim of the current study was to define whether other RecQ paralogs, besides AtRECQ4A, are involved in DNA crosslink repair. For this we obtained and analyzed mutants of At*HRQ1*, At*RECQ2* and At*RECQ3*. Homologs of all three genes have been reported to be involved in CL repair in other organisms like yeast (ScHrq1), human (HsWRN) and flies (DmRECQ5).

Materials and Methods

Plant lines and growth conditions

For the characterization of HRQ1 in *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia-0), two T-DNA insertion lines, *hrq1-1* (SALK_072230) and *hrq1-2* (GABI_080A06), from the SALK and GABI-Kat collections, respectively, were used (Alonso *et al.*, 2003; Rosso *et al.*, 2003). In the course of the AtRECQ2 and AtRECQ3 characterization, the mutant lines *recq2-1* and *recq3-1* were established by Cas9-mediated mutagenesis of Arabidopsis wild-type (WT) plants as previously described (Steinert *et al.*, 2015). For double-mutant analyses the previously described *fan1-1* (GABI_815C08), *mus81-1* (GABI_113F11), *rad1-1* (SALK_096156), *rad5A-2* (SALK_047150) and *recq4A-4* (GABI_203C07) lines from the SALK and GABI-Kat collections were used (Hartung *et al.*, 2006, 2007; Chen *et al.*, 2008; Yoshiyama *et al.*, 2009; Herrmann *et al.*, 2015). For line establishment, plants were grown in the glasshouse as previously reported (Schröpfer *et al.*, 2014). For sensitivity assays and propidium iodide staining, plants were grown in axenic culture, as described before (Schröpfer *et al.*, 2014).

PCR-based genotyping of T-DNA mutant lines

Genotyping of T-DNA mutant lines was performed in two PCR reactions. The primer pair for identifying T-DNA insertions was chosen to amplify the transition between insertion and adjacent genomic DNA. A second primer pair identified WT alleles and

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spanned the T-DNA insertion area. Elongation time was chosen to be short enough so only the WT allele without an insertion could be amplified. Primer pairs for the identification of *hrq1*-mutants were designed as follows: *hrq1-1* WT: HRQ-F1 5′-TCGTGACCTAAAGAAAGCC-3′ and HRQ-R1 5′-TTTAAG CAAGCCCTCCAAG-3′; *hrq1-1* T-DNA: Lbd1 5′-TCGGA ACCACCATCAAACAG-3′ and HRQ-R1; *hrq1-2* WT: HRQ-F1 and 080A08 5′-ACTTCAACGTGAACTACCTGTCCT-3′; *hrq1-2* T-DNA: GABI 5′-ATAATAACGCTGCGGACATCT ACATTTT-3′ and 080A08. Genotyping of the mutant lines *fan1-1*, *mus81-1*, *rad1-1*, *rad5A-2* and *recq4A-4* was performed as previously described (Hartung *et al.*, 2006, 2007; Chen *et al.*, 2008; Yoshiyama *et al.*, 2009; Herrmann *et al.*, 2015).

Sensitivity assays

To characterize the function of proteins in DNA repair, sensitivity assays with mutant lines were performed as previously described (Hartung *et al.*, 2007). Statistical analysis was performed using a two-tailed *t*-test with unequal variances.

Propidium iodide staining

The analysis of dead cells in the root meristem following genotoxin treatment was conducted with propidium iodide staining, as previously described (Herrmann *et al.*, 2015). Analysis of cell death was performed using a confocal laser scanning microscope (LSM700, Carl Zeiss), and only dead transiently amplifying (TA) cells within the anterior 200 μm of the root tip were included in the analysis.

Results

AtRECQ2 and AtRECQ3 are not involved in CL repair in Arabidopsis

The two RecQ helicases, AtRECQ2 (At1g31360) and AtRECQ3 (At4g35740), from Arabidopsis were identified a while ago and whilst they are well characterized biochemically, *in vivo* analysis has not yet been carried out (Hartung *et al.*, 2000). As no suitable T-DNA mutant lines for both genes were available, we performed a CRISPR/Cas9-mediated mutagenesis of both genes for further *in vivo* analyses. Therefore, we used the Cas9 ortholog from *Staphylococcus aureus*, as recently described (Steinert *et al.*, 2015). For At*RECQ2* mutagenesis, a target sequence in exon 4 (protospacer 5′-TTGTGTACAAGGCACTTGAA-3′) and for At*RECQ3* a target in exon 5 (protospacer 5′-TCCAGAATTGAT TGCGACGA-3′) of the gene was chosen. For AtRECQ2, the mutant line *recq2-1* was established, harboring a 25 bp deletion (Fig. 1a). For AtRECQ3, the mutant line *recq3-1,* with a deletion of 35 bp, was obtained (Fig. 1b). Both mutations led to a frameshift within the open reading frame of the respective genes and thereby generated premature stop codons. Mutations were verified on mRNA level by Sanger sequencing of cDNA (Supporting Information Fig. S1).

Both At*recq2* and At*recq3* mutants were viable and fertile in their homozygous state. No growth abnormalities could be detected. To elucidate the function of AtRECQ2 and AtRECQ3 in CL repair, sensitivity assays with crosslinking agents MMC and cisplatin were performed (Fig. 2). We determined the relative FW of 3-wk-old plants grown in liquid culture containing the respective genotoxin compared with untreated control plants of the same line. Interestingly, neither *recq2-1* nor *recq3-1* mutants showed any hypersensitivity against either CL agent. This leads to the assumption that neither RecQ helicase performs a significant role in CL repair in Arabidopsis.

Characterization of the *hrq1* helicase mutants in Arabidopsis

The helicase HRQ1 was identified in plants and fungi as a result of *in silico* analyses and resembles the human RecQ helicase RECQ4 (Barea *et al.*, 2008). To characterize the Arabidopsis homolog of HRQ1, two T-DNA insertion mutants were analysed. The genomic locus of At*HRQ1* localizes on chromosome 5 (At5g08110) and includes 20 exons and 19 introns on a length of

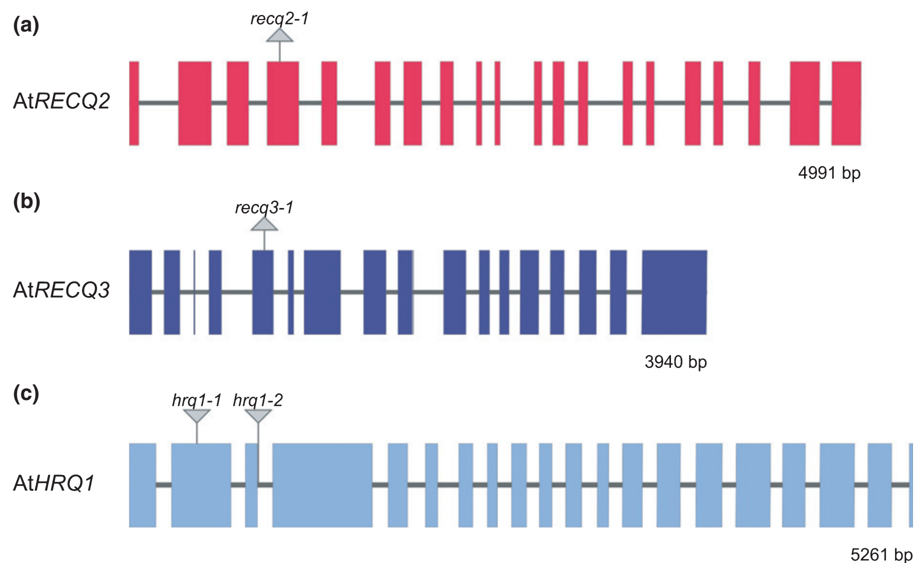

Fig. 1 Genomic structure of At*RECQ2*, At*RECQ3* and At*HRQ1*. (a) At*RECQ2* consists of 4991 bp with 20 exons and 19 introns, and the CRISPR/Cas9-induced 25 bp deletion of *recq2-1* is situated in exon 4. (b) At*RECQ3* comprises 3940 bp, including 18 exons and 17 introns. The *recq3-1* mutant holds a CRISPR/Cas9-induced deletion of 35 bp in exon 5. (c) The *HRQ1* gene from Arabidopsis has an overall length of 5261 bp. The T-DNA insertion of *hrq1-1* is located in exon 2 and the insertion of *hrq1-2* is in exon 3.

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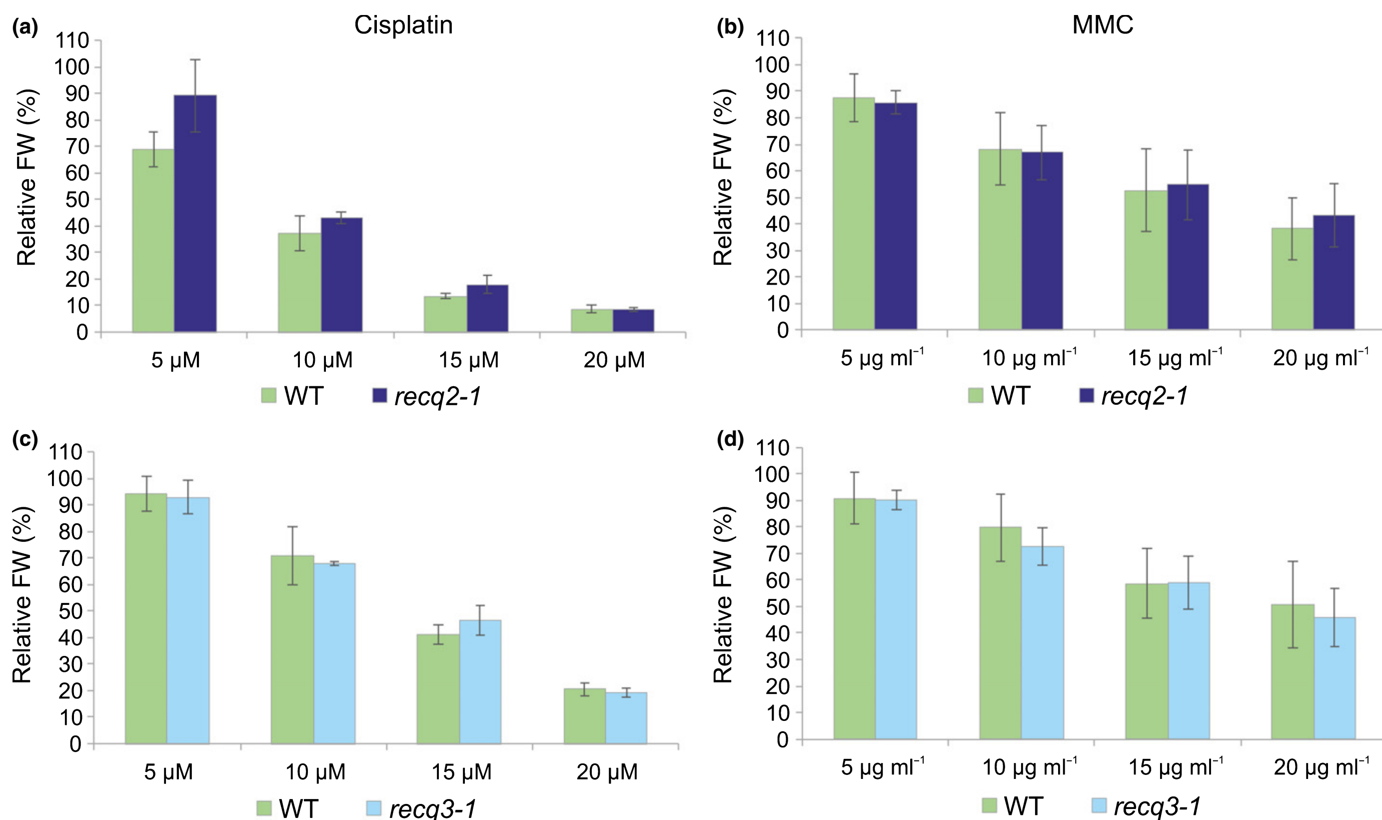

Fig. 2 Sensitivity of At*recq2-1* and At*recq3-1* mutants against cisplatin and mitomycin C (MMC). The FW of Arabidopsis *recq2-1* and *recq3-1* mutants, as well as the wild-type (WT), was determined after genotoxin treatment and was related to that of untreated control plants. At least three independent assays were performed and mean values ± SD (error bars) were calculated. The *recq2-1* mutant line did not show any significant hypersensitivity against all tested concentrations of cisplatin (a) or MMC (b) compared with the WT. In the *recq3-1* mutant line, no reduction of FW, compared with the WT, after cisplatin (c) or MMC (d) treatment could be determined.

5261 bp. The first T-DNA insertion mutant At*hrq1-1* (SALK_072230) harbors an insertion in the second exon, while the second mutant At*hrq1-2* (GABI_080A06) contains an insertion in the third exon (Fig. 1c). Both insertions were verified by sequencing (Fig. S2). To confirm the functional deficiency of HRQ1 in both mutants, relative gene expression was examined in comparison to WT plants. Therefore, expression 5′ and 3′, as well as spanning the respective insertion, was analyzed by quantitative real-time PCR (Fig. S3; see Methods S1). In *hrq1-1*, expression 5′ of the insertion was reduced to 15% of WT expression, while no expression was detected spanning or 3′ of the insertion. The *HRQ1* expression pattern in *hrq1-2* both 5′ and 3′ of the insertion was nearly unaffected compared with the WT, although no expression could be measured spanning the insertion.

AtHRQ1 is involved in intra- and interstrand DNA CL repair

Owing to the reported involvement of HRQ1 homologs from yeast in the repair of DNA CLs, it was of special interest to investigate if this function is conserved for plants. Therefore, both T-DNA insertion mutants were tested in comparison to the WT with sensitivity assays against the CL agents MMC and cisplatin. Compared with WT plants, both *hrq1-1* and *hrq1-2* mutant lines showed a significant reduction in FW after treatment with 10, 15 and 20 µM cisplatin (Fig. 3a,c). Furthermore, treatment with 5, 10, 15 and 20 µg ml^{-1} MMC also resulted in a significantly reduced FW of both mutant lines compared with WT plants (Fig. 3b,d). Taken together, both *hrq1* T-DNA lines exhibit comparable hypersensitivity against CL agents, thus demonstrating an involvement of AtHRQ1 in the repair of DNA CLs.

AtHRQ1 is involved in the repair of aberrant replication intermediates in root meristems

For the characterization of AtHRQ1 in the repair of replication-associated DNA damage, root tips of both At*hrq1* mutant lines were treated with the CL agents cisplatin and MMC. Depending on cell cycle phase, CLs are capable of interfering with replication by blocking the replication fork. Accumulation of fork blocking lesions can ultimately lead to programmed cell death. The highly replicative root meristem is especially suitable for the analysis of such DNA damage affecting replication (Curtis & Hays, 2007). For the analysis of dead cells in the root meristem, 4-d-old plantlets of both *hrq1* mutants and the WT were incubated in liquid medium with 3 µg ml^{-1} MMC or 40 µM cisplatin for 24 h. Control plantlets were incubated in medium without genotoxin. Roots were then stained with propidium iodide and the

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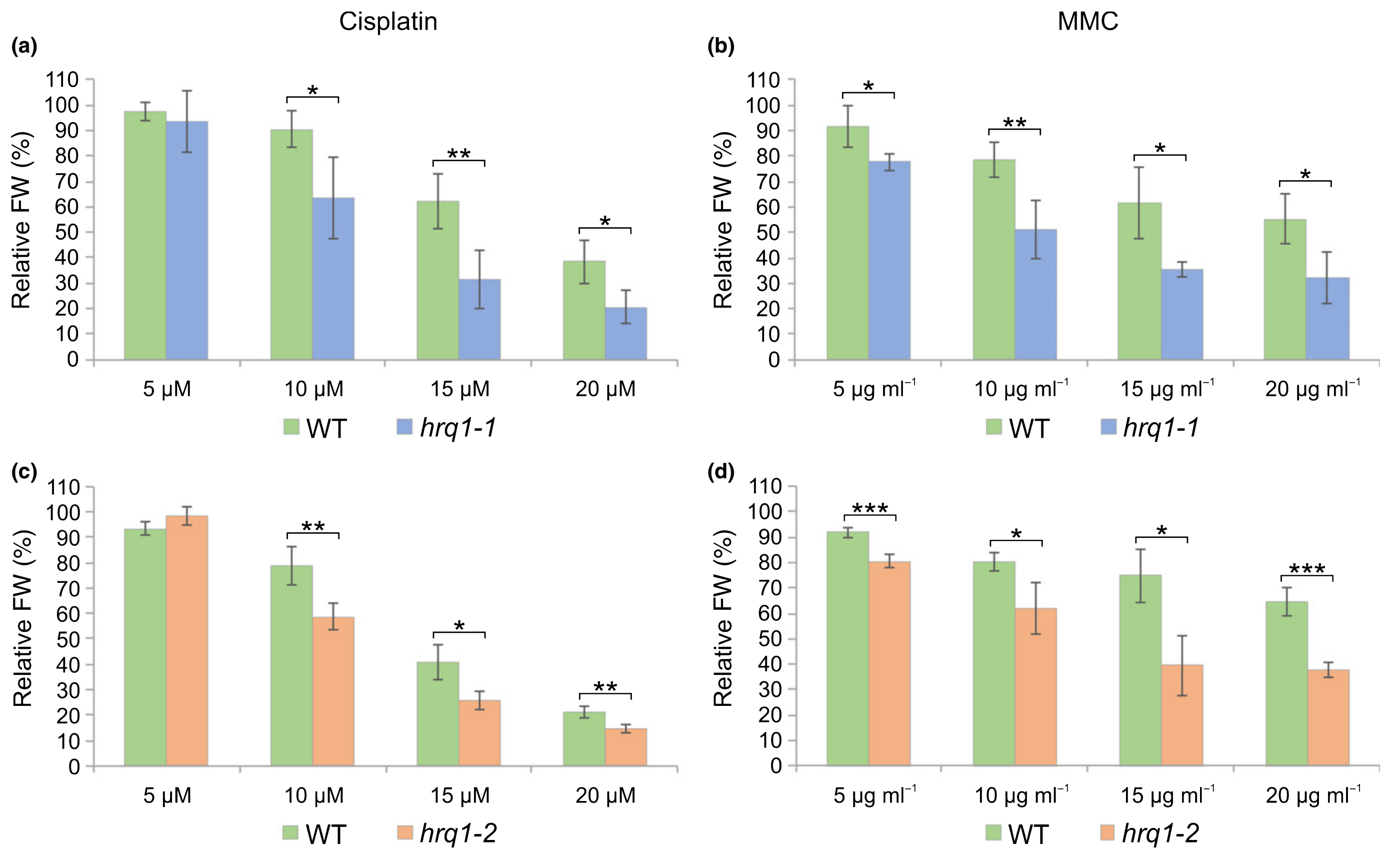

Fig. 3 Sensitivity of At*hrq1* mutants against cisplatin and mitomycin C (MMC). The FW of both Arabidopsis T-DNA mutants, *hrq1-1* and *hrq1-2*, as well as the wild-type (WT) was determined after genotoxin treatment and set in relation to untreated control plants. At least four independent assays were performed and mean values ± SD (error bars) were calculated. The cisplatin treatment with concentrations of 10, 15 and 20 µM led to a significant reduction of FW in both *hrq1-1* (a) and *hrq1-2* (c) compared with the WT. (b, d) All concentrations of MMC led to hypersensitivity of both *hrq1* mutants at a significant level. Statistical differences were calculated using a two-tailed *t*-test with unequal variances: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

number of dead TA cells per root was determined with a laser scanning microscope (Fig. 4). Without induction of DNA damage, no dead cells could be identified in both *hrq1* mutants, comparable to the WT. After the induction of replicative stress with MMC and cisplatin, both mutant lines showed a significantly increased number of dead cells per root in comparison with WT plants. While the WT exhibited one dead cell after MMC or cisplatin treatment on average, *hrq1-1* and *hrq1-2* featured two to three dead cells per root tip. This indicates a role of AtHRQ1 in the maintenance of genome stability and the repair of aberrant replication intermediates in the root meristem.

AtHRQ1 shares a common pathway with the NER endonuclease AtRAD1

For an extensive characterization of the HRQ1-mediated function in CL repair, we further analyzed the epistatic interactions with known CL repair factors from Arabidopsis. Therefore, double mutants were generated by crossbreeding and examined concerning their sensitivity to the CL agents cisplatin and MMC. As both *hrq1* single mutants exhibited comparable phenotypes in CL sensitivity, double-mutant analyses were performed using merely the *hrq1-1* mutant.

The endonuclease AtRAD1 together with AtRAD10 represents the functional homolog to the human endonuclease complex HsXPF-HsERCC1 in plants. HsXPF-HsERCC1 is involved in the NER pathway and is responsible for the DNA incisions after damage recognition (de Laat *et al.*, 1999). For the Arabidopsis homolog AtRAD1, an involvement in NER could be confirmed (Fidantsef *et al.*, 2000). As yeast Hrq1 was demonstrated to act in NER, the determination of interactions in Arabidopsis were of special interest (Groocock *et al.*, 2012; Choi *et al.*, 2014). Therefore, we generated the *hrq1-1rad1-1* double mutant and analyzed its sensitivity to the CL agents cisplatin and MMC in comparison to both single mutants and the WT (Fig. 5a,b). The *rad1-1* single mutant exhibited a strong hypersensitivity against both crosslinkers. The application of 2.5 µM cisplatin or 2.5 µg ml^{-1} MMC led to a severe reduction in relative FW of *rad1-1* to 37 and 27% of the untreated control, respectively. The hypersensitive phenotype of the *hrq1-1* single mutant is only visible at higher concentrations, as demonstrated previously. Compared with the WT, the *hrq1-1rad1-1* double mutant showed a significant reduction of FW after treatment with all tested concentrations of cisplatin and MMC. Thereby, no significant difference in FW of the *rad1-1* single mutant could be determined. This

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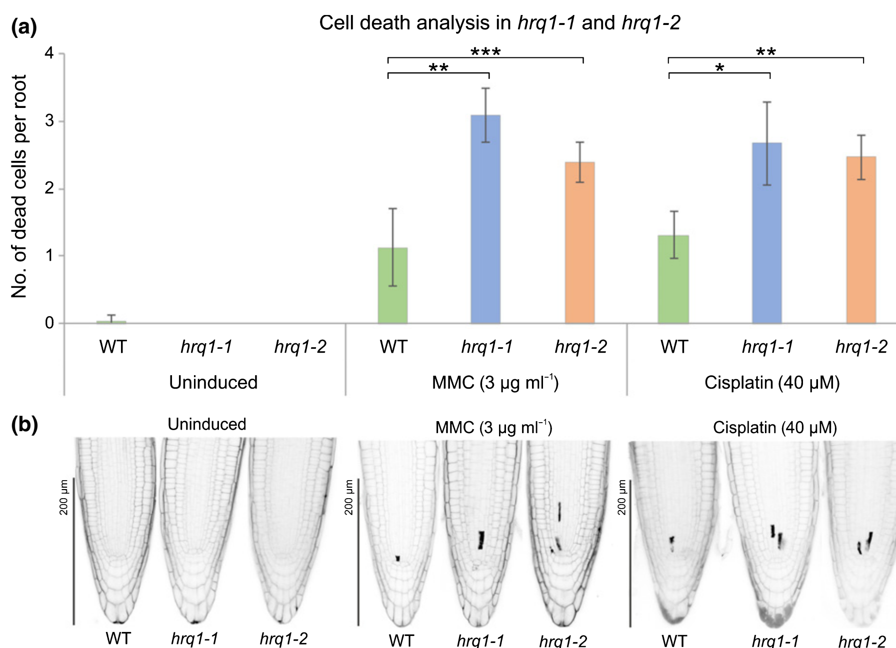

Fig. 4 Cell death analysis in root tips of At*hrq1-1* and At*hrq1-2*. To analyze the repair of replication-associated DNA damage in the root meristem, propidium iodide staining of Arabidopsis *hrq1-1*, *hrq1-2* and wild-type (WT) root tips was performed after treatment with cisplatin or mitomycin C (MMC). Each assay was performed at least three times, with 10 root tips per line (a). For each line, the average number of dead cells per root, including ± SD (error bars), were determined. (b) Additionally, representative root tips of the different plant lines are depicted. Untreated control plants of *hrq1-1*, *hrq1-2* and the WT showed comparable behavior with no dead cells. Both treatment with 3 μg ml^{-1} MMC and 40 μM cisplatin led to a significant increase in dead cells in *hrq1-1* and *hrq1-2* compared with the WT. The number of dead cells per root was hereby increased twofold in both mutant lines. Statistical differences were calculated using a two-tailed *t*-test with unequal variances: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

indicates a common involvement of AtHRQ1 and AtRAD1 in the repair of DNA CLs.

AtHRQ1 acts independently of the replicative repair endonuclease AtMUS81 in CL repair

MUS81 is part of a highly conserved endonuclease complex with EME1 in eukaryotes and is involved in the resolution of diverse branched replicative and meiotic recombination intermediates (Interthal & Heyer, 2000; Boddy *et al.*, 2001; Abraham *et al.*, 2003). AtMUS81 acts in CL repair in a pathway distinct from AtRECQ4A and AtRAD5A (Mannuss *et al.*, 2010). To elucidate the relationship between AtHRQ1 and AtMUS81, we generated an *hrq1-1 mus81-1* double mutant and tested its sensitivity against cisplatin and MMC (Fig. 5c,d). Both single mutants exhibited strong MMC sensitivity at all tested concentrations. Interestingly, the *hrq1-1 mus81-1* double mutant presented an additive effect with a hypersensitive phenotype, significantly greater than both single mutants (Fig. 5d). Cisplatin treatment of the double mutant resulted in an identical result. While the *mus81-1* single mutant exhibited a strongly reduced FW at all tested concentrations, the *hrq1-1* single mutant was not significantly affected. Nevertheless, the *hrq1-1 mus81-1* double mutant featured a significantly reduced FW compared with both single mutants (Fig. 5c). Thus, our results strongly suggest that AtHRQ1 indeed acts in a pathway separate from AtMUS81 in DNA CL repair.

AtHRQ1 and AtRECQ4A are involved in a common pathway in the repair of intrastrand but not interstrand CLs

Understanding the role of AtHRQ1 in relation to the only other known RecQ helicase involved in CL repair in plants was of particular interest. AtRECQ4A is a partner in the conserved RTR (RECQ4A/TOP3α/RMI1/2) complex and is involved in DNA damage repair via HR (Hartung *et al.*, 2007, 2008; Bonnet *et al.*, 2013; Schröpfer *et al.*, 2014). To elucidate the relationship between the two helicases AtHRQ1 and AtRECQ4A in CL repair, we created the *hrq1-1 recq4A-4* double mutant and analyzed its sensitivity against both cisplatin and MMC (Fig. 5e,f). Cisplatin induces mostly intrastrand crosslinks that affect only one DNA strand and leave the complementary strand as a repair template, whereas MMC mainly causes interstrand crosslinks that are highly toxic by connecting both DNA strands. While the *hrq1-1 recq4A-4* double mutant exhibited significant sensitivity after treatment with 1, 2.5, 5 and 10 μM cisplatin, sensitivity of the double mutant was equivalent to the *recq4A-4* single mutant for all tested concentrations (Fig. 5e). Whereas the *hrq1-1* mutant displays strong MMC sensitivity against all tested concentrations, only a minor sensitivity of *recq4A-4* was detected after treatment with 5, 10 and 15 μg ml^{-1} MMC. Treatment with all tested MMC concentrations led to hypersensitivity of the double mutant, which was, at concentrations of 10 and 15 μg ml^{-1}, significantly increased compared with the sensitivities of both single mutants (Fig. 5f). Therefore, surprisingly, the involvement of both helicases in the repair of inter- and intrastrand CL seem to

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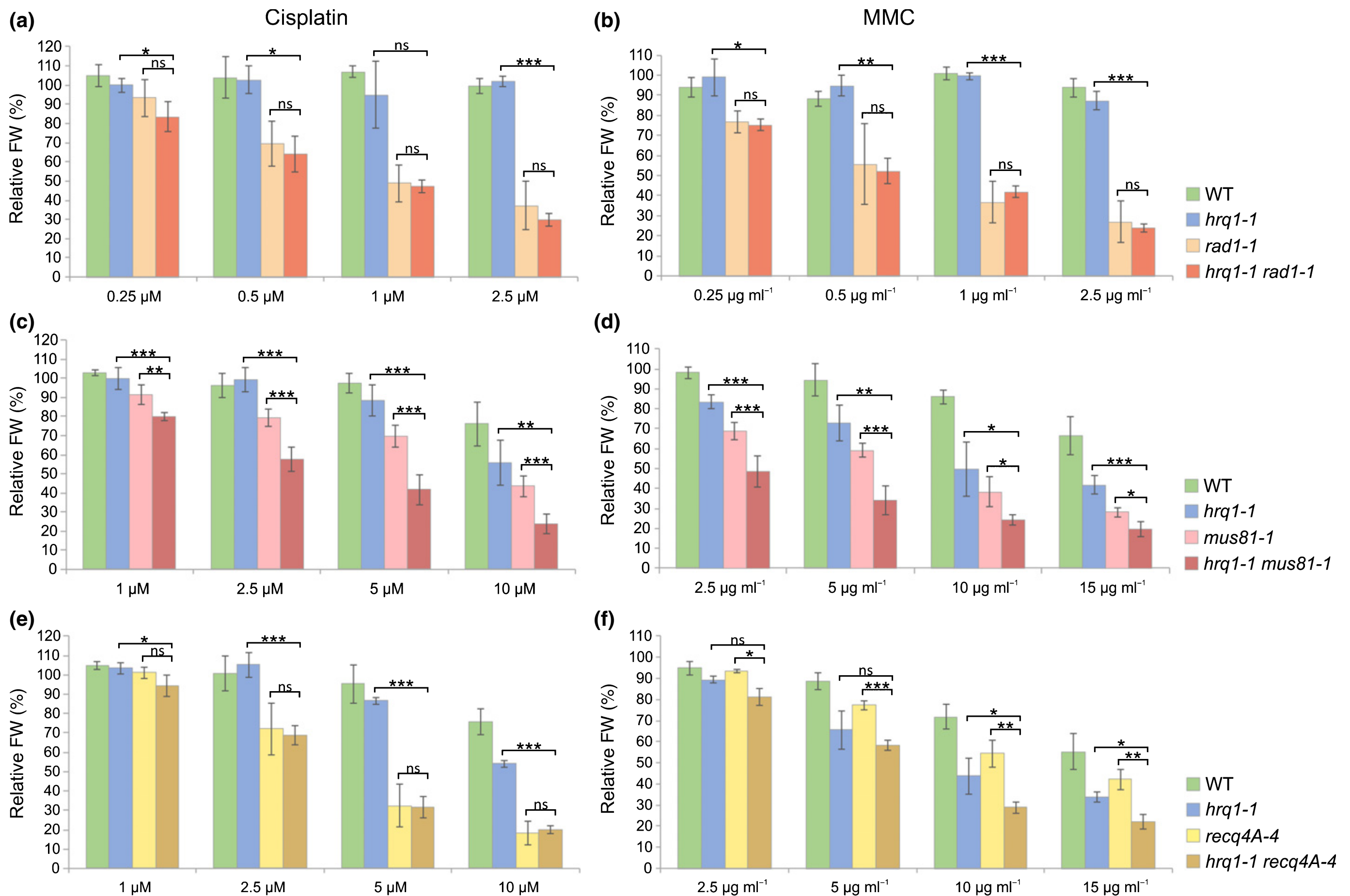

Fig. 5 Sensitivity of At*hrq1-1* double mutants with At*rad1-1*, At*mus81-1* and At*recq4A-4* against cisplatin and mitomycin C (MMC). The FW of Arabidopsis *hrq1-1 rad1-1*, *hrq1-1 mus81-1* and *hrq1-1 recq4A-4* double mutants, the respective single mutants and the wild-type (WT) was determined after genotoxin treatment and related to that of untreated control plants. At least three independent assays were performed and mean values ± SD (error bars) were calculated. The *hrq1-1 rad1-1* double mutant exhibited a relative FW comparable to the *rad1-1* single mutant at all tested concentrations of cisplatin (a) and MMC (b). In the *hrq1-1 mus81-1* double mutant treatment with both cisplatin (c) and MMC (d) resulted in a decreased FW compared with the single mutants. (e) After cisplatin treatment, the *hrq1-1 recq4A-4* double mutant showed a relative FW comparable to the *recq4A-4* single mutant. (f) Treatment with 10 and 15 µg ml^{-1} MMC led to a significant reduction of FW in the *hrq1-1 recq4A-4* double mutant compared with both single mutants. Statistical differences were calculated using a two-tailed *t*-test with unequal variances: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

differ. AtHRQ1 and AtRECQ4A cooperate functionally in intrastrand CL repair; however, they can at least partly complement for each other in interstrand CL repair.

AtHRQ1 acts in a pathway with the nuclease AtFAN1 in the repair of both interstrand and intrastrand CLs

The nuclease FAN1 was shown to be involved in the FA pathway of CL repair in mammals. Therefore, a role for FAN1 in unhooking the DNA lesion by endonucleolytic incisions on both sides of the CL was postulated (Kratz *et al.*, 2010; Liu *et al.*, 2010; MacKay *et al.*, 2010). Recently in plants, an independent function of both nucleases, AtFAN1 and AtMUS81, in interstrand CL repair could be demonstrated (Herrmann *et al.*, 2015). To test if the helicase AtHRQ1 is involved with AtFAN1 in CL repair, we created the double mutant *hrq1-1 fan1-1* and tested its sensitivity against cisplatin and MMC in comparison to both single mutants and the WT (Fig. 6a,b). We were able to confirm the hypersensitivity of *fan1-1* against MMC but so far no hypersensitive phenotype against cisplatin has been reported (Herrmann *et al.*, 2015). Only lower cisplatin concentrations were used before, and these did not lead to hypersensitivity of *fan1-1*, but we have now demonstrated that *fan1-1* exhibits a significant cisplatin sensitivity compared with WT plants at concentrations of 15 and 20 µM (Fig. 6a). This indicates a role for AtFAN1 in the repair not only of interstrand but also of intrastrand CLs. The *hrq1-1 fan1-1* double mutant exhibited a significantly increased sensitivity compared with the WT against cisplatin, at concentrations of 15 and 20 µM (Fig. 6a). The sensitivity of the double mutant to cisplatin was not significantly different from that of both single mutants. While *fan1-1* mutants were sensitive to MMC at concentrations of 5, 10 and 15 µg ml^{-1}, *hrq1-1* single mutants additionally showed hypersensitivity after 2.5 µg ml^{-1} MMC treatment. The *hrq1-1 fan1-1* double mutant exhibited no additional sensitivity to MMC, at all tested concentrations (Fig. 6b). This leads to the hypothesis that AtHRQ1 and

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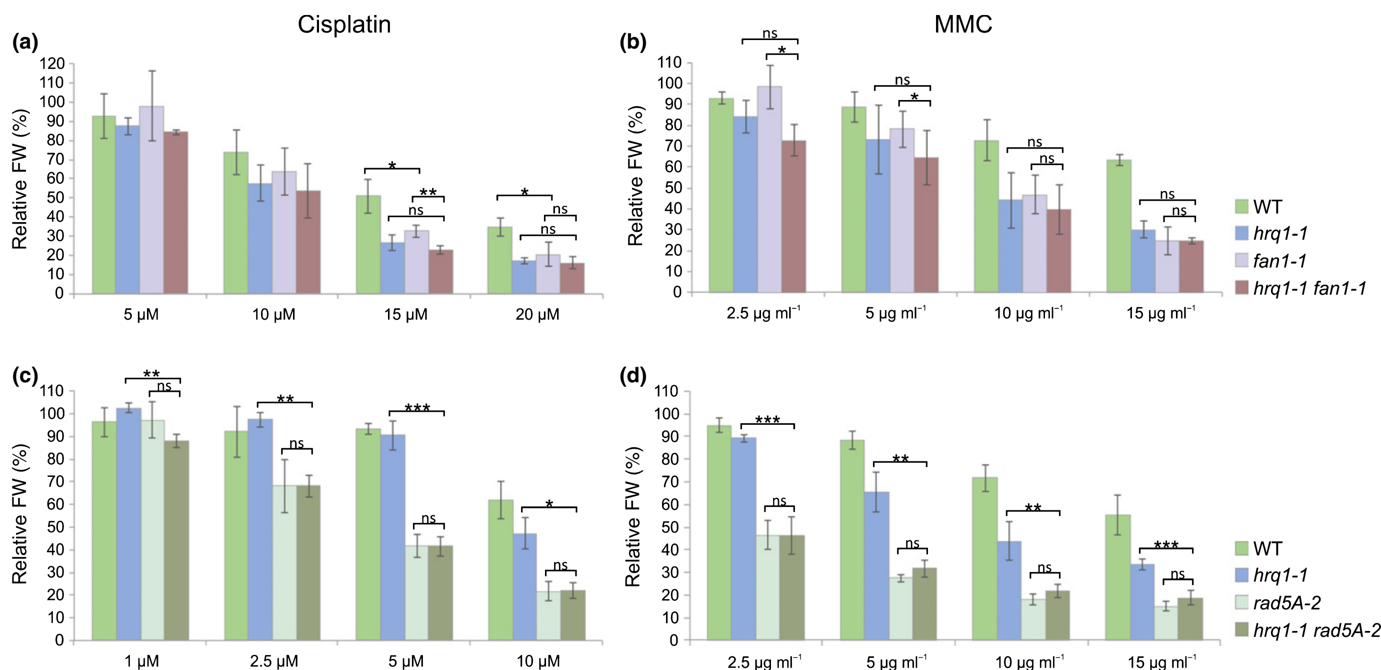

Fig. 6 Sensitivity of Arabidopsis *hrq1-1 fan1-1* and *hrq1-1 rad5A-2* double mutants in response to cisplatin and mitomycin C (MMC). The FW of the double mutants, the respective single mutants and the wild-type (WT) was determined after genotoxin treatment and related to that of untreated control plants. At least three independent assays were performed and mean values ± SD (error bars) were calculated. In the *hrq1-1 fan1-1* double mutant, after treatment with cisplatin (a) and MMC (b), a relative FW comparable to the *hrq1-1* single mutant could be determined. The relative FW of the *hrq1-1 rad5A-2* double mutant after cisplatin (c) and MMC (d) treatment was comparable to that of the *rad5A-2* single mutant. Statistical differences were calculated using a two-tailed *t*-test with unequal variances: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

AtFAN1 might cooperate functionally in the repair of interstrand and intrastrand CLs in the same pathway.

AtHRQ1 is epistatic to the PRR ATPase AtRAD5A

We previously demonstrated that the RecQ helicase AtRECQ4A acts independently of AtRAD5A in CL repair (Mannuss *et al.*, 2010). The ATPase AtRAD5A is involved both regulatorily and mechanistically in the DNA damage tolerance pathway and thereby enables the error-free repair of blocked replication forks (Chen *et al.*, 2008; Mannuss *et al.*, 2010; Kobbe *et al.*, 2016; Klemm *et al.*, 2017). To elucidate the role of AtHRQ1 in relation to AtRAD5A, we generated the *hrq1-1 rad5A-2* double mutant. The *rad5A-2* single mutant exhibits strong sensitivity against both cisplatin and MMC. The double mutant showed significant hypersensitivity against cisplatin at 2.5, 5 and 10 µM concentrations and thus matched the *rad5A-2* single mutant at all tested concentrations (Fig. 6c). MMC treatment with all tested concentrations led to a hypersensitivity of the double mutant comparable to that of the *rad5A-2* single mutant (Fig. 6d). Consequently, surprisingly, and in contrast to AtRECQ4A, AtHRQ1 is involved in a common pathway with AtRAD5A in inter- and intrastrand CL repair.

Discussion

Helicases are enzymes of the utmost importance for the maintenance of genome stability in every organism and comprise *c.* 1% of all coding sequences (Knoll & Puchta, 2011). The helicase HRQ1 was identified through *in silico* analyses, whilst searching for HsRECQ4 homologs in plants and fungi (Barea *et al.*, 2008). The functional characterization of yeast Hrq1 demonstrated a role in DNA repair and the preservation of genome stability (Groocock *et al.*, 2012). Here, we were able to demonstrate that the HRQ1 homolog in Arabidopsis is involved in CL repair. Furthermore, we could classify AtHRQ1 into the CL repair network in Arabidopsis, revealing relations that are seemingly unique to plants.

AtRECQ2 and AtRECQ3 do not contribute to CL repair

In contrast to the known functions of several RecQ helicases like AtRECQ4A, we could not find any indication of a role of the two RecQ paralogs, AtRECQ2 and AtRECQ3, in CL repair. Biochemical analyses led to the assumption that AtRECQ2 acts as the functional homolog of the helicase part from HsWRN (Kobbe *et al.*, 2008). AtRECQ2 was shown to interact with AtWRNexo, a protein containing an exonuclease domain homologous to the domain found in HsWRN (Hartung *et al.*, 2000). It was speculated that both proteins together form the functional homolog to HsWRN. The mutation of the human WRN helicase results in the severe progeroid disease, Werner's syndrome (Yu *et al.*, 1996). Werner's syndrome cells are sensitive to crosslinking agents and the helicase function of HsWRN was shown to be required for the processing of interstrand CLs *in vitro* (Poot *et al.*, 2001; Zhang *et al.*, 2005). Thus, the *in vivo*

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function of AtRECQ2 seems to differ from its homolog, HsWRN. Biochemical analyses of AtRECQ3 demonstrated specific functions of the protein, not comparable to a single human RecQ helicase. Nevertheless, taking into account both the domain structure and the biochemical properties, HsRECQ5b was postulated as the human RecQ helicase most similar to AtRECQ3 (Kobbe *et al.*, 2009). A role for RECQ5 homologs in crosslink repair has been reported, as *RECQ5*-depleted *Drosophila melanogaster* flies were hypersensitive against cisplatin, a phenotype also shown for chicken DT40 cells, in addition to a sensitivity against MMC (Maruyama *et al.*, 2012; Hosono *et al.*, 2014). As no involvement of AtRECQ3 in CL repair could be demonstrated, characteristics of both helicases seem to differ despite their similar biochemical characteristics.

Taking HRQ1 into account, Arabidopsis harbors eight RecQ helicase genes, compared with five genes in humans. We can now show that, besides AtRECQ4A, the HsRECQ4 helicase homolog AtHRQ1 also has a functional role in CL repair in plants. Nevertheless, other helicases might act as a backup in the absence of both enzymes. Therefore, we cannot exclude the possibility that helicases like AtRECQ2 and AtRECQ3, which do not induce a sensitivity phenotype when knocked out, still perform a minor 'hidden' role in CL repair. The simultaneous mutation of multiple RecQ helicases in Arabidopsis might provide further insight into the functions of the different proteins.

AtHRQ1 is involved in CL repair

Our results strongly suggest a role of AtHRQ1 in the repair of both intrastrand and interstrand CLs. Interestingly, the CL repair functions of HRQ1 homologs vary in different organisms. While Sc*hrq1* mutants exhibit strong MMC sensitivity and only little sensitivity against cisplatin, conditions are different in fission yeast, where cells are highly sensitive to both crosslinkers (Groocock *et al.*, 2012; Bochman *et al.*, 2014). Analyses from human RECQ4-deficient fibroblasts also demonstrated only modest cisplatin sensitivity (Jin *et al.*, 2008). As HRQ1 from Arabidopsis seems to be equally important in the repair of both classes of CLs, this implies an involvement different from HsRECQ4 or ScHRQ1. Furthermore, we demonstrated a function of AtHRQ1 in the repair of aberrant replication intermediates in the root meristem, which highlights its important function in the maintenance of genome stability.

Classification of AtHRQ1 into the CL repair network of Arabidopsis

As we were able to demonstrate that AtHRQ1 is involved in the repair of interstrand and intrastrand CLs, it was of great interest to rank this protein into the complex CL repair network in plants. With the generation of double mutants between AtHRQ1 and the already characterized CL repair factors AtRAD1, AtMUS81, AtRECQ4A, AtFAN1 and AtRAD5A, we aimed to define whether AtHRQ1 acts in common or different pathways.

The nuclease RAD1 forms the functional homolog of HsXPF, a classic factor of NER (de Laat *et al.*, 1999; Fidantsef *et al.*, 2000; Gallego *et al.*, 2000). In this pathway, the excision of the affected single strand occurs 5′ and 3′ of the lesion mediated by endonucleolytic incisions through HsXPF-HsERCC1 (Staresincic *et al.*, 2009; Fagbemi *et al.*, 2011). In Arabidopsis, mutants of *RAD1* were shown to be highly sensitive to CL agents (Klemm *et al.*, 2017). NER is a main repair mechanism for intrastrand CLs. In addition, NER factors are also involved in the repair of interstrand CLs, where nucleases like HsXPF are involved in the initial unhooking of the CL (Zhang & Walter, 2014). Our results hint at a common involvement of AtHRQ1 and AtRAD1 in the repair of interstrand and intrastrand CLs. The human homolog RECQ4 is able to interact with the NER factor XPA and colocalizes with it in response to UV irradiation (Fan & Luo, 2008). For SpHRQ1, a genetic interaction between the homologs of both NER factors HsXPF and HsXPA could be confirmed (Groocock *et al.*, 2012). Moreover, analyses from baker's yeast further verified a conserved role for HRQ1 in NER by an direct interaction of Hrq1 with the HsXPC homolog Rad4 (Choi *et al.*, 2014). Thus, the classification of HRQ1 into a common pathway with NER seems to be conserved for all currently characterized homologs.

The nuclease AtMUS81 and its complex partner AtEME1 act as a Holliday junction resolvase and is thereby involved in the repair of CLs in parallel to the RecQ helicase AtRECQ4A and the ATPase AtRAD5A (Hartung *et al.*, 2006; Geuting *et al.*, 2009; Mannuss *et al.*, 2010). By *in vitro* analyses in yeast and mammals, a double Holliday junction intermediate processing activity could be demonstrated for MUS81 (Constantinou *et al.*, 2002; Doe *et al.*, 2002). As double mutants of AtMUS81 and the helicases AtRECQ4A or AtFANCM exhibit lethal phenotypes, this highlights the importance of AtMUS81 in the repair of replication-dependent DNA damage (Hartung *et al.*, 2008; Dangel *et al.*, 2014). A parallel involvement of AtMUS81 and AtFAN1 further hinted at an involvement of AtMUS81 in the processing of complex DNA structures. As a result, we recently postulated that AtMUS81 acts in a backup pathway for the removal of different types of aberrant recombination intermediates (Herrmann *et al.*, 2015). It was now of special interest to elucidate whether this backup function is also applicable in the absence of the newly characterized helicase AtHRQ1. Indeed, we demonstrated a parallel involvement of AtHRQ1 and AtMUS81 in the repair of both interstrand and intrastrand CLs. For SpHrq1, an even more drastic effect was observed in the absence of Mus81, as double mutants exhibit a lethal phenotype (Groocock *et al.*, 2012). In plants, a surplus of repair pathways might prevent such a drastic effect, as we did not find growth aberrations in *hrq1 mus81* double mutants. Here, helicases like RECQ4A might make up for the loss of HRQ1. In any case, the parallel involvement of HRQ1 and MUS81 in DNA repair appears to be evolutionarily conserved between plants and fungi.

The RecQ helicase AtRECQ4A acts as a member of the so-called RTR complex in plants and hence forms the functional homolog of HsBLM and ScSgs1 (Hartung *et al.*, 2007; Knoll *et al.*, 2014). AtRECQ4A was shown to define one of three main pathways for the repair of intrastrand CLs, where it acts in parallel to AtRAD5A and AtMUS81 (Mannuss *et al.*, 2010). A

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function for AtRECQ4A in the repair of interstrand CLs was recently demonstrated via the analysis of double mutants with the helicase AtFANCM (Dangel *et al.*, 2014). Further analyses classified AtRECQ4A into a common interstrand CL repair pathway with the nuclease AtFAN1 (Herrmann *et al.*, 2015). To determine the relationship of the two helicases, AtHRQ1 and AtRECQ4A, in CL repair, we analyzed the double mutant according to its sensitivity against cisplatin and MMC. While the At*recq4A* single mutant exhibits strong cisplatin hypersensitivity, even at a concentration of 2.5 μM, in *hrq1-1* mutants significant sensitivity only manifests starting from concentrations of 10 μM. Thus, AtRECQ4A seems to be the main RecQ helicase involved in intrastrand CL repair, with a minor role of AtHRQ1. In the double mutant, a relative FW comparable to that of the *recq4A* single mutant was shown, thus implying a common involvement of AtHRQ1 and AtRECQ4A in intrastrand CL repair. Remarkably, for yeast Hrq1, a parallel function to Sgs1 (Rqh1 in *Schizosaccharomyces pombe*) after cisplatin treatment was demonstrated (Groocock *et al.*, 2012; Choi *et al.*, 2013). A strong growth defect of *hrq1 sgs1* double mutants further hints at independent functions of both helicases in maintaining genome stability. In contrast to this, for the human HRQ1 homolog RECQ4, a physical and functional interaction with HsBLM has been proposed (Singh *et al.*, 2012). Thus, the common involvement of AtHRQ1 and AtRECQ4A in intrastrand CL repair resembles the circumstances apparent in mammals, while it differs from those in yeast. However, the situation in Arabidopsis appears to be different in response to interstrand CL repair, as *hrq1-1* mutants exhibit a strong MMC sensitivity, while only a subordinate involvement of AtRECQ4A in interstrand CL repair was postulated. Interestingly, in the double mutant, a significantly reduced FW compared with both single mutants was determined after treatment with 10 and 15 $\mu g\,ml^{-1}$ MMC. This can be taken as a hint that both helicases are able to complement each other, to a certain extent, in the repair of interstrand CLs. These results resemble the findings from baker's yeast, where the functions of Hrq1 and Sgs1 in interstrand CL repair were also not epistatic (Bochman *et al.*, 2014). Here, in accordance with our findings from Arabidopsis, a major role for Hrq1 in the repair of interstrand CLs, was postulated, with only a backup function for Sgs1. Such a situation seems to be conserved between plants and fungi.

The FA-associated factor FAN1 is an essential factor of CL repair in humans. Interestingly, the nuclease is not conserved in all eukaryotes, as homologs were identified in *S. pombe* and *A. thaliana* but not in *D. melanogaster* or *Saccharomyces cerevisiae* (Smogorzewska *et al.*, 2010; Fontebasso *et al.*, 2013; Herrmann *et al.*, 2015). For the FAN1 homolog from Arabidopsis, the involvement in interstrand CL repair was recently confirmed, where a function upstream of two subpathways defined by the RecQ helicase RECQ4A and the PRR-associated ATPase RAD5A, but separate from the other nuclease AtMUS81, was suggested (Herrmann *et al.*, 2015). We now further demonstrated a cisplatin-sensitive phenotype for At*fan1* mutants, which implies an additional function of the nuclease in intrastrand CL repair. A similar sensitivity was already described for *fan1* mutants in DT40 chicken cells and *S. pombe*, implying a conserved involvement of FAN1 in intrastrand CL repair (Yoshikiyo *et al.*, 2010; Fontebasso *et al.*, 2013). As cisplatin also induces interstrand CLs in minor amounts, we cannot completely rule out the possibility that the observed effect relies on the involvement of FAN1 in interstrand CL repair. Our results further indicate that HRQ1 and FAN1 act together in a joint CL repair pathway. This links HRQ1 with the FA repair pathway in Arabidopsis. For ScHrq1, an involvement in CL repair parallel to the FA-like pathway was postulated and, to our knowledge, no involvement of HsRECQ4 in the FA pathway was previously demonstrated (Rogers *et al.*, 2017). Thus, the involvement of AtHRQ1 in an FA-associated pathway seems to be a unique characteristic in plants. Biochemical analyses of ScHrq1 indicated the binding and unwinding of bubble-like DNA substrates, hinting at a possible role in the unwinding of a DNA bubble around interstrand CLs (Rogers *et al.*, 2017). This could support the nucleolytic incisions on both sides of the lesion by a nuclease like FAN1.

For the repair of replication-blocking lesions, the PRR is an important pathway. Two different branches can be distinguished: the error-prone and error-free pathways. The yeast Rad5 ATPase was shown to be involved, both mechanistically as a translocase in the regression of the replication fork, and regulatorily as an E3

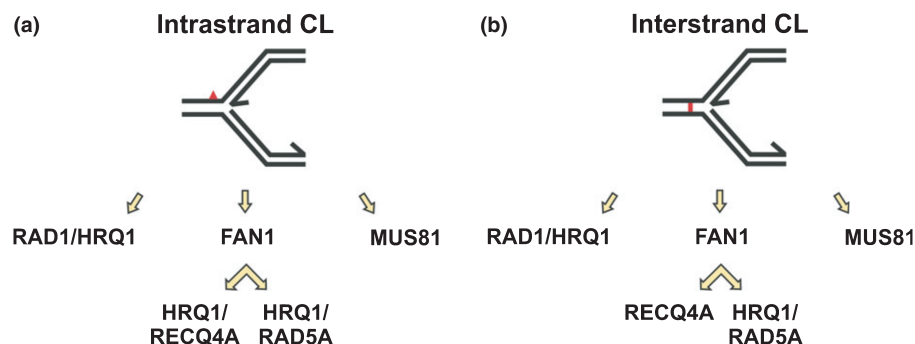

Fig. 7 Model of crosslink (CL) repair pathways with HRQ1 in *Arabidopsis thaliana*. (a) In intrastrand CL repair, HRQ1 acts in a pathway with RAD1 but separate from MUS81. HRQ1 further participates in a pathway with FAN1, contributing to both subpathways defined by RECQ4A and RAD5A. (b) In interstrand CL repair, HRQ1 and RAD1 also share a common pathway. While HRQ1 acts in a pathway with FAN1 epistatic to RAD5A, we assume that HRQ1 and RECQ4A might act in parallel, thereby possibly filling in for each other. MUS81 defines a pathway independent of HRQ1.

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ubiquitin ligase in the polyubiquitination of PCNA, preceding the error-free pathway choice (Ulrich & Jentsch, 2000; Blastyák *et al.*, 2007). AtRAD5A defines a pathway in the repair of DNA CLs separate from the helicase RECQ4A and the nuclease MUS81 (Mannuss *et al.*, 2010). Furthermore, we recently demonstrated that AtRAD5A acts independently of NER, single-strand break repair, microhomology-mediated end-joining and the ATM-mediated DNA damage response (Klemm *et al.*, 2017). Therefore, it was surprising to find a common involvement of AtHRQ1 and AtRAD5A in DNA CL repair. Previous analyses revealed a function for AtRAD5A downstream of the nuclease AtFAN1 in CL repair and we were now able to classify AtHRQ1 in the same pathway. These findings were unexpected, as the yeast homologs of HRQ1 were shown to act in pathways separate from PRR (Groocock *et al.*, 2012; Choi *et al.*, 2014). For human RECQ4, precise analyses concerning the interaction with PRR are missing, but a model postulating a function in parallel to PRR seems to be consistent with phenotypes of RTS patients (Groocock *et al.*, 2012). This would imply a plant-specific interaction of the three factors, AtHRQ1, AtFAN1 and AtRAD5A, in DNA CL repair.

Our findings enabled us to integrate AtHRQ1 into the complex network model of DNA CL repair in *A. thaliana* (Fig. 7). HRQ1 contributes to a common pathway with the NER factor RAD1 in the repair of both inter- and intrastrand CLs. MUS81 appears to mediate a backup pathway independent of HRQ1. We showed that HRQ1 and FAN1 act together in CL repair, thus linking a HsRECQ4 homolog and the FA pathway. While HRQ1 seems to be involved in the two subpathways defined by RAD5A and RECQ4A in intrastrand CL repair, HRQ1 and RECQ4A do not act epistatically but might complement for each other in the repair of interstrand CLs. The epistasis of HRQ1 and RAD5A in intra- and interstrand CL repair strongly indicates an involvement of HRQ1 in PRR, which seems to be plant-specific.

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Author contributions

S.R., A.K. and H.P. designed the experiments; S.R., J.E., A.S. and N.J.H. conducted the experiments; S.R., A.D., J.E., A.S., N.J.H., A.K. and H.P. analyzed data; and S.R., A.D. and H.P. wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 cDNA analysis of *recq2-1* and *recq3-1* mutant lines.

Fig. S2 T-DNA insertion loci sequences of *hrq1-1* and *hrq1-2* mutant lines.

Fig. S3 At*HRQ1* expression in *hrq1-1* and *hrq1-2* mutant lines.

Methods S1 Gene expression analysis via qRT-PCR.

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