

The role of *AtMUS81* in DNA repair and its genetic interaction with the helicase *AtRecQ4A*

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ABSTRACT

The endonuclease *MUS81* has been shown in a variety of organisms to be involved in DNA repair in mitotic and meiotic cells. Homologues of the *MUS81* gene exist in the genomes of all eukaryotes, pointing to a conserved role of the protein. However, the biological role of *MUS81* varies between different eukaryotes. For example, while loss of the gene results in strongly impaired fertility in *Saccharomyces cerevisiae* and nearly complete sterility in *Schizosaccharomyces pombe*, it is not essential for meiosis in mammals. We identified a functional homologue (*AtMUS81/At4g30870*) in the genome of *Arabidopsis thaliana* and isolated a full-length cDNA of this gene. Analysing two independent T-DNA insertion lines of *AtMUS81*, we found that they are sensitive to the mutagens MMS and MMC. Both mutants have a deficiency in homologous recombination in somatic cells but only after induction by genotoxic stress. In contrast to yeast, no meiotic defect of *AtMUS81* mutants was detectable and the mutants are viable. Crosses with a hyperrecombinogenic mutant of the *AtRecQ4A* helicase resulted in synthetic lethality in the double mutant. Thus, the nuclease *AtMUS81* and the helicase *AtRecQ4A* seem to be involved in two alternative pathways of resolution of replicative DNA structures in somatic cells.

INTRODUCTION

The genomic integrity of organisms is permanently challenged by extrinsic factors, such as irradiation and chemicals, as well as by intrinsic cellular processes, such as transcription and replication. To maintain a stable and proper inheritable genome structure, organisms have developed different pathways to cope with such challenges. For example, during DNA replication the cellular DNA is especially vulnerable

to damage, and several repair pathways or read-through mechanisms are active. One way to deal with DNA damage such as blocked replication forks is regression and annealing of the nascent strands to form a Holliday junction structure (1). The leading strand can thus be extended with the nascent lagging strand as template, and as a result replication can continue. Furthermore, stalled replication forks can be restored by fork regression and resolution into an intact duplex and a free double strand end. The free double strand end can then be repaired by homologous recombination, involving resection of one strand of the free end, invasion of the linear duplex and creation of a new replication fork (2). This process is called replication restart, which is well known in prokaryotes and might also operate in eukaryotes (3–5).

MUS81 is a highly conserved endonuclease and together with *EME1* (also referred to as *MMS4* in *Saccharomyces cerevisiae*) it is involved in the resolution of 3' flap structures and Holliday-like DNA junctions. Loss of the protein results in sensitivity to DNA damaging agents in yeast and mammals. Interestingly, the role of the protein in meiosis differs drastically between eukaryotes. Whereas in *Schizosaccharomyces pombe* loss of *MUS81* results in complete sterility, the mutant is partially fertile in *S.cerevisiae* and fully fertile in mammals (6–9). Moreover, the substrate specificity of the *MUS81/EME1* complex is also in debate as cleavage of intact or nicked Holliday junctions differs between preparations of native protein complexes and heterologously expressed *MUS81* protein (6,10–12). In any case, the *MUS81/EME1* complex cleaves different synthetic DNA substrates *in vitro* such as replication forks and 3' flaps (6,10,11,13–16).

The *MUS81* protein is conserved throughout all analysed eukaryotes but not eubacteria. It is structurally related to the human nucleotide excision repair gene *XPF1* and yeast *RAD1* (17). The C-terminal part of the protein where its endonuclease function is coded and also one of two Helix–hairpin–Helix (HhH) motifs is located is especially conserved (9,18). In contrast, the N-terminal part of *MUS81* is not well conserved except for a polymerase beta domain including the first HhH motif. The second partner of the protein complex, *EME1*, shows functional conservation between different organisms but no significant sequence conservation (10,19,20). In all organisms analysed so far, *EME1* is essential

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for the endonuclease function of MUS81 and the interaction between both proteins is mediated by the conserved C-terminal portion of MUS81 (6).

MUS81 is structurally related to RAD1. In plants, functional homologs of the RAD1/RAD10 complex have been found and investigated regarding their role in excision repair and recombination (21–25). Recently, Dubest and colleagues could show that both plant homologues (AtRAD1 and AtERCC1) remove non-homologous DNA tails in synapsed recombination intermediates and that AtERCC1 is required for mitotic recombination (22).

A MUS81 gene can be found in the Arabidopsis genome (At4g30870). As part of our analysis we characterised the role of AtMUS81 in DNA repair and recombination with the help of two independent T-DNA insertion mutant lines. Moreover, the mutants showed normal fertility indicating that the protein is not essential for meiosis. A synthetic lethality of MUS81 double mutants has been shown for *mus81/rqh1* of *S.pombe* as well as for *mus81/sgs1* in *S.cerevisiae* (17,26), both proteins representing the typical RecQ helicase of the respective organism.

RecQ genes are conserved throughout all kingdoms of life but they show a tendency of complexation regarding their number and functions in multicellular eukaryotes (27). The function of RecQ as suppressor of illegitimate recombination in *Escherichia coli* has also been shown for the unique yeast homologues SGS1, for the *S.pombe* homologue RQH1 and the mammalian RecQ homologues BLMs, WRN and RecQ4 (28–33). As there are seven different RecQ like helicase genes present in the Arabidopsis genome, the question which of them represents a functional SGS1 homolog could not be answered so far. In this study we discovered in an AtRecQ4A insertion mutant background (27,34) that MUS81 deficiency leads to a synthetically lethal phenotype, demonstrating for the first time that the phenomenon applies also to multicellular eukaryotes.

MATERIALS AND METHODS

RNA isolation and analysis of the MUS81 cDNA

RNA from young Arabidopsis plantlets was isolated using the RNeasy Plant Mini Kit from Qiagen (Hilden, Germany) according to the instructions of the manufacturer. Reverse transcription and RT-PCR was performed according to the SMART protocol from Clontech (Heidelberg, Germany) using 50–100 ng of mRNA. The 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed following a protocol from Matz *et al.* (35). The cDNA fragments so produced were sequenced and aligned to the genomic database of TAIR (The Arabidopsis Information Resource). The full-length cDNA is present in the database (AB177892).

Identification of T-DNA insertion mutant lines

Seeds of two different T-DNA insertion mutant lines were ordered, one from the GABI collection (line GABI_113F11) 113F11) (36) and the other one from the SALK collection (line SALK_107515) (37). The seeds, which derived from heterozygous mother plants, were cultivated in soil and PCR assays with primers left and right from the postulated T-DNA

insertion were used to screen 3–4 week old plants (ps1 and pr1 for line 1; for line 2 ps3 and pr2, respectively). Plants homozygous for the T-DNA insertion were propagated further. The exact integration sites were determined by PCR using primers specific for the left or right border of the respective T-DNA and gene specific ones. The PCR products were purified, then sequenced by the company GATC (Konstanz, Germany). The primers used were: ps1 (5'-AGTGAATCTGATAGTGAGTG-3') ps2 (5'-ACAAGAGCTTATTGATGCTG-3') ps3 (5'-GACTTGGACACTCTAAGAG-3') ps4 (5'-AGCTACAAATACAGGTCAAG-3') pr1 (5'-GCAGCATCAATAAGCTCTTG-3') pr2 (5'-GGATTTAAGGTGGATCCATC-3') pr3 (5'-AAGCAAGAGACAAAAGCGTTG-3').

MUS81 from the moss *Physcomitrella patens*

The sequencing of the moss *P.patens* is nearly finished but the sequence has not yet been assembled until now. We therefore searched the raw data for a MUS81 homolog and found it scattered in eight different shotgun sequences. These eight sequences were manually assembled into a contig that covers the whole of the MUS81 gene homolog. The trace sequences used were in order of the MUS81 coding sequence from start to stop: gnl/ti/1000306239; 1035644626; 1003362000; 1006156885; 859646437; 1035671766; 784299639; 1036040620.

Mutagen assays

Homozygous *mus81* plants from both lines were sterilised using 4% NaOCl solution and plated on germination medium (GM)-containing petri dishes. After 7 days the small seedlings were transferred into 6-well plates containing 5 ml pure liquid GM per well or liquid GM with different amounts of the respective mutagen bleomycin, MMS or MMC. Ten seedlings were transferred in each well. After 13 days in the respective mutagen solution, each of the 10 seedlings were taken out together and dried in aluminium foil overnight at 55°C. Finally, the dried seedlings were weighted on a fine scale balance.

HR assay

Verified and already propagated plants homozygous for the T-DNA insertions in the *MUS81* gene were used for crossings with a HR-reporter line, line 651 (38). After crossing and propagation of the heterozygous F1 generation, the F2 generation was screened by PCR for double homozygous plants and also for plants in which the *MUS81* wild type gene and the homozygous reporter construct locus were combined due to segregation. The latter plants were used as internal controls for the recombination assays. For HR assays, the seedlings were treated in the same manner as for the mutagen assays, but instead of transferring them to 6-well plates after 7 days, 30–35 seedlings each were transferred into halved petri dishes containing 10 ml of either pure liquid GM, liquid GM with 5 µg bleomycin or 1 µg MMC, respectively. After five additional days in liquid culture the seedlings were transferred to a staining solution (39). After 2 days in staining solution, the seedlings were incubated in 70% ethanol for 12 h and subsequently the blue sectors on each plant were counted using a binocular microscope. The HR assays were repeated eight times

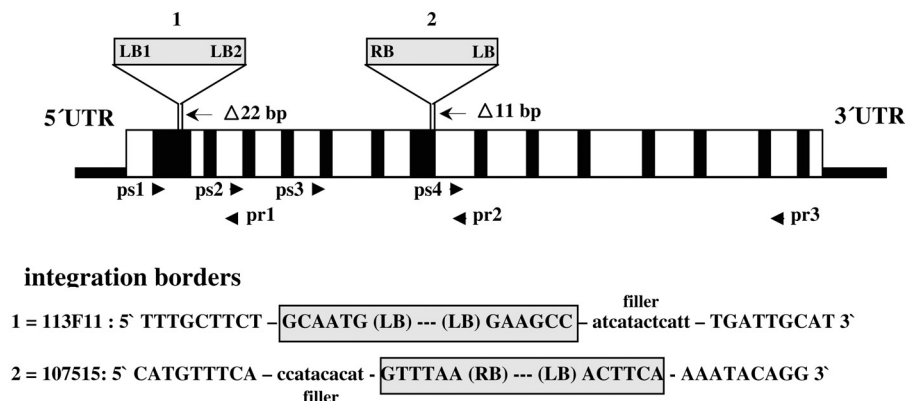
AtMUS81 (At4g30870) T-DNA insertion loci

Figure 1. Location of T-DNA insertion in two *AtMUS81* mutants and schematic *MUS81* protein sequences from different model organisms. The positions of the T-DNA insertions in the different lines are shown as bars above the gene. In *Atmus-81* the presence of two LBs indicates that an insert consists of at least two T-DNA in inverted orientation. The border sequences have been defined by sequencing of both sides using one border and one gene specific primer. The exons of *AtMUS81* are shown in white and the introns in black. The respective T-DNA border sequence is shown in grey boxes and the filler-sequence as small letters.

independently for the bleomycin treatment and four to five times for the MMC treatment.

The mean induction factor (mIF), which is shown in Table 2, results from averaging the IF of each independent experiment. Per experiment an individual IF was defined as relation between the mean HR events per plant of the sample that was treated with a DNA damaging agent and the mean HR events per plant of the untreated sample.

RESULTS

Characterisation of the functional *MUS81* ORF (At4g30870)

We isolated a full-length cDNA of *AtMUS81* which corresponds to the gene At4g30870 using 5'- and 3'-RACE techniques (35). The full-length cDNA is 2303 nt in size, containing 146 nt 5'-untranslated region (5'-UTR) and 177 nt 3'-UTR, respectively. The resulting open reading frame is 1980 nt long, coding for a protein of 659 amino acids. An alignment of the isolated cDNA with the genomic DNA revealed a total of 15 exons and 14 introns (Figure 1). The Kozak sequence of the start codon environment is perfectly conserved ($-3 = G$ and $+4 = G$) (40). The sequence is in accordance with the database entry AB177892.

According to the ClustalW alignment, the 659 aa long *AtMUS81* protein possesses an overall identity of 19.8–21.2% compared with *MUS81* from *S.pombe*, *S.cerevisiae* and *Homo sapiens*, respectively. This is slightly lower than is found in alignments of *MUS81* from *S.pombe* and *S.cerevisiae* (24.7%), *S.pombe* and *H.sapiens* (22.7%) or *S.cerevisiae* and *H.sapiens* (23.4%). Nevertheless, it is in the same range and comparing only the conserved C-terminal part of *MUS81* (240 amino acids long) we found 28.2% identity between *Arabidopsis thaliana* and *H.sapiens* *MUS81* and 28.6% between *S.pombe* and *S.cerevisiae*, which is within a very close range. The whole protein identity between *AtMUS81* and *MUS81* from *Oryza sativa* (clone NT079923 gene P0466H10.20 and corresponding full-length cDNA

AK111411), which serves as model organism for monocotyledonous plants, is 45.4 and 60.9% in the conserved C-terminus, respectively (F. Hartung, unpublished data). The moss *P.patens*, whose genome is not assembled yet, also contains a *MUS81* homolog that is scattered over several shotgun clones. We assembled eight of these clones into a contig covering a full-length gene homologous to *AtMUS81* (see Materials and Methods). The protein of this assembled clone is shown in Figure 2 in a ClustalW alignment together with *AtMUS81* and *OsMUS81*. The moss protein shows ~37% identity over the full-length sequence and 50% in the conserved C-terminus to *MUS81* from both *Arabidopsis* and rice, respectively. Using an earlier described method to compare the intron positions with respect to the coded protein sequence (41), we found a very similar exon/intron structure between the rice and *Arabidopsis* *MUS81* genes. Rice possesses an additional intron (and so 15 introns in total), which is located in the exon 6 if we follow the *Arabidopsis* exon numbering. The remaining 14 introns are in the same positions and phases as the introns in *Arabidopsis*, respectively.

In Figure 1 the schematic structure of the *AtMUS81* gene is depicted and in Figure 2 a ClustalW alignment of *MUS81* from *Arabidopsis*, rice and moss is shown. The T-DNA insertion mutants described later are shown as bars and the sequence surrounding the insertion locus is shown for both lines (Figure 1). The primers used for genotyping the T-DNA insertions and expression analyses are shown as directional arrows.

Three different domains can be identified within the *AtMUS81* protein, namely a polymerase beta domain (SSF47802), the nuclease domain related to ERCC4 (Pfam: PF02732) and a domain similar to the RuvA C-terminal domain (SSF46929) as shown in Figure 2.

The Pol beta (N-terminal) and the ERCC4 endonuclease domain (C-terminal) are common to all investigated *MUS81* proteins from 10 different organisms (data not shown), whereas the RuvA domain can only be found in *AtMUS81*, and neither *O.sativa* nor *P.patens* *MUS81* possess this domain (Figure 2). The Pol beta domain contains one of two HhH



Figure 2. Comparison of the MUS81 homologues from Arabidopsis, rice and moss. A ClustalW alignment of the three proteins was performed on the EBI Server in Heidelberg using ClustalW version 1.83. The RuvA domain is unique for AtMUS81, whereas the Pol beta and ERCC4 domains are found in all three plant MUS81 proteins. Amino acids identical in all three or in two proteins are highlighted below the aligned sequences by asterisks or colons respectively. (At) *Arabidopsis thaliana*; (Os) *Oryza sativa*; (Pp) *Physcomitrella patens*.

motifs, which are also found in all investigated proteins (Figure 3B).

In Figure 3 an alignment of AtMUS81 and seven other MUS81 proteins with respect to their endonuclease domain (Figure 3A) and to their HhH motifs is given (Figure 3B). The endonuclease domain can be also found in human XPF protein, which is not shown here. All animal MUS81 proteins possess an insertion of 5–10 amino acids in the endonuclease domain, which is not present in the plant or fungal proteins (Figure 3A).

Characterization of a second, non-functional MUS81 ORF in Arabidopsis (At5g39770)

The MIPS database annotates a second MUS81-like gene in silico as At5g39770, predicted to encode a protein of 1242 amino acids. The ORF appears to be chimeric because it codes mainly for sequences which are not related to MUS81. Only 307 amino acids from the predicted sequence are homologous to the C-terminus of the MUS81 protein expressed from At4g30870. However, when we examined the genomic region of the putative ORF, an additional 150 amino acids that are homologous to At4g30870 could be found. This putative protein of ~450 amino acids contains a total of three in-frame stop codons positioned in two different exons. We also tested primers binding to different areas of the predicted mRNA of this putative MUS81 homolog in different cDNA preparations. As we could not amplify any partial cDNA that corresponds to this predicted gene (data not shown), we suggest that the sequence represents a non-functional pseudogene.

Phenotypic analysis of the AtMUS81 insertion mutants

Two different insertion mutant lines for MUS81 (At4g30870) were available in the SIGnAL T-DNA insertion database (<http://signal.salk.edu/cgi-bin/tdnaexpress>). One was from the TAIR collection (SALK_107515) and the other one

from the GABI collection (113F11). The respective insertion locus of the two lines is shown in Figure 1. The T-DNA insertion is located in the first intron (GABI line) and in the seventh intron (SALK line) of a total of 14 introns (Figure 1). In the GABI line a small deletion of 22 bp in total occurred and the T-DNA insertion is at least doubled back by back with two left borders pointing outwards. A filler sequence of 14 nt is located at the 3'-LB site. In the SALK line only 11 bp of the intron 6 were deleted due to the T-DNA integration event and a filler sequence of 10 nt can be found at the 5'-RB site. In both cases the insertion is in front of the conserved C-terminal part of the MUS81 protein. Both lines were confirmed as MUS81 insertion lines as no expression of mRNA could be detected in RT-PCR experiments using primers spanning the insertion site (Figure 1, Primers ps1/pr1 and ps3/pr2, respectively). However, in both lines mRNA expression behind the T-DNA insertion was detectable by means of RT-PCR using primer pairs ps2 and pr3 for line 1 and ps4 and pr3 for line 2. In our semi-quantitative analysis the amount of truncated mRNA was similar to the amount of AtMUS81 mRNA in wild type for line 1 whereas it was reduced to about half for line 2 (data not shown).

Both MUS81 mutants grow normally and produce seeds in comparable amounts to the wild type Col-0. This was tested for at least three generations and no difference in growth and fertility of the mutants was found. Furthermore, pollen staining (Alexander staining) of both mutant lines exhibited fully viable pollen mother grains (data not shown). Therefore, MUS81 seems to have no essential role in meiosis of *A.thaliana*.

MUS81 is involved in DNA repair in somatic cells

We used different mutagens to induce genotoxic stress in the mutant lines and found a strong sensitivity of both insertion

A**Endonuclease domain (ERCC4)**

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At 399 EAYDVIL-ILDDREKFATKGS--RSRNIVENICSEFNKIEVRRRLVPGDCIWIARHKYLE
Dr 309 GSYDIVL-CVDLCETT--GGSSVRKQELVKELQ-RNSVTFDVRKLNVGDFLWARERVTP
Xt 320 GDFNILL-CVDFIETT--GGAHRKQELVTELR-RNGVNFVDRKLHIGDFLWIAQEKVQP
St 415 GQFDVIL-CVDNCETTGIGGKYGRKHVLLKELQ-KNGVNLDVRKLQVGDFLWVAREKGS
Hs 265 GEYRVLL-CVDIGETR--GGG--HRPELLRELQ-RLHVTHTVRKLHVGDFVVWAQETNPR
Dd 673 IKFKNIKCTIDNREVK----SVTERDYICNKLN-ERGINAQVKKLELGDFVVWAIDEHDN
Sp 290 SNCTVFL-LIDTREIR----SPLDRNLIIDKLTNDFGVNCQVRSLELGDALWVARDMESG
Sc 346 GDFEVFP-IIDHREIK----SQSDREFFSRAFE-RKGMKSEIRQLALGDIIWVAKNKNTG

At 456 T-----EYVLDFIAERKNVDDMRSSIRDNRYRDQKLRLQRSGFKKLIYIEGDPN
Dr 366 VPGQLRPPVGKELVLDYIEERKRMDDLCGSIIDGRFREQKFRLKRCGLRKPIYLVEECGS
Xt 377 VPGQLRIPQARELVLDYVVERKRMDDLCGSIIDGRFREQKFRLKRCGLRRPYLVEDHGS
St 474 VPGLLQMPRRREAVLDYVVERKRMDDLCSSIQDGRFREQKFRLKQCGLHPVYLVEDFGS
Hs 320 DP-----ANPGELVDHIVERKRLDDLCSSIIDGRFREQKFRLKRCGLERRVYLVEEHGY
Dd 729 -----EWLLNYIEERKRVDDLSSSIIDGRYKEQKFRLSKSGCDNIYLIEGIVS
Sp 346 Q-----EVLDFVVERKRYDDLVASIKDGRFREQKARLKKSGIRSVTYILEESSY
Sc 401 L-----QCVLNTIVERKRLDDLALSIRDNRFEQKNRLEKSGCEHKYYLIEETMS

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B**HhH Motif 1**

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At 59  KDLSQIKGFGKWMVKLMKGY
Dr 64  KIL-QNFGDGICKILDERLQ
Xt 64  KIL-QNFGDSICKMLDDRLE
St 64  KIL-DNFGDKICSMLDKKLE
Hs 64  KIL-QHFGDGLCRMLDERLQ
Dd 111 KECEVLNGFGPSLTKKINDY
Sp 12  SQALALKGIGPTICAKLEKK
Sc 51  TDLKKVRGIGNTIIKRLDTK

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HhH Motif 2

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At 585  TISDVFAIQLMQVPQVTEEIAIAVLDMYPTLLSLASAY
Dr 523  TVREVFARQLMQISGVSGDKAAAVLKHYSTVSSLQAY
Xt 531  TVKEVFARQLMQISGVSGEKAAAILEKYSTPASLMSAY
St 628  TVTETFAKQLLQFGGMSAEKALALTQIYQTPSDLTRAY
Hs 470  SVREVFARQLMQVRGVSGEKAAALVDRYSTPASLLAAY
Dd 884  KLIEFFATQLIQIPGCSAEKAHSITQVYPTPMSLYLAL
Sp 494  TVGDIFIRMLTIKGISASKAEIQKKYTFMHLFEAY
Sc 554  TVGELTHVLMLVKGISLEKAVAIQEIFPTLNKILMAY

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Figure 3. Alignment of the amino acid sequence of the two conserved domains of MUS81 from different organisms. (A) The C-terminal endonuclease domain of eight different MUS81 proteins. (B) The two Helix–hairpin–Helix motifs of the same eight MUS81 proteins. Amino acids are highlighted as follows. The grey boxes indicate positions in which particular amino acids are conserved. The amino acids written in bold type are identical to the consensus sequence of an earlier alignment from Interthal and Heyer (9). Colourless boxes indicate a small insertion that is present only in MUS81 from animals. Abbreviations of the organisms are: (At) *A.thaliana*; (Dr) *Danio rerio*; (Xt) *Xenopus tropicalis*; (St) *Strongylocentrotus purpuratus*; (Hs) *Homo sapiens*; (Dd) *Dictyostelium discoideum*; (Sp) *Schizosaccharomyces pombe*; (Sc) *Saccharomyces cerevisiae*. The alignments were done according to Interthal and Heyer (9).

lines to methyl methane sulfonate (MMS) and mitomycin C (MMC) but not to bleomycin, a double strand break-inducing agent. Both T-DNA insertion lines already showed growth reduction and bleaching at 40 p.p.m. MMS or 5 µg/ml MMC in comparison to the control lines (see Figure 4 and Table 1).

A statistical quantitation of the effect of the mutagens in 5–6 independent experiments is shown in Table 1. At 40 p.p.m. MMS the mutant lines show nearly 50% growth reduction whereas the wild type Col-0 is only reduced in its dry weight by 25%. At 100 p.p.m. MMS the mutants are reduced to 18% and Col-0 to 31%. At 5–10 µg/ml MMC both *MUS81* mutant lines show 45% growth reduction whereas the wild type plantlets show only 17% reduction. At 5–10 µg/ml this growth reduction is significantly stronger than for the wild type plantlets. At higher concentrations of MMC (15–20 µg/ml) both wild type and mutant plantlets are so strongly harmed that there is no longer a significant difference.

MUS81 and homologous recombination

To analyse the behaviour of *MUS81* insertion lines with respect to homologous recombination, we crossed both lines

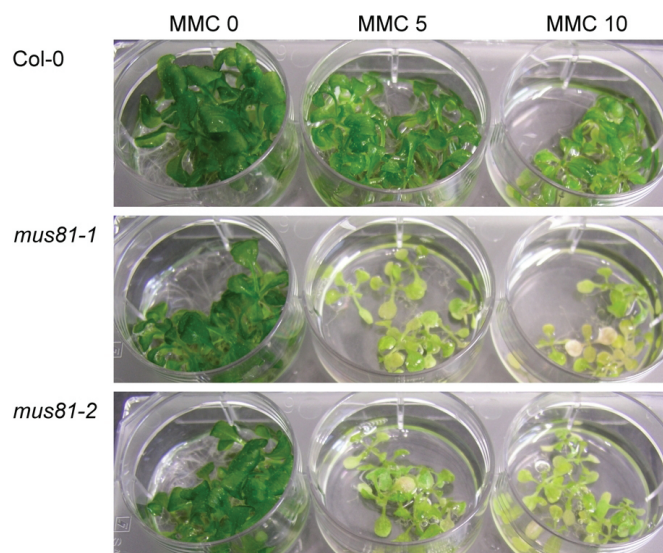


Figure 4. Mutagen treatment of two *MUS81* knockout lines. Both knockout lines treated with various amounts of MMC are shown in comparison to Col-0. The *mus81-1* and 2 plantlets exhibited a strong sensitivity at 5 and 10 µg/ml MMC visible as growth reduction and bleaching.

Table 1. Growth sensitivity of *MUS81* mutants to MMS or MMC, respectively

Plant line	% Dry weight	% Dry weight	% Dry weight	% Dry weight
MMS	20 p.p.m.	40 p.p.m.	60 p.p.m.	100 p.p.m.
Col-0	85.0 ± 11.0 (6)	74.5 ± 9.9 (6)	67.1 ± 10.5 (6)	32.7 ± 6.4 (6)
<i>mus81-1</i>	76.2 ± 7.4 (6)	54.0 ± 5.1 (6)	37.8 ± 4.5 (6)	18.1 ± 2.3 (6)
<i>mus81-2</i>	79.8 ± 11.1 (6)	54.3 ± 3.7 (6)	36.6 ± 5.9 (6)	18.2 ± 3.0 (6)
MMC	5 µg MMC	10 µg MMC	15 µg MMC	20 µg MMC
Col-0	83.2 ± 3.7 (5)	70.2 ± 6.5 (5)	49.6 ± 9.3 (5)	40.6 ± 9.7 (5)
<i>mus81-1</i>	56.0 ± 10.5 (6)	42.9 ± 6.9 (6)	34.7 ± 6.2 (6)	29.7 ± 7.6 (6)
<i>mus81-2</i>	55.0 ± 3.6 (6)	48.7 ± 5.8 (6)	36.3 ± 4.1 (6)	33.9 ± 5.4 (6)

The mutagen sensitivity is shown as growth reduction, measured in percentage of the plantlets dry weight compared to their respective growth of 100% in germination medium containing no mutagen. For each measurement 10 plantlets were pooled. The ± values represent the standard deviation and the numbers shown in brackets represent the performed experiments. p.p.m. = parts per million.

with the reporter line 651. The line 651 harbours two parts of the bacterial β -glucuronidase (GUS) gene as an inverted repeat (38). Inter- and intrachromosomal homologous recombination by crossover both result in a functional GUS gene (42). Because in this assay system the interrupted GUS reporter gene is inserted in a different Arabidopsis ecotype (C24 versus Col-0), it was absolutely important to use a restored *MUS81* line from the same crossing as a control line.

After isolation of *mus81/651* double mutants, we determined the HR frequency without and after induction of DSBs by the mutagens MMC and bleomycin. The results of 4–16 independently repeated HR assays are summarised in Table 2. The distribution of blue sectors resulting from HR events is shown for one representative experiment in Figure 5. Both mutant lines exhibited a phenotype in which their HR induction (IF) upon mutagen treatment is reduced to nearly 50% or less in 651 background. The control line (resulting from the backcross of *mus81-1* with the line 651) in which the *MUS81* gene is unaffected showed a basic level of 1.8 spots per plant (spp) and a 41.5-fold bleomycin HR induction factor (mean of 11 experiments). The mutant lines showed no significant deviation for non-induced HR. However, the induction was only 22-fold for *mus81-2* and 9.4-fold for *mus81-1* after the bleomycin treatment (Figure 5 and Table 2).

A similar behaviour was observed with MMC. The control line showed a 23.6-fold induction under the influence of 1 µg/ml MMC (mean of five experiments). In these assays *mus81-1* and *mus81-2* again showed induction factors which are 50–75% lower than in the wild type.

Genetic interaction of AtMUS81 and AtRecQ helicases

The *MUS81* endonuclease and RecQ helicases are both postulated to process recombinogenic DNA replication intermediates. The proteins act in independent pathways, and production of double mutants resulted in a synthetic lethal phenotype in *S.pombe* as well as in *S.cerevisiae* (17,26). In both organisms the knockout of the single RecQ homologue led to a hyperrecombination phenotype. Although seven different RecQ-like genes are present in the Arabidopsis genome (27) there is only one gene known so far whose mutation leads to a hyperrecombination phenotype, namely *AtRecQ4A* (34). We therefore crossed the *AtMUS81* insertion mutant line 113F11 with the insertion mutant of *AtRecQ4A* (GABI

collection, line 203C07) and analysed the offspring of these crossings. Furthermore, we crossed the same *AtMUS81* insertion mutant with an insertion mutant line of *AtRecQ2*, which, on the other hand, did not show enhanced recombination frequencies (F. Hartung and S. Suer, unpublished data) (43). As described, the RecQ insertion mutants and the *AtMUS81* insertion mutants exhibited no visible phenotype. Analysis of F2 plants from the different crosses showed viable and phenotypically unaffected double mutants resulting from the cross with *recq2* only. In contrast, the crossing *recq4A/mus81-1* yielded several small and severely affected plants, both in soil and on agar plates containing GM (Figure 6). PCR analysis of these plants revealed that they were indeed double homozygous for the mutated genes whereas the normally growing plants were either homozygous/heterozygous or without homozygous mutants for both genes (data not shown). In total we analysed 80 plants grown in soil for *MUS81/RecQ4A*, 3 of which were double homozygous (3.7%), and 264 plants grown on agar plates, of which 13 (4.9%) were double homozygous (Table 3). In the case of *mus81/recq2* and *mus81/recq4A* grown in soil, all plants were genotyped by PCR using primers specific for the insertion loci (line 1 and 2 in Table 3). In the case of the *mus81/recq4A* plants grown on agar all poorly growing plantlets (13) and a subset of 37 of the 264 normal growing plantlets were genotyped by PCR. The numbers were in the range of the expected Mendelian segregation for two independent hemizygous mutants (6.25%, see χ^2 values). The double mutants grew poorly and died within 4–5 weeks regardless if they were cultivated in soil or on agar. The strict correlation between genotype and phenotype indicates that the specific combination of *MUS81* and *RecQ4A* deficiency is synthetically lethal in *A.thaliana*.

DISCUSSION

Isolation of AtMUS81 T-DNA insertion mutants

In this study we isolated the full-length cDNA of a plant *MUS81* ortholog and analysed two T-DNA insertion mutants from this gene At4g30870. Both mutants were unaffected in growth and fertile but they exhibited a strong sensitivity to the mutagens MMS and MMC. The T-DNA insertion of *mus81-1* is near the start of the gene, interrupting the pol beta domain, whereas in *mus81-2* it is in the middle part of the gene. Because both lines show expression behind the

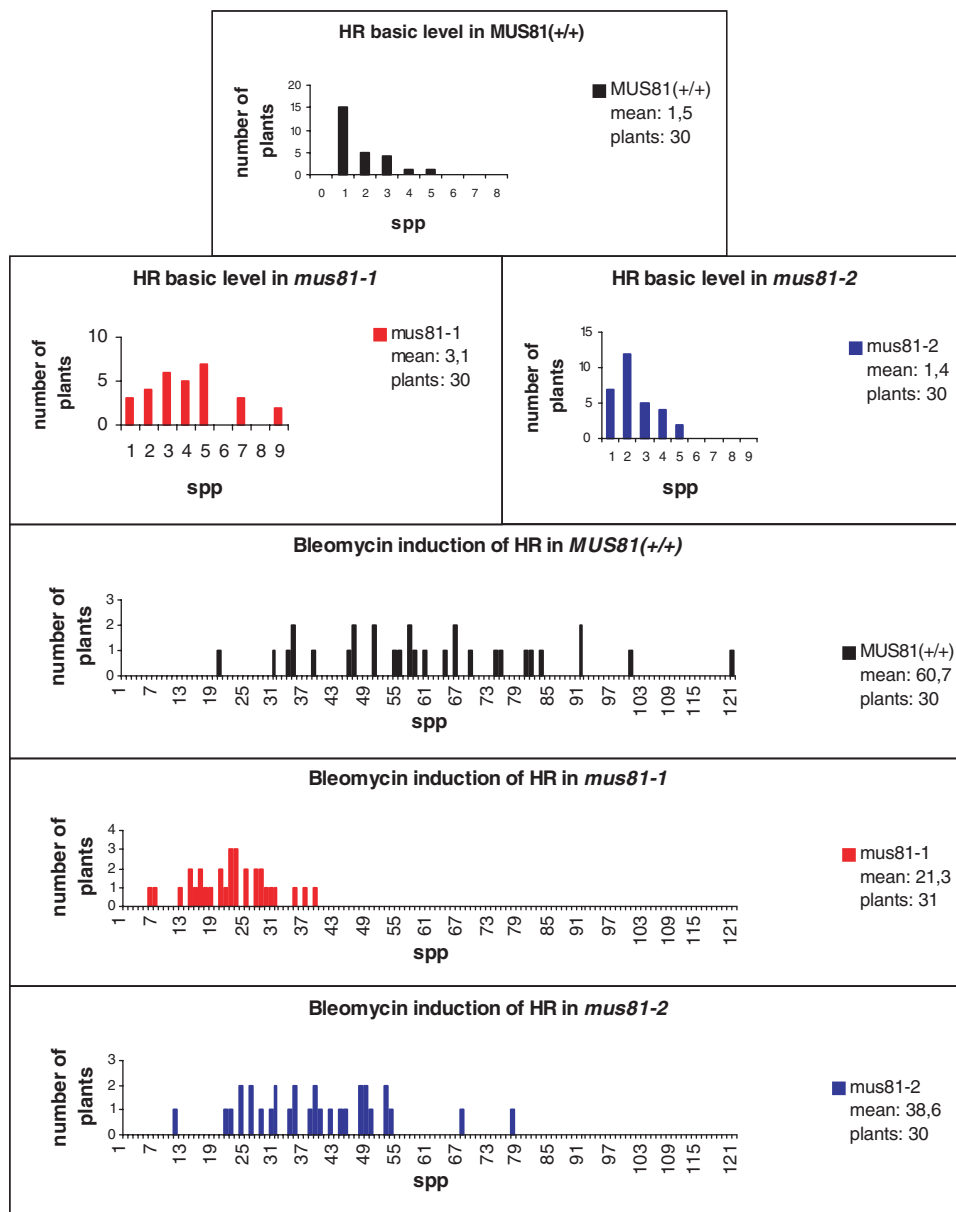


Figure 5. Distribution of β -glucuronidase-expressing sectors in the control line and both *MUS81* mutant lines with and without bleomycin treatment in one representative experiment. The *mus81* mutant plants clearly have fewer sectors and therefore less homologous recombination than the control when treated with bleomycin. spp = sectors per plant.

Table 2. Combined intra- and interchromosomal homologous recombination level of two *MUS81* mutants with and without induction by bleomycin or MMC

Plant line	Control spp (SD)	5 μ g BLE spp (SD)	mIF-BLE spp (SD)	1 μ g MMC spp (SD)	mIF-MMC spp (SD)
<i>MUS81</i>	1.8 \pm 0.6 (16)	60.5 \pm 19.1 (11)	41.5 \pm 13.6 (11)	52.4 \pm 20.8 (5)	23.6 \pm 9.7 (5)
<i>mus81-1</i>	3.2 \pm 1.1 (13)	25.1 \pm 7.6 (8)	9.4 \pm 1.4 (8)	23.3 \pm 2.0 (5)	6.0 \pm 1.5 (5)
<i>mus81-2</i>	1.6 \pm 0.4 (12)	33.7 \pm 7.8 (8)	22.0 \pm 2.9 (8)	21.5 \pm 5.3 (4)	11.9 \pm 1.6 (4)

The HR level was measured by counting blue sectors of 30 plants per assay either under non-induced or mutagen induced conditions. The numbers and standard deviations (SDs) are the result of at least four independent experiments each (up to 16, shown in brackets). A single example experiment is shown in Figure 5. mIF = mean Induction Factor, spp = sectors per plant.

insertion locus, the resulting truncated mRNAs are different and might affect the phenotype. Nevertheless, the phenotypic differences were not statistically significant for either the mutagen sensitivity or the HR-assays. The gene structure of

AtMUS81 and the mutagen sensitivity of the mutants are in overall accordance with the previously reported yeast and human *MUS81* genes, with one exception regarding the functional domains of the protein.



Figure 6. Double mutants of AtRecQ4A and AtMUS81 grown on germination medium. A two week old control seedling (Col-0) and three different 2-week-old seedlings carrying homozygous T-DNA insertions for both AtRecQ4A and AtMUS81 are shown. The two week old double mutant seedlings show growth defects of various severities. Each image was taken at the same time point under a binocular microscope using a 7-fold magnification.

Table 3. Statistical analysis of siblings from crosses between *mus81* and *recq* like genes

Crossed lines	Plants	Visible phenotypes	Double homozygous	Percent	χ^2
<i>mus81/recq2</i>	38	none	2	5.3	0.06
<i>mus81/recq4A</i>	80	3	3	3.7	0.80
<i>mus81/recq4A</i> ^a	264	13	13	4.9	0.49

The percentage of homozygous siblings is within the expected statistical range using the χ^2 -test.

^aGrown on agar plates.

MUS81 from *S.pombe* was originally reported to act together with MMS4 as a true Holliday Junction (HJ) resolvase, a conclusion that was not generally accepted (6,10–13,44–46). Nevertheless, all studies indicate that a nicked HJ can be efficiently processed by the MUS81 complex (8,47). Interestingly, the AtMUS81 protein possesses a RuvA domain whereas all other described MUS81 proteins only have the polymerase beta and the ERCC4 nuclease domain, which are also present in the Arabidopsis protein. The RuvA domain could therefore be involved in branch migration of HJs as described (48). The Arabidopsis MUS81 protein seems to have gained this new functional module in between the polymerase beta and ERCC4 domains. Surprisingly, the RuvA domain is neither present in the rice nor in the moss ortholog of MUS81, so it remains unclear if this newly gained RuvA module is indeed involved in HJ branch migration. This question can be addressed only by means of biochemical analysis.

The *Atmus81* mutants are mutagen sensitive

We found a strong sensitivity to the mutagens MMS and MMC but not Bleomycin for both of the *mus81* mutant lines, which corresponds to the phenotype of yeast *mus81* mutants (9). The sensitivity of both lines was measured as a reduction in their dry mass compared to wild type plants and showed a 40–50% reduction. This may actually be an underestimation of the effect because in our assay the plants were grown for a week on normal medium before they were transferred for the next 13 days to mutagen-containing liquid medium. In a recent report Bleuyard *et al.* (49) plated seeds directly on mutagen-containing medium which resulted in an even stronger growth inhibition of *Atrcc2* and *Atrad51c* mutants from Arabidopsis. As both assays are not directly comparable it will be interesting to test under similar experimental conditions whether *Atmus81* lines or the *Atrcc2* and *Atrad51c* are more sensitive to mutagens and whether the proteins are involved in the same DNA repair pathway.

The role of AtMUS81 in meiotic and mitotic recombination

It seems that AtMUS81 in contrast to yeast MUS81 has no indispensable role in meiosis as both insertion lines surprisingly showed no detectable meiotic impairment. The normal pollen production and fertility of the AtMUS81 mutants both argue strongly against an important meiotic role for MUS81 in Arabidopsis. Similarly, an essential meiotic function for MUS81 in mammals has not yet been reported (50,51). It seems that MUS81 in multicellular organisms is involved in

mitotic rather than in meiotic recombination. Nevertheless, the observation that both *AtMUS81* insertion lines have no detectable meiotic impairment does not exclude a role of the protein in a minor pathway of meiotic recombination. We cannot formally exclude that although no functional mRNAs are produced, transcripts downstream of the insertion locus could be translated into truncated proteins that still possess some residual activity. Two different crossover (CO) pathways in Arabidopsis have recently been proposed by Copenhaver *et al.* (52) and supported by Higgins *et al.* (53). The authors of this excellent study on a MSH4 knockout line suggest that Arabidopsis possesses two crossover pathways as in *S.cerevisiae* (53). This suggestion supports Copenhaver *et al.* (52) theoretical considerations made in 2002 about the existence of such a second CO pathway. Higgins *et al.* (53) found a delayed meiotic progression and synapsis in the MSH4 mutant and furthermore an ~85% reduction in chiasmata frequency during metaphase I. The residual 15% chiasmata are randomly distributed and not connected to specific bivalents. This observations fit to the data from Börner *et al.* who estimated that ~15% of COs in yeast arise from a pathway other than class I, and the data from de los Santos *et al.* (44) who found only a modest decrease in CO frequency in *S.cerevisiae* *MUS81* mutants (54). If we assume that the *AtMSH4/MSH5* complex might partially takeover functions of the *AtMUS81* complex, only a minor meiotic impairment—undetectable in our analyses—might occur in the *AtMUS81* knockout lines. Therefore, our data *per se* do not exclude that a residual chiasmata frequency in Arabidopsis depends on *MUS81* action.

The recombination substrate used in somatic cells in this study is based on an inverted repeat of non-functional parts of the β -glucuronidase genes (38). The marker can be restored by a conservative recombination reaction that results in a crossover (55). We did not detect a significant effect of mutation of the *AtMUS81* gene on the basic level of homologous recombination in somatic cells. Therefore, independent of the question as to whether or not HJs have to be processed in this reaction, it seems that *AtMUS81* is not involved in this process. However, *AtMUS81* seems to be required for homologous recombination after application of genotoxic stress. In both mutant lines we detected a statistical significant decrease in HR induction upon mutagen treatment. It seems that under these conditions the endonuclease activity of *MUS81* is needed to process repair intermediates that are then channelled in a HR pathway. These kinds of intermediates seem to be different to the recombination intermediates mentioned above and might not arise in reasonable amounts during normal growth of the plant cell. It could well be that they arise only after the cell has accumulated a bigger number of DNA insults and other nucleases like *RAD1/RAD10* (=XPF/ERCC1 in mammals) are titrated out by the increase in DNA damage. The *RAD1* nuclease is related to *MUS81*, acting in a complex together with *RAD10* on recombination intermediates (56). *AtRAD1* as well as *AtERCC1* insertion mutants have a strong defect in HR events in which protruding ssDNA tails are produced (21,22). One could speculate that *MUS81* is involved in HR in somatic cells only after the activity of the *RAD1/RAD10* complex becomes rate limiting in a step by which a free 3' end is produced, which is needed for further processing as primer on a homologous

matrix. It will be therefore of great interest to define the role of the *mus81/rad1* or *erccl* double mutants in Arabidopsis.

Genetic interactions between *AtMUS81* and *AtRecQ4A*

It has been previously speculated that *MUS81* is involved in cleavage of the leading strand during DNA replication and is therefore able to facilitate reinitiation of stalled replication forks (12,14). Interestingly, a synthetic lethal phenotype in *S.pombe* as well as in *S.cerevisiae* is obtained when a *mus81* deletion is combined with the loss of the RecQ helicases *SGS1* or *RQH1*. This is in line with the fact that several RecQ helicases are also involved in the repair of stalled replication forks (57,58). If the respective helicase is knocked out, a hyperrecombination phenotype becomes apparent. This phenotype has not only been reported for the RecQ homologues *SGS1* of *S.cerevisiae* (31), *RQH1* in *S.pombe* (30) but also for the BLM protein of mammals (28). Thus, the unresolved DNA structures are channelled in a repair pathway that results in an elevated crossover level and uses mainly sister chromatid exchange (32). The easiest explanation of our finding that the *AtRecQ4A* and *AtMUS81* double mutant is not viable is that both proteins are involved in parallel pathways that dissolve stalled replication forks. The cells can survive if either one or the other pathway is available, but as soon as both pathways are blocked further replication is not possible. We believe that the *RecQ4A* gene has a similar function in Arabidopsis as *SGS1* in yeast and *BLM* in human. It has been reported before that insertion mutants of *AtRecQ4A* also exhibit a hyperrecombination phenotype (34). When other RecQ homologues of plants were analysed, no hyperrecombination phenotype could be detected (e.g. for *AtRecQ2*, F. Hartung, S. Suer and H. Puchta, unpublished data) (59).

In contrast to *AtRecQ4A* the deletion of *AtMUS81* does not lead to a hyperrecombination phenotype. This indicates that stalled replication intermediates produced in an *AtMUS81* mutant are not channelled preferentially into a crossover pathway. Otherwise we would have seen an enhancement of recombination events with our crossover-specific assay. It might well be that such replicative damage will be processed rather by the *AtRecQ4A* helicase and channelled into a non-crossover pathway. Indeed, in mammals *BLM* together with *Topo3A* can resolve double HJs exclusively to non-crossovers which is dependent on the HRDC domain of the *BLM* protein (32,60). Alternatively, the *AtRecQ4A*-processed intermediate might be repaired by non-homologous end-joining, as this pathway is the most prominent mode of DSB repair in Arabidopsis (61). If the *RecQ4A* based pathway is blocked (as in the double mutant *mus81/recq4A*), then the intermediates cannot be processed properly, leading to the lethality observed. To our knowledge this is the first genetic evidence of a synthetic lethality of both mutants in a multicellular eukaryotic organism.

As there is a genetic conservation of the *MUS81* and *RecQ* pathways involved in the resolution of replicative intermediates from yeast via plants to humans, it will be very interesting to examine whether this conservation is also reflected in the biochemical properties of the respective plant factors.

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