AtRAD5A is a DNA translocase harboring a HIRAN domain which confers binding to branched DNA structures and is required for DNA repair in vivo

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SUMMARY
DNA lesions such as crosslinks represent obstacles for the replication machinery. Nonetheless, replication can proceed via the DNA damage tolerance pathway also known as postreplicative repair pathway. SNF2 ATPase Rad5 homologs, such as RAD5A of the model plant Arabidopsis thaliana, are important for the error-free mode of this pathway. We were able to demonstrate before, that RAD5A is a key factor in the repair of DNA crosslinks in Arabidopsis. Here, we show by in vitro analysis that AtRAD5A protein is a DNA translocase able to catalyse fork regression. Interestingly, replication forks with a gap in the leading strand are processed best, in line with its suggested function. Furthermore AtRAD5A catalyses branch migration of a Holliday junction and is furthermore not impaired by the DNA binding of a model protein, which is indicative of its ability to displace other proteins. Rad5 homologs possess HIRAN (Hip116, Rad5; N-terminal) domains. By biochemical analysis we were able to demonstrate that the HIRAN domain variant from Arabidopsis RAD5A mediates structure selective DNA binding without the necessity for a free 3’OH group as has been shown to be required for binding of HIRAN domains in a mammalian RAD5 homolog. The biological importance of the HIRAN domain in AtRAD5A is demonstrated by our result that it is required for its function in DNA crosslink repair in vivo.

Keywords: DNA damage tolerance, postreplicative repair, DNA crosslink repair, HIRAN domain, DNA translocase, Arabidopsis thaliana, Rad5, DNA-binding domain, replication fork, At5g22750.

INTRODUCTION
DNA lesions during the S-phase of the cell cycle endanger genomic integrity and proper replication. The mechanisms of the DNA damage tolerance (DDT) pathways allow lesion bypass without the requirement of direct lesion repair (Svoboda and Vos, 1995; Pagès and Fuchs, 2003). In Saccharomyces cerevisiae, DDT is regulated by the ubiquitination of proliferating cell nuclear antigen (PCNA) (Ulrich and Jentsch, 2000; Xiao et al., 2000; Broomfield et al., 2001; Hoege et al., 2002; Ulrich, 2005; Andersen et al., 2008). The monoubiquitination of PCNA by Rad6/Rad18 leads to translesion synthesis (TLS), which is error prone. The error-free DDT pathway involves template switching and is initialized by the polyubiquitination of PCNA mediated by the E3 ubiquitin ligase Rad5, which recruits Mms2/Ubc13 to Rad6/Rad18. There are two not mutually exclusive modes of error-free DNA damage bypass. One mode involves replication fork regression, whereby the new leading strand anneals to the new lagging strand. The resulting four-way junction is called a chicken foot structure; due to the previous uncoupled and ongoing synthesis of the nascent lagging strand, this strand serves as the template for leading strand synthesis. The chicken foot structure is subsequently reversed, restoring the replication fork. The second error-free DDT mode involves template switching via strand invasion and D-loop (displacement loop) formation in a homologous recombination-like mechanism. In vitro, Rad5 physically interacts with branched DNA substrates and catalyses the regression and reversal of model replication forks via its ATP-dependent translocase activity (Blastyák et al., 2007) and also catalyses strand invasion and D-loop formation (Burkovics et al., 2014). In vivo investigations showed that blocked replication forks cannot be restarted in cells lacking Rad5 and that abnormal DNA structures arise. Both the
ubiquitin ligase and the ATPase activity are involved in restarting blocked replication forks (Minca and Kowalski, 2010). Recently it was shown that the Walker B motif of the helicase domain not only plays a role for the ATPase activity of Rad5, but is also required for PCNA polyubiquitination (Choi et al., 2015). In summary, Rad5 is not only involved in the error-free DDT pathway via its ubiquitin ligase activity but might also participate in both proposed error-free DDT modes through physical interactions with DNA.

DNA damage tolerance is conserved in eukaryotes. In humans, there are two functional Rad5 orthologs that are also involved in DDT: SNF2 histone linker PHD RING helicase, first described as a potential tumor suppressor protein, and helicase-like transcription factor (HLTF) (Sood et al., 2003; Motegi et al., 2006, 2008; Unk et al., 2006). Both human homologs interact with human RAD18, UBC13, and PCNA and with each other (Motegi et al., 2006, 2008). In vitro, HLTF is able to process replication forks, including the displacement of associated proteins, and catalyses D-loop formation; in vivo, HLTF restarts stalled replication forks (Blastyák et al., 2010; Achar et al., 2011; Burkovics et al., 2014). Several Rad5 homologs are found in the genome of the model plant Arabidopsis thaliana; however, only RAD5A is involved in DNA repair. AtRAD5A shares the domain organization of yeast Rad5 and human HLF (Chen et al., 2008) (Figure 1a). Investigations of RAD5A-deficient plants revealed reduced plant growth on media containing DNA crosslinking agents and methylating genotoxins, which can block replication. Additionally, RAD5A is involved in double-stranded break repair via homologous recombination (Chen et al., 2008; Mannuss et al., 2010). REV3 is one of several known proteins involved in TLS in Arabidopsis (Sakamoto et al., 2003; Kobbe et al., 2015). The analysis of rad5a and rev3 single and double mutant lines have demonstrated that both the error-free and the error-prone pathways of DDT are conserved in Arabidopsis (Wang et al., 2011). RAD5A seems also to be involved in the regulation of DDT in plants, as Arabidopsis PCNA1 and PCNA2 are ubiquitinated due to the RING domain and ubiquitin ligase function of RAD5A (Strzalka et al., 2013).

The RING domain is flanked by the two helicase subdomains with the seven helicase motifs typical of superfamily 2 helicases (SF2) (Figure 1a). The helicase domain is involved in ATP binding and hydrolysis and therefore in DNA translocation, causing regression and reversion of a stalled replication fork (Blastyák et al., 2007; Unk et al., 2010). In the N-terminus an HIRAN (Hip116, Rad5p, N-terminal) domain is identified by bioinformatic algorithms. The HIRAN domain was first described as a domain that exists as a stand-alone unit in several bacteria and prophages or is a module of proteins with other domains known to be involved in DNA processing in eukaryotes. On this basis, it was postulated that the HIRAN domain is involved in specific interactions with DNA (Iyer et al., 2006). This assumption was recently confirmed for the HIRAN domain of human HLF by structural and biochemical data (Hishiki et al., 2015; Kile et al., 2015). Both groups were able to resolve the structure of this HIRAN domain in complex with DNA. Additionally it was shown, that HLF is necessary for restraining the further progression of replication forks under dNTP shortage in vivo probably by catalysing fork regression and that the HIRAN domain is crucial for this function (Kile et al., 2015). Also data from Schizosaccharomyces pombe Rad8 (the Rad5 homolog in S. p.) implicate the HIRAN domain in DNA damage response in vivo (Ding and Forsburg, 2014).

Interestingly, the residues defined for HLF for interactions with DNA are not conserved in the HIRAN domain of AtRAD5A and AtRad5A was not yet biochemically characterized. Therefore, in this study, we analyzed the biochemical properties of Arabidopsis thaliana RAD5A and investigated the function of its HIRAN domain variant in vitro and in vivo.

RESULTS
RAD5A of Arabidopsis thaliana is a double-stranded DNA-dependent ATPase able to regress replication forks with preference for forks with a leading strand gap
RAD5A of Arabidopsis thaliana was expressed in Escherichia coli and purified via a C-terminal Strep-Tag II and gel filtration. RAD5A-KT425,426AA, with two amino acid substitutions of lysine and threonine in the Walker A motif that as shown for HsHLTF (MacKay et al., 2009) or ScRad5 (Chen et al., 2005; Minca and Kowalski, 2010), abolishes the ATPase activity, and was prepared in the same manner.

We could show that RAD5A hydrolyzed ATP in the presence of double-stranded DNA, but not in the absence of DNA or when using single-stranded DNA, which would be in line with it being a double-stranded DNA translocase (Figure 1b). The ATPase-deficient variant RAD5A-KT425,426AA showed negligible ATP hydrolysis, indicating the absence of contaminating bacterial proteins such as helicases in the protein preparation.

As Rad5 homologs have been shown to be able to regress replication forks we asked if also AtRAD5A is able to perform this reaction. So far, most fork regression assays for Rad5 homologs were performed with replication forks without significant gaps at the fork. However in nature single-stranded regions occur. While undisturbed replication is correlated with a lagging strand gap, at stalled forks uncoupling of replication leads to leading strand gaps. Therefore we compared the ability of RAD5A to process replication forks with a leading strand gap, a lagging strand gap and without a significant gap. The gap length was chosen to be 30 nt. AtRAD5A is able to regress replication forks (Figures S2 and 1c) and – in line with one of its proposed functions in DDT namely the regression of stalled replication forks – replication forks with a leading strand gap are processed most efficiently at 8.5 nM of enzyme 23% (±2%) for the fork regression product compared with 11% (±2%) for the fork with lagging strand gap and 2% (±1%) for the fork without significant gap. With the negative control (KT-AA) 0% (±0%), 2% (±3%) and 0% (±1%) were detected respectively. Values in brackets represent standard deviation after correction for background without enzyme.

RAD5A is able to catalyse branch migration of Holliday junctions unaffected by protein blockades
A double-stranded DNA translocase is assumed to translocate dsDNA using the hydrolysis of a nucleoside triphosphate as a source of energy. This reaction can lead to the remodeling of DNA structures as shown, or to the removal of proteins bound to the DNA or both. We decided to further analyze the possible translocase activity of RAD5A within the biologically relevant context of a Holliday junction. Holliday junctions are central intermediates in homologous recombination and additionally mimic the chicken foot structure that arises in error-free DDT. In vivo data obtained with rad5a mutant plants suggest functions of RAD5A in those two pathways (Chen et al., 2008; Mannuss et al., 2010). The Holliday junction substrate (Gari et al., 2008) used is partially mobile (pmHJ) because of two homologous and two heterologous arms, and processing by a dsDNA translocase via branch migration yields two double-stranded DNA products (Figure S1c). Indeed, RAD5A was able to process this pmHJ substrate via branch migration (Figure 2a,b). Because of the homology, pmHJ is also forming the two duplex structures slowly by DNA breathing in the absence of enzyme. Binding of RAD5A-KT425,426AA stabilizes the structure and leads to the apparent ‘formation’ of pmHJ in the quantification (Figure 2b). In contrast, a model Holliday junction without sequence homology (X0-HJ) (Figure S1c) could not be processed by RAD5A, indicating its inability for strand separation and branch migration over long heterologous regions (Figure 2a). Branch migration products were observed when using an intermediate Holliday junction with a homologous core but heterologous ends (X26-HJ), albeit in reduced amounts compared with a Holliday junction with homologous arms (Figure 2a,b).

Using pmHJ as the preferred Holliday junction substrate, we assessed whether the branch migration activity of RAD5A is dependent on the presence of (deoxy)ribonucleoside triphosphates (Figure S3). Although ATP or dATP were the preferred cosubstrates, other (d)NTPs could also be used but led to reduced branch migration activity. Interestingly, common ATP analogs, which are often used as negative controls, were also used for branch migration if the anhydride linkage between the β-phosphate position and γ-phosphate position was intact (ATP–α-S, ATP–γ-S, ApCp). Further ATP analogs with modified linkage of the γ-phosphate to the β-phosphate (AppCp, AMPPNP) could not be used for branch migration (Figure S3).

As dsDNA translocases may also be involved in the removal of bound proteins, we investigated the branch migration activity of RAD5A on a protein-bound Holliday junction by creating a partially mobile Holliday junction with one BamHI binding sequence (pmHJ–BamHI, Figure S1c). Modified BamHI–E111A, in which glutamate-111 is replaced by alanine, can bind to the BamHI recognition site but is not able to cut the DNA (Dorner et al., 1999; Engler et al., 2001). First, we determined the optimal BamHI–E111A concentration, such that the binding sequence of pmHJ–BamHI was nearly saturated after preincubation with BamHI–E111A (Figure S4). Interestingly, branch migration of RAD5A was not affected by the obstacle induced by BamHI–E111A: pmHJ–BamHI was processed

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identically, irrespective of whether BamHI–E111A was pre-bound or not (Figure 2c,d). To compare these findings with another branch migration-catalysing enzyme, we performed the same experiments with the helicase RECQ4A-420-983; this catalytic active core fragment of Arabidopsis thaliana RECQ4A is an ATP-dependent helicase that can process synthetic replication forks by fork regression and branch migration (Schröpfer et al., 2014). In contrast to RAD5A, the activity of RECQ4A-420-983 following preincubation with BamHI–E111A was reduced (Figure 2c,e). Thus, the unaffected branch migration activity of RAD5A by a protein obstacle is not a common feature of proteins processing Holliday junctions, and the results suggest that RAD5A is directly involved in the disassociation of proteins bound to DNA.

The HIRAN domain of Arabidopsis RAD5A is different from the characterized HIRAN domain of human HLTF

The structure of the HIRAN domain of human HLTF in complex with single-stranded DNA was recently solved by three groups (Achar et al., 2015; Hishiki et al., 2015; Kile et al., 2015) and the importance of specific amino acids was analyzed. Kile et al. showed that most of the amino acids involved in DNA interaction (indicated by numbers in Figure 3) were conserved in HIRAN domains of proteins from different domains of life so that they concluded that the HIRAN domain evolved from a general nucleic acid binding architecture to a domain that specifically binds to 3’ ends. Indeed they also identified the HIRAN domain of SM3L1 (At5g05130) of Arabidopsis thaliana. However this protein was not yet shown to be involved in DNA metabolism, in line with our own preliminary data that the growth of a T-DNA insertion mutant in this gene is not sensitive to the genotoxins bleomycin and mitomycin C. However, as already stated in the introduction, we identified a functional homolog of ScRAD5 and HsHLTF, namely AtRAD5A (At5g22750) (Chen et al., 2008). A multiple sequence alignment suggests that in plants at least two different HIRAN domain variants exist (Figure 3). The HIRAN domain of SM3L1 homologs is similar to the characterized HIRAN domain of human HLTF, but for AtRAD5A homologs the amino acids identified for interaction with the 3’ end of DNA are not conserved. Without further information it is unclear if the HIRAN domain variant of Arabidopsis RAD5A will have the same properties as the characterized one of HsHLTF and this situation could lead to different biochemical properties of the whole proteins. Therefore we wanted to assess the biochemical properties of the RAD5A HIRAN domain.

The HIRAN domain-containing N-terminus of RAD5A preferentially binds to branched DNA structures without the need of a free 3’ hydroxyl group

To investigate the potential DNA-binding function of the HIRAN domain variant of Arabidopsis RAD5A, we purified an N-terminal fragment of RAD5A that contains the HIRAN domain (RAD5A-1-242) and performed electrophoretic mobility shift assays (EMSA) with different DNA substrates (Figure 4a). To guarantee comparability and to exclude potential sequence dependency, the DNA structures all shared the same radioactively labeled oligonucleotide as a common origin. Interestingly branched DNA structures (spliced arm, 3’ and 5’ flaps, replication fork and Holliday junction) were the preferred substrates for binding by RAD5A-1-242 compared to single stranded, double-stranded or 3’ or 5’ overhang substrates. As the replication fork is the central element of DDT we continued with this substrate and asked, whether a free 3’ OH group at the junction point is important for efficient DNA binding. For

![Image](image-url)
the HIRAN domain of human HLTF it was shown that the 3' hydroxyl group of single-stranded DNA was necessary for the binding of the HIRAN domain to this single-stranded DNA (Hishiki et al., 2015; Kile et al., 2015). However a 3' phosphate group at the junction point on the leading strand did not impair DNA binding of the Arabidopsis RAD5A HIRAN variant (Figure 4b).

As binding by the HIRAN domain variant was not impaired by a blocked 3' end we next asked if the fork DNA (Hishiki et al., 2015; Kile et al., 2015). However a 3' phosphate group at the junction point on the leading strand did not impair DNA binding of the Arabidopsis RAD5A HIRAN variant (Figure 4b).
regression activity of the full-length protein is influenced. Using the replication fork with leading strand gap as analyzed before (Figure 1c) either with or without the fluorophore TAMRA as a potential blockage of the 3’ end of the leading strand we did not detect significant differences in fork regression (Figure 4c). This result supports the hypothesis that the HIRAN domain variant of Arabidopsis RAD5A is recruiting RAD5A to branched DNA structures irrespectively of the presence of a 3’ OH group.

The HIRAN domain is essential for DNA repair in vivo

To define the role of the HIRAN domain in vivo, we transformed rad5a-mutant plants with constructs encoding RAD5A or RAD5A-ΔHIRAN. The coding sequence was placed under control of the natural RAD5A promoter and terminator. The plants were challenged with the genotoxic agent cisplatin for sensitivity assays. The structures preferentially formed by cisplatin are intrastrand crosslinks (Eastman, 1985). After treatment with 5 μM cisplatin, the rad5a-2 mutant exhibited a strong impairment in growth that was one-fifth that of the wild type (wt) (Figure 5a). Four independent lines carrying the full-length RAD5A ORF had a relative dry weight comparable to wt (rad5a-2 RAD5A #1-#4). However, rad5a-2 mutant lines transformed with the construct encoding RAD5A-ΔHIRAN (rad5a-2 RAD5A-ΔHIRAN #1-#4) showed strong hypersensitivity comparable with the rad5a-2 mutant. Taken together, these results demonstrate that the function of RAD5A in DNA repair in vivo is dependent on the presence of its HIRAN domain.

To analyze whether the respective proteins were indeed expressed in planta at comparable levels as wild type RAD5A, we performed western blotting. RAD5A proteins encoded by the complementation constructs were detected in plantlets at quantities similar to that in wild type (Figure 5b).

DISCUSSION

In this study, we biochemically characterized RAD5A of Arabidopsis thaliana and demonstrated that the conserved HIRAN domain variant is indeed a DNA-binding domain with a preference for the binding of branched DNA without the need of a 3’ hydroxyl group. Deletion of the HIRAN domain of Arabidopsis RAD5A leads to an impaired DNA damage response. It was already known by in vivo investigations of plants lacking RAD5A that this enzyme is required for repairing DNA crosslinks and methylation damage. These kinds of DNA damage can hinder replication and cause stalling of the replication fork leading to the assumption that the role of Arabidopsis RAD5A is similar to that of Rad5 in DDT in Saccharomyces cerevisiae (Chen et al., 2008). Uncoupling of replication synthesis leads to a leading strand gap and, interestingly, RAD5A catalyses fork regression of such a replication fork with a leading strand gap better than one with lagging strand gap or without gap at the junction point. Also HLTF was shown to regress replication forks with a leading strand gap (Achar et al., 2011) but mostly replication forks without gaps at the junction were used and the processing of the different replication fork substrates by Rad5 homologs were not compared (Blastyák et al., 2007, 2010; Achar et al., 2011, 2015). However the preference demonstrated for RAD5A of Arabidopsis is in line with a direct function of Rad5 homologs in the error-free mode of DDT.

Continuing studies demonstrated a further involvement of Arabidopsis RAD5A in the repair of double-stranded breaks via homologous recombination (Chen et al., 2008; Mannuss et al., 2010). Both the model of error-free DDT and the model of homologous recombination involve cruciform DNA intermediates (Holliday junctions), which need to be processed to canonical double-stranded DNA. As shown in this study with recombinant RAD5A and a partially mobile Holliday junction, RAD5A can process such cruciform DNA structures via branch migration.

Thus, similar to yeast Rad5 and human HLTF, Arabidopsis RAD5A is most likely not only involved in the control of
plant DDT, as recently demonstrated by the ability of RAD5A to function as an E3-ubiquitin ligase that polyubiquitinates Arabidopsis PCNA1 and PCNA2 (Strzalka et al., 2013), but also in the error-free pathway of DDT by processing and resolving the chicken foot intermediate to restart replication after stalling and bypassing the lesion through fork regression (Ulrich and Jentsch, 2000; Hoege et al., 2002; Branzei et al., 2004; Blastyk et al., 2007, 2010; Motegi et al., 2008; Unk et al., 2008, 2010; Masuda et al., 2012).

In contrast to a Holliday junction with sequence homology (representing the situation in nature), a static Holliday junction without sequence homology (X0-HJ; Ip et al., 2008) cannot be processed by RAD5A but is used as a co-substrate for ATP hydrolysis, indicating that RAD5A has no canonical strand-separating helicase activity.

We showed that ATP hydrolysis is dependent on the presence of double-stranded DNA. In contrast, Arabidopsis RAD5A does not hydrolyze ATP in the presence of single-stranded DNA. In agreement, human HLTF is also not able to hydrolyze ATP in the presence of oligonucleotide-based single-stranded DNA (Blastyk et al., 2010). Further biochemical analysis showed that in contrast to Saccharomyces cerevisiae Rad5 and human HLTF, a broad spectrum of (d)NTPs can be used by RAD5A (Blastyk et al., 2007, 2010). Nonetheless, ATP and (d)ATP are the preferred nucleoside triphosphates.

During branch migration, the activity of RAD5A is not impaired by prebound BamHI-E111A as a model of a blockade by DNA-bound proteins. We also wanted to compare this behavior with other helicases or translocases involved in the DNA damage response and recombination in Arabidopsis. We have previously characterized AtRECQ2 (Kobbe et al., 2008), AtRECQ3 (Kobbe et al., 2009), AtRECQ4A-420-983 (Schröpper et al., 2014) and AtSRS2 (Blanck et al., 2009) HsBLM has already been analyzed with respect to BamHI-E111A displacement (Sommers et al., 2014), and BamHI was found to impair the translocation of BLM. AtRECQ4A is the functional homolog of human BLM (Hartung et al., 2007), the RecQ helicase that is defective in the human disease Bloom syndrome. Therefore, we chose to use AtRECQ4A-420-983 in the present study. In contrast to AtRECQ4A-420-983, RAD5A’s branch migration activity is not affected by the presence of BamHI-E111A, which is in line with it belonging to the family of Snf2-like proteins. Snf2-like proteins are described as chromatin remodelers that must address proteins bound to DNA during translocation (Flaus et al., 2006).

As shown in Figure 1(a) Rad5 homologs have an N-terminal HIRAN domain. Although this domain was already described in 2006 on a bioinformatic level with functions proposed on the basis of the occurrence of HIRAN domains (Iyer et al., 2006), different groups solved the structure of the HIRAN domain of human HLTF in complex with DNA only in 2015 and characterized the human HLTF HIRAN domain variant biochemically (Achar et al., 2015; Hishiki et al., 2015; Kile et al., 2015). While the focus of two studies was on the HIRAN domain as a single-stranded DNA-binding domain with interactions with the 3’ OH end of single-stranded DNA (Hishiki et al., 2015; Kile et al., 2015) the third study showed that while single-stranded DNA is bound, still a replication fork is bound preferentially (Achar et al., 2015).

We show, that in Arabidopsis at least two HIRAN domain variants exist (Figure 3). Interestingly the HIRAN domain variant of the functional Arabidopsis Rad5 homolog, RAD5A, is different with respect to some amino acids shown to be important for DNA interaction by the human HLTF HIRAN variant. Therefore we analyzed the DNA-binding properties of the HIRAN domain variant of Arabidopsis RAD5A.

Also the HIRAN domain of RAD5A binds better to a replication fork than to single-stranded DNA as does the HIRAN domain of HLTF (Achar et al., 2015). Binding of the HLTF HIRAN domain, to the replication fork does not need a single-stranded DNA region (Achar et al., 2015). As in our study, the replication fork used in the study of Achar et al. (2015) did not expose single-stranded DNA. However before, the HTLF HIRAN domain was shown to require single-stranded DNA with a 3’ OH end for binding (Hishiki et al., 2015; Kile et al., 2015). Therefore the study by Achar et al. extended and specified the binding spectrum of the HIRAN domain of HTLF. However the effect of the presence of the 3’ OH group at the junction point of the replication fork was not further analyzed. However a 3’ OH at the junction point is important for DNA binding (Hishiki et al., 2015; Kile et al., 2015). We showed for the HIRAN domain variant of Arabidopsis RAD5A that a substitution of the 3’ OH by a 3’ phosphate group did not interfere with the binding of the HIRAN domain to the replication fork. Also fork regression by the full-length protein was not impaired by a substitution of the 3’ OH by the fluorophore TAMRA. Furthermore we were able to show that the HIRAN domain variant of Arabidopsis RAD5A does not need an accessible end at all for binding as also a static Holliday junction was bound with high efficiency.

The HIRAN domain of Schizosaccharomyces pombe Rad8 (the Rad5 homolog in S. p.) (Ding and Forsburg, 2015) and HTLF (Achar et al., 2015) are required for DNA repair.

To demonstrate the importance of the RAD5A HIRAN domain variant, we complemented rad5a-mutant plants with RAD5A and RAD5A::HIRAN. The rad5a-RAD5A::HIRAN plants show no complementation and a rad5a-like DNA repair phenotype, illustrating the importance of the HIRAN domain for repairing DNA crosslinks, which can cause replication fork stalling. Strictly speaking, deletion of the HIRAN domain might not only abolish the ability...
AtRAD5A to bind to branched DNA structures but might also perturb interactions with other cellular proteins, which might also contribute to the detected defect in DNA repair. In any case, our western blot data exclude the possibility that the phenotype is merely due to the misfolding of the mutant protein because this would most probably result in imminent degradation. Thus, although the HIRAN domain of Arabidopsis RAD5A is not conserved with respect to certain amino acids shown to be involved in protein-DNA interactions by human HLTF, it is important for DNA repair in vivo. This action could be mediated by binding of the HIRAN domain to defined branched DNA structures, thus guiding the protein to provide a correct and precise protein localization.

Thus, although with the present sets of data one cannot finally conclude whether or not the human HLTF HIRAN domain variant might bind some branched DNA structures also without the necessity of a 3’ OH group as it is the case for the RAD5A HIRAN variant, our findings generalize the potential role of structure specific DNA binding even by different HIRAN domain variants and support the proposed function of Arabidopsis RAD5A in DDT.

EXPERIMENTAL PROCEDURES

Cloning of expression vectors

The coding sequence for RAD5A (accession no. AK228695, AED93070.1) was cloned into a modified PET-Duet-1 vector (Novagen; Merck KGaA, Darmstadt, Germany), giving rise to an expression construct with the following additional N- and C-terminal sequences: MGSSHHHHHHHSQDPN

SSARLOVD(1029)GTLEVLFQGPTGTSAWSHPOFEK. The StreptII-Tag is shown in bold. For RAD5A-KT425,426AA, point mutations were introduced in the Walker A box, leading to –LGAAVM- instead of –LGKTVM-. For RAD5A-

ΔHIRAN, 119 amino acids were deleted, starting at aa position 107, leading to –VSGL[119]INS- instead of –VSG[119]INS-. This corresponds to the HIRAN domain as described (Iyer et al., 2006). The N-terminal RAD5A fragment was expressed with the following sequence: MGSSHHHHHHHSQDPN

M[240]T

Gari et al., 2008) with an AIDA Image Analyzer 4.5 (Raytest).

Recombinant protein preparation

RAD5A and its variants were expressed in ER2566 (NEB) and purified via the StreptII-Tag followed by gel filtration. RAD5A-1-242 was purified via the StreptII-Tag. Details can be found as in Methods S1. Fractions were mixed with an equal volume of 100% glycerol, and fractions of interest were evaluated by SDS-PAGE and branch migration activity and pooled. Aliquots of the purified protein were stored at –80°C for further characterization. All recombinant RAD5A variants were purified in the same manner. The protein concentration was determined by colloidal Coomassie-stained SDS-PAGE using BSA (BioRad, Hercules, CA, USA) as the standard.

DNA substrates

Appropriate oligonucleotides (Constantinou et al., 2001; Gari et al., 2008; Ip et al., 2008; Bétous et al., 2013) (Figure S1) were labeled with [γ-32P]-ATP (6000 Ci/mmol, Hartmann Analytic) and T4 polynucleotide kinase (NEB, Ipswich, MA, USA) and subsequently purified using Illustra MicroSpin G-25 columns (GE Healthcare, Pittsburgh, PA, USA). For the building up of substrates having either a 3’ TAMRA or a 3´ phosphate modification T4 PNK 3’ phosphatase minus (NEB) was used.

The replication fork substrates were prepared similarly to the HJ substrates following the protocol described (Bétous et al., 2013) with the exception of elution of the substrates after gel extraction by diffusion at 4°C overnight in annealing buffer.

For the HJ substrates used for the data in Figure 2 oligonucleotides types A (labeled) and B as well as C and D were annealed by slowly decreasing the temperature after heating for 5 min at 95°C; oligonucleotides used for the generation of double-stranded DNA were prepared in the same manner. AB and CD were then annealed at 37°C for 30 min followed by cooling to room temperature.

The substrates used for the EMSAs shown in Figure 4(a, b) were prepared by slowly decreasing the temperature after heating for 5 min at 95°C of equimolar mixtures of the constituting oligonucleotides (Figure S1d) in annealing buffer as used for the preparation of the replication fork substrates.

Translocase assay

Reactions were performed in translocase reaction buffer (40 mM Tris–HCl pH 8, 20 mM NaCl, 1.35 mM MgCl2, 50 μg ml−1 BSA, 6 mM DTT, 1.8 mM ATP) with 150 pm radioactively labeled HJ or RF. RAD5A enzyme was used in the indicated concentrations in a 20 μl reaction. To pre-bind BamHI–E111A (NEB) to pmHJ_BamHI-E111A, 5 nm BamHI-E111A was added for 15 min at 30°C. The translocase reaction was initiated with the addition of RAD5A enzyme and was incubated at 37°C for 30 min.

The reactions were terminated with a one-third volume of stop solution (50 mM EDTA, 0.6% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, and 20% glycerol). To determine background values, parallel reactions were performed without enzyme. After native TBE-PAGE at 4°C, the radioactive DNA was visualized by autoradiography using the CR-35-Bio Scanner (Raytest, Straubenhardt, Germany) and quantified (Kobbe et al., 2008, 2009; Blanck et al., 2009) with an AIDA Image Analyzer 4.5 (Raytest).

ATPase assay

An ATPase assay was performed with 5.75 nm of enzyme at 37°C for 30 min in ATPase-reaction buffer (40 mM

adding four times the volume of a solution containing 10% and the proteins were separated by 8% SDS-PAGE. Western

and Bent, 1998). T1-Transformants were selected on solid into Arabidopsis plants via the floral dip method (Clough

Agrobacterium (Hajdukiewicz

EMSA-reaction buffer (40 mM Tris/C0

50 μM MgCl2, 20 mM Tris–HCl pH 8, 20 mM NaCl, 50 μg ml⁻¹ BSA, 6 mM DTT) with 50 nm DNA substrate, 5 nm ATP and 0.4 nm (γ-32P)-ATP as a tracer. The reactions and analysis were performed as described for the translocase assay using 20% TBE-PAGE.

Electrophoretic mobility shift assay

EMSA reactions were performed at 37°C for 10 min in EMSA-reaction buffer (40 mM Tris–HCl pH 8, 20 mM NaCl, 50 μg ml⁻¹ BSA, 6 mM DTT) with 75 pm radioactively labeled DNA substrate (supplied in one-tenth volume of annealing buffer); 0, 86, 172, 259 and 345 nm of RAD5A-1-242 were used in a 20 μl reaction. After adding one-third of the volume of loading solution (0.1% bromophenol blue, and 40% glycerol), the analysis was performed as described above.

In vivo analysis

Genomic sequences of the promoter (763 bp upstream of the start codon) and terminator (518 bp downstream of the stop codon) regions of RAD5A and the coding sequences of RAD5A and RAD5A-ΔHIRAN were cloned into pPZP221 (Hajdukiewicz et al., 1994). Using the GV3101::pMP90 Agrobacterium strain, the constructs were transformed into Arabidopsis plants via the floral dip method (Clough and Bent, 1998). T1-Transformants were selected on solid GM selection medium (60 mg L⁻¹ gentamycin), and a segregation analysis was used to identify single locus lines in generation T2. Homozygous T-DNA containing plants were selected on selection medium in generation T3 and subsequently used in sensitivity assays, as described (Mannus et al., 2010).

Immunologic detection of protein expression of complementation constructs

Three-day old Arabidopsis seedlings were grown in liquid nitrogen. The proteins in the cell powder were extracted by adding per 100 mg of powder 1 ml low salt buffer (20 mM Tris–HCl pH 8, 5 mM MgCl2, 1 mM DTT, 1 x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1 ml urea buffer (8 M urea, 20 mM Tris–HCl pH 8, 5 mM MgCl2, 20 mM DTT, 1 x protease inhibitor cocktail (Sigma-Aldrich)). Both after the addition of low salt buffer and urea buffer the sample was treated for 5 min in a cold ultra sound bath. After centrifugation for 30 min at 16 000 g, the proteins in the supernatant were precipitated overnight at –20°C by adding four times the volume of a solution containing 10% trichloroacetic acid dissolved in acetone and 20 mM DTT. The pellet after centrifugation was washed twice with acetone containing 20 mM DTT and dissolved in 30 mM Tris pH 8.5, 7 M urea, 2 M thiourea, and 65 mM CHAPS. Sample buffer was added and after denaturation for 30 min at 80°C, and the proteins were separated by 8% SDS-PAGE. Western blotting was then performed. An anti-RAD5A antibody was generated in rabbits against denatured recombinant full-

length RAD5A by BioGenes. Serum antibodies were purified using Protein A HP SpinTrap columns (GE Healthcare). Detection was carried out using an anti-rabbit IgG HRP-linked secondary antibody (CST, Danvers, MA, USA) and the SignalFire Elite ECL reagent (CST).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online ver-

cion of this article.

Figure S1. Overview of the substrates used.

Figure S2. Phosphorimager data demonstrating fork regression.

Figure S3. Translocase activity dependence on different (deoxy) nucleoside triphosphates and ATP analogs.

Figure S4. Titration of BamHI-E111A.

Methods S1. Detailed purification protocol.

REFERENCES


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