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Homologous recombination *in planta* is stimulated in the absence of Rad50

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Chromosomal double-strand DNA breaks must be repaired; in the absence of repair the resulting acentromeric (and telomere-less) fragments may be lost and/or the broken DNA ends may recombine causing general chromosomal instability. The Rad50/Mre11/Xrs2 protein complex acts at DNA ends and is implicated in both homologous and non-homologous recombination. We have isolated a *rad50* mutant of the plant *Arabidopsis thaliana* and show here that it has a somatic hyper-recombination phenotype *in planta*. This finding supports the hypothesis of a competition between homologous and illegitimate recombination in higher eukaryotes. To our knowledge, this is the first direct *in vivo* support for the role of this complex in chromosomal recombination in a multicellular organism and the first description of a mutation of a known gene leading to hyper-recombination in plants.

INTRODUCTION

Understanding of the mechanisms used by eukaryotic cells to repair DNA double-strand breaks (DSBs) has greatly advanced in recent years. One of the most notable aspects of this is the demonstration of the involvement of two general classes of recombination processes, those involving DNA sequence homology between the two partners (homologous recombination, HR) and those independent of such homology (non-homologous recombination or non-homologous end joining, NHEJ). Specific protein factors have been identified as being involved in one or other of these processes (reviewed by Jeggo, 1998; Tsukamoto and Ikeda, 1998; Paques and Haber, 1999).

The preferred mechanism of recombinational DNA repair differs between organisms. NHEJ is much more frequent than HR in mammalian and plant cells, whereas yeast cells rely almost entirely on homology-based recombinational DNA repair.

Mutants showing defects in DSB repair and recombination fall mostly into two general classes: those involved primarily in HR (*rad51/52/54/55/57/59*) or NHEJ (ku, DNA-PK, lig4, Xrcc4) (reviewed by Critchlow and Jackson, 1998; Paques and Haber, 1999); however, one protein complex, Rad50/Mre11/Xrs2, has been implicated in both HR and NHEJ pathways. Yeast mutants lacking this complex are X-ray sensitive and show a weak hyper-HR and strong hypo-NHEJ phenotype in mitotic yeast cells (Malone and Esposito, 1981; Gottlieb *et al.*, 1989; Malone *et al.*, 1990; Schiestl *et al.*, 1994; Milne *et al.*, 1996; Moore and Haber, 1996; Tsukamoto *et al.*, 1996, 1997; Lewis *et al.*, 1999). *In vivo* studies of the roles of this complex come essentially from yeast as animal cells carrying knockouts of the *RAD50/MRE11* genes are not viable (Xiao and Weaver, 1997; Luo *et al.*, 1999; Yamaguchi-Iwai *et al.*, 1999).

In recent years, considerable advances have been made in the understanding of recombination processes in plants, pointing to a conservation of recombination and DSB repair mechanisms between plants and animals (reviewed in Britt, 1999; Gorbunova and Levy, 1999; Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999).

We have recently isolated and characterized the *Arabidopsis thaliana RAD50* homologue (DDBJ/EMBL/GenBank accession No. AF168748) and shown that *rad50* mutant plants are viable, but are sterile and hypersensitive to the radio-mimetic agent methylmethane sulfonate (Gallego *et al.*, 2001). The fact that this plant *rad50* mutant is viable has permitted us to examine *in vivo* the chromosomal recombination processes of a multicellular organism in the absence of a member of this crucial complex.

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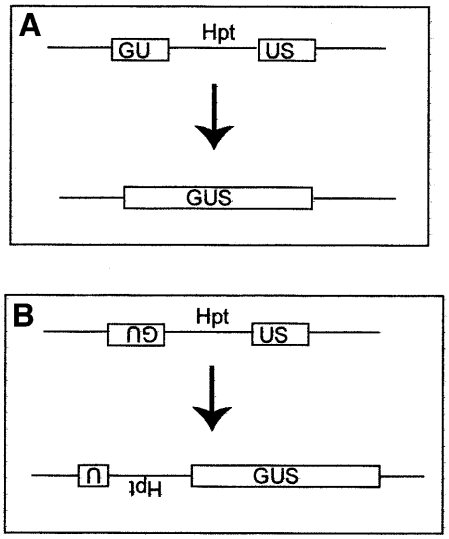

Fig. 1. Schematic representation of recombination substrates. The recombination substrate consists of two fragments of *GUS* (*uidA*) gene separated by a hygromycin selectable marker gene. The GUS sequences have an overlap of 618 bp (indicated by 'U') either in direct (**A**) or inverted orientation (**B**). Hpt, hygromycin phosphotransferase gene.

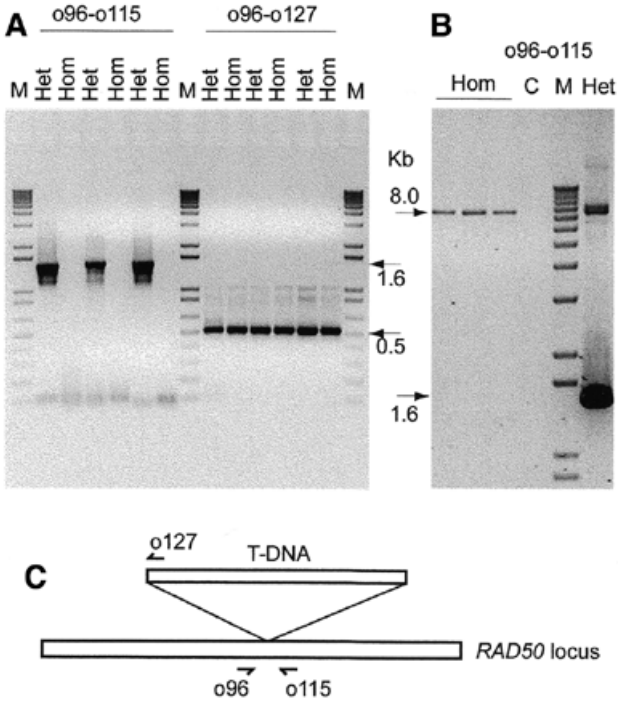

Fig. 2. Identification of individual heterozygote (*rad50/RAD50*) or homozygote (*rad50/rad50*) plants. (**A**) Total DNA amplified with primers o96 and o115 in the *RAD50* sequence, spanning the T-DNA insertion site. Hot-Start *Taq* polymerase (Qiagen) was used for the amplification. The 1.6 kb expected product for the wild-type *RAD50* gene is amplified only in the heterozygote plants. Positive control PCR of total DNA amplified with primers located in the *RAD50* gene (o96) and in the T-DNA (o127) shows the expected 0.5 kb band from amplification of the mutant *rad50* gene in all plants. (**B**) Long PCR of total DNA with the same pair of primers used in (A). Homozygote plants show only the 8 kb expected band, while heterozygotes also show the 1.6 kb band, due to amplification of the wild-type gene. (**C**) Diagram indicating the positions of the oligonucleotide primer sequences relative to the *RAD50* gene and inserted T-DNA in the mutant locus (not to scale). M, molecular weight marker; Het, heterozygote; Hom, homozygote; C, negative control.

RESULTS AND DISCUSSION

We have tested the hypothesis that, in plant cells where DSBs are predominantly repaired by NHEJ, inhibition of this pathway will result in stimulation of HR. The frequency of HR was measured using an *in planta* recombination assay (Swoboda *et al.*, 1994), with a reporter transgene consisting of two overlapping fragments of the *GUS* (*uidA*) gene separated by a hygromycin selectable marker. The *GUS* sequences have an overlap of 618 bp and recombination between the two overlapping DNA sequences can produce a functional *GUS* gene (Figure 1). Sectors of cells expressing GUS activity are detectable by their blue colour after histochemical staining. Heterozygous *rad50* mutant plants and GUS recombination reporter plants were crossed, and the frequency of recombination events was monitored in F3 progeny plants homozygous for the recombination reporter and homo- or heterozygous for the *rad50* mutation (see Methods). PCR analysis was used to confirm the *rad50* genotype of individual plants and examples of this PCR verification are presented in Figure 2.

Mutant *rad50/rad50* and heterozygote *rad50/RAD50* seedlings, each homozygous for the GUS recombination substrate in either inverted or direct repeat configuration, were stained for GUS activity. Numbers of recombination events per plant were determined visually under a dissecting microscope as blue spots and sectors. Previous work has confirmed that these blue-staining spots result from recombination (Swoboda *et al.*, 1994). In two experiments, a total of 112 heterozygote ($Rad50^{+}$) and 120 mutant ($rad50^{-}$) plants were tested for the direct repeat recombination. Figure 3 shows examples of stained mutant (A–C) and heterozygote (D–F) plants. A single population with 59 heterozygote and 55 mutant plants was tested for inverted repeat recombination. Proportions of plants with a given number of spots per plant are shown in Figure 4 and the data are summarized in Table I. For both the direct and inverted repeat populations it can be clearly seen that the homozygote *rad50* mutant plants have considerably more recombination spots than the heterozygote controls. This increase is 9- to 10-fold in the direct repeat recombination and 8-fold in the inverted repeat recombination. Statistical analysis of these results confirms in all cases that the heterozygote and mutant plants belong to different populations with respect to the number of recombination events per plant (Table I; Methods).

Direct repeat structures may recombine productively via gene conversion/reciprocal exchange (GC) or single-strand annealing (SSA) mechanisms, whilst productive recombination events in the inverted repeats occur through GC. The $rad50^{-}$ mutant plants show an 8- to 10-fold stimulation of recombination in both the direct and inverted repeat tester lines. Thus, the detected increase in recombination in the *rad50* mutant plants is presumably

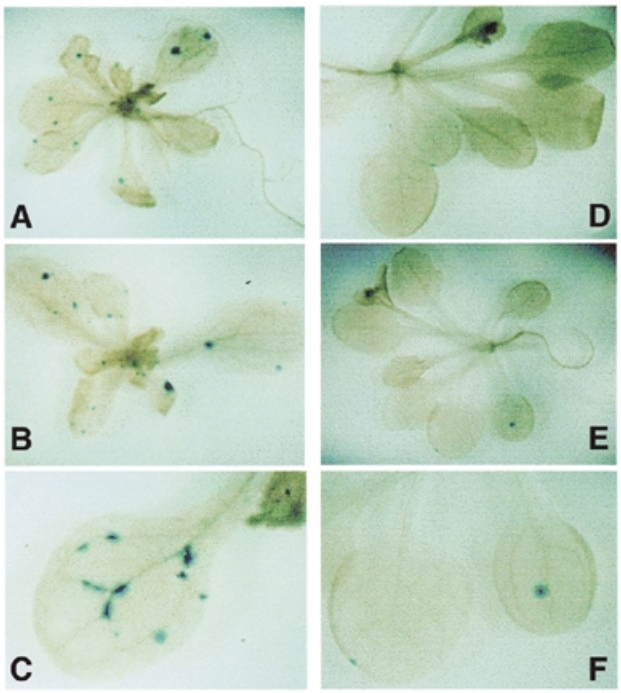

Fig. 3. Examples of blue spots after GUS staining in *rad50* mutant (**A**–**C**) and heterozygote (**D**–**F**) plants.

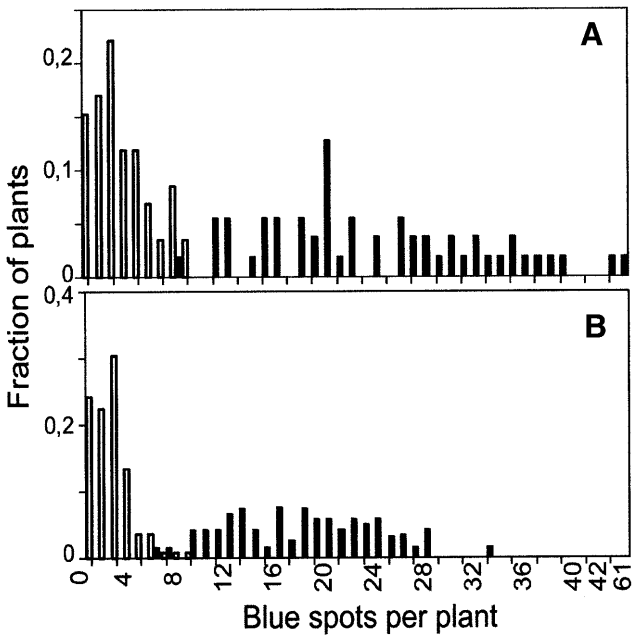

Fig. 4. Frequency distribution histogram showing the proportions of plants with a given number of blue spots in the inverted repeat (**A**) and pooled direct repeat (**B**) populations. Heterozygotes (*rad50/RAD50*) are shown as open bars and homozygotes (*rad50*/*rad50*) as filled bars.

due to an increase in GC. This mitotic hyper-recombination phenotype is reminiscent of that seen in yeast *rad50* mutants, which show 5- to 10-fold increased spontaneous mitotic recombination (Malone and Esposito, 1981; Gottlieb *et al.*, 1989; Malone *et al.*, 1990). The inviability of mammalian *rad50* and *mre11* mutants has precluded such testing of recombination in animal cells (Xiao and Weaver, 1997; Luo *et al.*, 1999), with one exception: using a conditional *mre11* mutant, Yamaguchi-Iwai *et al.* (1999) have shown that gene targeting is reduced in the absence of Mre11 protein in a conditional mre11^{-}, hyper-recombinational chicken DT-40 cell line. Repression of *MRE11* expression in these cells leads to an accumulation of chromosomal DSBs, accumulation at the G_2/M phase and cell death in 4–5 days. The gene targeting assay was carried out 3 days after *MRE11* repression and *MRE11* expression re-induced 12 h later. This assay measures the proportion of stably transformed cells that have integrated the transforming DNA at the homologous chromosomal locus, versus those in which the DNA had integrated elsewhere. Given the presence of significant numbers of random chromosomal DSBs in these dying chicken cells and the highly recombinogenic nature of chromosomal DSBs, we believe it is difficult to make a direct comparison between our chromosomal recombination assay and the gene targeting assay of Yamaguchi-Iwai *et al.* (1999). We are currently undertaking work to test directly whether the mitotic hyper-recombinational phenotype described here for the *Arabidopsis rad50* mutant is accompanied by elevated frequencies of gene targeting in these plants.

Table I. Recombination in *rad50* lines

Line	Heterozygote			Heterozygote				χ^2	
	n	N	X	n	N	X	Stimulation	Homoz.	Heteroz.
1406-2 (DR)	56	94	1.7	59	925	15.7	9	47.6	48.3
1406-8 (DR)	56	101	1.8	61	1103	18.1	10	53.3	56.0
1415-9 (IR)	59	168	2.9	55	1260	22.9	8	69.4	51.3

n, number of plants analysed.
N, total number of blue spots (recombination events).
X, mean number of recombination events per plant.
χ^2 (1-df) values for the null hypothesis that all plants from a given line are from the same population with respect to number of blue spots. Non-parametric statistical analysis was carried out as described in Methods. All values are highly significant, permitting the rejection of the null hypothesis in all cases.

VanDyck *et al.* (1999) have recently presented a model for the channelling of recombination into either HR or NHEJ pathways via the competition between the Rad52 and Ku proteins, respectively, for a common substrate (see Haber, 1999). This substrate results from the Rad50/Mre11/Xrs2-dependent resection of the DSB to produce single-stranded extensions. Yeast *rad50* mutants show increased mitotic recombination (Malone and Esposito, 1981; Gottlieb *et al.*, 1989; Malone *et al.*, 1990) and are deficient in NHEJ (Schiestl *et al.*, 1994; Moore and Haber, 1996; Tsukamoto *et al.*, 1996). In this context, the hypo-NHEJ and hyper-HR phenotype of these mutants implies that, at least in yeast, the Ku-dependent NHEJ pathway has greater dependence on the prior (or concomitant) action of the Rad50/Mre11/Xrs2 complex than the Rad52-dependent HR pathway. The resulting reduction in the efficiency of NHEJ in the absence of Rad50 would make more substrate available for HR. Our description of a hyper-recombination phenotype in the *Arabidopsis rad50* mutant raises the attractive possibility that this situation also exists in plants and possibly other multicellular organisms.

METHODS

Plants. Recombination tester lines were produced by *Agrobacterium*-mediated transformation (Clough and Bent, 1998) of *A. thaliana* ecotype 'Columbia' plants, and two lines homozygous for a single chromosomal copy of the reporter construct were selected (data not shown). The recombination substrates (Tinland *et al.*, 1994) consist of two fragments of the β-glucuronidase (GUS) gene with 618 bp of overlap, either in direct (pGU.US; line 1406) or inverted orientation (pU'G'.US; line 1415) (Figure 1).

The two GUS tester lines were crossed to the heterozygous *rad50/RAD50 Arabidopsis* mutant. The mutant *rad50* allele in this line results from a T-DNA insertion into the *RAD50* gene and has been described elsewhere (Gallego *et al.*, 2001). Resulting F1 progeny, heterozygous for the *rad50* mutant allele (kanamycin resistant) and hemizygous for the GUS recombination tester substrate (hygromycin resistant), were allowed to self-fertilize to produce F2 seed. Individual F2 plants were allowed to self-fertilize to produce F3 seed and samples of this seed were screened for the segregation of the kanamycin and hygromycin resistance markers to identify the F2 plants homozygous for the GUS recombination substrate (homozygous for the hygromycin resistance marker) and heterozygous for the mutant *rad50* allele (heterozygous for the kanamycin resistance marker).

Recombination assays. F3 seed from plants homozygous for the GUS recombination substrate and heterozygous/homozygous for the mutant *rad50* allele was allowed to germinate on agar plates containing 50 mg/l kanamycin (the mutant *rad50* allele is marked by kanamycin resistance). Kanamycin-resistant seedlings (*RAD50/rad50* and *rad50/rad50*) were stained for GUS activity at 3–4 weeks of age. The GUS histochemical staining was carried out as described by Jefferson (1987) and Swoboda *et al.* (1994). The number of blue spots or sectors, each resulting from a recombination event, on each seedling was then determined visually under a dissecting microscope. For each line, ~50 *rad50/RAD50* heterozygotes and 50 *rad50/rad50* homozygotes were tested (see Table I). Initial visual identification of the *rad50/rad50* homozygote seedlings at this stage was based on the fact that they are slightly smaller than the heterozygotes and their leaves are rougher. The *RAD50* genotype of the individual plants was then confirmed using PCR as described below.

PCR analysis. PCR analyses were used to determine the *RAD50* genotype of individual plants. Genomic DNA was extracted from a single seedling leaf according to Berthold *et al.* (1993). PCR and long PCR conditions have been described previously (Gallego *et al.*, 2001). Two primer pairs were used: o96 (5'-GAGCTG-TGAAGCTAGAAAGAATGAACTTGCAGGTG) and o115 (5'-CCC-ATCCAGGTTTGTAGTTG), which span the T-DNA insertion in the mutant *rad50* allele and amplify 1.6 or 8 kb fragments from the wild-type or mutant alleles, respectively; and o96 and a primer in the T-DNA sequence (o127: 5'-CTGATACCA-GACGTTGCCCGCATAA), which amplify a 500 bp fragment from the mutant *rad50* allele. Positions of the primers on the mutant *rad50* locus are shown schematically in Figure 2C.

Statistical analysis. The data on the number of recombination events per plant were analysed non-parametrically (with the kind advice of Dr E.J. Louis, University of Leicester, UK). All plants analysed from a given line were ranked by the number of blue spots for each set of plants analysed. On the null hypothesis that the plants analysed from each line belong to the same distribution, half should be below the mid-point of the ranking and half above. χ^2 (1 degree of freedom) values were calculated for the heterozygote and homozygotes from each line (see Table I).

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