AtGEN1 and AtSEND1, two paralogs in Arabidopsis thaliana, possess Holliday junction resolvase activity

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Summary of the most important findings:

Two Arabidopsis proteins resolve intermediary structures of DNA recombination and repair by symmetrically oriented incisions and provide new mechanistic insight in processing of nicked structures.
Footnotes

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ABSTRACT

Holliday junctions are physical links between homologous DNA molecules that arise as central intermediary structures during homologous recombination and repair in meiotic and somatic cells. It is necessary for these structures to be resolved to ensure correct chromosome segregation and other functions. In eukaryotes, including plants, homologs of a gene called GEN1 have been identified that process Holliday junctions in a manner analogous to the HJ resolvases of phages, archaea, and bacteria. Here, we report that Arabidopsis thaliana, a eukaryotic organism, has two functional GEN1 homologs instead of one. Like all known eukaryotic resolvases, AtGEN1 and AtSEND1 both belong to class IV of the Rad2/XPG family of nucleases. Their resolvase activity shares the characteristics of the EcRuvC paradigm for resolvases, which involves resolving Holliday junctions by symmetrically oriented incisions in two opposing strands. This leads to ligatable products without the need for further processing. The observation that the sequence context influences the cleavage by the enzymes can be interpreted as hint for the existence of sequence specificity. The two Arabidopsis paralogs differ in their preferred sequences. The precise cleavage positions observed for the resolution of mobile nicked Holliday junctions suggest that these cleavage positions are determined both by the substrate structure and the sequence context at the junction point.
INTRODUCTION

To counter the effects of endogenous and exogenous factors that threaten the genome integrity efficient mechanisms have evolved to ensure the faithful transmission of genetic information (Tuteja et al., 2001). Double-strand breaks, induced by conditions such as ionizing radiation or replication fork stalling, are among the most deleterious lesions (Jackson and Bartek, 2009). To protect the genome from to consequences of these lesions, the cells have ancient double strand break repair mechanisms including the homologous recombination pathway (HR). The HR mechanism is also of great importance in the intentional genetic recombination during sexual reproduction. A key intermediate in HR is the so-called Holliday junction (HJ), a structure that was first suggested in the context of a gene conversion model in fungi (Holliday, 1964) and later shown to arise in somatic and meiotic cells (Szostak et al., 1983; Schwacha and Kleckner, 1995; Cromie et al., 2006; Bzymek et al., 2010).

HJs are structures consisting of the four DNA strands of two homologous DNA helices, e.g., homologous chromosomes or sister chromatids. They arise through invasion of one single strand from each of the two helices into the other double strand. This results in two continuous strands (one per helix) and two strands that cross from one helix into the other. Schematics often depict the HJs with a parallel orientation of the helices, in which the crossing strands cross each other as was originally postulated (Holliday, 1964). However, HJs based on oligonucleotides have been shown to adopt an antiparallel conformation (reviewed in Lilley, 2000). In this configuration, the junction resembles the letter H in a lateral view, and the crossing strands actually perform U-turns. The crossing strands represent physical links between the two DNA strands involved. If a replication fork is restored via HR-mediated repair during mitosis, the resulting Holliday junction usually involves the two sister chromatids of one chromosome (Li and Heyer, 2008). In meiosis, the physical links in shape of HJs arise due to meiotic crossover between homologous chromosomes. In either case these links must be resolved to ensure unperturbed cell survival.

The importance of resolving the HJs for the survival of cells and organisms is highlighted by the phenotypes described for mutants defective for the known pathways of HJ resolution. One of these pathways is the resolution by canonical HJ resolvases, enzymes that cleave the two opposing strands of a HJ in perfectly symmetric positions relative to the junction point, which results in readily ligatable nicked duplex products (Svendsen and Harper, 2010). This property distinguishes the canonical HJ resolvases from the noncanonical resolvases (see below).
The main resolvase of *E. coli* is EcRuvC, which is part of the *E. coli* “resolvasome” (RuvABC complex) (Otsuji et al., 1974; Sharples et al., 1990; Sharples et al., 1999). In this complex, a HJ is sandwiched between two RuvA tetramers (Panyutin and Hsieh, 1994). Two RuvB complexes form ATP-dependent motors of branch migration, with two opposing helical arms of the junction threaded through their central openings. For the resolution of the HJ, one RuvA tetramer is replaced by a RuvC homodimer. This homodimer positions two active sites at the center of the junction, poised to cleave the junction point, if a preferred consensus sequence of the form 5'-(A/T)TT↓(G/C)-3' is encountered. The requirement for this correct sequence is quite strict: even a single base change can lead to a drastic reduction of the cleavage efficiency (Shah et al., 1994). Isolated EcRuvC is also active *in vitro* and binds only HJ structures with high specificity. This binding is independent of the sequence context, but the cleavage depends on the specific sequence (Iwasaki et al., 1991; Benson and West, 1994; Dunderdale et al., 1994). The exact cleavage position has been determined to be either one nucleotide 3' or 5' from the junction, or at the junction point (Bennett and West, 1996; Shida et al., 1996; Osman et al., 2009). The well-characterized EcRuvC is often referred to as a paradigm of canonical HJ resolution.

Eukaryotes have evolved a more complex interplay of different HJ resolution pathways (Schwartz and Heyer, 2011; Zakharyevich et al., 2012). The RTR complex, consisting of a RecQ helicase (AtRECQ4A in Arabidopsis, HsBLM in human, ScSgs1 in yeast), a type IA topoisomerase (AtTOP3A in Arabidopsis, HsTOPOIIIα in human, ScTop3 in yeast) and the structural protein RecQ-mediated genome instability 1 (AtRMI1 in Arabidopsis, HsRMI1 in human and ScRmi1 in yeast), mediates the so-called dissolution pathway. The crossing points of a double HJ (dHJ) are brought together by branch migration catalyzed by the helicase followed by decatenation catalyzed by the topoisomerase (Wu and Hickson, 2003; Hartung et al., 2007a; Mankouri and Hickson, 2007; Hartung et al., 2008; Yang et al., 2010). In addition to the catalytic activities, a functional RTR complex also requires structural functions based on protein-protein interactions, for which RMI1 plays an essential role (Mullen et al., 2005; Chen and Brill, 2007; Bonnet et al., 2013; Schröpfer et al., 2014). Dissolution leads to noncrossover products and is therefore a major mechanism in somatic yeast cells (Gangloff et al., 1994; Ira et al., 2003; Matos et al., 2011). In Arabidopsis, the loss of RTR component function leads to elevated rates of HR as well as sensitivity to UV light and methylmethane sulfonate (MMS) (Bagherieh-Najjar et al., 2005; Hartung et al., 2007a; Bonnet et al., 2013). Mutants of *AtRMI1* and *AtTOP3A* exhibit severe and unique meiotic phenotypes (Chelysheva et al., 2008; Hartung et al., 2008). This meiosis I arrest is dependent on HR, but the exact nature of the recombination intermediates that are involved remains unclear (Li et al., 2004; Hartung et al., 2007b; Knoll et al., 2014).
Dissolution acts in parallel with a second pathway mediated by the structure-specific endonuclease MUS81 as shown by the fact that the additional mutation of ScSgs1/AtRECQ4A leads to synthetic lethality (Mullen et al., 2001; Hartung et al., 2006; Mannuss et al., 2010). Single mutants of MUS81 in yeast, human, Drosophila and Arabidopsis are sensitive to DNA-damaging agents that perturb replication forks and show reduced HR following induction of double strand breaks (Boddy et al., 2001; Hanada et al., 2006; Hartung et al., 2006). The MUS81 homologs form heterodimers with the noncatalytic subunit EME1 (ScMms4 in Saccharomyces cerevisiae). SpMus81-Eme1 was the first nuclear endonuclease reported to be capable of resolving HJs (Boddy et al., 2001). The Arabidopsis complexes can be formed with the two different subunits, AtEME1A or AtEME1B (Geuting et al., 2009). AtMUS81-EME1A/B, like the fission yeast ortholog, preferentially cleaves nicked HJs (nHJs) and 3'-flaps but also show weaker activity on intact HJs in vitro (Boddy et al., 2001; Osman et al., 2003; Geuting et al., 2009; Schwartz and Heyer, 2011). Mus81 homologs are key players in meiotic crossover generation (Osman et al., 2003; Berchowitz et al., 2007; Higgins et al., 2008). While crossover formation is solely dependent on SpMus81 in fission yeast, this function was shown to be shared with ScYen1 in budding yeast (Osman et al., 2003; Blanco et al., 2010; Ho et al., 2010; Tay and Wu, 2010). Tightly regulated by ScCdc5-dependent hyperphosphorylation at the end of prophase I, the main activity of ScMus81-Mms4 is timed to coordinate with the formation of chiasmata and HJs that link the homologous chromosomes. This role in meiosis I is demonstrated by the failure of chromosome segregation at the end of meiosis I in ScMus81 mutants (Matos et al., 2011). Interestingly, the chromosomes could be segregated at the end of meiosis II, due to the presence of ScYen1. In contrast to canonical HJ resolvases, the hallmark of the MUS81-EME1 cleavage mechanism is the asymmetry of the second incision relative to either a first incision or a preexisting nick. This difference classifies MUS81-EME1 as a noncanonical resolvase. Its products need further processing by gap-filling or flap-cleaving enzymes to allow religation (Boddy et al., 2001; Geuting et al., 2009).

In very recent studies, HsMUS81-EME1 was found to constitute an essential canonical HJ resolvase with HsSLX1-SLX4, in which a first incision is made by HsSLX1-SLX4 followed by the enhanced action of the HsMUS81-EME1 subunits on the resulting nHJ (Garner et al., 2013; Wyatt et al., 2013). HsSLX1-SLX4 had previously been described as a canonical resolvase albeit producing only a low level of symmetrically cut ligatable products (Fekairi et al., 2009).

In addition to the mechanisms described above, an activity resembling that of EcRuvC had long been known to be present in mammalian cell-free extracts. In 2008, the group of Steven C. West succeeded in identifying the first nuclear proteins analogous to the EcRuvC paradigm: ScYen1 and HsGEN1 (Ip et al., 2008). These proteins are members of the large...
and well characterized Rad2/XPG family of nucleases. The Rad2/XPG family consists of the XPG endonucleases of the nucleotide excision repair (class I), the FEN1 replication-associated flap endonucleases (class II), the EXO1 exonucleases of recombination and repair (class III) and class IV, containing the (putative) eukaryotic Holliday junction resolvases. The last class was introduced after the identification of the rice single strand endonuclease 1 (OsSEND-1) based on sequence homology. The Class IV members show a domain composition homologous to FEN1 and EXO1, with no spacer region between their XPG-N and -I domains, while the primary structure of these domains is more similar to the sequence of XPGs’ nuclease domain (Furukawa et al., 2003).

While all Rad2/XPG homologs share a common cleavage mechanism as observed for the typical 5′-flap substrate (Tsutakawa et al., 2011; Tsutakawa and Tainer, 2012), the striking evolutionary difference between classes I, II, and III on the one hand and the HJ resolvases (class IV) on the other hand is the ability of class IV members to form homodimers in vitro at their preferred substrate, the Holliday junctions (Rass et al., 2010). The homodimer configuration ensures the presence of two active sites positioned on the opposing strands of the HJ, as is necessary for resolution. The mode of eukaryotic HJ resolution is largely similar to the bacterial paradigm: (i) cleavage occurs one nucleotide in the 3’ direction of a static junction point, equivalent to the main cleavage site on 5′-flaps, (ii) the incisions occur with almost perfect point symmetry, (iii) the incisions result in readily ligatable nicked duplexes and (iv) certain sites within a migratable HJ core are preferred, providing evidence for a (yet to be determined) sequence specificity (Ip et al., 2008; Bailly et al., 2010; Rass et al., 2010; Yang et al., 2012).

In the absence of MUS81-EME1/Mms4, the proteins HsGEN1, ScYen1 and CeGEN-1 have been shown to play a role in response to replication-associated perturbations such as MMS- and UV-induced DNA damage (Bailly et al., 2010; Blanco et al., 2010; Tay and Wu, 2010; Gao et al., 2012; Muñoz-Galván et al., 2012). It is also likely that these proteins provide a backup mechanism in mitosis and meiosis, ensuring proper chromosome segregation after a failure of other mechanisms including MUS81-EME1/Mms4 (Blanco et al., 2010; Matos et al., 2011).

While canonical HJ resolvases in animals and fungi are a current topic of great interest, very little is known about these proteins in plants. In rice, two members of the Rad2/XPG class IV have been described: OsSEND-1, the founding member and OsGEN-like (OsGEN-L). OsSEND-1 was shown to digest single-stranded circular DNA and its expression is induced upon MMS-induced genotoxic stress, while OsGEN-L is implicated in late spore development (Furukawa et al., 2003; Moritoh et al., 2005). The authors of both studies proposed putative homologs in other plants, and the gene locus At1g01880 of Arabidopsis, coding for the
protein AtGEN1, is considered the ortholog of HsGEN1 and ScYen1 (Ip et al., 2008). However, currently only OsGEN-L has been further investigated and described to possess \textit{in vitro} properties similar to both the Rad2/XPG nucleases and EcRuvC. This protein demonstrates a well-defined 5'-flap activity as well as a poorly characterized ability, similar to that of EcRuvC, to resolve mobile HJs (Yang et al., 2012).

Thus, of the two members of the Rad2/XPG class IV of plants only one has so far been analyzed with respect to a possible HJ resolvase activity. However, Arabidopsis expression data show that both proteins are expressed in plants and do not reveal marked differences (Laubinger et al., 2008). In this study, the goal was therefore to characterize the \textit{in vitro} activities of not only AtGEN1 but also AtSEND1, focusing on the idea that \textit{A. thaliana} and (seed) plants in general might encode not one but actually two HJ resolvases with functional homology to EcRuvC.

**RESULTS**

**GEN1 and SEND1 Homologs Are Conserved Among Seed Plants**

Searches in the \textit{A. thaliana} genome for sequence homologs of the previously characterized human and yeast resolvases resulted in two hits corresponding to gene loci At1g01880 and At3g48900, which share 34 % amino acid similarity in their conserved N-terminal regions. Their domain structure and sequence similarity place them in the Rad2/XPG family of nucleases, subclass IV, as previously postulated based on partial protein sequences (Furukawa et al., 2003; Moritoh et al., 2005). Sequence similarities to OsGEN-L and OsSEND-1 enabled us to assign AtGEN1 to At1g01880 and AtSEND1 to At3g48900. 

\textit{AtGEN1} and \textit{AtSEND1} code for proteins of 599 amino acids (aa) and 600 aa, respectively, with the typical organization of all class IV members: an N-terminal XPG domain (XPG-N), an internal XPG domain (XPG-I) separated by a short spacer and followed by a partially overlapping helix-hairpin-helix (HhH2) domain (Fig. 1A). The sequence of the N-terminal half of the proteins is conserved between animals, fungi and plants, while the C-terminal half shows little to no sequence similarity except among plant paralogs (Supplemental Information 1). Both AtGEN1 and AtSEND1 share between ~18 to 28 % identity on the amino acid level with their homologs from \textit{Caenorhabditis elegans}, \textit{Drosophila melanogaster}, \textit{S. cerevisiae}, and human. No sequence conservation could be found to non-eukaryotic HJ resolvases (data not shown).

To determine whether the presence of two Rad2/XPG class 4 members, hence two putative canonical HJ resolvases, is a common feature among plants, we investigated the conservation of GEN1 and SEND1 in several plant genomes. The corresponding phylogenetics of selected species are depicted in Figure 1B. While animals and fungi have
one resolvase (GEN1/Yen1), a second resolvase gene (SEND1) was found in most plants with a higher organization than moss. The plant orthologs share an average identity of 37 to 59%, while paralogs share an average identity of 22 to 28%, dependent on the evolutionary distances among the species. GEN1 and SEND1 form two separate clades in the tree. In Picea, as a representative of conifers, we were only able to confirm the presence of one homolog. A similar situation was found in Malus domestica, Prunus persica and Fragaria vesca. It is not clear whether these organisms have eliminated one paralog or if the absence is due to the quality of the sequencing data, as is the case in Carica papaya. By BLAST analysis, we verified that the genome of Physcomitrella patens contains only one gene, which is located in the GEN1 clade. Thus, a gene duplication event prior to development of seed plants might have led to the evolution of two paralogous genes, which might both encode functional Holliday junction resolvases.

To investigate the possible functions of both paralogs, we cloned the respective cDNAs, and sequencing confirmed that the gene models provided by The Arabidopsis Information Resource (TAIR) are correct. The overexpressed recombinant full length AtGEN1 and AtSEND1 were purified using C-terminal affinity tags (Supplemental Fig. S1) that left the N-terminus unaltered. The latter criterion proved to be required because initial constructs with a N-terminal His-tag were inactive (data not shown), whereas the proteins used in this study exhibit a robust nuclease activity on various substrates. Proteins featuring an amino acid exchange in their nuclease domain (D75A for AtGEN1 and D76A for AtSEND1) showed no such activities and were used as controls for purification quality (Supplemental Fig. S2).

**Structure-Specific Cleavage of Flapped DNAs**

Because AtGEN1 and AtSEND1 are members of the Rad2/XPG family, we first tested their activity on 5'-flaps, the shared substrate of all described nucleases within this superfamily. Both plant homologs showed a robust activity with a specific cleavage site in the 5'-overhang strand exactly one nucleotide 3' of the branch point (Fig. 2A, Fig. S3), the identical location to the preferred cleavage site of the FEN1, XPG, and GEN1 homologs. Testing of four different 5'-flaps, each featuring one of the four strands of the X0-HJ (see below) as the flap strand, revealed that each of the Arabidopsis proteins exhibit a weaker activity toward one of the four substrates (Fig. 2, A and B, Fig. S3). For AtGEN1, the weaker activity was demonstrated toward 5'-flap3, while AtSEND1 shows a slightly reduced activity on 5'-flap2 and shows an additional cleavage site two nucleotides 3' of the branch point (Fig. S3). This argues for an influence of the sequence around the branch point (Fig. 2C) on the activities of AtGEN1 and AtSEND1, with different sequence preferences for the two paralogs.

A 3'-flap proved not to be a suitable substrate for either AtGEN1 or AtSEND1 (Fig. 2D). However, a model replication fork, which can be considered as a flapped structure with a
double-stranded flap, was well recognized by both enzymes. Incision mapping showed that
AtGEN1 preferentially cleaves strand 1 exactly at the branch point, while AtSEND1
preferentially cleaves 1 nt in 3' direction of the branch point (Fig. 2E). AtSEND1 shows a less
pronounced preference for this main cleavage site as indicated by the fact that the two
neighboring positions are also cleaved in approximately 40 % of cases. Cleavage in strand 2
was not detected with either AtGEN1 or AtSEND1 (data not shown), indicating a preference
for cleavage of the lagging strand matrix as described for HsGEN1 and OsGEN-L (Rass et
al., 2010; Yang et al., 2012).

**GEN1 and SEND1 Cleave HJs Near the Junction Point**

With respect to their putative roles as HJ resolvases, we tested whether AtGEN1 and
AtSEND1 cleave Holliday junctions. A common substrate for the characterization of Holliday
junction-processing enzymes is a static HJ like the X0-HJ because it is characterized by a
fixed junction point due to the heterologous sequences of the four contributing
oligonucleotides (X0 because of zero base pairs of homology around the junction point).
These static HJs are used for the determination of the cleavage position, although the overall
outcome of the reaction may differ compared to the natural situation of migratable HJs with
homologous sequences (see later). The main incisions by AtGEN1 and AtSEND1 are
introduced exactly one nucleotide 3' of the fixed junction point in seven of the eight cases
(Fig. 3, S4). The only exception is the incision by AtSEND1 in strand 2, which is displaced to
the position 2 nt 3' of the junction point (Fig. 3B, S4). Notably, this is the same strand that
produces the flapped arm of 5'-flap2, which shows the additional cleavage site described
above. These data suggest that AtGEN1 and AtSEND1 preferentially cleave the sugar-
phosphate backbone one nucleotide 3' of the junction point as is the case with several HJ
resolvases (Ip et al., 2008; Osman et al., 2009; Bailly et al., 2010; Yang et al., 2012).

An additional similarity to previously characterized HJ resolvases like EcRuvC and OsGEN-
L, the most closely related of the characterized resolvase, is the apparent asymmetric
distribution of activity on the four strands of a static X0-HJ (Osman et al., 2009; Yang et al.,
2012). Both Arabidopsis proteins favor strands 1, 2, and 3 over strand 4. This asymmetry is
especially pronounced for AtGEN1 (Fig. 3A) but is also apparent with AtSEND1 (Fig. 3B). A
similar pattern has been observed with OsGEN-L (Yang et al., 2012) and is, in case of
EcRuvC the result of the well characterized sequence specificity of the bacterial HJ
resolvase (Osman et al., 2009). This observation suggests the potential for sequence
preference of the plant resolvases, which will be further evaluated.
Symmetrical Cleavage Action as Resolvases Leads to Ligatable Products

The spatial structure of a static HJ such as the X0 may resemble that of a naturally occurring HJ, but the main difference is the heterologous sequence that fixes the junction point. We therefore tested another HJ substrate, the X26, which comprises a homologous core of 26 base pairs that mimics the natural character of a migratable junction. AtGEN1 and AtSEND1 were both able to resolve the X26. The resulting major product migrates at the position of nicked duplex DNA in native gel electrophoresis (Fig. 4A), while a minor product is a faster migrating species equivalent to a short duplex (Fig. 4B). Analysis of the product lengths for each strand of the junction reveals cleavage patterns almost perfectly symmetrical with respect to the junction point (Fig. S5). These results are summarized in Figure 4, C and D. AtGEN1 introduces multiple incisions in both cleavage axes throughout the homologous core with three pronounced cleavage sites at 32, 31, and 27 nt in strands 1 and 3 and a single main cleavage position at 33 nt in strands 2 and 4 (Fig. 4C, Fig. S5). As the HJs were labeled at their 5’ ends the sizes of the cleavage products (as determined by sequencing gel analysis) are indicative for the cleavage positions. A cleavage product of 32 nt means, that the enzyme cut the sugar-phosphate backbone between nucleotides 32 and 33 as visualized by the arrowheads in the schematic drawings and named here a cleavage site at position 32. In contrast, the cleavage pattern of AtSEND1 is more concentrated around the center of the X26 with only one main position (30 nt) accompanied by a single secondary position (31 nt) in strands 1 and 3 and two main incisions (33 and 32 nt) in strands 2 and 4 (Fig. 4D, Fig. S5). Because even a cleavage pattern with perfect symmetry alone does not constitute proof of HJ resolvase activity according to the EcRuvC paradigm, we tested whether the activities resulted in ligatable nicked duplex products as suggested by the native gels and the pattern symmetry. For that purpose, we performed a religation experiment as was recently performed for other eukaryotic HJ resolvases (Ip et al., 2008; Yang et al., 2012). The asymmetric X26-S features three 30 bp- and one 23 bp-long helical arms in its centered conformation. Radioactive labeling of strand 2S enables us to detect a ligation-specific product of 60 nt (Fig. 5). For both AtGEN1 and AtSEND1, this ligation product is detected in reactions containing the T4 DNA ligase. This ligation product can only arise due to symmetrical resolution of the HJ, proving that the symmetrical cleavage patterns of AtGEN1 and AtSEND1 do not arise due to statistically distributed nicking events. Instead, both enzymes are able to act as canonical HJ resolvases.

Resolution Takes Place Within the Life Time of an Enzyme-Substrate Complex

The action of a canonical HJ resolvase is also characterized by a rapid succession of the two incisions. These may either take place simultaneously or as two sequential events in which the complex of HJ and resolvase remains intact, thus stabilizing the junction structure. The...
assay system described in Fig. 6A, using a plasmid-based cruciform structure as a HJ substrate, allows us to distinguish between nicking and resolution (Lilley and Markham, 1983; Rass et al., 2010). In contrast to the substrate described for the analysis of human GEN1, our plasmid, called pIR9, also included a portion of the sequence of the homologous core of the X26 as part of the inserted inverted repeat (see Materials and Methods).

In the reactions using the negative controls AtGEN1-D75A and AtSEND1-D76A no significant cleavage was observed, indicative for the fact that the cleavage observed in the reactions with AtGEN1 and AtSEND1 are catalyzed by the proteins of interest (Fig. 6B, also see Supplemental Fig. S2). Both Arabidopsis resolvases show a similar activity, with AtGEN1 reaching a plateau after 20 minutes, while AtSEND1 takes 60 minutes (Fig. 6B). The linearized plasmid, the product of the enzymes' resolvase activities, constitutes up to 70% of the total products for AtGEN1 (at 10 min) and roughly half of all products for AtSEND1. The remaining products were nicked circular plasmids, which run the slowest in the gel. These products indicate the presence of a nicking activity for both enzymes.

The Cleavage Pattern at Nicked Holliday Junctions Is Condensed

HJ resolvases are known to act on nicked Holliday junctions, which may play a role in vivo as an early recombination intermediate. To date, all in vitro experiments using nHJs have been carried out with static junctions, which fix the nick at a specific position. Usually the nick has been positioned at the structurally preferred cleavage site. In this study, we tested two nHJs with homologous cores based on the migratable X26, thus providing the enzymes with a more natural imitation of an nHJ. The nHJs in Fig. 7 are shown such that that the discontinuity is placed in the junction center because Pöhler et al. (1994) proposed that this was the manner in which the junction would migrate. As Figure 7 summarizes, the main cleavage activities can be detected in strand 1 opposite of the discontinuity (nick). The pattern of cleavage of strand 1 of the nHJs by AtGEN1 is further condensed compared to the cleavage of the intact X26 (compare Fig. 4C and Fig. 7, A and C). Because AtSEND1 cleaved strand 1 of the X26 mainly at position 30, a prominent condensation cannot be detected (compare Fig. 4D and Fig. 7, B and D). Interestingly, in the nX-32, the incisions by AtSEND1 are displaced to positions 32 and 33 (Fig. 7D). Taken together, for both AtSEND1 and AtGEN1, the main incisions in strand 1 are either directly opposite the nick in the center of the junction (position 30 in the nX-30; position 32 in the nX-32) or one nucleotide 3' of the junction center (position 31 in the nX-30; position 33 in the nX-32).

Although no cleavage can be detected in strand 2, incisions are made in strand 4. The extent of cleavage of strand 4 was in general less than cleavage in strand 1, but differed depending on the enzyme and the respective nHJ. However, this detection of cleavage in strand 4
without corresponding incisions in strand 2 is not consistent with a resolution-like activity of
the enzymes. Therefore, we endeavored to unravel the source of this cleavage pattern.

**Resolvase Activity Is Cryptic at Nicked Holliday Junctions**

nHJs with homologous cores and therefore the potential to migrate are clearly more natural
models than static nHJs. Furthermore, it is clear that testing two similar substrates leads to
more globally valid data. We therefore chose to test two nHJs, nX-30 and nX-32, based on
the previously obtained data. We have already shown that AtGEN1 and AtSEND1 differ in
their cleavage efficiency toward several substrates. The efficiency depends on the precise
sequence used, possibly due to different sequence specificities. Because the X26 is cut at
position 30 of strand 3 only by AtSEND1, testing only the nX-30 with the nick at this position
would have given an incomplete and biased view. Therefore, we decided to test two different
nHJs. The choice of the position of the discontinuities in strand 3 was based on the cleavage
patterns of AtGEN1 and AtSEND1 at the X26, as illustrated in Fig. S6. Briefly, the nX-30 is a
X26 featuring a nick between nucleotides 30 and 31 in strand 3 and might represent a
favorable substrate for AtSEND1. The discontinuity of nX-32 is located between nucleotides
32 and 33 and provides a substrate that might be favored for AtGEN1.

As stated above, both enzymes make incisions in strand 4 but not strand 2 of the two tested
nHJs, which is inconsistent with a resolvase-like activity. The activity of AtGEN1 on strand 4
is considerably more pronounced for the nX-30 substrate compared to the nX-32. The
cleavage activity of AtSEND1 in strand 4 is detectable, but low, with both substrates. We
analyzed the outcome of cleavage by AtGEN1 and AtSEND1 on the nHJs by native gel
electrophoresis (Supplemental Fig. S7). The observed patterns of the native product
structures for the two enzymes and the two different nHJs differ in quantity but are markedly
similar in quality. Specifically, when the substrates are labeled on strand 1 or 4, a short
duplex (sD) and a long duplex that most likely corresponds to the nicked duplex product (nD)
are present. In contrast, when the nHJs are labeled on strands 2 or 3, only nD products can
be detected. The identity of the short duplex in lanes 4* (indicating that the nHJs are labeled
at strand 4) can be easily explained by the activities of both AtGEN1 and AtSEND1 on strand
4 of the nHJs: the observed incisions in strand 4 of the nHJs (Fig. 7) in the presence of a
discontinuity in strand 3 leads to the separation of a duplex region composed of the
oligonucleotide 3b and the 5'-terminal region of oligonucleotide 4. This is equivalent to the
separation of arm 4 from the nHJ and would leave a replication fork-like structure. Indeed,
minor amounts of such a replication-fork like structure are visible for AtGEN1 with the nX-30.
The considerably smaller amount of this RF structure compared to the amount of arm 4
suggests that the RF structure is only an intermediate, which is further processed. We
therefore named this activity “Ref-I” for the involved replication fork intermediate. Because
strand 2 of the nHJs is not cleaved (Fig. 7), the processing of the replication fork-like structure most likely involves cleavage of strand 1. This would also be consistent with the exclusive cleavage of a replication fork in the lagging strand matrix as shown above. Indeed, a significant amount of an sD product, corresponding to arm 1 of the nHJ, is present when the nHJs are labeled on strand 1 (sD1*, Fig. 8). Separately prepared nicked duplex structures were not processed by either AtGEN1 or AtSEND1 (data not shown), supporting the idea that sD1* products can only arise from processing of a replication fork intermediate.

In addition to this Ref-I activity, we can assume that both enzymes possess a resolution-like activity because we can detect a nicked duplex product composed of arms 1 and 4 (scheme of native product structures in Fig. S7 = nDs in lanes 1* or 4*, nD1* in Fig. 8). The proposed composition of this duplex is as described in the previous paragraph and Fig. 8 because strand 2 is not incised (Fig. 7). Incisions by AtGEN1 and AtSEND1 only occur in strand 1 and 4. Because incisions in strand 4 lead into the Ref-I pathway that separates arm 1 from arm 4, the presence of the unseparated arms 1 and 4 (nDs in lanes 1* or 4*) indicates exclusive cleavage in strand 1, and thus represents a resolvase-like activity.

Both activities, resolvase-like and Ref-I activity, lead to cleavage of strand 1 and were previously analyzed together. Therefore, we wanted to know if specific cleavage positions in strand 1 can be attributed to one activity or the other. Because the sD1* product arises only via the Ref-I activity, and the nD1* product is exclusively the product of the resolution-like activity, we separated the two species by native gel electrophoreses, isolated the DNA and analyzed the respective cleavage positions and their relative quantities on a sequencing gel (Fig. 8, A and C). Interestingly, no prominent differences are visible in the cleavage patterns of AtSEND1 for nD1* and sD1* (Fig. 8, A and C). This indicates that both the Ref-I activity and the resolvase-like activity have the same cleavage patterns. The same is true for AtGEN1 with the nX-32 (Fig. 8C). However, for AtGEN1 with the nX-30, the cleavage event that is directly opposite the discontinuity (position 30) occurs almost exclusively via the Ref-I pathway, while the incision that occurs one nucleotide 3’ of the junction center is the result of both Ref-I and resolvase-like activity (Fig. 8A).

To gain further insights into the contributions of the two activities, we also constructed the different replication forks that we expect to be intermediates of the Ref-I pathway and analyzed the quantity and position of the cleavage events (Fig. S8). The data obtained is in good accordance with the cleavage positions and estimated quantities determined after gel extraction of the sD1* bands (Fig. 8, A and C). With the data presented above, we were able to define the contributions of the two pathways for the enzyme-derived products of the nHJs: the main resolution-like pathway and the secondary Ref-I pathway (Fig. 8, B and D). Considering only the resolution-like pathway, it is obvious that the main incision of AtGEN1 in
strand 1 of the nX-30 is one nucleotide 3’ of the junction point (Fig. 8B). The incision one nucleotide 3’ of the junction point is reminiscent of the behavior towards the static X0 HJ (Fig. 3). In contrast, in the case of the nX-32, AtGEN1 primarily incises strand 1 directly opposite the discontinuity in strand 3 (Fig. 8D), which would correspond to the cut of a canonical resolvase and lead to ligatable products. AtSEND1, on the other hand, shows two main cleavage sites, both directly opposite the centered nick and dislocated one nucleotide 3’ of the junction point. Thus, the differences detected for the two different nHJs are not as strong for AtSEND1 as they are for AtGEN1. Still, the situation described for AtGEN1 is inversely mirrored by AtSEND1: the nX-30 is preferentially cut into ligatable products by an incision directly opposite the discontinuity (Fig. 8B), while the main incision in the nX-32 is one nucleotide 3’ of the junction point (Fig. 8D).

Incorporating the results of the analysis of both the nX-30 and the nX-32, the picture is as follows: if, on the one hand, the discontinuity leading to a nicked Holliday junction is located at a preferred incision site for the Arabidopsis resolvase, the enzyme will preferentially cut directly opposite the nick, which should lead to ligatable products. If, on the other hand, the discontinuity leading to a nicked Holliday junction is at a position that is not a preferred incision site, the enzyme will preferentially cut one nucleotide 3’ of the junction point as observed with static HJs for the Arabidopsis resolvases and resolvases from other organisms (Ip et al., 2008; Osman et al., 2009; Bailly et al., 2010; Yang et al., 2012).

DISCUSSION

The ability to resolve HJs is conserved throughout all living organisms although resolution represents analogous rather than homologous evolution (West, 2009). The underlying principle is universal: resolution involves two diametrically opposed incisions that separate the two DNA helices into easily ligatable double strands.

Plants Possess Two Paralogs of Canonical HJ Resolvases

OsGEN-L, the homolog of HsGEN1 and AtGEN1, was the first functional HJ resolvase to be described in plants (Yang et al., 2012). Additional members of the same family include OsSEND1 and its Arabidopsis ortholog AtSEND1 (Furukawa et al., 2003; Moritoh et al., 2005). In this study, we have shown that AtGEN1, AtSEND1, and their respective orthologs are conserved throughout the spermatophyta and in lycophytes. In contrast, the more ancient mosses carry only one gene coding for a canonical resolvase, homologous to AtGEN1 and AtSEND1. This leads us to postulate that a gene duplication took place in the early development of higher plants, after separation from the mosses.

Our biochemical data further support the concept that AtGEN1 and AtSEND1 are indeed functional enzymes of paralogous origin. Both gene products are canonical HJ resolvases
that show strong similarities to both the eukaryotic Rad2/XPG family members and the EcRuvC paradigm of resolution. They also possess additional properties that might represent a gain-of-function variant compared to other eukaryotes (see below). This makes A. thaliana the first organism to be shown to have two functional paralogous resolvases.

**The Resolvases of Arabidopsis Show Broadened Substrate Specificities**

In contrast to EcRuvC, HsGEN1, ScYen1, and CeGEN-1, both Arabidopsis resolvases are active on a broad range of substrates with no marked preference for HJs (Fig. 9) (Benson and West, 1994; Ip et al., 2008; Bailly et al., 2010). This is somewhat reminiscent of the broad substrate specificity observed for the phage resolvases T7 endo I and T4 endo VII (Dickie et al., 1987; Benson and West, 1994). These different substrate specificities are summarized in Supplemental Table S1. The broad substrate specificity is also similar to the ability of HsSLX1-SLX4 to act on these structures, although this human protein recognizes 3’-flaps (Wyatt et al., 2013), which are not substrates for AtGEN1 and AtSEND1. The 5’-flap activities of AtGEN1 and AtSEND1 seem identical to that of HsGEN1 as well as HsFEN1, further supporting the postulated universal cleavage mechanism for the superfamily (Tsutakawa et al., 2011).

**Possible Sequence Specificities**

Sequence specificity, present to various degrees, is a common feature of nucleases, for example the type II restriction enzymes and the bacterial resolvases such as EcRuvC (Shah et al., 1994; Kovall and Matthews, 1999). Furthermore, it is known that the three-dimensional structure in which a HJ is bound by a resolvase may exert some influence on the cleavage decision. Free HJs in solution exist as an equilibrium of different three-dimensional structures, with the two opposite forms of the stacked X-structure as extremes and the open planar form as the central intermediate (reviewed in Lilley, 2000). Which of the two possible stacked X-conformations a HJ adopts depends on the sequence surrounding the junction point (Altona, 1996; Lilley, 2000). Therefore, the selectivity for a tertiary structure of the junction might be confused with sequence specificity. For example, the specificity of AtGEN1 and AtSEND1 for certain positions within the homologous core of the migratable X26 could stem from a preference for certain junction conformations. However, for most characterized HJ resolvases, the three-dimensional conformation of the junction is altered upon binding to the enzyme. The resulting structures often feature unstacked helical arms and resemble the open planar conformation to various degrees (Bennett and West, 1995; Declais and Lilley, 2000; Ceschini et al., 2001; Bierlümfpfel et al., 2007). Therefore, the structural influence of the sequence composition on the cleavage preference could be diminished.

We found that AtGEN1 and AtSEND1 showed distinct preferences using four 5’-flap substrates with different sequence compositions. A similar behavior has not been described...
for HsGEN1, ScYen1, OsGEN-L nor, to our knowledge, for FEN1. However using different substrates the existence of sequence specificities can be assumed (Ip et al., 2008; Bailly et al., 2010; Yang et al., 2012). Marked preferences for cleavage of certain strands of a static Holliday junction by EcRuvC was proposed to stem from the enzyme's well characterized sequence specificity (Osman et al., 2009) and was also observed for AtGEN1 and AtSEND1. Furthermore, the presence of distinct cleavage patterns for AtGEN1 and AtSEND1 within the mobile core of the X26 might be due to structural or sequence specificities (see above). Many canonical HJ resolvases, including T7 Endo I, EcRuvC, and the eukaryotic resolvases, have been shown to act with sequence specificity on oligonucleotide based HJs or alpha-structures (Picksley et al., 1990; Shah et al., 1994; Ip et al., 2008; Bailly et al., 2010; Yang et al., 2012).

While the specific consensus sequences for EcRuvC and, in part, for the phage enzymes are known, further investigation using more substrates with different sequences will be needed in case of the eukaryotic HJ resolvases.

Resolution of Nicked Holliday Junctions Reveals Common Resolvase-like Characteristics of AtGEN1 and AtSEND1

Despite the masking effect of the Ref-I activity, we were able to discern an inherent property of HJ resolvases in the activity of AtGEN1 and AtSEND1 towards nHJs: the structure-dependent cleavage one nucleotide 3' of the junction point. Although such an activity would not lead to ligatable products without further processing, this function retains the resolvase character in cleaving the strand opposite the nick and in the structure-specific position. We therefore designated this activity as resolvase-like.

It has been proposed that a mobile nicked Holliday junction would adapt a conformation with the nick exactly at the junction point as depicted in Figure 7 (Pöhler et al., 1994). If the Arabidopsis resolvases act on a nicked HJ in which the junction point is centered at the nick but the sequence differs from their putative sequence preference, both enzymes favor incisions at the position displaced by one nucleotide 3' from the nick and hence, from the junction point. This position, 1 nt 3' of the junction point, is exactly the cleavage position that is defined by the structure of the junction, as observed for the activities of AtGEN1, AtSEND1, EcRuvC, and the other eukaryotic resolvases on 5'-flaps and static HJs (Ip et al., 2008; Osman et al., 2009; Bailly et al., 2010; Yang et al., 2012). Interestingly, AtSEND1 also shows a somewhat broadened cleavage pattern if its sequence preference is not met, as is the case with the nX-32, strand 4 of the static X0, and 5'-flap2. On the other hand, AtSEND1 may resolve a nHJ more often canonically than AtGEN1, even if the sequence around the junction point deviates from its putative preference. Mus81-Eme1 from Schizosaccharomyces pombe is known to depend on the exact fitting of nucleotides into the
active site, facilitated by junction flexibility, for efficient second-strand cleavage (Chang et al., 2008; Osman et al., 2009). Applied to AtSEND1, this property might explain how AtSEND1 achieves the higher canonical resolution rate with the nHJ: because the nick enhances the flexibility of the junction, one could imagine that this flexibility is used to align the bases directly adjoining to the opposite site of the nick into the active site, as if it is searching for a better cleavage site. In summary, the processes that occur to resolve nHJs are not a matter of simple cleavage, but a complex interplay of at least structure and sequence specificity, if the latter is a property of the respective enzyme.

**AtGEN1 and AtSEND1 as Members of the HJ Processing Toolkit**

The unfaithful resolution into non-ligatable products or via the Ref-I mechanism by AtGEN1 and AtSEND1 *in vitro* might be considered detrimental for DNA repair and cell survival *in vivo*. The first possibility would necessitate further processing and the second case would reintroduce a double-strand break. Because an intact mobile HJ is predominantly resolved in a canonical manner by AtGEN1 and AtSEND1, it is unclear whether nHJs are physiologically relevant substrates. If they are, the need for processing to ensure ligatability might pose a relatively minor problem because nHJs occur early during repair processes (Schwartz and Heyer, 2011). In contrast, the activity of AtGEN1 and AtSEND1 on intact mobile HJs is in good agreement with findings from yeast. ScYen1 was shown to act late during meiosis and mitosis, thus serving as a backup mechanism if previous repair pathways such as dissolution or ScMus81-Mms4-mediated cleavage should fail to do the job (Matos et al., 2011).

The products from the action of AtGEN1 and AtSEND1 on the static and nicked Holliday junctions observed in this study are similar to the pattern seen for OsGEN-L (Yang et al., 2012). We therefore think it is likely that OsGEN-L acts on HJs in a manner similar to that of AtGEN1 and AtSEND1, including the presence of the alternative Ref-I activity. Another example of a similar activity is HsSLX1-SLX4. During preparation of this manuscript, a functional interaction of HsMUS81-EME1 and HsSLX1-SLX4, which together form a heterotetrameric *bona fide* HJ resolvase, was described (Wyatt et al., 2013). Despite earlier findings showing that HsSLX1-SLX4 can act on its own as a canonical HJ resolvase, these authors describe the heterodimer HsSLX1-SLX4 as a nicking enzyme (Fekairi et al., 2009; Wyatt et al., 2013). This is based on a low yield of ligatable products compared to HsGEN1 and the substantial quantity of nicking products, assayed using a plasmid-based HJ similar to the one used in the present study.

In comparison, both AtGEN1 (up to 70 %) and AtSEND1 (50 %) show substantially higher percentages of resolution products in the plasmid assay than the 20 % seen with HsSLX1-SLX4. On the other hand, they possess more nicking activity than HsGEN1, which acts almost exclusively as a resolvase (Rass et al., 2010). Another very recent work showed that
HsSLX1-SLX4 defines one of three parallel pathways of HJ processing: the other two are resolution by HsGEN1 and dissolution (Garner et al., 2013). Strikingly, although AtGEN1 and AtSEND1 are orthologs of HsGEN1, several rounds of BLAST analyses with SLX4 from S. cerevisiae and the human ortholog (also known as HsBTBD12) failed to identify a putative SLX4 ortholog in A. thaliana (unpublished data). If Arabidopsis possesses a SLX4-homolog, it is most likely heavily altered and therefore might be unlikely to fulfill the same functions as in other organisms. Thus A. thaliana might be missing the important scaffold protein known in humans to interact with HsMUS81, HsSLX1, and HsXPF-ERCC1 and to enhance their activities (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009; Andersen et al., 2011).

Our data define AtGEN1 and AtSEND1 as enzymes with in vitro properties intermediate between HsGEN1 and HsSLX1-SLX4 with respect to resolution and their alternative activity. Thus, AtGEN1 and AtSEND1 provide another example of duplicated proteins that have been conserved in plant evolution, which have some properties similar to their mammalian counterparts but also differ in some aspects from those and from each other (Hartung et al., 2007a; Geuting et al., 2009; Knoll and Puchta, 2011; Schröpfer et al., 2014). While the basic principles of pathways in DNA repair and recombination are conserved, plants feature differences in the detailed organization of the pathways.

CONCLUSIONS

We suggest that in contrast to mosses and animals like humans, which have one GEN1 homolog, seed plants have two functional, canonical Holliday junction resolvases as we have shown here for Arabidopsis thaliana in vitro. The two evolutionarily related paralogs have similar biochemical properties, e.g., with respect to their relatively broad substrate spectra and processing mechanisms, but they differ slightly in their possible sequence preferences. This allowed us to unravel a common, inherent mechanism in processing of nicked Holliday junctions by the two paralogs AtGEN1 and AtSEND1. Because the two paralogs are evolutionarily conserved, it is likely that they both play important, possibly non-redundant, roles in the nucleic acid metabolism of the cell.

MATERIALS AND METHODS

Bioinformatics Analyses

BLAST analyses were conducted with the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Multiple Sequence Alignments were performed using the MUSCLE (Multiple Sequence Comparison by Log- Expectation) algorithm at the EMBL-EBI (http://www.ebi.ac.uk). MEGA 6.0 (Tamura et al., 2013) was used
to calculate the cladogram. A more detailed description of tools, databases, and parameters used is provided online (Supplemental Methods 1).

Cloning of AtGEN1 and AtSEND1

All primers and oligonucleotides used for cloning are listed in Supplemental Table S2. Total RNA was extracted from two week-old A. thaliana (ecotype columbia) seedlings and reverse transcribed into cDNA. For sequencing, the cDNA was amplified using primers 1 + 2 (AtGEN1) and 3 + 4 (AtSEND1) and subcloned into pGEM-T Easy (Promega Corporation, Madison, USA). Restriction sites for BamHI and KpnI were then attached to the full length ORFs by PCR primers 5 + 6 (AtGEN1) and 7 + 8 (AtSEND1). The coding sequences for the nuclease-deficient proteins AtGEN1 D75A and AtSEND1 D76A were created by overlap extension PCR using the pGEM-T Easy clones as templates and primers 5, 6, 9, 10 and 7, 8, 11, 12, resulting in an exchange from aspartate to alanine at position 75 (AtGEN1) and 76 (AtSEND1), respectively.

For the expression constructs, the vector pETDuet1 (EMD Millipore, Billerica, USA) was modified (oligonucleotides 13-18, Table S2, Supplemental Methods 2) to yield recombinant proteins featuring an unchanged/native N-terminus and a C-terminus with a PreScission cleavage site (PS) +His-tag+StrepII-tag (final additional C-terminal amino acid sequence: GTLEVLFQGPTGHHHHHHLESGTSAWSHPQFEK).

Overexpression and Purification

Heterologous protein expression was performed in E. coli strain ER2566 (NEB) liquid cultures cotransformed with the vector pTf16 (Takara Bio Inc). Chaperone expression was induced with 0.5 mg/mL L-arabinose at the inoculation start. Once the cultures reached an OD600 of 0.7 after growth at 28 °C and 200 rpm, they were cooled to 16 °C for 30 minutes. The expression of the target proteins was then induced with 0.2 mM IPTG for three hours at 16 °C and 200 rpm. The cells were harvested by centrifugation.

All purification steps were performed at 4 °C. The pellets from 2.2 liters of liquid culture were thawed and resuspended in 75 mL of buffer A (100 mM Tris-HCl, 500 mM NaCl, 1 mM dithiothreitol [DTT], and 0.1 % [v/v] Tween20, pH 8.0). The cells were lysed by incubation with 0.1 mg mL⁻¹ lysozyme for 30 min on ice followed by sonication. The lysate was centrifuged for 30 min at 40,000 g, and the supernatant was filtered using a GF/PET membrane and supplemented with 1 µg mL⁻¹ Avidin. This clear lysate was loaded at a flow rate of approximately 1 mL min⁻¹ on self-packed 6 mL streptactin (Strep-Tactin® Superflow®, IBA) gravity flow columns equilibrated with buffer A. After washing with 41.4 mL of buffer A, the proteins were eluted in three steps: 1x 2.34 mL, 1x 9 mL and 1x 3 mL of buffer B (buffer A with 3 mM desthiobiotin). The second fraction, containing most of the protein, was
supplemented with 20 mM imidazole and loaded on a HisTrap FF crude column (GE Healthcare) at a flow rate of 0.25 mL min⁻¹. All of the following steps were conducted at a flow rate of 1 mL min⁻¹. An initial washing step with 45 mL buffer C (100 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, and 5 % [v/v] glycerol, pH 8.0) + 0.1 % (v/v) Triton X-100 was followed by another wash with 15 mL of buffer C. The proteins were eluted with buffer D (100 mM Tris-HCl, 500 mM NaCl, 400 mM imidazole, and 5 % [v/v] glycerol, pH 8.0), the peak fractions were pooled and their buffer was exchanged for buffer A using PD10 columns (GE Healthcare) according to the manufacturer’s instructions. The final fractions were supplemented with 50 % (v/v) glycerol and stored in aliquots at -80 °C.

The purified proteins were identified by colloidal Coomassie-stained (Neuhoff et al., 1988) SDS-PAGE gels and western blot. Quantification was performed with the ImageJ software (http://rsb.info.nih.gov/ij/) using BSA (Bio-Rad) as a standard.

DNA Substrates

Most substrates were based on oligonucleotides (Supplemental Table S3, Supplemental Table S4). The Holliday junction substrates X0, X26 and X26-S as well as the 3'-flap and 5'-flap4 were composed of the same oligonucleotides as described elsewhere (Ip et al., 2008) but a different protocol was used. For the nX-30, nX-32, 3'-flap, and 5'-flaps, one 5'-32P-labeled oligonucleotide was annealed with a fourfold molar excess of unlabeled oligonucleotides in 1xPNK-buffer (70 mM Tris-HCl, 10 mM MgCl₂, and 5 mM DTT, pH 7.6) by heating to 95 °C for 5 min followed by cooling to room temperature (RT). In case of X0, X26, X26-S, and the replication forks oligonucleotides constituting strands 1 and 4 were first annealed in an equimolar ratio by heating to 95 °C for 5 min followed by cooling to RT. Similarly, strands 2 and 3 were annealed in a separate reaction. In a second step, the previously annealed splayed arm structures were combined and incubated at 37 °C for 30 min followed by another 30 min at RT. All substrates (except the X26-S) were purified by 10 % native TBE polyacrylamide gel electrophoresis (PAGE) and electroelution into TB-MgCl₂ buffer (44.5 mM Tris-Base, 44.5 mM boric acid, and 5 mM MgCl₂) using D-Tube Dialyzers (Merck). All HJs (except the X26-S) were labeled on one oligonucleotide for each HJ, resulting in four differently labeled substrates per HJ.

To create pIR9, pAT153 (MoBiTec) was linearized by EcoRI digestion, and the single-strand overhangs were converted to blunt ends using mung bean nuclease (NEB). After religation, a plasmid without the EcoRI site was obtained and verified by sequencing. The inverted repeat was created by self-annealing of oligonucleotide 19 (Supplemental Table S2) and inserted into the BamHI site of the vector just described. Oligonucleotide 19 was designed with overhangs corresponding to a BamHI digest, sequences of the homologous core of the X26 Holliday junction (underlined in Supplemental Table S2) and the inverted repeat sequence
(italics) and central EcoRI site (bold) used for pIRbke8 (Lilley and Markham, 1983). The formation of the Holliday junction was favored by incubation at 37 °C in HJ extrusion buffer (50 mM Tris-HCl, 50 mM NaCl, 0.1 mM EDTA, pH 7.5) before performing the assays.

**Endonuclease Assays**

The standard reactions (20 µL) contained 1.5 nM 32P-labeled substrate and 30 nM protein in 1x reaction buffer (25 mM HEPES-KOH, 5 mM Tris-HCl, 50 mM NaCl, 2 mM MgCl₂, 1.05 mM DTT, 0.1 mg mL⁻¹ BSA, 0.005 % [v/v] Tween20, and 5 % [v/v] glycerol, pH 8.0). An additional 50 mM NaCl (final concentration 100 mM) was added to samples containing AtSEND1. Unless otherwise indicated, the reactions were incubated at 37 °C for 30 min and terminated by the addition of 10 µL of native stopping solution (50 mM EDTA, 0.6 % [v/v] SDS, 20 % [v/v] glycerol, 0.1 % [v/v] xylenxyanole FF, 0.1 % [v/v] bromophenol blue, and 0.95 mg mL⁻¹ proteinase K) or 20 µL denaturing stopping solution (89 mM Tris-HCl, 7 M urea, 12 % [v/v] Ficoll, 0.8 % [v/v] SDS, 0.02 % [v/v] xylenxyanole FF, 0.01 % [v/v] bromophenol blue, and 1 mg mL⁻¹ proteinase K, pH 8.0) followed by further incubation at 37 °C for 15 min.

For the ligation experiments, T4 ligase (Fermantas) was added to a final concentration of 40 units µL⁻¹, and the incubation was continued for 30 min at RT. The reactions were stopped by the addition of an equal volume of denaturing stopping solution and incubated for another 15 min at 37 °C.

The 32P-labeled reaction products were separated either by 12 % native TBE-PAGE, 20 % denaturing TBE-PAGE (containing 7 M urea) or sequencing gels with 7 M urea 13 % TBE-PAGE followed by autoradiography. Quantification was carried out using a CR 35 Bio for phosphorimaging and the Advanced Image Data Analyzer (AIDA) software (raytest, Straubenhardt, Germany).

For assays with plasmid pIR9, the reactions containing 4.5 nM plasmid and 90 nM enzyme were incubated under the same conditions as for the oligonucleotide-based substrates. Aliquots were taken at each time point. The reaction products were separated on vertical 1 % TAE agarose gels run under native conditions and visualized using GelStar™ (Lonza Group Ltd, Basel, Switzerland) staining. To determine the proportion of supercoiled plasmids extruding the cruciform structure, the samples were digested with EcoRI under the respective assay conditions. The fraction of plasmid containing the HJ structure was defined as 100 % of the total substrate DNA.

**Accession numbers**

The Arabidopsis Genome Initiative locus identifiers for AtGEN1 and ATSEND1 are At1g01880 and At3g48900, respectively.
SUPPLEMENTAL MATERIAL

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Quantification of the purified recombinant proteins.

**Supplemental Figure S2.** Structures of the recombinant proteins and negative controls.

**Supplemental Figure S3.** Cleavage mapping at static 5’-flaps reveals a single main cleavage site.

**Supplemental Figure S4.** Cleavage mapping at the static HJ X0.

**Supplemental Figure S5.** Cleavage mapping at the mobile HJ X26.

**Supplemental Figure S6.** Rational for the design of nX-30 and nX-32.

**Supplemental Figure S7.** Cleavage pattern at two different nHJs analyzed by native gel electrophoresis reveals both a canonical resolution activity and an activity involving a replication fork intermediate (Ref-I).

**Supplemental Figure S8.** Cleavage pattern with different replication fork-like structures sustains the hypothesis of a replication fork intermediate in the Ref-I pathway, that exists beside the classical resolvase-like pathway in the resolution of a nicked HJ.

**Supplemental Table S1.** Examples of characterized canonical HJ resolvases

**Supplemental Table S2.** PCR primers and oligonucleotides used in cloning

**Supplemental Table S3.** Oligonucleotide combinations used for model substrates in the *in vitro* assays

**Supplemental Table S4.** Oligonucleotide sequences used for model substrates in the *in vitro* assays

**Supplemental Methods 1:** Bioinformatics Analysis

**Supplemental Methods 2:** Cloning of Expression Constructs

**Supplemental Information 1.** Alignment used for the calculation of the phylogenetic tree shown in Fig. 1

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FIGURE LEGENDS

Figure 1. Eukaryotic HJ resolvases. A, Domain structure of subclass IV of the Rad2/XPG superfamily. All members share a common N-terminal domain organization, while the C-terminal half contains no defined functional domains. XPG-N and XPG-I domains constitute the nuclease domain and mediate metal ion coordination by conserved amino acids. The HhH2 domain is implicated in binding of double-stranded DNA. B, The evolutionary history was inferred using the Maximum Parsimony method. Tree 1 out of the 2 most parsimonious trees (length = 2351) is shown. AtMUS81 was defined as outgroup. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

Figure 2. Structure-specific cleavage of flapped DNA structures. A, Incision mapping at four static 5'-flaps (1 to 4, indicated by the respective colors) with different sequence compositions. The dots mark the labeled 5'-end of the cleaved flapped strand. The length of the arrowheads and percentages give the 31 nt product as the portion of the total DNA content. B, AtGEN1 and AtSEND1 cleave the four 5'-flaps with different efficiencies: AtGEN1 is less active with 5'-flap3 and AtSEND1 with 5'-flap2 (native gel electrophoresis). C, Sequence context of the flapped strands around the junction point. The main and secondary cleavage sites of AtGEN1 and AtSEND1 are indicated by arrows. D, AtGEN1 and AtSEND1 do not cleave 3'-flaps as shown by denaturing gel electrophoresis of the 5'-labeled flapped strand. E, Cleavage of a replication fork (RF2) by AtGEN1 and AtSEND1 occurs exclusively in the matrix strand of lagging strand synthesis and preferentially around the branch point of the junction. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotides measured from the 5' end, are indicated at or in the arrowheads. The dotted lines demarcate the migratable homologous core of the replication fork.

Figure 3. Schematic summary of incision mapping at the static HJ X0 with A, AtGEN1 and B, AtSEND1. Cleavage events (Supplemental Fig. S4) were quantified and the relative frequencies are represented by the lengths of the arrows. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotide measured from the 5' end, are indicated at or in the arrowheads. Cleavage products that represent less than 5% of the cleavage events are not shown.
Figure 4. Symmetric cleavage pattern at a Holliday junction providing a migratable, homologous core (HJ X26). Four versions of the X26 were used as substrates, differing in which strand was labeled (numbers with asterisks). A, Native gel electrophoresis of the reaction products of AtGEN1 and AtSEND1 compared to buffer controls (-). With the help of marker structures the reaction products were identified (nD = nicked duplex, sD = short duplex). B, Quantification of the results of independent experiments as shown in A. C, D, Schematic summary of incision mapping at the mobile HJ X26 with C, AtGEN1 and D, AtSEND1. The cleavage events (Supplemental Fig. S5) were quantified and the relative frequencies are represented by the lengths of the arrows. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotide measured from the 5' end, are indicated at the arrowheads. Cleavage products that represent less than 5% of the cleavage events are not shown. The lengths of the main products are indicated.

Figure 5. Ligation of cleavage products. A, Schematic illustration of the ligation assay principle. The asymmetric X26-S Holliday junction containing a label (red dot on red oligonucleotide) was used. Symmetric cleavage followed by ligation yields a longer labeled oligonucleotide of 60 nt (red and yellow segment). B, Resolution products of AtGEN1 and AtSEND1 are ligatable, as shown by a denaturing sequencing gel after performing the experiments according to the scheme in A.

Figure 6. Resolvase and nicking activities of AtGEN1 and AtSEND1 on plasmid pIR9. A, The plasmid pIR9 features an inverted repeat, which forms a HJ stabilized by negative supercoiling. A single incision leads to relaxation and reabsorption of the HJ if the junction is not stabilized by the DNA-protein complex. The resulting nicked circular (n.c.) plasmid therefore is a nicking product. Resolution into linear (lin.) products via two opposing incisions can either happen simultaneously or in a successive manner in which the HJ structure is maintained by the bound resolvase dimer. B, Separation of the nicking and resolution products from the uncleaved supercoiled (sc.) plasmid. For quantification, the percentage of the products was corrected for the background of nicked circular and linear DNA in the buffer reaction (-). The fraction of supercoiled plasmid resistant to EcoRI digestion was determined and set to 100%, representing the available portion of plasmids extruding the cruciform structure.

Figure 7. Nicked Holliday junctions are primarily cut in the strand opposite the nick but also in the strand that hybridizes with the 5’-end of the nick. Results of incision mapping of two
different nHJs treated with AtGEN1 or AtSEND1 are shown. A, AtGEN1 at nX-30. B, AtSEND1 at nX-30. C, AtGEN1 at nX-32. D, AtSEND1 at nX-32. The cleavage events were quantified and the relative frequencies are represented by the lengths of the arrows. Cleavage products that represent less than 5% of the cleavage events are not shown. The cleavage positions equivalent to the lengths of the main products are indicated (in nucleotides) in or at the arrowheads.

**Figure 8.** Nicked Holliday junctions are processed both via a resolution-like pathway and via a pathway involving a replication fork intermediate (Ref-I pathway). In the resolution-like pathway, the position of incision in the strand opposing the nick is dependent on the sequence context at the junction point. A, The nicked Holliday junction nX-30 was labeled on strand 1 and native gel electrophoresis after incubation with AtGEN1 or AtSEND1 reveals more products than the nicked duplex structure expected by a solely canonical HJ resolvase (RF = replication fork, nD1* = nicked duplex, sD1* = short duplex). The sD1* product is the result of an incision first in strand 4, yielding a replication-fork intermediate and then in strand 1 (see text). The nD1* and sD1* products were analyzed separately on sequencing gels to identify the exact cleavage positions for the two different pathways. Supporting data regarding the identity and processing of the replication fork intermediates is presented in Supplemental Figures S7 and S8. B, Assignment of the cleavage events for the nX-30 to the two different pathways. The portions of the arrowheads ascribed to resolvase-like (dark colors) and Ref-I (light colors) pathways are based on the quantifications shown in figures 7 and S8 (H – K). The cleavage positions, which are equivalent to the lengths of the 5’-cleavage products, are indicated in or at the colored arrowheads. The relative frequencies are represented by the lengths of the arrows. Cleavage products that represent less than 5% of the cleavage events are not shown. C, as in A but with the nX-32. D, Assignment of the cleavage events for the nX-32 to the two different pathways as in B.

**Figure 9.** Summary of tested substrate types and detected activities. All types of oligonucleotide-based substrates used in the *in vitro* experiments are shown and the corresponding activities of AtGEN1 (violet) and AtSEND1 (green) are presented in a simplified manner. The length of the arrows is a rough approximation of the enzymes' activities on all tested subtypes (e.g., replication forks RF1 to RF3) to present a more general picture than shown in the detailed data presentation before. The schematic of the mobile nicked Holliday junctions is to be read as follows: AtGEN1 cleaves strand 1 of the nX-32 exactly opposite the discontinuity in strand 3 (continuous arrow), leading to religatable products, while for the nX-30, AtGEN1’s inherent preference to cleave at a certain site in...
relation to the junction point leads to an incision 1 nt 3' of the junction point (dashed arrow).
For AtSEND1 the is situation is inverted: nX-30 cleavage I. Cleavage of strand 4 of the nHJs (small arrow) leads to a replication fork intermediate, which is processed as shown for the RF-substrate type. Note that the Ref-I activity can also be observed at the static X0 and to a limited degree also at the intact X26.
Figure 1. Homology of the (putative) eukaryotic HJ resolvases. A, Domain structure of subclass IV of the Rad2/XPG superfamily. All members share a common N-terminal domain organization, while the C-terminal half contains no defined functional domains. XPG-N and XPG-I domains constitute the nuclease domain and mediate metal ion coordination by conserved amino acids. The HhH2 domain is implicated in binding of double-stranded DNA. B, The evolutionary history was inferred using the Maximum Parsimony method. Tree 1 out of the 2 most parsimonious trees (length = 2351) is shown. AtMUS81 was defined as outgroup. The consistency index is (0.674715), the retention index is (0.628385), and the composite index is 0.430328 (0.429981) for all sites and parsimony-informative sites (in parentheses). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random-sequence addition method (10 replicates).
Figure 2. Structure-specific cleavage of flapped DNA structures. A, Incision mapping at four static 5'-flaps (1 to 4, indicated by the respective colors) with different sequence compositions. The dots mark the labeled 5'-end of the cleaved flapped strand. The length of the arrowheads and percentages give the 31 nt product as the portion of the total DNA content. B, AtGEN1 and AtSEND1 cleave the four 5'-flaps with different efficiencies: AtGEN1 is less active with 5'-flap3 and AtSEND1 with 5'-flap2 (native gel electrophoresis). C, Sequence context of the flapped strands around the junction point. The main and secondary cleavage sites of AtGEN1 and AtSEND1 are indicated by arrows. D, AtGEN1 and AtSEND1 do not cleave 3'-flaps as shown by denaturing gel electrophoresis of the 5'-labeled flapped strand. E, Cleavage of a replication fork (RF2) by AtGEN1 and AtSEND1 occurs exclusively in the matrix strand of lagging strand synthesis and preferentially around the branch point of the junction. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotides measured from the 5' end, are indicated at or in the arrowheads. The dotted lines demarcate the migratable homologous core of the replication fork.
Figure 3. Schematic summary of incision mapping at the static HJ X0 with A, AtGEN1 and B, AtSEND1. Cleavage events (Supplemental Fig. S4) were quantified and the relative frequencies are represented by the lengths of the arrows. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotide measured from the 5' end, are indicated at or in the arrowheads. Cleavage products that represent less than 5% of the cleavage events are not shown.
Figure 4. Symmetric cleavage pattern at a Holliday junction providing a migratable, homologous core (HJ X26). Four versions of the X26, differing in the position of the radioactive label, were used as substrates. A, Native gel electrophoresis of the reaction products of AtGEN1 and AtSEND1 compared to buffer controls (-). The numbers with asterisks indicate which oligonucleotide strand carried the radioactive label. With the help of marker structures the reaction products were identified (nD = nicked duplex, sD = short duplex). B, Quantification of the results of independent experiments as shown in A. C, D, Schematic summary of incision mapping at the mobile HJ X26 with C, AtGEN1 and D, AtSEND1. The cleavage events (Supplemental Fig. S5) were quantified and the relative frequencies are represented by the lengths of the arrows. The cleavage positions, which are equivalent to the lengths of the cleaved oligonucleotide measured from the 5' end, are indicated at the arrowheads. Cleavage products that represent less than 5% of the cleavage events are not shown. The lengths of the major products are indicated.
**Figure 5.** Ligation of cleavage products. A, Schematic illustration of the ligation assay principle. The asymmetric X26-S Holliday junction, composed of the oligonucleotides 1 (dark blue), the radioactively labeled 2S (red, 53 nt), 3S (light blue), and 4 (yellow), was used. Symmetric cleavage followed by ligation yields a longer labeled oligonucleotide of 60 nt (red and yellow segment). B, Resolution products of AtGEN1 and AtSEND1 are ligatable, as shown by a denaturing sequencing gel after performing the experiments according to the scheme in A.
Figure 6. Resolvase and nicking activities of AtGEN1 and AtSEND1 on plasmid pLR9. A, The plasmid pLR9 features an inverted repeat, which forms a HJ stabilized by negative supercoiling. A single incision leads to relaxation and reabsorption of the HJ if the junction is not stabilized by the DNA-protein complex. The resulting nicked circular (n.c.) plasmid therefore is a nicking product. Resolution into linear (lin.) products via two opposing incisions can either happen simultaneously or in a successive manner in which the HJ structure is maintained by the bound resolvase dimer. B, Separation of the nicking and resolution products from the uncleaved supercoiled (sc.) plasmid. For quantification, the percentage of the products was corrected for the background of nicked circular and linear DNA in the buffer reaction (-). The fraction of supercoiled plasmid resistant to EcoRI digestion was determined and set to 100 %, representing the available portion of plasmids expressing the cruciform structure.
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