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An SMC-like protein is required for efficient homologous recombination in *Arabidopsis*

Tesfaye Mengiste, Ekaterina Revenkova[1], Nicole Bechtold[2] and Jerzy Paszkowski[3]

Friedrich Miescher Institute, PO Box 2543, CH-4002 Basel, Switzerland and [2]INRA, Station de Génétique et d'Amélioration des Plantes, route de St Cyr, 78026 Versailles, France

[1]Present address: Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland

[3]Corresponding author
e-mail: jurek@fmi.ch

In plants, the observed low frequency of gene targeting and intrachromosomal recombination contrasts markedly with the efficient extrachromosomal recombination of DNA. Thus, chromatin accessibility can have a major influence on the recombination frequency of chromosomal DNA *in vivo*. An *Arabidopsis* mutant hypersensitive to a range of DNA-damaging treatments (UV-C, X-rays, methyl methanesulfonate and mitomycin C) is also defective in somatic intrachromosomal homologous recombination. The wild-type gene encodes a protein closely related to the structural maintenance of chromosomes (SMC) family involved in structural changes in chromosomes. Although loss of SMC function is lethal in other eukaryotes, growth of the *Arabidopsis* mutant is normal in the absence of genotoxic treatments. This suggests a surprisingly specialized function for this protein in plants, and provides the first *in vivo* evidence for the involvement of an SMC protein in recombinational DNA repair. It is possible that SMC-like proteins in plants alleviate suppressive chromatin structure limiting homologous recombination in somatic cells.

Keywords: *Arabidopsis thaliana*/homologous recombination/mutants/SMC

Introduction

Two pathways are known to be involved in the repair of DNA double-strand breaks (DSBs): non-homologous end joining (NHEJ) and homologous recombination. The proteins involved in the two processes are evolutionarily conserved but the relative contributions of these repair pathways vary in different biological systems. In multicellular eukaryotes, such as nematodes, insects, mammals and higher plants, the prevalent mechanism is NHEJ. In bacteria and unicellular eukaryotes, such as yeast, homologous recombination is more frequent. In contrast to NHEJ, homologous recombination repairs damage precisely and thus the end products of this reaction are predictable. This precision has been exploited for the modification of chromosomal genes (gene targeting) in a number of biological systems. Although gene targeting could be an important technology to study gene function, its effectiveness in higher plants is unsatisfactory due to the low frequency of homologous recombination. This technical disadvantage is amplified by our ignorance of plant factors important for recombination. *Arabidopsis* mutants with altered frequencies of homologous recombination have been obtained recently (Masson and Paszkowski, 1997), but the nature of the genes affected is not known. Conversely, the plant orthologues of yeast recombination genes have been isolated (Kobayashi *et al.*, 1993; Klimyuk and Jones, 1996; Doutriaux *et al.*, 1998) but their functions in plants are unknown. Thus, no plant proteins involved in homologous recombination have yet been identified.

Yeast mutants deficient in homologous recombination are often hypersensitive to γ- and X-ray radiation and to chemicals mimicking radiation, such as methyl methanesulfonate (MMS) (Petes *et al.*, 1991; Game, 1993; Haber, 1995). This connection has also been observed recently in plants (Masson and Paszkowski, 1997; Masson *et al.*, 1997). Using this genetic approach, we have identified the first molecular component required for intrachromosomal homologous recombination in somatic cells of a higher plant.

Results

A population of *Arabidopsis thaliana* mutagenized by transferred DNA (T-DNA) (Bechtold *et al.*, 1993) was screened for individuals hypersensitive to MMS as described (Masson *et al.*, 1997; Albinisky *et al.*, 1999; Revenkova *et al.*, 1999). Of 2300 T3 families, three segregated MMS-hypersensitive plantlets. One mutant family was subjected to the detailed studies described here. Thirty randomly selected plants of this family were grown to maturity and their progenies examined for a genetic link between T-DNA-encoded traits and MMS hypersensitivity. Genetic and molecular analysis of the tagged locus in F_3 single plant progenies supported the conclusion that MMS hypersensitivity is a single recessive Mendelian trait linked to a T-DNA insertion.

To determine whether the mutation resulting in MMS hypersensitivity also increases sensitivity to other DNA-damaging treatments, mutant seedlings were challenged with increasing doses of X-ray or UV-C radiation, or were treated with the DNA cross-linking agent mitomycin C (MMC). The mutant seedlings were clearly more sensitive than the wild-type to all three genotoxic treatments (Figure 1A–C). The mutation was named *mim* (hypersensitive to MMS, irradiation and MMC). The sensitivity of *mim* plantlets to increased osmolarity, oxidative stress, elevated temperature or exogenously added hormonal mediators of stress (abscisic acid and ethylene) was compared with that of the wild-type. With the exception of elevated temperature, the responses of *mim* plantlets to

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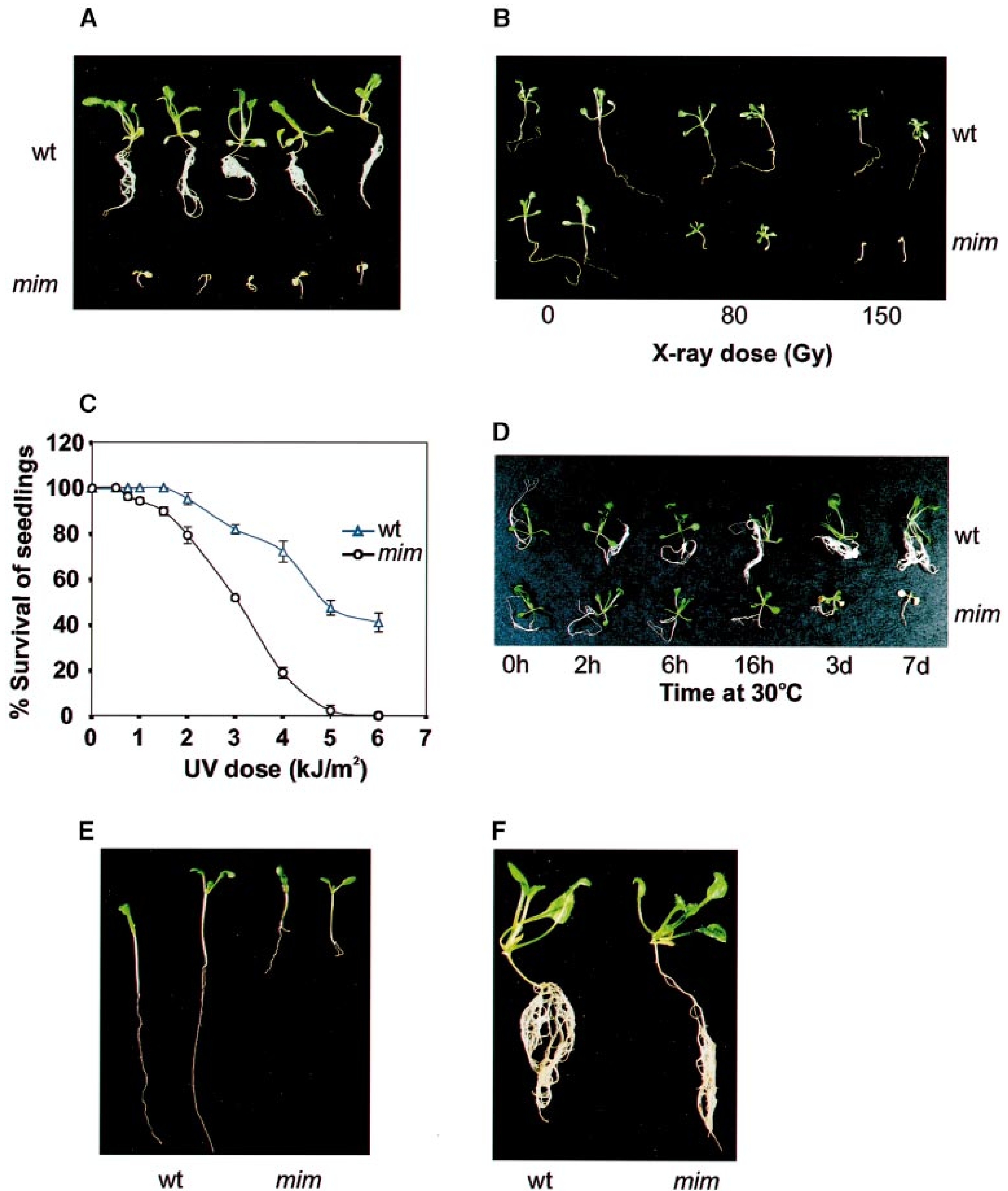

Fig. 1. Phenotypes of the *mim* mutant. Seeds were surface sterilized and plated on plastic plates (Sterilin, square, 10 cm) containing 60 ml of germination medium (Masson and Paszkowski, 1992). Germination was synchronized by cold treatment at 4°C in the dark for 48 h and transferred to a growth chamber with 16 h of light at 24 μE/m^2/s (Osram Natura de Luxe) and 22.5°C and 8 h of darkness at 16°C. Plates were kept in a vertical position. Five-day-old seedlings were (**A**) transferred to liquid nutrient medium in the presence of 100 p.p.m. MMS as described (Albinisky *et al*., 1999; Revenkova *et al*., 1999), (**B**) exposed to X-ray radiation using a Philips 300 kV/9 mA generator at a rate of 5 Gy/min at the doses indicated, (**C**) irradiated with UV-C light (254 nm lamp OSRAM HNS 55W ORF) or (**D**) incubated at 30°C for the periods indicated. In all cases, seedlings were grown further for 4 weeks after treatment. (**E**) Ten-day-old seedlings and (**F**) 4-week-old seedlings grown under standard growth conditions.

all other treatments were not distinguishable from those of the wild-type (Figure 1, and data not shown). Thus *mim* can be clearly distinguished from mutants affected in responses to general stress. In addition, *mim* plants do not appear to be suffering from pleiotropic mutations causing generally weak plants. The *mim* plants were fertile and showed no major developmental abnormalities. However, the growth of *mim* roots was retarded compared

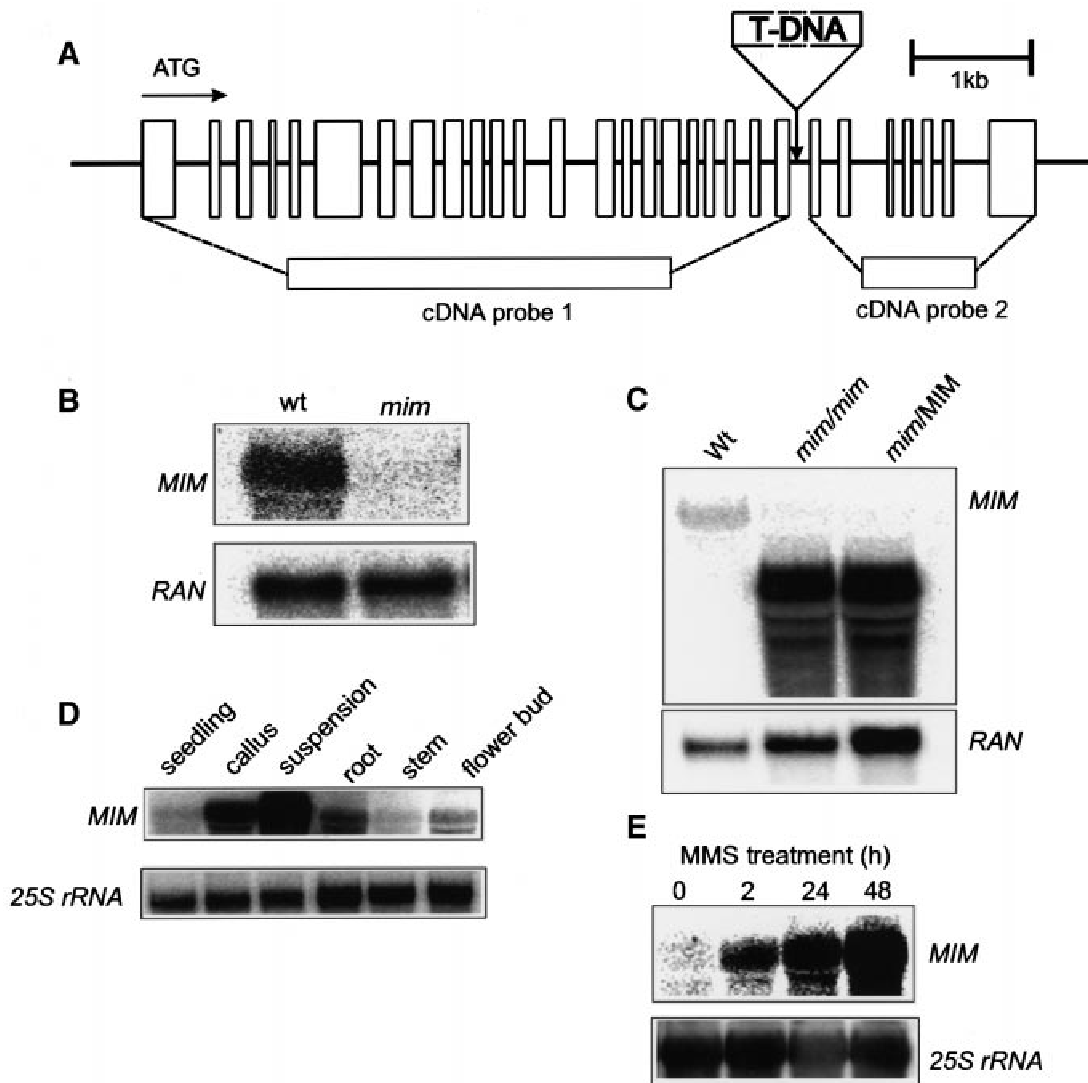

Fig. 2. Organization of the *mim* locus and transcriptional regulation of the *MIM* gene. (**A**) Structure of the *MIM* gene and position of the T-DNA insert. Exons are shown as open boxes (T-DNA is not drawn to scale). (**B**) Northern blot of total RNA (10 μg) extracted from wild-type and *mim* mutant callus hybridized with a fragment of *MIM* cDNA spanning the first 22 exons left of the T-DNA insert (cDNA probe 1). (**C**) Northern blot of total RNA (5 μg each) of wild-type callus and homozygous or heterozygous *mim* seedlings hybridized to a *MIM* cDNA fragment spanning seven exons right of the T-DNA insert (cDNA probe 2). (**D**) Northern blot of total RNA (10 μg) extracted from different plant tissues, callus and suspension culture cells, hybridized to a full-length *MIM* cDNA. (**E**) Northern blot of total RNA (5 μg each) extracted from wild-type suspension culture cells treated before harvest with MMS (100 p.p.m.) for the periods indicated. Cells in lane 0 were not treated with MMS. Blots were hybridized to a full-length *MIM* cDNA probe. All blots were re-hybridized to the constitutively expressed *RAN* gene (Haizel *et al*., 1995) (B and C) or to 25S rDNA (D and E) as loading controls. Blots hybridized to the MIM probe in (E) was subjected to shorter exposure than in (D). Total RNA preparation and hybridization of the blots were as described (Church and Gilbert, 1984).

with the wild-type, but only during a specific phase shortly after germination, when very rapid growth of the primary root occurs (Figure 1E). Later, in the slower growth phase, the mutant seedlings reached a size similar to the wild-type (Figure 1F). The sensitivity of the mutant to elevated temperature was also restricted to the particular period of fast growth.

To identify the mutated gene, plant DNA flanking the right T-DNA border was obtained by plasmid rescue (Bouchez *et al.*, 1996) and used as a probe to screen an *Arabidopsis* genomic library. Three overlapping λ phage clones spanning 10.5 kb of genomic sequence were isolated and sequenced. To determine the sequence of the *MIM* gene transcript, a cDNA library (Elledge *et al.*, 1991) was screened with a 4.2 kb genomic fragment tagged by the T-DNA in the mutant. The longest cDNA clone of 2.25 kb used as a probe on Northern blots detected a single transcript of 3.7 kb in the wild-type (Figure 2B). To assemble the cDNA corresponding to the full-length *MIM* mRNA, RT–PCR and rapid amplification of cDNA ends (RACE) (Frohman *et al.*, 1988) were used. Alignment of *MIM* cDNA with the genomic sequence revealed the presence of 29 exons (Figure 2A). This is rather unusual for *Arabidopsis*, where 30% of the genes are intronless and the majority have no more than five exons (Delseny and Cooke, 1998). The highest number of exons found so far in *Arabidopsis* (25) occurs in the gene coding for the large subunit of RNA polymerase II (Delseny and Cooke, 1998). The T-DNA in the *mim* mutant is inserted in the twenty-second intron (Figure 2A). The *MIM* locus was mapped, using an ordered yeast artificial chromosome (YAC) genomic library of *Arabidopsis* (Creusot *et al.*,

1995), to the bottom of chromosome 5 between the markers *LFY3* and *SEP5A* (Schmidt *et al.*, 1997). Southern blots performed under stringent hybridization conditions showed a single copy of the *MIM* gene. A genomic clone containing the *MIM* gene flanked by 2 kb of a putative promoter sequence was used for complementation of the *mim* mutant. Of 17 independent transformants, 12 showed the wild-type phenotype in MMS, UV, X-ray and MMC sensitivity tests. Slow root growth and thermosensitivity were also corrected, suggesting that the mutation in the *MIM* gene is solely responsible for the *mim*-specific phenotypes (data not shown).

The *MIM* transcript was of low abundance in RNA extracted from 2-week-old seedlings and stems, but showed increased levels in young inflorescences, rapidly growing roots and particularly in callus and suspension culture cells (Figure 2D). Thus, accumulation of the *MIM* transcript correlated well with cell division activity. Considering the DNA damage hypersensitivity of the mutant and a possible role for the *MIM* gene product in DNA repair, expression of the *MIM* transcript after genotoxic treatment was studied. RNA extracted from MMS-treated suspension culture cells had a higher level of the transcript than untreated controls (Figure 2E). This was already apparent after 2 h of MMS exposure, and the transcript accumulated further upon continued MMS treatment. The 5′ end of the transcript (left of the T-DNA) covering two-thirds of the *MIM* sequence (Figure 2A) was absent in the *mim* mutant (Figure 2B). In contrast, the 3′-terminal section of the *MIM* transcript was expressed as multiple, truncated RNAs (Figure 2C) in both *mim/mim* and *mim*/*MIM* plants. Since the phenotype was not correlated with the presence of these RNA species, these short transcripts are not functional and are probably initiated within the T-DNA insert. Thus, the large T-DNA insertion of ~6 kb caused highly aberrant transcription at the *mim* locus and loss of *MIM* gene function.

The *MIM* cDNA encodes a protein of 1055 amino acids (Figure 3A) with extensive homology to proteins of the SMC family (structural maintenance of chromosomes) (reviewed in Strunnikov, 1998) (Figure 3B and C). SMCs are considered to be crucial modulators of chromosome architecture and are involved in chromosome condensation and segregation (Hirano and Mitchison, 1994; Saitoh *et al.*, 1994; Saka *et al.*, 1994; Strunnikov *et al.*, 1995), sister chromatid cohesion (Guacci *et al.*, 1997; Michaelis *et al.*, 1997), transcriptional repression (Chaung *et al.*, 1994) and possibly homologous recombination (Lehmann *et al.*, 1995; Jessberger *et al.*, 1996). The SMC protein family has five conserved structural features: an N-terminal globular domain with an NTP-binding motif (Walker A type), two α-helical regions with potential to form a coiled-coil structure separated by a hinge, and a second globular domain in the C-terminal region harbouring the DA-box, a signature motif for the SMC family (Strunnikov *et al.*, 1993), which includes an NTP-binding motif (Walker B) (Walker *et al.*, 1992). Mutational analysis showed that the DA-box or NTP-binding domains are required for SMC function (Chaung *et al.*, 1994; Strunnikov *et al.*, 1995). The *MIM* gene product is the first plant protein with all these structural attributes (Figure 3D).

The closest relative of *MIM* is the *rad18* gene of *Schizosaccharomyces pombe* with an overall amino acid identity of 26.8%. The sequence conservation is even higher (47% identity) in a 121 amino acid region spanning the N-terminal NTP-binding site, and in 53 amino acids around the DA-box motif (54% identity) (Figure 3B and C). Importantly, the *rad18* gene is epistatic to *RHP51* (*S.pombe* homologue of *RAD51*), which is involved in homologous recombination (Lehmann *et al.*, 1995). However, an effect of *rad18* mutation on recombination has not been demonstrated directly.

To address the question of whether the *MIM* gene product is involved in homologous recombination in *Arabidopsis*, we crossed homozygous *mim* plants with a line transgenic for a recombination substrate derived from the β-glucuronidase (*GUS*) gene (Figure 4A) (Swoboda *et al.*, 1994). The frequency of intrachromosomal recombination could be assessed from the number of somatic sectors which express the *GUS* gene restored by a recombination process. Since the *mim* mutation and the recombination substrate were genetically unlinked, genotypes homozygous for the recombination substrates and homozygous or wild-type for the *mim* mutation could be recovered simultaneously in the F_3 generation. In order to minimize the influence of other segregating traits on the recombination frequency, we used 22 independent F_3 families homozygous for the *mim* mutation (GU-US/GU-US, *mim/mim*) and 20 homozygous for the wild-type gene (GU-US/GU-US, *MIM/MIM*) as a control. A total of 30–50 seedlings from each family were scored for somatic intrachromosomal homologous recombination events by assaying for GUS activity essentially according to Jefferson *et al.* (1987). In each case, the frequency of intrachromosomal recombination in the *mim/mim* mutant background was consistently lower. The average reduction was 3.9-fold relative to the wild-type control plants (Figure 4B). This, together with the DNA damage hypersensitivity of *mim*, implies that the MIM protein is involved in recombinational DNA repair in *Arabidopsis*.

Discussion

We have described an *Arabidopsis* mutant hypersensitive to a range of DNA-damaging treatments which is deficient in intrachromosomal homologous recombination in somatic cells. The affected gene encodes a protein with all the structural properties characteristic of the SMC family of proteins. These proteins are crucial for structural changes of chromatin affecting various activities of the DNA template (transcription, repair) (reviewed in Koshland and Strunnikov, 1996; Jessberger *et al.*, 1998; Strunnikov, 1998; Hirano, 1999). The *mim* mutant phenotype is consistent with a defect in DSB repair (Petes *et al.*, 1991; Game, 1993; Shinohara and Ogawa, 1995). The *mim* plants are hypersensitive to MMC, MMS, UV-C and X-rays. MMC causes DNA cross-links, which represent a unique class of DNA lesions repaired in yeast by components of the excision and recombination repair pathway (Jachymczyk *et al.*, 1981). Inactivation of RAD54, a member of the recombinational DNA repair pathway in vertebrate cells, causes radiation hypersensitivity and reduces the level of homologous recombination (Bezzubova *et al.*, 1993; Essers *et al.*, 1997). Hamster cell lines (irs1 and irs1SF) which display broad-spectrum mutagen sensitivity (ionizing radiation, UV, ethyl methane-

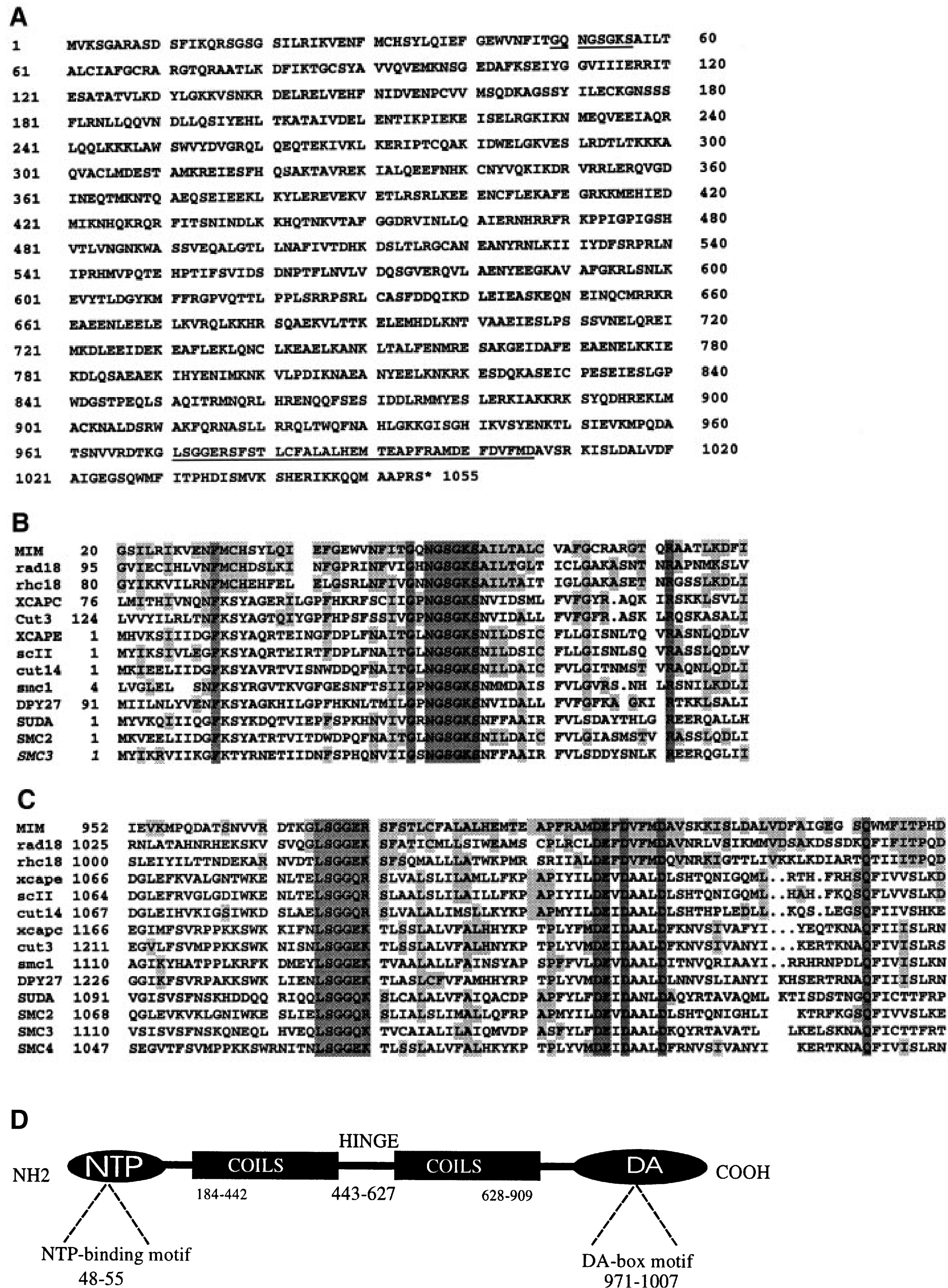

Fig. 3. Comparison of primary and secondary structure of the MIM protein. (**A**) The amino acid sequence encoded by the MIM cDNA is shown as a single letter code. The NTP-binding motif and the DA-box motif are underlined. Alignments of the N- (**B**) and C-terminal (**C**) sequences of MIM and various SMC proteins. The sequences compared are: MIM (*A.thaliana*, DDBJ/EMBL/GenBank accession Nos AF120932 and AF120933 for genomic and cDNA sequences, respectively), RHC18, SMC1, SMC2, SMC3, SMC4 (*S.cerevisiae*), SCII (chicken), Rad18, Cut3, Cut14 (*S.pombe*), XCAP-E, XCAP-*C (Xenopus laevis*), SUDA (*Aspergillus nidulans*) and DPY-27 (*Caenorhabditis elegans*). Dark shading indicates amino acids conserved in all entries, and light shading shows amino acids identical to the MIM sequence. The N-terminal sequence of SMC4 was not available. (**D**) Structural features of the MIM protein: globular domain harbouring the NTP-binding motif (NTP), two α-helices likely to form coiled coils (COILS), a hinge region (HINGE) and a C-terminal globular domain harbouring the conserved DA-box motif (DA) with a predicted helix–loop–helix structure. The amino acid positions that mark the approximate motif boundaries are indicated. The probability of coiled-coil structures in MIMp was analysed as described (Lupas, 1991).

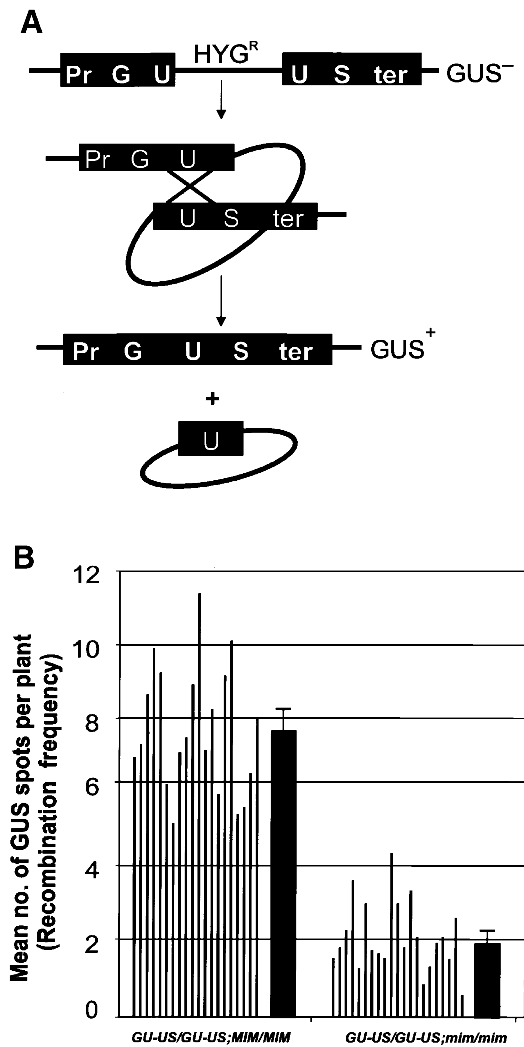

Fig. 4. Intrachromosomal recombination assay. (**A**) A recombination substrate derived from the *Escherichia coli* β-glucuronidase (*GUS*) gene integrated into the *Arabidopsis* line N1DC1 No.11 as a single copy (Swoboda *et al*., 1994) including a promoter (Pr) and terminator (ter) linked to the hygromycin phosphotransferase resistance gene (HYG^R). (**B**) Homologous recombination frequencies in *Arabidopsis* lines homozygous for the recombination substrate and the wild-type allele of *MIM* (*GU-US/GU-US*; *MIM/MIM*) and lines harbouring the *mim* mutation (*GU-US/GU-US*; *mim/mim*). Recombination frequency was determined by scoring the number of somatic recombination events in 30–50 single plant progeny (F_3) seedlings (thin black bar) of both genotypes above. Wide bars represent the mean recombination frequencies for 20 and 22 F_3 populations of *GU-US/GU-US*; *MIM/MIM* and *GU-US/GU-US*; *mim/mim* genotypes, respectively. Standard errors are indicated.

sulfonate and MMC) can be complemented by the human Rad51 family genes *XRCC2* and *XRCC3*, which are implicated in homologous recombination (Liu *et al.*, 1998). Thus, the sensitivity of *mim* to agents provoking DSBs (MMS, UV-C and X-rays) suggests a recombinational repair defect. The two phenotypes of the *mim* mutant not directly related to DNA-damaging treatment (the slower root growth during germination and the sensitivity to higher temperature) may nevertheless be associated with a deficiency in post-replication recombinational repair that could become a limiting factor in rapidly cycling cells. This characteristic of the *Arabidopsis* mutant is in accordance with the phenotype of a *Bacillus subtilis* SMC deletion mutant that can be rescued by lengthening the cell cycle on synthetic media or by lowering the temperature (Britton *et al.*, 1998; Moriya *et al.*, 1998).

The predicted structure of the MIM protein is remarkably similar to that of members of the SMC protein family. SMC proteins are putative ATPases conserved among Bacteria, Archaea and Eukarya (reviewed in Saitoh *et al.*, 1995; Koshland and Strunnikov, 1996; Jessberger *et al.*, 1998; Strunnikov, 1998; Hirano, 1999). The most distinctive structural feature of an SMC protein is the presence of the five domains described for MIM (Figure 3D). Both the N- and C-terminal domains share a high level of sequence conservation among SMC proteins. The structure of the MIM polypeptide, the sizes of the hinge and coiled-coil regions and amino acid sequences at the N- and C-terminals all match other SMCs. However, subtle differences in sequence around the N- and C-terminal domains are apparent between MIM and SMC proteins, and phylogenetic analysis based on these regions confirms slight divergence of the MIM protein from the group of *bona fide* SMCs (data not shown). This is in agreement with a recent comprehensive phylogenetic analysis that recognized four eukaryotic SMCs (SMC1, SMC2, SMC3 and SMC4) (Melby *et al.*, 1998). A further heterogeneous group of proteins was classified as SMC-related, which includes proteins that resemble SMCs. However, compared with this group, MIM, Rad18 and RHC18 represent a separate subfamily closest to the *bona fide* SMCs. This justifies a separate sub-family including MIM within the SMC superfamily, as proposed by Lehmann *et al.* (1995) for Rad18 and RHC18.

SMC proteins have been implicated in homologous recombination. *In vitro*, SMC proteins can perform recombination-related activities such as DNA renaturation (Jessberger *et al.*, 1996; Sutani and Yanagida, 1997). SMC proteins are essential components of the bovine recombination complex (RC-1), which is able to carry out recombination of DNA substrates and repair of gaps and deletions *in vitro* (Jessberger *et al.*, 1996). It has been suggested, based on epistasis analysis, that the *S.pombe* Rad18 and its *Saccharomyces cerevisiae* homologue RHC18 (the closest relatives of MIM) are involved in a recombinational repair pathway (Lehman *et al.*, 1995). However, all eukaryotic SMCs tested so far are essential for viability; homozygous lack-of-function mutations, including the *S.pombe rad18*, are lethal (Lehmann *et al.*, 1995; for review, see Strunnikov, 1998). Since recombinational proficiency is not essential for yeast survival, multiple functions of Rad18 may be postulated. Thus, direct *in vivo* evidence for the involvement of SMC proteins in homologous recombination was missing. In this context, it is rather surprising that homozygous *mim* plants develop normally and that their hypersensitivity is confined to genotoxic stress. This allowed for the first *in vivo* evidence for the involvement of proteins with SMC features in recombinational DNA repair and indicated that the MIM protein has a more specialized function than those of other known SMCs.

Although the exact role of SMCs and SMC-like proteins in DNA repair in general, and in recombination in particular, remains to be determined, it is thought that structural features of chromatin conferred by SMCs are required for recognition of DNA damage and the recruitment of repair

complexes. In plants, suppression of chromosomal recombination is in direct contrast to the efficient extrachromosomal recombination of naked DNA (Puchta and Hohn, 1996). Thus, chromatin remodelling may be a crucial step regulating *in vivo* accessibility of chromosomal DNA for recombination in plants, and our data suggest that SMC-like proteins play an active role in this process.

Materials and methods

Mutant screening and phenotypic characterization

A total of 2300 T-DNA mutagenized lines of *Arabidopsis* generated at the Institute National de la Recherche Agronomique (INRA), Versailles, France were screened for individuals hypersensitive to MMS (Fluka) as described (Revenkova *et al.*, 1999). Plant growth conditions and sensitivity tests for MMS, MMC, abscisic acid, salinity (NaCl), oxidative stress (Rose Bengal, 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein), anti-oxidant (*N*-acetyl-L-cysteine), osmotic stress (mannitol) and temperature were as described (Masson *et al*., 1997; Albinisky *et al.*, 1999; Revenkova *et al.*, 1999).

Cloning of the MIM gene and corresponding cDNA

Plant DNA flanking the T-DNA insert in the *mim* mutant was rescued as described (Bouchez *et al.*, 1996). A 32 bp oligonucleotide, representing the rescued plant sequence (5′-CTGCAGATCTGTTTATGTTAAAGCT-CTTTGTG-3′) was synthesized and end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and was used to probe a λ-ZAPII *Arabidopsis* genomic library (Stratagene). A λ-YES cDNA library (Elledge *et al.*, 1991) was screened with a probe derived from a *MIM* genomic clone flanking the T-DNA insertion in the mutant. Four partial cDNA clones of different lengths were obtained which are transcripts of the same gene. To obtain a full-length cDNA, RNA from suspension culture cells was reverse transcribed using an SP1 primer (5′-AATGACTCTGT-CCCCTCCAAATG-3′) corresponding to the sequence proximal to the 5′ end of the longest partial cDNA clone. The synthesized cDNA was purified (High PCR Purification Kit, Boehringer Mannheim) and amplified with a second specific primer SP2 (5′-ATGTTCGAGGTTATG-AATCTTTG-3′) and a primer (FP1) designed from the genomic sequence (5′-CTGGGTCGGGTTCGATTCTGAG-3′). Sequencing of the PCR product confirmed a 1.2 kb extension of the *MIM* transcript beyond the partial cDNA. The start of the transcript was determined by the RACE procedure (Frohman *et al.*, 1988) (Boehringer Mannheim) with an antisense-specific primer RP1 (5′-GACTCAGTTATCCTGCGTTCG-3′) and two subsequent primers RP2 (5′-GGACAACGGCATAGCTGCAT-CCAG-3′) and RP3 (5′-GGCAGCACGCTGAGTCCCTCTCGC-3′) deduced from the RT–PCR product.

Mapping of the MIM gene

To identify the map position of the *MIM* gene in the *Arabidopsis* genome, the locus disrupted by the T-DNA in the mutant, the *MIM* cDNA was hybridized to membranes carrying the CIC YAC library for *Arabidopsis* (Creusot *et al.*, 1995). The membrane was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Hybridization conditions were as described (Church and Gilbert, 1984).

Intrachromosomal recombination assay

Four-week-old *mim* mutant and wild-type seedlings containing intrachromosomal recombination substrates (Swoboda *et al*., 1994) were subjected to histochemical X-gluc assay as described (Jefferson *et al.*, 1987).

DNA and RNA blot analysis

DNA was extracted as described (Dellaporta *et al.*, 1983) and separated electrophoretically after endonucleolytic digestion. DNA fragments were transferred to nylon membranes (Hybond N, Amersham) using standard protocols (Sambrook *et al.*, 1989). Total RNA was purified using the plant RNeasy Plant Mini kit (Qiagen) according to the supplier's instructions. After standard gel separation and blotting, filters were hybridized as described (Church and Gilbert, 1984).

Complementation of the mim mutant

The genomic clones covering the *MIM* gene and the 5′ and 3′ regulatory signals were assembled in a pAdlox vector (Hardy *et al.*, 1997). The insert was then moved to a binary vector (pHygy5) harbouring a T-DNA with hygromycin phosphotransferase as a selectable marker (E.Revenkova and J.Paszkowski, unpublished). The binary plasmid was introduced into *Agrobacterium tumefaciens* strain C58CIRif^R containing the pGV3101 Ti plasmid (Van Larebeke *et al.*, 1974) and this was used to transform plants by vacuum infiltration (Bechtold *et al.*, 1993). Screening for transformants and analysis of the T2 generation were performed as described previously (Mengiste *et al.*, 1997).

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