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Two roles for Rad50 in telomere maintenance

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We describe two roles for the Rad50 protein in telomere maintenance and the protection of chromosome ends. Using fluorescence *in situ* hybridisation (FISH) and fibre-FISH analyses, we show that absence of AtRad50 protein leads to rapid shortening of a subpopulation of chromosome ends and subsequently chromosome-end fusions lacking telomeric repeats. In the absence of telomerase, mutation of *atrad50* has a synergistic effect on the number of chromosome end fusions. Surprisingly, this 'deprotection' of the shortened telomeres does not result in increased exonucleolytic degradation, but in a higher proportion of anaphase bridges containing telomeric repeats in *atrad50/tert* plants, compared to *tert* mutant plants. Absence of AtRad50 thus facilitates the action of recombination on these shortened telomeres. We propose that this protective role of Rad50 protein on shortened telomeres results from its action in constraining recombination to sister chromatids and thus avoiding end-to-end interactions.

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Introduction

Telomeres, the nucleoprotein structures of the ends of linear chromosomes, play essential roles in chromosome stability. In general, telomeres are composed of tandem repeats of a short sequence, $(TTAGGG)_n$ in vertebrates (Moyzis *et al*, 1988) and $(TTTAGGG)_n$ in *Arabidopsis* (Richards and Ausubel, 1988), although this does not hold for all organisms studied (reviews by Cech, 2004; Tomaska *et al*, 2004; Fajkus *et al*, 2005). Notwithstanding the role of telomeres in 'hiding' chromosome ends from DNA double-strand break repair (DSBR) and recombination, many DSBR proteins play essential roles in the maintenance and function of telomeres (reviews by d'Adda di Fagagna *et al*, 2004; Gallego and White, 2005; Slijepcevic and Al-Wahiby, 2005).

The Rad50 protein, with Mre11 and Xrs2/Nbs1, forms part of the highly conserved MRN complex, which plays key roles in the metabolism and repair of DNA breaks (Connelly and Leach, 2002; D'Amours and Jackson, 2002; Lisby and Rothstein, 2004; Stracker *et al*, 2004), as well as in telomere structure and function (d'Adda di Fagagna *et al*, 2004; Gallego and White, 2005; Zhang *et al*, 2006). The Rad50 protein consists of a globular head with nucleotide binding domains and a long coiled-coil tail, which has been shown to bridge DNA molecules (Anderson *et al*, 2001; de Jager *et al*, 2001; Hopfner *et al*, 2001) via interactions between two Rad50 tails through their zinc-hook domains (Hopfner *et al*, 2002; Wiltzius *et al*, 2005). Rad50 is required both for telomere length homeostasis and telomere capping in yeast cells (Nugent *et al*, 1998; Mieczkowski *et al*, 2003) and in mammals the Rad50 protein is present at telomeres through its interaction with TRF2 (Zhu *et al*, 2000). It has recently been shown that MRN is essential for capping *Drosophila* telomeres (Bi *et al*, 2004; Ciapponi *et al*, 2004), showing that the role of this complex is not specific to telomerase-mediated telomere structures.

The results presented here concern the role of the AtRad50 protein in telomere metabolism in *Arabidopsis* plants. By using fluorescence *in situ* hybridisation (FISH) and fibre-FISH in preparations from dividing cells, we found that *atrad50* mutant plants present frequent end-to-end chromosome fusions lacking telomeric repeats. In the absence of telomerase, mutation of *atrad50* has a synergistic effect on the number and type of chromosome end fusions. Surprisingly, FISH analyses show a higher proportion of anaphase bridges containing telomeric repeats in *atrad50/tert* plants, as compared to the single *tert* mutant plants. We conclude that the AtRad50 protein plays an essential role in telomere capping through mechanisms that differ between normal and shortened telomeres.

Results

High frequencies of mitotic anaphase bridges in atrad50 mutant plants

Meiotic defects result in the complete sterility of *atrad50* mutant plants (Gallego *et al*, 2001; Bleuyard *et al*, 2004). Mutant plants are thus always first generation and it is not possible to follow the effects of the absence of the AtRad50 protein across multiple generations. In order to get around this constraint, we have previously worked with cell-suspension cultures and shown that absence of AtRad50 leads to rapid loss of telomeric DNA (Gallego and White, 2001). Notwithstanding the rapidity of this loss in mutant cell cultures, this effect is not detectable by telomere restriction fragment (TRF) analyses on genomic DNA from (first generation) *atrad50* mutant plants, presumably owing to the presence of a mixed population of degraded and normal telomeres for each individual chromosome end in the different cells of the plant (Gallego and White, 2001). Such loss is, however, expected to result in chromosome and chromatid fusions and these should be detectable in mitotic cells of mutant plants as anaphase bridges.

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Table I Quantification of mitotic anaphases with visible chromosome bridges in 12 wild-type and 12 *atrad50* mutant plants

	Wild type			*atrad50*		
Plant No.	Anaphases	With bridges	Plant No.	Anaphases	With bridges	Fraction
1	340	0	1	340	38	0.11
2	300	0	2	300	34	0.11
3	250	0	3	100	14	0.14
4	165	0	4	300	57	0.19
5	300	0	5	300	28	0.09
6	200	0	6	100	15	0.15
7	200	0	7	200	23	0.12
8	200	0	8	300	34	0.11
9	200	0	9	247	23	0.09
10	200	0	10	80	13	0.16
11	200	0	11	300	36	0.12
12	200	0	12	300	39	0.11
Total	2755	0	Total	2867	354	
					Mean	0.13 (0.03)

The mean (standard deviation) fraction observed is given at bottom left.

Screening of mitotic anaphases shows significantly higher levels of chromosome bridges in *atrad50* mutant plants compared to wild-type controls. Mitotic figures were analysed from pistil cells of 12 different *atrad50* mutant plants. Of 2867 anaphases, 354 showed visible chromosome bridges (12.35%), with a mean percentage of bridge-containing anaphases in each plant of 12.75% (s.d. = 2.75%). No anaphase bridges were detected in 2755 anaphases from 12 wild-type plants (Table I). These results are in agreement with the data recently published by the Riha laboratory showing high levels of such bridges in the *Arabidopsis atmre11*-3 mutant (Puizina *et al*, 2004) and with our previous report of rapid telomere shortening in *atrad50* mutant cell cultures (Gallego and White, 2001).

Loss of telomeric DNA in atrad50 chromosome fusions

The effect of absence of AtRad50 (or AtMre11) is far more drastic than that of the absence of telomerase, with such levels of anaphase bridges only observed after the sixth or seventh generation *tert* mutants (Riha *et al*, 2001). What is the origin of these fusions, given the absence of observable telomere shortening in the *atrad50* mutant plants by TRF analyses? On the hypothesis that a subpopulation of chromosome ends has undergone drastic shortening, it is to be expected that these shortened chromosome ends will be overrepresented in the population of fused chromosomes. As the microscope permits us to observe individual fusions in individual cells, we quantitated the presence or absence of telomeric DNA in chromosome bridges in mitoses using FISH on mitotic cells from *atrad50* mutant plants.

Two sorts of probes were used for FISH analyses: a telomeric DNA repeat probe consisting of a tetramer of the TTTAGGG *Arabidopsis* telomeric repeat sequence (the same probe as that used for TRF analyses) and mixture of nine chromosome-end-specific subtelomeric BAC probes identified from the *Arabidopsis* genome resource, TAIR (http://www.arabidopsis.org). The BAC probes permit identification of nine of the 10 chromosome ends of *Arabidopsis* (the 10th has a long stretch of subtelomeric rDNA repeats and is thus not suited to this approach). FISH analysis of mitoses in *atrad50* mutant plants showed hybridisation to subtelomeric probes in 34 of 64 anaphase bridges analysed (Figure 1).

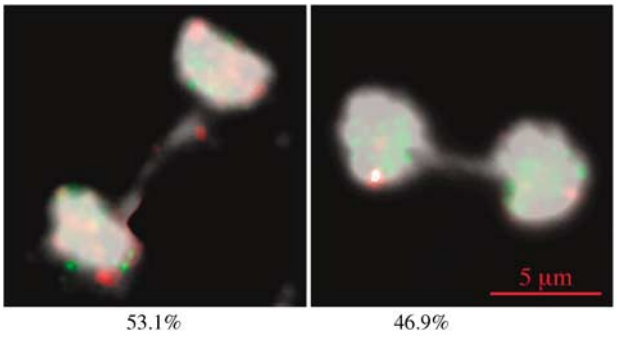

Figure 1 End-to-end chromosome fusions in *atrad50* plants. DAPI-stained mitotic nuclei from *atrad50* plants analysed by FISH using telomeric repeat (green) and the nine subtelomeric (red) fluorescent probes. 53.1% (34/64) of chromosomal bridges showed hybridisation with the subtelomeric probe, whereas the others (30/64) showed no hybridisation to the FISH probes. No telomeric repeat signals were detected associated to the subtelomeric signals in the bridges.

Thus, at least 53% of the observed chromosome fusions include a chromosome end. Interestingly, no telomeric repeats were detected associated to the subtelomeric signals. The FISH detection limits for this probe are estimated to be several hundred base pairs. We hypothesise from this observation that the absence of AtRad50 protein induces a rapid and drastic loss of telomeric repeats and that these chromosome ends are then recognised and repaired as double-strand breaks (DSBs).

To confirm the absence of telomeric repeat sequences in *atrad50* end-to-end chromosome fusions, we increased the resolution of the analysis by using FISH on extended DNA fibres (fibre-FISH). The exact lengths and positions of the subtelomeric BACs are known, as are the distances to the ends of their respective chromosomes. Figure 2 presents the results of hybridisation of extended DNA fibres from *atrad50* plants to a mix of all nine subtelomeric probes and the telomeric repeat probe as well as the positions, sizes and the distances to the beginning of the telomeric repeats of each of the nine subtelomeric BACs on the five *Arabidopsis* chromosomes. Our results show the expected degree of stretching of 2 kb/μm (Michalet *et al*, 1997). The lengths of

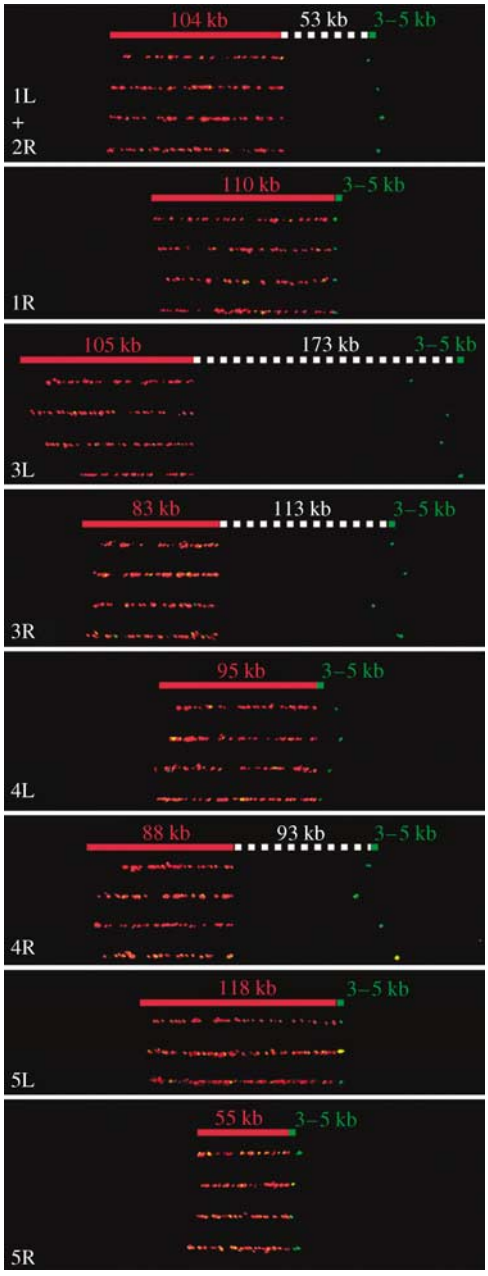

Figure 2 Validation of telomeric repeat (green) and the nine subtelomeric BAC (red) probes by fibre-FISH hybridisation of *atrad50* genomic DNA. Each panel shows a schematic with predicted lengths above the fibre-FISH images for a given chromosome end (indicated at the bottom left of each panel). Correspondence between predicted sizes from the *Arabidopsis* genome sequence and the fibre-FISH measurements confirms the specificity and the validity of FISH probes.

the different subtelomeric signals and the distances to the telomeric repeats permit individual identification of seven of the nine probed chromosome ends, with chromosome 1 left end being indistinguishable from that of the right end of chromosome 2. The fibre-FISH measurements are given in Table II, although probes for ends 1R and 5L are similar in length and position relative to the telomeric sequences, the mean lengths differ significantly from each other (Student's *t*-test: $t = 4.69$; $P = 0.18$). Chromosome 2L was not analysed as it consists of an rDNA repeat array.

Table II Means and standard deviations (in parentheses) of measured lengths in kb of chromosome ends from fibre-FISH analyses (see Figure 2)

End	SubTel	Gap	Tel	N
1L and 2R	107.6 (3.8)	63.5 (7.4)	3.1 (1.0)	11
1R	108.8 (2.8)	—	2.9 (1.4)	4
3L	89.1 (12.5)	155.6 (12.7)	2.9 (0.6)	4
3R	75.4 (2.9)	109.0 (8.4)	2.9 (1.4)	5
4L	94.2 (4.0)	—	2.6 (0.7)	5
4R	86.3 (7.6)	89.7 (10.8)	3.2 (1.1)	4
5L	115.4 (0.2)	—	3.2 (0.7)	3
5R	55.7 (4.2)	—	3.3 (1.1)	4

Except for chromosome ends 1L and 2R, all ends are individually identifiable from the subtelomeric BAC probe hybridisation (SubTel) and the distance (Gap) of this to the telomeric repeat sequence (Tel) at the end of the chromosome. Chromosome 2L was not analysed as it consists of an rDNA repeat array. The number of fibres analysed in each case is given (*N*).

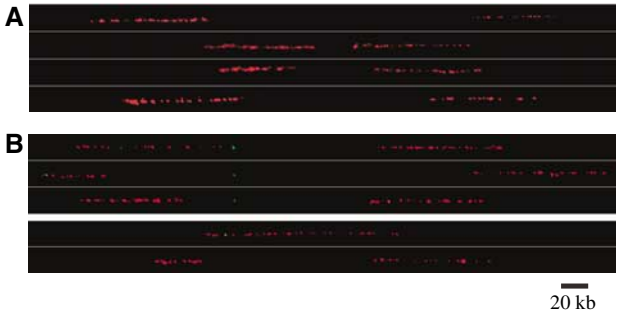

Figure 3 Absence of telomeric repeats in *atrad50* end-to-end chromosomal fusions. Fibre-FISH of genomic DNA from *atrad50* (**A**) and *tert* (**B**) plants, hybridised with telomeric repeat (green) and the nine subtelomeric BAC (red) probes. Chromosomal fusions are characterised by the alignment of two subtelomeric signals on single fibre and any telomeric signals appear between the two subtelomeric signals. In contrast to the *tert* mutant (B), no telomeric repeat signals were observed in the *atrad50* chromosomal fusions (A).

Chromosome end-to-end fusions are identified as two subtelomeric signals juxtaposed in the same DNA fibre. Figure 3A shows several examples of these, none of which show detectable telomeric repeat signal between the two subtelomeric regions. In total, we identified eight end-to-end chromosome fusions from *atrad50* mutant plants and none presented any detectable telomeric repeat signal.

In the absence of telomerase, *Arabidopsis* plants show progressive loss of telomeric repeats finally giving rise to the production of anaphase bridges from generation 6 (Riha *et al*, 2001). Using a polymerase chain reaction (PCR) approach, Heacock *et al* (2004) found end-to-end fusions both with and without telomeric repeats in tert mutant plants. We have carried out similar PCR analyses, confirming these results on *tert* mutant plants (data not shown). Parallel experiments failed to amplify chromosome-end fusion junctions from the *atrad50* mutant plants, in agreement with our data showing more important losses of telomeric and subtelomeric DNA at fusion junctions in these plants (data not shown).

In order to confirm these data and to be able to compare it directly with the *atrad50* data (above), we carried out fibre-FISH on DNA fibres from nuclei of *Arabidopsis* generation 6 *tert* mutant plants. Figure 3B shows that chromosome fusions

both with and without detectable telomeric repeats were observed in the *tert* mutants (10 fusions analysed). These results contrast clearly with the absence of detectable telomeric repeats in chromosome end fusions of the *atrad50* mutant plants. We thus conclude that the majority of chromosome ends participating in chromosome fusions in the *atrad50* mutant plants have thus been subjected to variable and extensive terminal deletions. Unlike the progressive and relatively uniform loss of telomeric repeats that occurs in *tert* mutants, some chromosomes abruptly lose their telomeres in a single generation *atrad50* mutants, resulting in significant genomic instability.

AtRad50 protects shortened telomeres in the absence of telomerase

AtRad50 protein is thus essential for the protection of telomeres in *Arabidopsis* and the absence of this protein leads to significant terminal degradation of chromosomes and frequent chromosome fusions. The resulting dicentric chromosomes and bridge–breakage–fusion cycles give rise to significant genomic instability. Given the known roles of the Rad50 protein in DNA DSBR and in telomere maintenance, we posed the question of whether the severe chromosome degradation/fusion phenotype of the *atrad50* mutant is solely due to the role of AtRad50 in maintaining the telomeric cap structure, or whether this protein (and presumably the MRN complex?) plays other roles in chromosome-end protection?

In order to answer this question, we analysed the potential role of AtRad50 function on short telomeres generated in the telomerase mutant. Successive generations of telomerase mutant plants show progressively shorter telomeres and fourth or fifth telomerase mutant generation plants start to show anaphase bridging. We constructed a telomerase mutant *tert-/atrad50* heterozygote line and analysed the frequency of mitotic anaphase bridges in *tert−/ATRAD50+* and *tert−/atrad50−* sister plants in successive generations. This approach thus permitted the study of the effect of the removal of the AtRad50 protein in plants with successively shorter telomeres.

Figure 4 presents the results of this analysis for third, fourth and fifth telomerase mutant generation plants. Mitotic figures from pistil cells were analysed for consecutive generations of the *atrad50/tert* double-mutant plants and *atrad50* and *tert* single-mutant plants. For each generation and mutant, 200–300 anaphases were examined from pistils isolated from 5–6 individual plants. 7.5–9.7% of anaphases from *atrad50* mutant plants show bridged chromosomes independent, as expected, of the generation analysed. In telomerase mutant generation 1, similar levels of chromosome bridges were observed in *atrad50* single-mutant and *atrad50/tert* double-mutant plants. However, in telomerase mutant generation 3, *atrad50/tert* plants presented 16.7% of anaphases with bridges, whereas the single *tert* mutant plants show 1.7% and *atrad50*, 7.4%. Thus, *atrad50/tert* double-mutant plants presented twice as many chromosome bridges than that expected from simple addition of the individual effects of each single mutation. A similar synergistic effect was observed in telomerase mutant generation 4, with 21.6% of fusions in *atrad50/tert* double-mutant plants compared to 8.7% for *atrad50* and 1.6% in *tert* single mutants. These results thus clearly show a synergistic effect of the

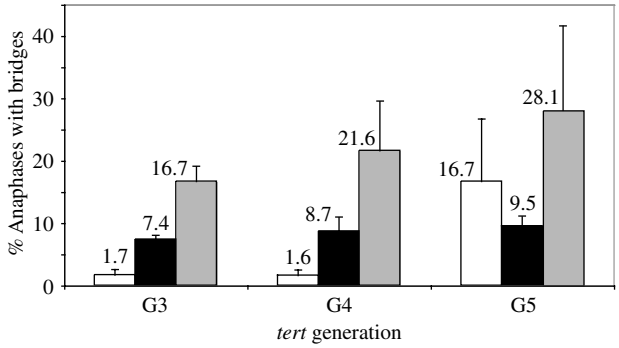

Figure 4 Synergistic effect of *atrad50* and *tert* mutations on the frequency of mitotic anaphases presenting at least one chromosome bridge. Data for *tert* (unfilled), *atrad50* (black) and double *tert/atrad50* (grey) mutants are shown. For each generation and mutant, approximately 250 anaphases were examined from 5 to 6 experiments of mitotic cells from flower pistils.

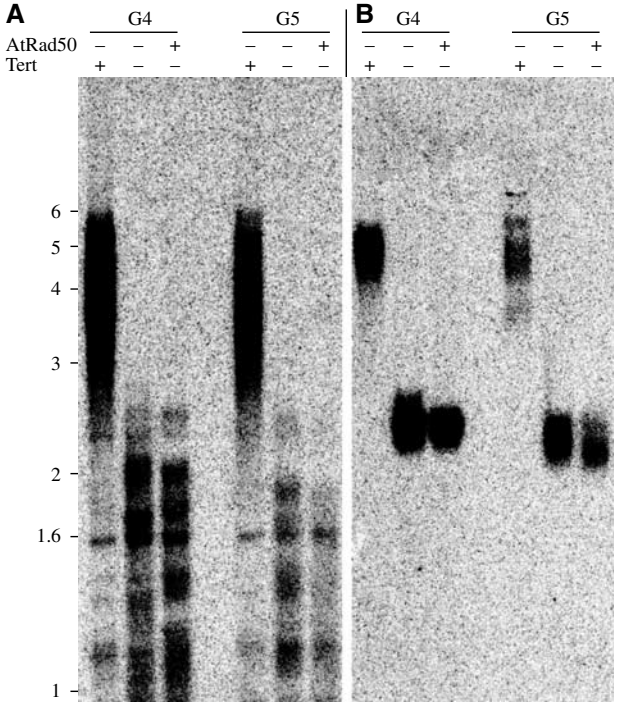

Figure 5 TRF analysis of bulk telomere lengths in DNA from flower buds from *atrad50*, *tert* and *atrad50/tert* double mutants of *tert* mutant generations 4 and 5. Southern analysis of Mbo1-digested total DNA using the telomeric repeat probe (**A**) and by the chromosome II–subtelomeric-specific probe (**B**). Positions of DNA size markers (kb) are shown to the left of the panels.

two mutations, with absence of AtRad50 protein exacerbating the effects of the telomere shortening produced by the absence of telomerase. The observed synergism of the two mutants supports a dual protective role for AtRad50 on normal and on shortened telomeres.

Two hypotheses can be proposed to explain these observations: either the higher levels of fusions seen in the double mutant result from an increased rate of telomeric repeat loss in the absence of AtRad50, or that the shortened telomeres in the telomerase mutant are 'protected' from the action of recombination by AtRad50 protein. In order to distinguish between these two hypotheses, we carried out TRF analysis of

the telomerase mutant generation 4 and 5 plants, in the presence or absence of the AtRad50 protein (Figure 5). No acceleration of telomere repeat loss is observed in double *tert*/*atrad50* mutant plants with respect to single *tert* mutants. Thus in cells with shortened telomeres, the lack of the AtRad50 protein provokes telomere dysfunction without accelerated loss of telomeric repeats. This result argues in favour of an active role for AtRad50 on shortened telomeres in 'protecting' them from recombination.

To better understand the role of AtRad50 in the protection of shortened telomeres, we analysed the structure of the chromosome fusions from *atrad50*/*tert* and *tert* plants by FISH. Figure 6 shows FISH analysis of mitoses from generation 5 *tert* mutant plants and double-mutant *tert*/*atrad50* sister plants. 38.1% of mitotic anaphases from the telomerase mutant generation 5 plants show detectable telomeric repeat DNA sequence in the bridge. Surprisingly, the double-mutant sister plants showed an even higher level, with 48.2% of

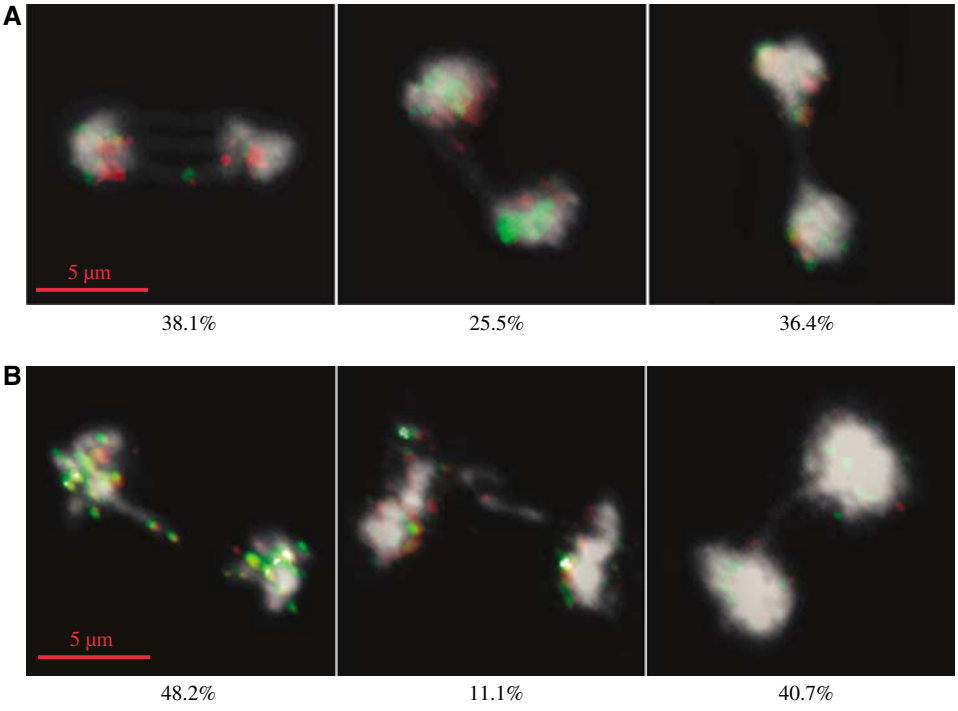

Figure 6 DAPI-stained mitotic nuclei from fifth *tert* generation: *tert* (**A**) and *tert*/*atrad50* (**B**) mutants analysed by FISH with telomeric repeat (green) and the nine subtelomeric BAC (red) fluorescent probes. (A) In the *tert* mutant, 38.1% (21/55) chromosome bridges showed subtelomeric and telomeric signals, 25.5% (14/55) only subtelomeric signal and 36.4% (20/55) neither telomeric nor subtelomeric signals. (B) In the double *tert*/*atrad50* mutant, 48.2% (13/27) chromosome bridges showed subtelomeric and telomeric signals, 11.1% (3/27) only subtelomeric signal and 40.7% (11/27) neither telomeric nor subtelomeric signals.

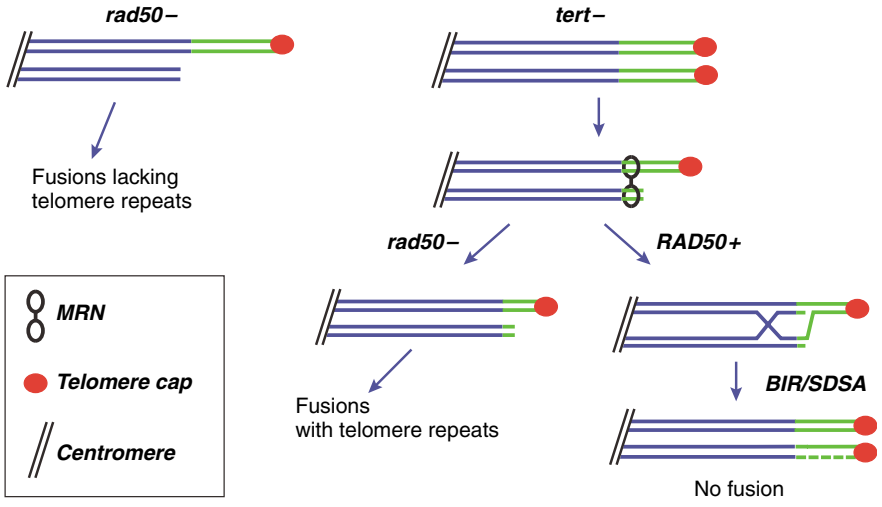

Figure 7 A model to explain the two roles of Rad50 in telomere maintenance. In the absence of Rad50, breakage or incomplete replication of some chromosome ends result in fused chromosomes lacking telomeric repeat DNA. Telomere erosion in the tert mutant results in uncapping of some telomeres, which are then recognised as DSBs by the cellular recombination machinery. MRN constrains this recombination to the sister chromatid via BIR or SDSA and thus favours repair of the telomere. In the absence of Rad50, this preference for the sister chromatid is lost and more chromosome fusions are found in the double tert/atrad50 mutant. Each line represents a DNA strand and each group of four lines represents a pair of chromatids. Telomere repeat sequence is green.

bridge-containing anaphases showing telomeric repeat DNA in the bridge. This result was confirmed in the generation 4 *tert/atrad50* mutant plants (50.7%). Thus, absence of AtRad50 protein accelerates the appearance of chromosome fusions in the *tert* mutant plants, but these fusions include telomere repeat DNA characteristic of the *tert* mutants, rather than the absence of telomere repeat DNA characteristic of *atrad50* mutants. Absence of the AtRad50 protein thus increases the probability of fusion of shortened telomeres (due to absence of telomerase).

Discussion

Through analysis of *Arabidopsis atrad50* mutants, both alone and in conjunction with the telomerase mutant, we describe two roles for the Rad50 protein in telomere maintenance. Absence of AtRad50 protein leads to rapid (within a single generation) shortening of a subpopulation of chromosome ends and subsequently chromosome-end fusions lacking telomeric repeats. In addition to this effect, in the telomerase mutant, absence of AtRad50 'deprotects' partially eroded chromosome ends, greatly stimulating the probability of their participation in chromosome fusions. This synergy in the double *tert/atrad50* mutant thus results in a mixed population of 'rad50-minus' and 'tert-minus' chromosome-end fusions, lacking or including telomeric repeats at the fusion points, respectively. Given that we do not observe any effect of the *atrad50* mutant on telomeric DNA erosion in TRF analysis of the *tert* and *tert/atrad50* mutants, the 'deprotection' of the shortened telomeres does not result in increased general exonucleolytic degradation, but seems to act specifically in facilitating the access of recombination to these shortened, but still functional telomeres.

Arabidopsis atrad50 plants show high levels of chromosome fusions lacking telomere repeat DNA sequences at the fusion points. Such rapid chromosomal instability appearing in first generation has also been observed in *Arabidopsis atm atr* double-mutant plants (Vespa *et al*, 2005). Based on specific PCR amplification to detect chromosomal end-fusions, these authors suggested that telomeres are rapidly degraded in these mutants before fusion. Null mutations in either *MRE11* or *RAD50* in mice are early-embryonic lethal, preventing cytological analysis of chromosomes. However, mice homozygous for the hypomorphic mutation *rad50s* show low levels (0.1–0.2 per metaphase) of end-to-end fusions presenting telomeric repeats (Bender *et al*, 2002). A role for Rad50 in telomere protection has also been reported in yeast and *Drosophila* (Mieczkowski *et al*, 2003; Bi *et al*, 2004; Ciapponi *et al*, 2004), indicating that this essential role of the Rad50 protein in telomere maintenance is conserved in most eukaryotic cells.

The rapid loss of telomere repeats of only a subpopulation of chromosomes in *Arabidopsis atrad50* plants is reminiscent of the telomeric rapid deletion (TRD) observed in yeast and mammals (Lustig, 2003). TRD results from the telomeric t-loop structure being resolved by homologous recombination (HR) to yield a shortened telomere in one step (Li and Lustig, 1996; Bucholc *et al*, 2001; Wang *et al*, 2004). Arguing against this interpretation, however, is the dependence of TRF2ΔB-induced t-loop HR on Nbs1 in mammals (Wang *et al*, 2004) and the fact that TRD in yeast is abrogated in the absence of Rad50 or Mre11 (Bucholc *et al*, 2001). Thus, HR-dependent rapid loss of telomeres in yeast and mammals is dependent on MRX (or MRN) complex function. Our preliminary studies have failed to detect the appearance of telomeric repeat circles in *atrad50* mutant plants (data not shown) and we are currently crossing *atrad50*, with mutants defective in components of HR pathways to clarify whether the loss of repeats in *atrad50* plants is HR dependent.

An alternative mechanism has been proposed to explain the telomere shortening in human cells deficient for the helicase WRN (Bai and Murnane, 2003; Bailey and Murnane, 2006). This invokes breakage owing to DNA replication fork collapse in a subpopulation of telomeres to explain the stochastic loss of telomeric sequences at some telomeres, followed by chromosome fusions involving these damaged chromosome ends. This argument should also be applicable to the MRN complex and explain the *rad50* results reported here. Mre11 localises with chromatin in S-phase and this is enhanced by replication fork stalling in mammalian cells (Maser *et al*, 2001; Mirzoeva and Petrini, 2003) and Rad50 has been shown to localise to stalled replication forks in human cells (Robison *et al*, 2004). Mre11-dependent replication fork restart has been shown to act to avoid the accumulation of DSBs in replicating *Xenopus* oocyte extracts (Costanzo *et al*, 2001; Trenz *et al*, 2006). Furthermore, WRN protein localisation to gamma-H2AX at DNA DSBs is dependent upon MRN complex (Cheng *et al*, 2005).

Thus, absence of Rad50 protein can be expected to result in increased levels of chromosome and chromatid breaks, which would agree with the 49% of the anaphase chromosome bridges observed in *atrad50* plants with no detectable subtelomeric sequence hybridisation, even greater than the 36% seen in *tert* mutant plants. Additionally, a proportion could be explained by the fusion junction being hidden in the mass of the other chromosomes (i.e. not in the 'middle' of the bridge). Multiple rounds of breakage and fusion, following the initial event (in bridge–breakage–fusion cycles), would also result in such structures. The fibre-FISH analyses are based on the juxtaposition of two subtelomeric signals on one fibre and so are of no help here and further data are needed to distinguish between these arguments concerning the initiating events for the fusions. Notwithstanding, these propositions both provide potential mechanisms to explain the absence of telomeric repeat DNA in chromosome fusions seen in the *atrad50* mutant and the fact that the mutant plants show no concurrent generalised shortening of telomeric repeat DNA.

How is the AtRad50-dependent protection of telomeres mediated? In contrast to the telomere repeat DNA addition by telomerase in *Arabidopsis* and many other species, *Drosophila* telomeres are maintained by transposition of specialised
transposons (het-A and TART) (Mason and Biessmann, 1995; Pardue and DeBaryshe, 1999). Null mutations in the *Drosophila rad50* and *mre11* genes cause telomeric fusion without total loss of Het-A sequences (Bi *et al*, 2004; Ciapponi *et al*, 2004). In *Drosophila*, both *rad50* and *mre11* mutants present reduced levels of two telomere-associated proteins HOAP and HP1, both known to act in preventing telomere fusions (Bi *et al*, 2004; Ciapponi *et al*, 2004). The earlier telomere fusions we observe in *atrad50/tert* mutant plants, as compared with the *tert* mutant, could be due to a reduction of

TRF1 and/or TRF2 proteins in the absence of Rad50. A similar role in the protection of short telomeres has been described for the ATM protein in both plants and mammals (Chan and Blackburn, 2003; Qi *et al*, 2003; Vespa *et al*, 2005), where it has been proposed that loss of ATM drives short telomeres to be prematurely dysfunctional without any lost of telomeric repeats. One possible explanation for the similar phenotypes of *atm* and *rad50* mutants comes from the observation that ATM association to telomeres is dependent upon, or at least facilitated by, presence of the Rad50 protein (Takata *et al*, 2005). This hypothesis is further supported by the absence of an additive effect in telomere protection in double *atm/mre11* mutants in both *Drosophila* and yeast (Ritchie and Petes, 2000; Bi *et al*, 2004; Ciapponi *et al*, 2004).

Rad50 has been implicated in constraining recombination to sister-chromatids in *Schizosaccharomyces pombe* (Hartsuiker *et al*, 2001) and possibly in yeast and *Arabidopsis* (Malone *et al*, 1990; Moore and Haber, 1996; Bressan *et al*, 1999; Gherbi *et al*, 2001). Given this and structural and functional studies showing its role in tethering DNA molecules (Anderson *et al*, 2001; de Jager *et al*, 2001; Hopfner *et al*, 2001; Hopfner *et al*, 2002; Wiltzius *et al*, 2005), we speculate that the protective 'anti-fusion' role described in this work may indicate that, when a telomere shortens (in the *tert* mutant) to an extent to become a substrate for the recombination machinery of the cell, Rad50 acts to constrain this interaction to invasion of the sister chromatid. Extension of the invading end using the sister as template would re-establish the required telomere repeat length and thus 'heal' the shortened telomere (Figure 7). Such a mechanism could well be a 'safety-net' in normal cells. This use of the sister chromatid as a template to restore a shortened telomere is essentially the same mechanism as that proposed for the recombination-mediated (ALT) lengthening of telomeres in the absence of telomerase in animals (Bryan *et al*, 1995, 1997; Bailey *et al*, 2004; Londono-Vallejo *et al*, 2004) (review by Muntoni and Reddel, 2005) and similar to that of Rad50-dependent type II survivors in yeast (Teng and Zakian, 1999; Lundblad and Blackburn, 1993; Teng *et al*, 2000). Furthermore, the role of Rad50 in promoting this via constraint of recombination to sister chromatids is consistent with the mitotic hyperrecombination phenotype of *rad50* mutants in yeasts and also *Arabidopsis* (Malone *et al*, 1990; Moore and Haber, 1996; Bressan *et al*, 1999; Gherbi *et al*, 2001). Absence of Rad50 would thus increase the likelihood of the shortened chromosome end recombining in an end-to-end manner with another chromatid (of the same or another chromosome), yielding more frequent fusions and the resulting anaphase bridges.

In the light of the *Arabidopsis* data presented here, the Rad50 protein apparently plays a universal role in preventing telomere fusion, independently of their structure and the implication of telomerase. Absence of AtRad50 protein leads both to rapid shortening of a subpopulation of chromosome ends and also 'deprotects' partially eroded chromosome ends, greatly stimulating the probability of their participation in chromosome fusions. The synergy in the double *tert/atrad50* mutant thus results in a mixed population of chromosome-end fusions, lacking or including telomeric repeats at the fusion points. This 'deprotection' of shortened telomeres does not result in increased exonucleolytic degradation, but acts specifically in facilitating the access of recombination.

Materials and methods

Arabidopsis *mutants and TRF analysis*

Arabidopsis thaliana plants were grown in soil in the greenhouse under standard conditions. The *tert* (Fitzgerald *et al*, 1999) and *atrad50* (Gallego *et al*, 2001) *Arabidopsis* mutants have been described previously. The double *atrad50/tert* mutants were produced by crossing *atrad50* heterozygote with a *tert* homozygote (third mutant generation) using standard techniques. The *tert* mutant generations used in this work thus begin with the first *tert* mutant generation in the F2 plants from this cross. PCR genotyping was carried out as described for *tert* (Fitzgerald *et al*, 1999) and *atrad50* (Gallego *et al*, 2001). TRF analysis of telomere length in Mbo1-digested genomic DNA was as previously described (Gallego and White, 2001).

DAPI staining of mitoses

As described (Caryl *et al*, 2000), whole inflorescences were collected, fixed and mitotic chromosomes of fixed flower pistils were squashed on a slide. Slides were mounted using Vectashield (Vector Laboratories) mounting medium with 1.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) and observed by fluorescence microscopy, using a Zeiss AxioPlan2 microscope. Images were further processed and enhanced using Adobe Photoshop software.

Fluorescence in situ hybridisation

Arabidopsis BACs F6F3, F23A5, F17A22, F4P13, T20O10, F6N15, T19P19, F7J8 and K9I9 were labelled with biotin-16-dUTP using the BioPrime DNA labelling system (Invitrogen) and telomeric probe was labelled with digoxigenin-11-dUTP (Roche) by PCR using specific telomere primers $5'(TTTAGGG)_6 3'$. FISH experiments were performed with slides prepared as described above for DAPI staining according to Schubert *et al* (2001). For the detection of biotin-labelled probe, avidin conjugated with Texas Red (1:500, Vector Laboratories) followed by goat anti-avidin conjugated with biotin (1:100, Vector Laboratories) and avidin-Texas Red (1:500) were used. For the detection of digoxigenin-labelled probe, mouse anti-digoxigenin (1:125, Roche) followed by rabbit anti-mouse fluorescein isothiocyanate (FITC) (1:500, Sigma) and goat anti-rabbit conjugated with Alexa 488 (1:100, Molecular Probes) were used.

Isolation of nuclei and fibre-FISH

Isolation of plant nuclei was performed according to Zhong *et al* (1996) with minor modifications (Fransz P and Mathieu O, unpublished). Flower buds were frozen and ground to a fine powder in liquid nitrogen. The powder was transferred to a 50-ml centrifuge tube with 20 ml chilled nuclei isolation buffer (NIB: 10 mM Tris–HCl pH 9.5, 10 mM ethylene diaminetetra acetic acid, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1 mM spermine and 0.1% β-mercaptoethanol) and mixed gently on ice for 5 min. The mixture was filtered sequentially through 80 and 40 μm nylon filters and 1 ml of ice-cold NIB containing 10% Triton X-100 was added to the filtrate and gently mixed. The mixture was centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was decanted and the pellet was resuspended in NIB at 5×10^5 nuclei/150 μl, to which an equal volume of 1% (w/v) low-melting-point agarose (42°C) was added. Molecular combing and FISH were carried out as described by Michalet *et al* (1997). Images were acquired on a Zeiss epifluorescence microscope using Axiovision software. Measurements were performed using the same software and images were enhanced using Adobe Photoshop software.

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