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Brief report

Rapid repair of DNA double strand breaks in *Arabidopsis thaliana* is dependent on proteins involved in chromosome structure maintenance

Jaroslav Kozak[a], Christopher E. West[b], Charles White[c], José A. da Costa-Nunes[d], Karel J. Angelis[a,*]

[a] *Institute of Experimental Botany AS CR, Na Karlovce 1, 160 00 Praha 6, Czech Republic*
[b] *CPS, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK*
[c] *Génétique, Reproduction et Développement, UMR CNRS 6247 - Clermont Université - INSERM U931, 63177 Aubière cedex, France*
[d] *ISA, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal*

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ABSTRACT

DNA double strand breaks (DSBs) are one of the most cytotoxic forms of DNA damage and must be repaired by recombination, predominantly via non-homologous joining of DNA ends (NHEJ) in higher eukaryotes. However, analysis of DSB repair kinetics of plant NHEJ mutants *atlig4-4* and *atku80* with the neutral comet assay shows that alternative DSB repair pathways are active. Surprisingly, these kinetic measurements show that DSB repair was faster in the NHEJ mutant lines than in wild-type *Arabidopsis*.

Here we provide the first characterization of this KU-independent, rapid DSB repair pathway operating in *Arabidopsis*. The alternate pathway that rapidly removes the majority of DSBs present in nuclear DNA depends upon structural maintenance of chromosomes (SMC) complex proteins, namely MIM/AtRAD18 and AtRAD21.1. An absolute requirement for SMC proteins and kleisin for rapid repair of DSBs in *Arabidopsis* opens new insight into the mechanism of DSB removal in plants.

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 [3], *Tradescantia* stamen hairs [4] as well as more recently in growing root meristems of *Vicia faba* [5,6]. 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 knockout *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 AtSMC6/AtRAD18 protein MIM [16] and kleisin AtRAD21.1, whose yeast homologue is part of SMC1–3 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.

* Corresponding author. Tel.: +420 224322603; fax: +420 224322603.
E-mail address: angelis@ueb.cas.cz (K.J. Angelis).

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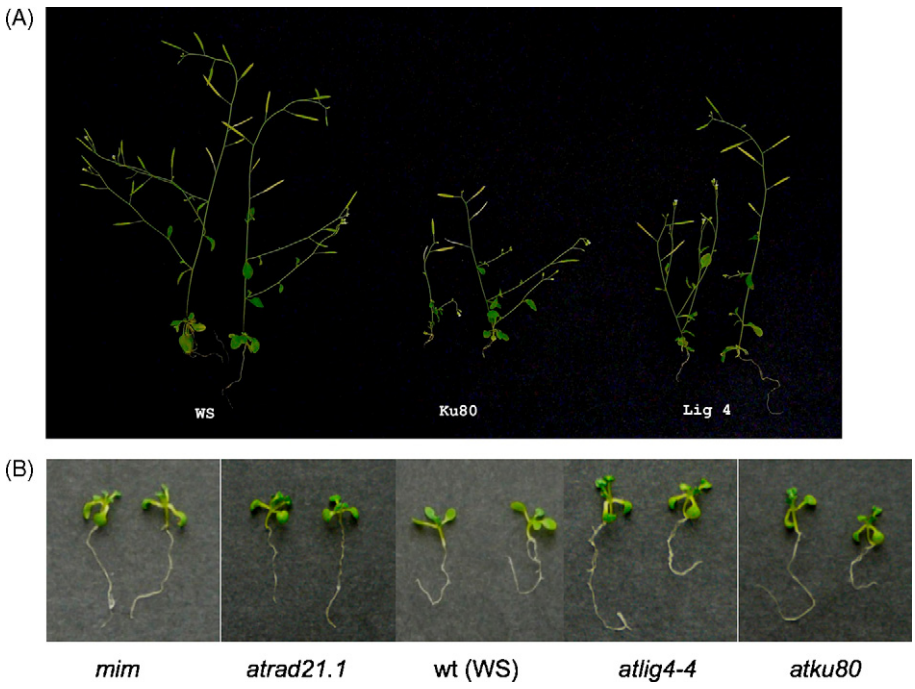

Fig. 1. Phenotype of *Arabidopsis* mutant lines. (A) Mature *atlig4-4* and *atku80* mutant lines showed minor growth defects when compared to the background *Arabidopsis* ecotype Ws after four weeks growing in soil. (B) No germination or growth defects were detected in the 10-day-old seedlings used for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

DSB fragmentation of nuclear DNA was measured in seedlings treated with indicated concentrations of Bleomycin for 1 h in liquid MS/2. In repair kinetic experiments, after the treatment with 50 μg/ml Bleomycin seedlings were thoroughly rinsed in H_2O, blotted on filter paper and either flash-frozen in liquid N_2 ($t = 0$) or left to recover on filter discs moist with MS/2 for the indicated repair times, before being frozen in liquid N_2. Part of the treated seedlings was also let to recover in liquid MS/2 for one week to see the effect of this dose on the growth vigor (Fig. 2B).

2.3. Comet assay

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

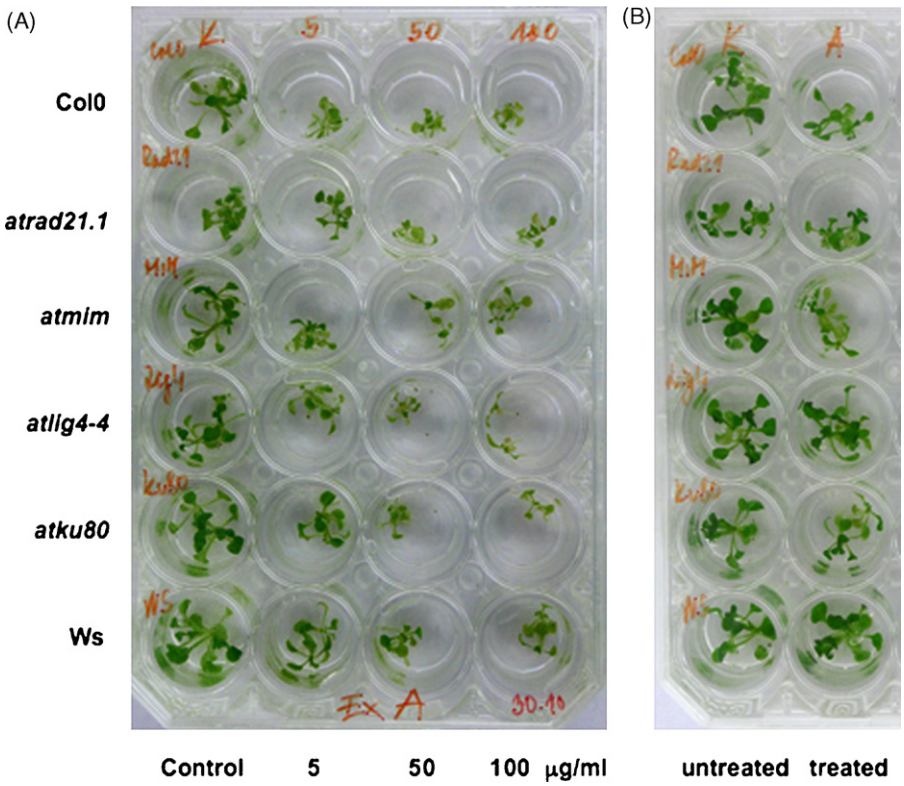

Fig. 2. Sensitivity of *Arabidopsis* lines (*Arabidopsis* Col0, *atrad21.1*, *atmim*, *atlig4-4*, *atku80* and *Arabidopsis* Ws) to Bleomycin treatment. (A) 10-day-old seedlings growing for following week in the presence of 5, 50 and 100 μg Bleomycin/ml in liquid MS/2 media. (B) 10-day-old seedlings treated similarly as for measurement of repair kinetics with 50 μg Beomycin/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:

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

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].

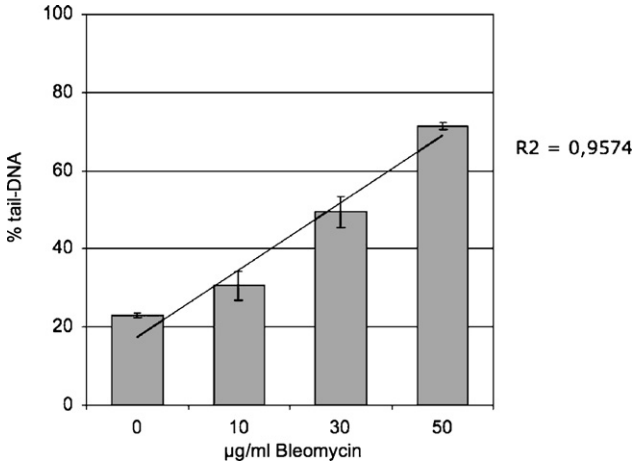

Fig. 3. Induction of DSBs by Bleomycin. 10-day-old seedlings of *Arabidopsis* Ws were treated with 10, 30 and 50 μg/ml Bleomycin for 1 h. Nuclei isolated from treated and untreated seedlings were analyzed by the neutral comet assay and evaluated for comet formation. The mean percentage of DNA in the comet tail for 300 comets for each concentration point are shown. (Error bars—standard error.) Induction of DSB is linear without any signs of saturation ($R^2 = 0.9574$) in the 0–50 μg/ml Bleomycin concentration range used.

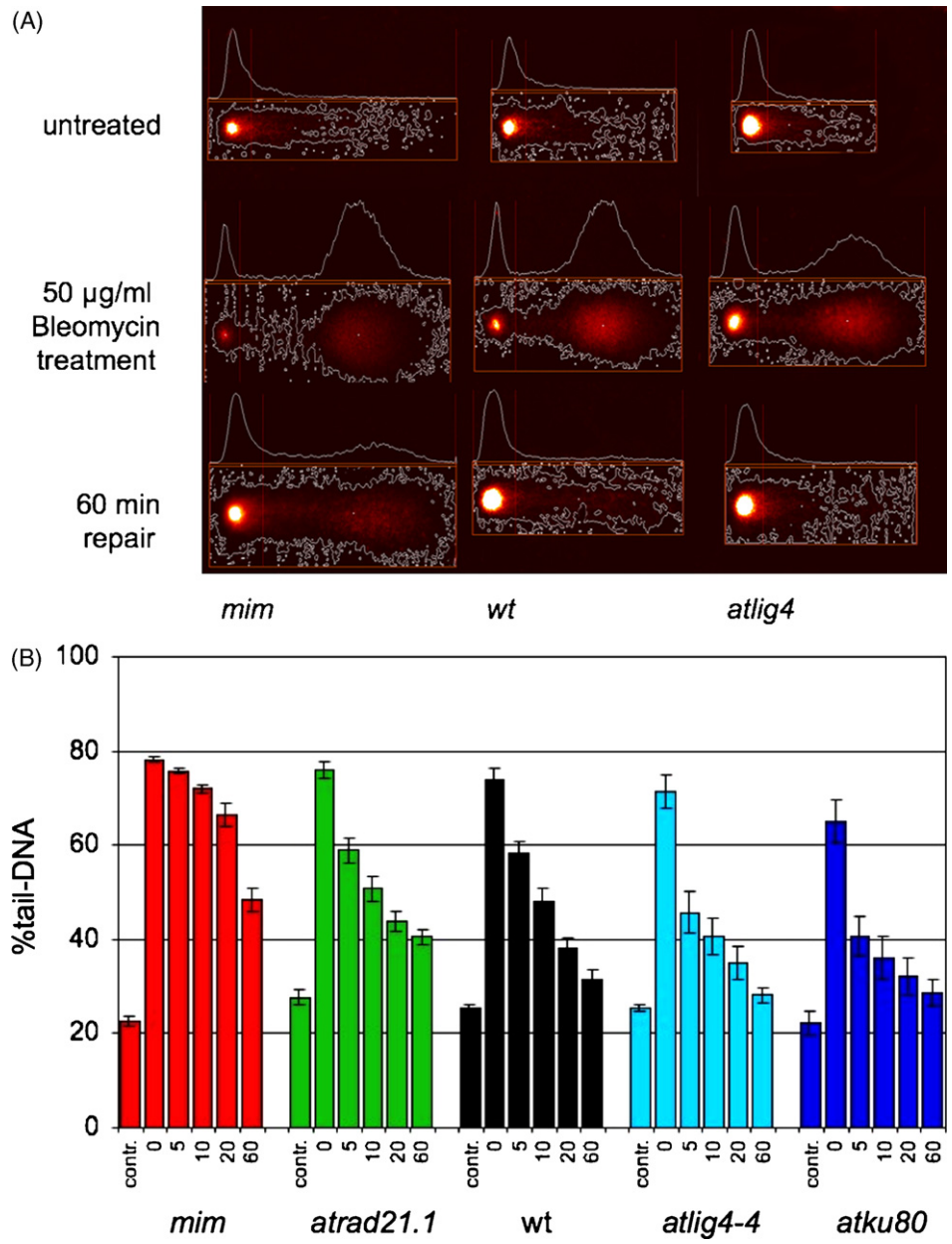

Fig. 4. Detection of DNA damage by comet assay. Comet slides were prepared from untreated and Bleomycin treated 10-day-old wild-type and mutant seedlings by the neutral comet protocol. Representative comet images (A) are shown for wild-type (Ws), *atmim* and *atlig4-4* mutants, untreated or treated and left to repair for 60 min. The extent of DSB repair is manifested by the fraction of DNA remaining in the comet tails after repair recovery (% tail-DNA) and quantified with the LIM software. Mean values of 300 comets are plotted on (B) to show time course of DSB repair in mutant lines over 1 h repair period. Background DNA damage in untreated (control) seedlings and damage after 1 h treatment with 50 μg/ml Bleomycin ($t = 0$) is similar in all lines. (Error bars—standard error.).

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

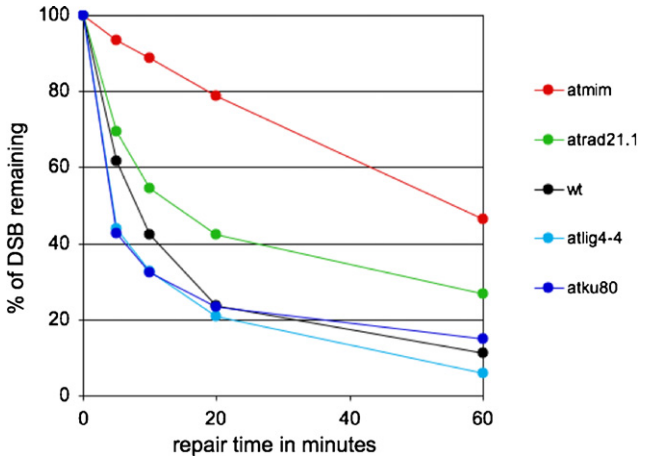

Fig. 5. Kinetics of DSB repair. Fractions of remaining DSBs were calculated for 0, 5, 10, 20 and 60 min repair time points after the treatment with 50 μg/ml Bleomycin. Maximum damage is normalised as 100% at $t=0$ for all lines. Wild-type, *atku80* and *atlig4-4* rapidly repair induced DSBs during the 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).

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 of *atmim*. Mutant *atmim* plants retained a significant level of DNA damage after 1 h of recovery, with around half of the original number of Bleomycin induced DSBs. However, some mutant lines did display variation in the rate of DSB repair during the first 5–10 min 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.

Table 1
Rate constants k and half-times $t_{1/2}$ of initial rapid DSB repair in *Arabidopsis* wild-type and mutant lines were determined by least-square linear regression of fraction of DSBs remaining for 0, 5 and 10 min of repair recovery (Fig. 5).

	k (min^{-1})	$t_{1/2}$ (min)	R^2
mim	0.0122	56.8	0.9958
atrad21.1	0.0630	11.0	0.9838
wild-type	0.0881	7.9	0.9932
atlig4-4	0.1221	5.7	0.9168
atku80	0.1244	5.6	0.9058

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 IgH 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]. SMCs 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

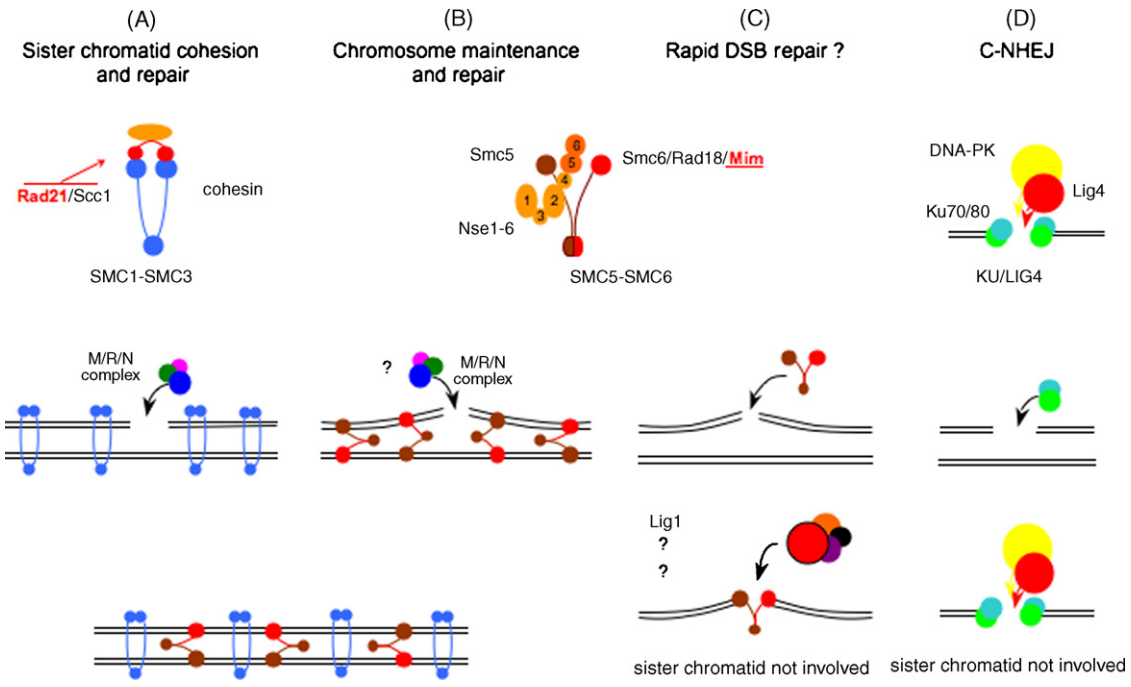

Fig. 6. Possible roles of Smc complexes in DSB repair. (A) The Smc1–3 cohesin complex stabilizes DSBs by enclosing sister chromatids in proteinaceous loops (in blue) and enables entry of the M/R/N complex for completing repair by HR. RAD21 and other kleisins are involved in circularization of Smc1–3 heterodimer (structure on top). In contrast, the Smc5–6 heterodimer forms a V-shaped complex with auxiliary proteins (structure on top), which interacts with DNA through its ends (brown). The complex can either bind together sister chromatids (B), or as suggested by Lehmann [36] stabilize DSBs by direct linking of both DNA ends (C). Stabilization of DNA ends would then parallel binding of KU70/80 heterodimer to DSB ends as a prerequisite for direct end-joining in the C-NHEJ pathway (D).

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 ($t_{1/2}$ 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 cohesion 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2008.11.012.

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