1. Introduction

Due to their sessile nature and need of sunlight, plants are particularly exposed to environmental genotoxins, which lead directly or indirectly via generation of reactive oxidative species (ROS) to DNA lesions including single (SSB) and double strand breaks (DSBs). DSBs are particularly critical lesions, because if unrepaired they lead to major karyotypic instability and cell death. Early studies showed that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1, 2], carrot cell cultures that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1,2], carrot cell cultures that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1, 2], carrot cell cultures that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1,2], carrot cell cultures that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1,2], carrot cell cultures that breaks induced in plant DNA by ionizing radiation are rapidly repaired in genomic DNA of seed embryos [1,2]. An important role for DNA ligases was proposed in pathways for the removal of X-ray induced DNA breakage, which requires the rejoining of the phosphodiester bond of the DNA backbone in the final stage of repair [7]. The study of DNA repair kinetics has benefited greatly from the single cell gel electrophoresis (comet) assay, which has been adapted to differentiate between SSB and DSB repair in genomic DNA [8–10]. This technique has been successfully applied in DNA repair studies of various plants such as V. faba [6, 11], tobacco [12] and Arabidopsis [13].

DSB repair is mediated either by homologous recombination (HR) catalyzed by the RAD52 epistasis group or by non-homologous end-joining (NHEJ), which involves the KU heterodimer and the DNA ligase 4/XRCC4 complex. The molecular components of these pathways are highly conserved amongst eukaryotes and recent studies have revealed the requirement for both HR and NHEJ in DSB repair in plants as reviewed in Bray and West [14] and Bleuyard et al. [15]. From these studies in plants and other eukaryotes, it was assumed that NHEJ was responsible for the majority of DSB repair in higher plants. Surprisingly, here we report that the NHEJ knock-out ku80 and lig4 Arabidopsis mutants repair DSBs very rapidly, with similar kinetics to wild-type plants. This demonstrates that rapid repair of the majority of DSBs in plant cells is independent of the evolutionarily conserved factors of the “classic” NHEJ (C-NHEJ) pathway. Furthermore, we have identified putative components of this rapid DSB repair pathway including the plant orthologue of structure maintenance of chromosome 1–3 cohesion complex [17].

Abbreviations: DSB, DNA double strand break; SSB, DNA single strand break; NHEJ, non-homologous end-joining; C-NHEJ, classical-NHEJ; SMC, structural maintenance of chromosomes; ROS, reactive oxidative species; UV, ultraviolet; IR, ionizing radiation.

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Fig. 1. Phenotype of Arabidopsis mutant lines. (A) Mature atlig4-4 and atku80 mutant lines showed minor growth defects when compared to the background Arabidopsis ecotype Ws after four weeks growing in soil. (B) No germination or growth defects were detected in the 10-day-old seedlings used for preparation of comet slides (atmim, atrad21.1, Arabidopsis Ws (wild-type), atlig4-4 and atku80).

2. Materials and methods

2.1. Arabidopsis lines

Arabidopsis insertion mutants of atmim [16], atrad21.1 [17] and atku80 [18] have been described previously. In this study we used the unpublished line atlig4-4 (Fig. 1A) that is phenotypically indistinguishable from previously described mutants in this gene [19,20]. For details on isolation and characterization of atlig4-4 see Supplementary information.

Interestingly none of mutant lines used in this study showed delays in germination or growth during the first 10 days after the onset of germination (Fig. 1B).

Control experiments with wild-type Arabidopsis were carried out using the same ecotype as the mutant: Arabidopsis Ws in experiments with mim, atku80 and atlig4-4 and Arabidopsis Col0 with atrad21.1. The comet data for Ws and Col0 were identical (see Supplementary information).

2.2. Bleomycin treatment

Seeds were germinated on Petri plates with MS/2 media (M0223, Duchefa, The Netherlands) solidified with 0.8% Plant Agar (P1001, Duchefa, The Netherlands) during 16/8 h day/night regime at 22 °C and 18 °C, respectively. Sensitivity and repair kinetics of Arabidopsis wild-type and mutant lines were measured using 10-day-old Arabidopsis seedlings (Fig. 1B). Prior to treatment, seedlings were gently transferred from agar to liquid medium in 5 cm Petri plates to avoid drying.

The phenotype was checked by continuous exposure of seedlings to 5, 50 and 100 µg/ml Bleomycin (Bleocin inj., Euro Nippon Kayaku GmbH, Germany) for one week in liquid MS/2 (Fig. 2A).

DSBs were detected by a neutral comet assay [21] as described previously [10,13]. In brief, approximately 100 mg of frozen tissue were cut with a razor blade in 300 µl PBS + 10 mM EDTA on ice and tissue debris removed by filtration through 50 µm mesh funnels (Partec, Germany) into Eppendorf tubes on ice. 50 µl of nuclei suspension were dispersed in 200 µl of melted 0.7% LMT agarose (15510-027, GibcoBRL, Gaithersburg, USA) at 40 °C and four 80 µl aliquots were immediately pipetted onto each of two coated microscope slides (in duplicates per slide), covered with a 22 mm × 22 mm cover slip and then chilled on ice for 1 min to solidify the agarose. After removal of cover slips, slides were immersed in lysing solution (2.5 M NaCl, 10 mM Tris–HCl, 0.1 M EDTA, 1% N-lauroyl sarcosinate, pH 7.6) on ice for at least 1 h to dissolve cellular membranes and remove attached proteins. The whole procedure from chopping tissue to placement into lysing solution takes approximately 3 min. After lysis, slides were twice equilibrated for 5 min in TBE electrophoresis buffer to remove salts and detergents. Comet slides were then subjected to electrophoresis at 1 V/cm (app. 12 mA) for 5 min. After electrophoresis, slides were placed for 5 min in 70% EtOH, 5 min in 96% EtOH and air-dried.

Comets were viewed in epifluorescence with a Nikon Eclipse 800 microscope after staining with GelRed stain (Biotium, Hayward, USA) and evaluated by the Comet module of the LUCIA cytogenetics software suite (LIM, Praha, Czech Republic).

2.4. Data evaluation

The fraction of DNA in comet tails (% tail-DNA) was used as a measure of DNA damage. Data for Arabidopsis Ws and Col0 and the
Fig. 2. Sensitivity of Arabidopsis lines (Arabidopsis Col0, atrad21.1, atmim, atlig4-4, atku80 and Arabidopsis Ws) to Bleomycin treatment. (A) 10-day-old seedlings growing for following week in the presence of 5, 50 and 100 μg Bleomycin/ml in liquid MS/2 media. (B) 10-day-old seedlings treated similarly as for measurement of repair kinetics with 50 μg Bleomycin/ml for 1 h and grown for additional week in liquid MS/2.

four lines (atmim, atrad21.1, atlig4-4 and atku80) analyzed in this study were measured in at least 3 independent experiments. In each experiment, the % tail-DNA was measured at 5 time points: 0, 5, 10, 20 and 60 min after treatment and in control seedlings without treatment. Measurements included 4 independent gel replicas of 25 evaluated comets totaling at least 300 comets analyzed per experimental point.

The percentage of damage remaining as plotted in Fig. 4 after a given repair time ($t_x$) is defined as:

$$\% \text{ damage remaining (} t_x \text{)} = \frac{\text{mean } % \text{ tail-DNA (} t_x \text{)} - \text{mean } % \text{ tail-DNA (control)}}{\text{mean } % \text{ tail-DNA (} t_0 \text{)} - \text{mean } % \text{ tail-DNA (control)}} \times 100$$

3. Results and discussion

3.1. Bleomycin induction of DSBs and their detection with comet assay

The radiomimetic antibiotic Bleomycin was used to induce DNA damage, subsequently detected by the neutral comet assay. In 10-day-old Arabidopsis wild-type seedlings we observed a linear increase of DNA damage ($R^2 = 0.9574$) in the 0–50 μg/ml dose range (Fig. 3). When treated with 50 μg/ml Bleomycin, the fraction of DNA migrating in comet tails was approximately 50% above the background level in all mutant and wild-type Arabidopsis lines used in this study (Fig. 4A and B). This shows that Bleomycin is capable of inducing DSBs with equivalent efficiency regardless of the plant genotype. Bleomycin, which belongs to a family of low-molecular-weight glycometallopeptides, functions as a catalyst activated by interaction with DNA and attachment of Fe(II) to produce ROS, SSBs and DSBs [22].

The comet assay carried out under neutral conditions directly detects DSBs present in nuclear DNA [21,23] and currently represents, in comparison to other methods [24], the quickest preparation method for their detection. Preparation of comet slides from isolated nuclei takes less than 5 min, making this method particularly suitable for measurement of rapid repair processes. Speed and unbiased detection of DSBs is an advantage over non-direct methods of DSBs quantification as e.g. γH2AX foci [25].
3.2. Genome integrity in untreated plants

In this study we have analyzed young seedlings in the early leaf development phase [26]. Surprisingly none of mutant lines showed a delay in germination or growth of the seedling during first 10 days after the onset of germination (Fig. 1B). However a delayed growth phenotype was manifested during later stages in all but atrad21.1 mutated lines [16–18]; for atlig4-4 and atku80 see Fig. 1A.

Levels of background DNA damage were similar in all mutant and wild-type seedlings, with approximately 20% DNA migrating in the comet tail (Fig. 4A and B). This is surprising as it suggests that the mutations do not lead directly to the accumulation of DSBs in nuclear DNA, despite the hypersensitivities of the mutant lines to induced DNA damage. In contrast to young seedlings, mature leaves of all mutants and wild-type Arabidopsis showed such extensive fragmentation of nuclear DNA (more than 90% tail-DNA) that it was not possible to measure differences between lines using this material (data not shown).

3.3. DSB repair kinetics in wild-type and mutant lines

A 1-h treatment of mutant and wild-type seedlings with 50 μg/ml Bleomycin caused a large shift in the migration of the genomic DNA. All lines displayed similar extents of fragmentation with 60–80% of DNA migrating in the comet tail (Fig. 4A and B). Nevertheless all tested lines effectively survive this dose for one week (Fig. 2B) as compared to survival of seedlings continuously exposed even to 10 times lower Bleomycin concentration for the same time (Fig. 2A). The repair kinetics of mutant and wild-type plants (Fig. 5) were determined through a time course of recovery
from Bleomycin treatment, with the extent of remaining DNA damage calculated from the percentage of DNA in comet tails (Fig. 4B) as defined in Section 2. Wild-type seedlings displayed very rapid repair with 80% of DSBs repaired during first 20 min (Fig. 5). Repair kinetics were biphasic with a very rapid initial phase responsible for the majority of DSB repair followed by a slower phase in which the remaining DNA damage was repaired. The later phase of slower repair represents either the repair of a subset of DSBs by an alternative pathway or, more likely, other types of DNA lesions induced by Bleomycin and detected by neutral comet assay such as close SSBs due to oxidative damage [27].

In order to determine the molecular basis for rapid DSBs repair, the parameters of repair kinetics for the initial 10 min period were determined in wild-type and mutant lines using a least-square analysis of the time course of recovery after Bleomycin treatment. A linear relationship on the semi-logarithmic scale indicates that rapid removal of DSBs from genomic DNA followed first order kinetics, suggesting involvement of single rather than two DNA molecules during the initial stage of DSB repair. The rate constant \( k \), half-life \( t_{1/2} \) and \( R^2 \) for the initial rates of repair of each mutant line are provided in Table 1.

Almost all DSBs were removed within 1 h of Bleomycin treatment in wild-type, atku80 and atlig4-4 plants (Fig. 5). In comparison to yeast and mammals, Arabidopsis DSB repair is very rapid with a \( t_{1/2} \) of less than 8 min. Contrary to wild-type, atrad21.1 and atmim have clearly slower initial DSB repair with a striking repair defect at atmim. The rate constants \( k \) and half-lives \( t_{1/2} \) were calculated by regression analysis for repair points 0, 5 and 10 min (see Table 1).

![Fig. 5. Kinetics of DSB repair. Fractions of remaining DSBs were calculated for 0, 5, 10, 20 and 60 min repair time points after the treatment with 50 μg/ml Bleomycin. Maximum damage is normalised as 100% at \( t = 0 \) for all lines. Wild-type, atku80 and atlig4-4 rapidly repair induced DSBs during the first 10 min. atku80 and atlig4-4 have even faster repair rates than wild-type, and this is most pronounced in the first 5 min. Contrary to wild-type, atrad21.1 and atmim have clearly slower initial DSB repair, with a striking repair defect in atmim. The rate constants \( k \) and half-lives \( t_{1/2} \) were calculated by regression analysis for repair points 0, 5 and 10 min (see Table 1).](image)

Table 1

<table>
<thead>
<tr>
<th></th>
<th>( k ) (min(^{-1}))</th>
<th>( t_{1/2} ) (min)</th>
<th>( R^2 )</th>
</tr>
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<td>min</td>
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<td>56.8</td>
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<td>atrad21.1</td>
<td>0.0630</td>
<td>11.0</td>
<td>0.9838</td>
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<td>wild-type</td>
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<td>5.7</td>
<td>0.9168</td>
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<td>atku80</td>
<td>0.1244</td>
<td>5.6</td>
<td>0.9058</td>
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After treatment (Fig. 5) resulting in significant differences in the kinetic data provided for the initial rapid phase of DSB repair (Table 1). Surprisingly mutants in the NHEJ pathway (atku80 and atlig4-4) displayed rapid DSB repair kinetics in the initial repair phase, even faster than in wild-type seedlings (\( t_{1/2} \) of 5.6, 5.7 and 7.9 min, respectively). These results provide the first evidence for a rapid pathway substituting for C-NHEJ in the repair of a majority of DSBs in plants.

In mammals the ligation of non-homologous ends during V(D)J recombination and Ig class switching also depends on Xrcc4 and DNA ligase IV (Lig4). Nevertheless two recent reports [28,29] suggest an alternative robust C-NHEJ independent DSB cut and paste recombination pathway. Yan et al. [28] described that LghI class switching in C-NHEJ deficient mouse B-cells uses a novel, non-classical, end-joining pathway. This alternative end-joining pathway also frequently joins the IgH locus to other chromosomes leading to translocations. Nevertheless the authors do not provide data on the extent and kinetics of this alternative pathway. However, recent studies have provided the first clues for the mechanism of alternative DSB repair pathways in plant, yeast and animals. A common feature of Ku-independent end-joining in eukaryotes is a greater dependence on microhomologies to facilitate repair, in pathways termed alternative-NHEJ, backup-NHEJ (B-NHEJ) or microhomology-mediated end-joining (MHEJ) [30–33]. While the molecular basis for this end-joining activity is poorly defined, in mammals this requires PARP-1, XRCC1 and DNA ligases 3 and/or 1, rather than Lig4 which is specific for C-NHEJ [32,33].

That other factors substitute for KU70/80, Lig4, and possibly for the entire C-NHEJ pathway in plants as in other organisms, implies the presence of a possible salvage mechanism effectively repairing one of the most critical DNA lesions by joining of DSB ends [34]. The main function of this repair pathway in plants could be the demand for rapid elimination of DSBs that accumulate during seed storage upon the onset of germination [2,7]. Nevertheless the relationship of both C-NHEJ and a substituting rapid pathway in repair of DSBs in Arabidopsis remain to be established.

In further analysis of this novel DSB repair pathway we studied repair capacity and kinetics in Arabidopsis mutant lines with impaired SMC complexes [35], which are involved in a broad spectrum of chromosome maintenance functions, including cohesion (Smc1–3), chromosome condensation (Smc2–4), and DNA recombination and repair (Smc5–6) [36]. SMC complexes were strong candidates to offset missing Ku proteins in stabilization of the DSB for repair.

Cohesins play an important role in the repair of DSBs. In yeast, mutants in the cohesin subunit SCC1/RAD21/MCD1 are hypersensitive to DNA-damaging agents [37]. It has been proposed that cohesin facilitates DNA repair by holding sister chromatids together locally at the DSB (Fig. 6A). Shortly after laser irradiation, proteins known to be involved in the repair of DSBs including both SMC and non-SMC components of cohesin complexes accumulate along the beam tracks [38].

RAD21/SCC1 is a member of the superfamily of Kleisin proteins, which interacts with N- and C-terminal domains of SMC proteins to form a ring-like structure [Fig. 6A]. Arabidopsis has three RAD21 gene homologues. The transcript level of AtRAD21.1 is increased specifically after induction of DNA damage and this protein plays a critical role in recovery from DNA damage during seed imbibition, prior to germination [17]. We show here that atrad21.1 mutant have a nearly two-fold defect on the initial rate of DSB repair when compared to wild-type plants (\( t_{1/2} \) 11.0 min and 7.9 min, respectively) and after 1 h 30% of total DNA damage remained unrepaired as compared to only 15% in wild-type seedlings. The effect of atrad21.1 on DSB repair is, however, less pronounced than that of atmim.

Of the several SMC mutant lines available, it was decided to test SMC6/MIM which has been shown to affect several aspects of...
chromosome biology [16]. The Smc5–6 complex was initially identified via a radiation-sensitive mutant of *Schizosaccharomyces pombe*. The rad18-X mutant was isolated in a screen for radiation sensitivity and was found to be sensitive to both UV and IR [39]. Models have been proposed in which the role of the Smc5–6 complex is to hold either sister chromatids or broken DNA ends together to facilitate repair of the DSB (Fig. 6B and C). De Piccoli et al. [40] showed that Smc5–6 complex is recruited to the DSB to support repair by homologous recombination between sister chromatids, similar to cohesin functions. In addition smc5-6 mutants suffer from high levels of gross chromosomal rearrangements. Based on the study of Pebernard et al. [41] there is no clear candidate for a RAD21/SCC1 related factor to bridge head domains. They propose that Smc5–6 may function without enclosing sister chromatids in a proteinaceous loop suggesting a dual function of the Smc5–6 complex in stabilizing DSB as depicted in Fig. 6B and C. This was first indicated by mutational analysis of smc6, in which the repair function was separated from the essential role in genome maintenance [42].

The *Arabidopsis* MIM protein belongs to the Smc5–6 complex and is an orthologue of Smc6. The corresponding mutant is hypersensitive to UV, IR and mitomycin C and shows a reduced frequency of intrachromosomal recombination [16]. In our studies, *atmim* mutant displayed the most severe reduction in the initial rate of DSB repair (t1/2 56.8 min) with first order kinetics consistent with complete abolition of the initial rapid repair, leaving only the slower repair pathway(s) (Fig. 4). This suggests that the initial rapid repair of DSBs has an absolute requirement for MIM. Interestingly, the *atmim* mutant only displayed a two-fold reduction in intrachromosomal recombination, showing that homologous recombination was still active in this line [43]. Hence, this can be taken as a further indication that even if HR is involved in the process of rapid DSBs repair, it is likely not solely responsible for removal of DSBs during early rapid stages of DSBs repair. Due to “non-ring” structure of the SMC5–6 heterodimer, the complex could provide stability by holding together sister chromatids at the sites of DSB, to facilitate recombinational repair (Fig. 6B). This structure would also allow stabilization of DNA ends of DSB in a way the KU proteins binding to DSB in the C-NHEJ repair pathway (Fig. 6D), thereby enabling access to repair factors for direct sealing of the DSB as depicted in Fig. 6C. Our preliminary results suggest that DNA ligase 1 could be one of the factors providing final ligation of DSBs as demonstrated by two-fold reduction of initial rate of repair in siRNA/Lig1 mutant (Waterworth et al. 2009, in preparation).

Data obtained with *atmim* suggests that the Smc5–6 complex is a key player in rapid, global DSB repair in nuclear DNA, rather than Smc1–3 components of the cohesin complex to which *atrad21.1* belongs.

4. Summary

By using the neutral version of the comet assay to directly quantify DSBs in nuclear DNA we have found that a previously uncharacterised pathway is responsible for the rapid repair of the majority of Bleomycin induced DSBs in nuclear DNA of *Arabidopsis*. This novel recombination mechanism is independent of the C-NHEJ pathway, but requires the SMC protein MIM to stabilize the DSB and a DNA ligase(s) other than LIG4 for ligation. These results provide the first biochemical and molecular characterization of an alternative DSB repair pathway, responsible for the recombination previously observed in *atlig4* and *atku80* mutant lines of *Arabidopsis* [20,30].

Conflict of interest

None.

Acknowledgements

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