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REDUCED FERTILITY AND MEIOTIC ABNORMALITIES IN LATE GENERATIONS OF TELOMERASE-DEFICIENT *ARABIDOPSIS THALIANA*

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Here we report the consequences of telomere erosion in *Arabidopsis thaliana*, studied by examining seed and pollen production and the course of male meiosis through the last five generations (G5–G9) of telomerase-deficient *Arabidopsis* mutants. We used a previously described mutant line in which telomerase activity was abolished by T-DNA insertion into the *TERT* gene encoding telomerase reverse transcriptase. Reduced fertility accompanied by morphological abnormalities occurred in G6, which produced on average 35 seeds per silique (vs. 43 in wild type) and worsened in G7 (30 seeds) and G8 (14 seeds), as did the morphological abnormalities. The last generation of *tert* mutants (G9) did not form reproductive organs. Analysis of meiosis indicated that the main cause of reduced fertility in the late generation *tert* mutants of *Arabidopsis* was the numerous chromosomal end-to-end fusions which led to massive genome rearrangements in meiocytes. Fusion of meiotic chromosomes began in G5 and increased in each of the next generations. Unpaired chromosomes (univalents) were observed in G7 and G8. The study highlights some differences in the meiotic consequences of telomere shortening between plant and animal systems.

Key words: Telomere, meiosis, telomerase-deficient *Arabidopsis thaliana*, chromosome fusions, aneuploidy.

INTRODUCTION

Meiosis is a specialized type of cell division which allows one of two genomes to be transmitted to progeny and contributes to genetic diversity in sexually reproducing eukaryotes. Parallel studies in yeast, worm, fly, mouse and *Arabidopsis* have shown that the basic meiotic molecular machinery is highly conserved among eukaryotes (for reviews see Kleckner, 2006; Neale and Keeney, 2006; Cromie and Smith, 2007; Hunt and Hassold, 2008; Vogt et al., 2008; Caryl et al., 2003).

Telomeres are the physical ends of eukaryotic chromosomes, composed of tandem repeats of a simple sequence (TTAGGG in vertebrates, TTTAGGG in *Arabidopsis*) and associated proteins, with a 3' overhang which forms a loop. Such a structure serves as a cap and protects the ends of eukaryotic linear chromosomes from degradation, end-to-end fusion, and mistaken recognition as DNA breaks (for recent reviews see Blackburn, 2005; de Lange, 2005; Blasco, 2007; Watson and Riha, 2010). Recently a novel mechanism of chromosome end protection by blunt-ended telomeres was confirmed in angiosperm plants (Kazda et al., 2012).

The plant telomere repeat sequence TTTAGGG was first characterized in *Arabidopsis thaliana* (Richards and Ausubel, 1988) and has since been found in the majority of plant species. In the phylogenetic clade of Asparagales (~6300 species), however, a single nucleotide change occurred in the canonical plant telomere sequence, causing a switch to the human TTAGGG sequence (Adams et al., 2001; Weiss and Schertan, 2002; Puizina et al., 2003; Sykorova et al., 2003; Weiss-Schneeweiss et al., 2004). Similarly, in the model algae *Chlamydomonas* the telomeres are composed of TTTTAGGG repeats (reviewed in Watson and Riha, 2010). Interestingly, Alliaceae (onions) lack typical telomere repeats (Fuchs et al., 1995) and the exact structure of telomeres in these plants remains unknown (Pich et al., 1996).

Telomere length is maintained by the enzyme telomerase, which adds telomere repeats onto chromosome ends de novo (Greider and Blackburn, 1985). The composition of the plant telomerase holoenzyme appears to be similar to vertebrate

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telomerase. Genes encoding telomerase reverse transcriptase (TERT) can be readily identified in plant genomes, and they are more related to the TERTs of vertebrates than to those in yeasts and ciliates. The RNA subunit of plant telomerase, TR, remains an enigma, since rapid divergence of the RNA subunit (TR) of telomerase has precluded their in silico identification in plant genomes (reviewed in Watson and Riha, 2010). Similar to its activity in humans, telomerase activity in plants is restricted to highly proliferative tissues and the germline (Fitzgerald et al., 1996; Riha et al., 1998).

At meiosis telomeres display remarkable behavior which involves their attachment and motility along the nuclear envelope (for reviews see Scherthan, 2007; Siderakis and Tarsounas, 2007). Telomere involvement in meiotic pairing/synapsis has been widely reported and documented in a variety of species. In almost all cases the involvement of telomeres in these events is thought to be closely associated with their binding to the inner surface of the nuclear envelope and their subsequent clustering within a limited area of the inner nuclear surface to produce the so-called bouquet configuration (Dernburg et al., 1995; Zickler and Kleckner, 1998; Cowan et al., 2001). It is generally supposed that the clustering of telomeres facilitates the colocalization of homologous chromosome ends by bringing them into a common nuclear subregion and making them all roughly codirectional (Cowan et al., 2001). This view is supported by numerous observations that synapsis, the formation of SCs, often begins at or near chromosome ends (von Wettstein et al., 1984). A secondary role of telomere clustering and pairing is that they may serve to pull other, more internal, homologous chromosome regions into rough alignment (Scherthan et al., 1996). In plants, coincident initiation of homolog pairing and synapsis during the telomere clustering (bouquet) stage of meiotic prophase has been reported in maize (Bass et al., 2000) and *Brachypodium* (Wen et al., 2012). However, the model plant *Arabidopsis* lacks the classical bouquet arrangement; their telomeres cluster around nucleoli. *Arabidopsis* meiotic telomere clustering is established prior to entry into prophase I at the nucleolus and is relaxed during leptotene when recombination is initiated (Armstrong et al., 2001). Thus, in *Arabidopsis* the classic bouquet stage probably is replaced by an association of telomeres with the nucleolus. However, the functional significance of this telomere clustering has not been examined.

Studies of mutations affecting telomere structure and function in yeasts, mouse and worm model organisms have revealed that loss of telomeric repeats leads to meiotic defects (for review see Scherthan, 2007). Although the telomeres in mice are extremely long (40–150 kbp), mice lacking telomerase activity ($TR^{-/-}$) undergo progressive telomere shortening (at a rate of approximately 5 kbp per generation) over six generations, eventually resulting in telomere-depleted chromosomes and chromosomal abnormalities (Blasco et al., 1997; Lee et al., 1998; Herrera et al., 1999; Rudolph et al., 1999). In generation 6 (G6) telomerase-deficient mice, premeiotic male germ cells undergo apoptosis shortly before or upon entering meiosis, whereas oocytes remain alive but produce mature oocytes with high rates of chromosomal aberrations and abnormal cell division of fertilized eggs (Hemann et al., 2001; Liu et al., 2002). Liu et al. (2004) showed that shortened telomeres of intermediate G4 telomerase-deficient mice caused impaired attachment to the nuclear envelope and synapsis, and decreased recombination. Meiocytes of late-generation telomerase-deficient females with shortened telomeres bred with early-generation males harboring relatively long telomeres exhibit severely impaired chromosome pairing and synapsis, and reduced meiotic recombination. These findings imply that functional telomeres are important in mammalian meiotic synapsis and recombination (Liu et al., 2004).

The telomeres of *A. thaliana* are relatively short, ranging from 2 to 4.5 kb in wild-type cells (Richards and Ausubel, 1988). Due to the dysfunctionality of telomerase (telomerase reverse transcriptase, $TERT^{-/-}$), the telomeres of *Arabidopsis tert* mutants are progressively shortened by 250-500 nucleotides per generation (Fitzgerald et al., 1999; Riha et al., 2001; Heacock et al., 2004). Riha et al. (2001) showed that *Arabidopsis thaliana* can survive up to ten generations without telomerase. The last five generations of telomerase-deficient plants endured increased levels of chromosome end-to-end fusions in somatic cells, which correlated with developmental anomalies in both vegetative and generative organs. Interestingly, late-generation mutants had extended life spans and remained metabolically active but partially or fully sterile (Riha et al., 2001).

In this study we monitored the reduction in fertility of late-generation telomerase-deficient *Arabidopsis* (G5–G9). We analyzed meiosis in meiocytes in order to identify the cause of infertility of these mutants.

MATERIAL AND METHODS

PLANT GROWTH AND MUTANTS

Plants were grown at 21°C in an environmental growth chamber under a 16 h photoperiod. The *tert* mutants (line 69) were generated as previously described (Riha et al., 2001).

Fig. 1. Comparison of wild-type plants (Col) and late-generation *tert* mutants of *Arabidopsis* (G5–G9). All plants were 6 weeks old. Three seeds were planted in each compartment. (**a**) Plants from G7 and G8 with poorly developed stem and flowers, (**b**) Plant from G9 arrested in vegetative development and unable to form reproductive organs.

MEIOSIS ANALYSIS

Meiotic figures were prepared from anthers of young floral buds according to Puizina et al. (2004). Whole inflorescences were fixed in ethanol/acetic acid (3:1) and transferred to 0.01 M citrate buffer, pH 4.6, and dissected pistils or anthers were transferred to an enzyme mixture containing 0.5% (w/v) Onozuka R-10 cellulase (Serva, Heidelberg, Germany) and 0.5% (w/v) pectolyase (Sigma, Vienna, Austria) in citrate buffer. The enzyme mixture was replaced by citrate buffer after 2 h incubation at 37°C, and slides were prepared by standard squashing technique in a drop of 60% acetic acid. Slides were stained with 2 mg/mL DAPI and mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA). Mitotic and meiotic nuclei were examined using an Axioimager D1 epifluorescence microscope (Carl Zeiss, Inc.). Images were recorded with an AxioCam MRm camera (Carl Zeiss, Inc.) using AxioVision ver. 4.4 (Carl Zeiss, Inc.)

ALEXANDER STAINING

Flower buds were fixed in Carnoy's fixative (alcohol/chloroform/acetic acid (6:3:1) for a minimum 2 h (buds can be stored up to several years in fixative at 4°C). The staining solution was prepared according to Peterson et al. (2010) and the following constituents were added in the order indicated and stored in the dark: 10 mL 95% alcohol, 1 mL Malachite green (1% solution in 95% alcohol), 50 mL distilled water, 25 mL glycerol, 5 mL acid fuchsin (1% solution in water), 0.5 mL orange G (1% solution in water), 4 mL glacial acetic acid and distilled water (4.5 mL) to a total volume of 100 mL. Following at least 2 h fixation, the buds were dissected under a dissecting microscope to release the anthers and pollen. Excess fixative was dried using filter paper. Several drops (1–4) of the stain solution were applied, and the slide was heated over an alcohol burner until the stain solution was nearly boiling (~30 s), covered with a coverslip, lightly pressed and sealed with nail polish.

RESULTS

MORPHOLOGICAL ABNORMALITIES

Plants of the late-generation *tert* mutants displayed morphological abnormalities and reduced germination of seeds. Comparison of wild-type plants (Col) and late-generation *tert* mutants of *Arabidopsis* (G5–G9) (Fig. 1a) showed that G5 *tert* mutants were morphologically indistinguishable from wild-type (Col) plants. G6 was the first generation of *tert* mutants to show slower growth, smaller rosettes, deformed yellowish leaves and other developmental abnormalities. Plants from G7 and G8 were characterized by dwarf growth, bent stems, and wrinkled rosette leaves with visible yellow necrotic spots. Plants from G9 had arrested vegetative development and formed no reproductive organs (Fig. 1b). Interestingly, some G8 plants looked greener and formed more rosette leaves than G7 plants (Fig. 1a),

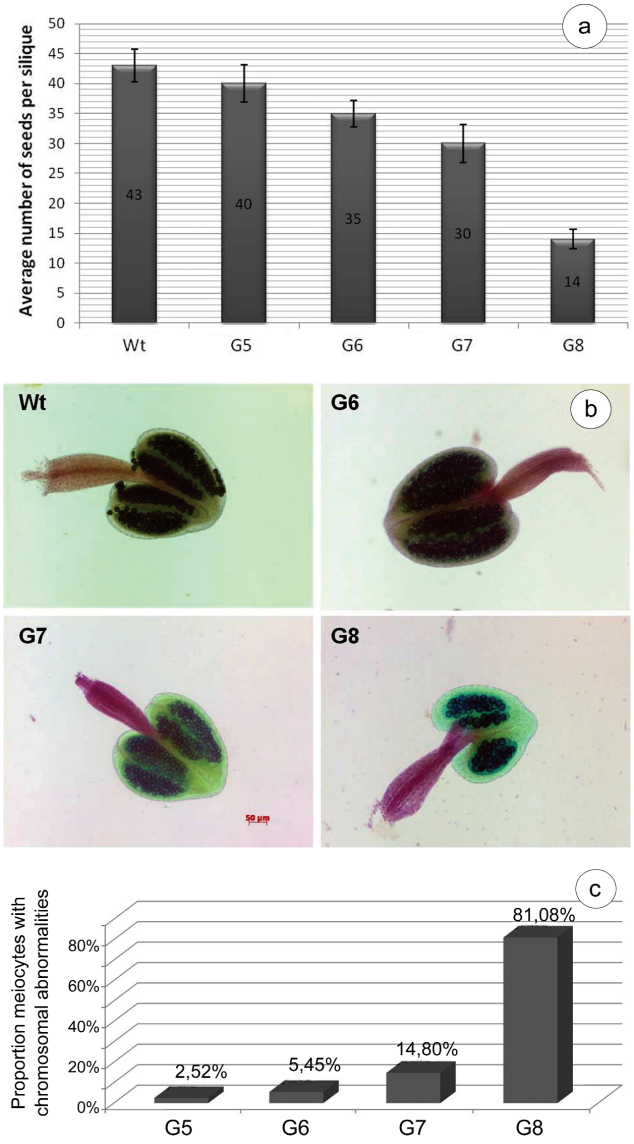

Fig. 2. (**a**) Average number of seeds per silique in wild-type (Col) and late-generation *tert* mutants of *Arabidopsis* (G5–G8). Dashes at top of columns denote standard deviations, (**b**) Stamens of wild-type plants (wt) and late-generation *tert* mutants of *Arabidopsis* (G6, G7, G8), Alexander staining. Cellulose cell walls of aborted (empty) pollen grains stained blue-green; cytoplasm of nonaborted (viable) pollen grains stained magenta-red. Arrow indicates abnormally large and probably aneuploid pollen grains, (**c**) Proportion of pollen mother cells with abnormalities detected in late generations of *Arabidopsis tert* mutants (G5–G8). Number of analyzed meiocytes is given in parentheses.

but those leaves and the general morphology of those plants were severely deformed. Such a result is consistent with findings from Riha et al. (2001), who reported that plants from G8–G10, despite their severely impaired growth, remained green and metabolically active for months after the wild-type plants died.

REDUCTION OF NUMBER OF SEEDS

The average number of seeds per silique in the wild-type (Col) plants was 43. The first signs of fertility decline were observed in G5, which had an average 40 seeds per silique. Fertility continued to decline: we counted 35 seeds in G6 and 30 in G7. The decline in seed production was particularly dramatic in G8, with only 14 seeds per silique (Fig. 2a).

REDUCTION OF POLLEN GRAIN VIABILITY

The seed production results are in line with the results on pollen grain viability. To distinguish viable from nonviable pollen grains, stamens of wild-type plants (Col) and late-generation *tert* mutants (G6-G8) were stained with Alexander's (1969) mixture, modified by Peterson et al. (2010). This routinely used technique stains the cellulose of cell walls green and the cytoplasm of viable pollen purple. Figure 2b shows that late-generation *tert* mutants (G6–G8) retained partial fertility, with a significantly smaller number of mature pollen grains.

MEIOTIC ABNORMALITIES IN POLLEN MOTHER CELLS

The analysis of meiosis included metaphase I, anaphase I, telophase I and metaphase II, anaphase II and telophase II (Fig. 3). No meiotic abnormalities were observed in wild-type control plants. By contrast, all four generations of mutants examined (G5–G8) showed meiotic abnormalities with varying frequency. The most common types of meiotic anomaly were chromosomal and/or chromatid fusions, visible as anaphase/telophase bridges and fragmentation (Fig. 3). The share of meiocytes with chromosomal abnormalities increased from 2.52% in G5 to 5.45% in G6 and 14.9% in G7. The increase in the number of meiocytes with chromosomal abnormalities was drastic in G8, where 81% of the meiocytes exhibited some type of meiotic abnormally, mainly chromosome fusions (Figs. 2c, 3). In addition to chromatid and chromosomal fusions we also observed unpaired chromosomes (univalents) during the 1st and 2nd meiotic divisions of G7 and G8 (Fig. 4), which were not observed in G5 and G6. The subsequent segregation of univalents involved random segregation events during meiosis II, which at the end of meiosis would result in the production of polyads containing microspores with unequal amounts of DNA. In some cases there were extramicrosporial chromosomal fragments outside microspores of uneven size and shape (data not shown).

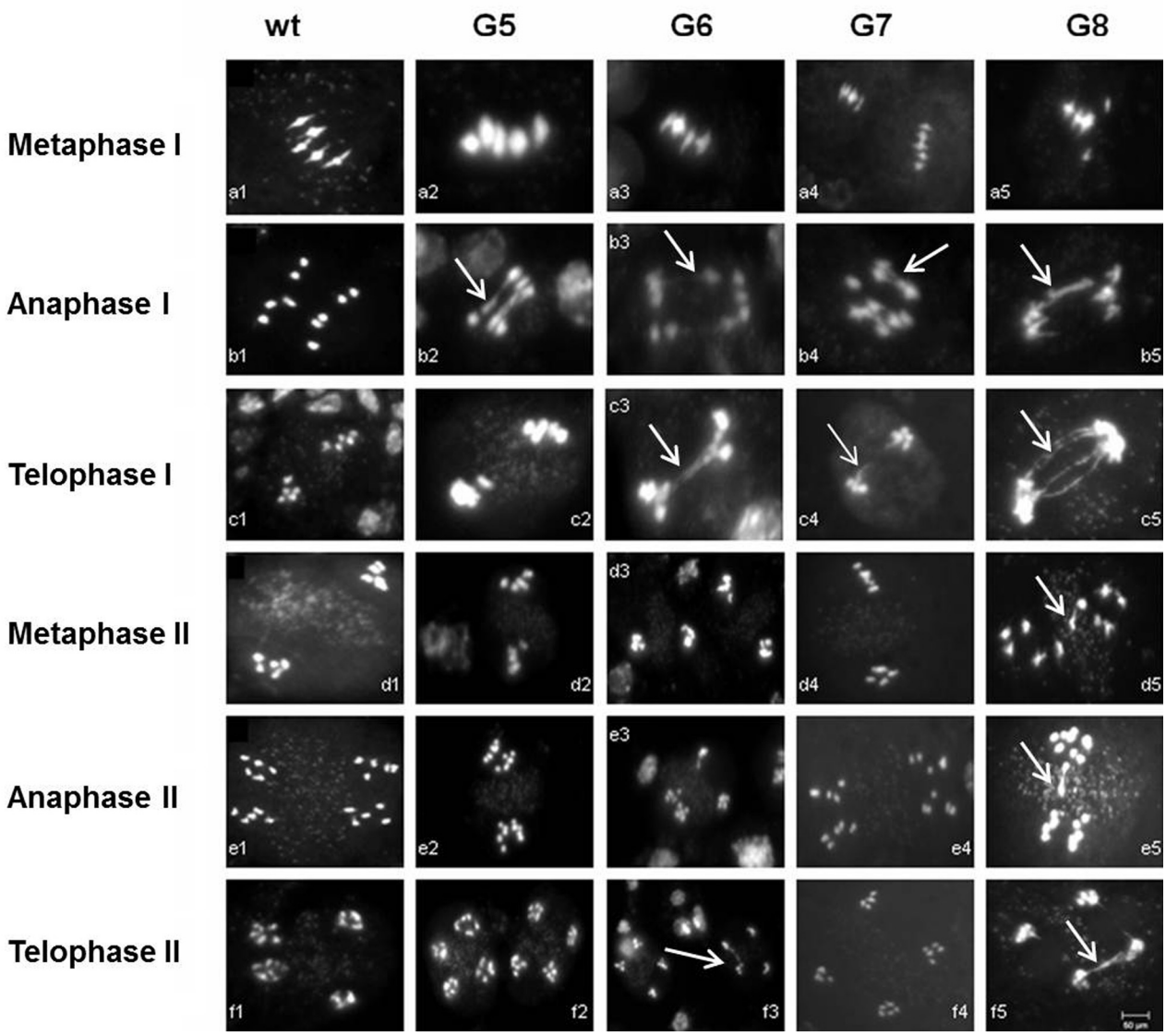

Fig. 3. Comparative analysis of chromosome morphology and segregation during meiosis I and II in DAPI-stained pollen mother cells (PMCs) of wild-type (**a1, b1, c1, d1, e1, f1**) and late-generation telomerase mutants (G5–G8). (**b2**) First abnormalities in *tert* meiosis were observed at anaphase I in G5, when chromosomes remained attached while segregating to opposing poles and formed chromatin bridges, (**b3, c3, b4, c4, b5, c5**) Chromosome/chromatid bridges (arrows) were frequently observed over the next three seed generations (G6, G7, G8). In addition to chromatin bridges, meiocytes of the last generations of telomerase-deficient plants exhibited dramatic meiotic abnormalities: (**d5**) Aneuploid meiocyte after first meiotic division with 11 chromosomes, (**e5**) Undivided chromosome(s) (arrow) lagging behind in the organellar band in anaphase II of G8, (**f3, f5**) Bridges between chromosomes/chromatids in meiosis II, (**f5**) Four groups of incompletely separated chromosomes in telophase II of G8, while filamentous chromatid bridges span along and across organellar band. Bar = 10 μm (**f5**) and all micrographs.

DISCUSSION

DECLINE IN FERTILITY

Here we have shown that telomere shortening and dysfunctions in late generations of *Arabidopsis tert* mutants resulted in massive meiotic defects which ultimately compromised seed and pollen production. The first signs of reduced fertility (both seed number and pollen viability) were observed in G6, and further deterioration ensued in later generations (G7–G9). Such results are consistent with observations reported by Riha et al. (2001): a decline in fertility correlated with morphological and developmental defects of late-generation *tert*

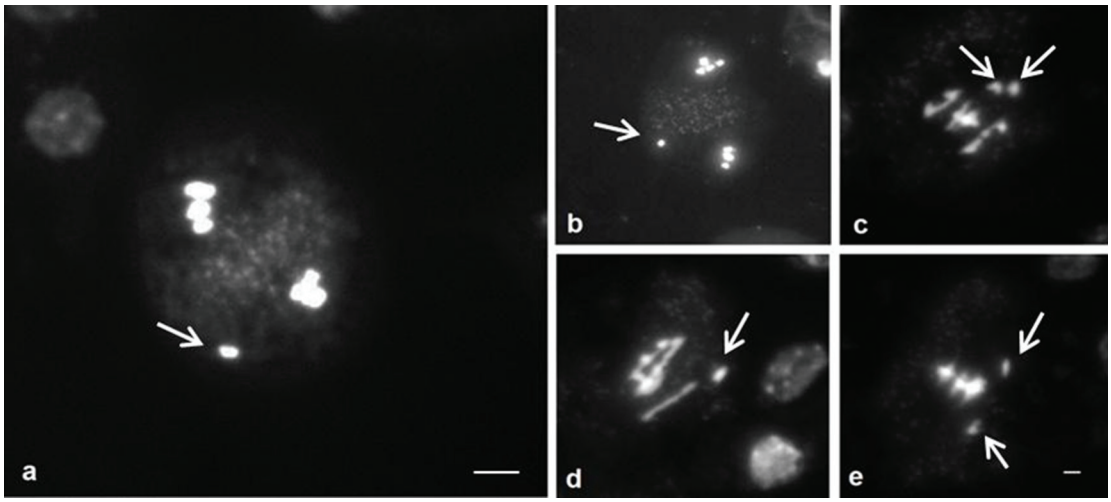

Fig. 4. Chromosome segregation defects in PMC spreads of *tert* mutants in G8. (**a, b**) Misplaced and randomly distributed chromosomes visible outside two well-separated metaphase II plates, a consequence of aberrant meiosis I, (**c**) Two unpaired chromosomes (univalents) observed at the metaphase-to-anaphase I transition. They lagged behind stretched chromosomes which began to separate at the metaphase plate, (**d**) Chromosome bridges were often associated with mis-segregating univalents. Chromatin bridging resulted in the formation of multiple interconnected chromosomes at the metaphase-to-anaphase I transition, (**e**) Metaphase I shows either two univalents (arrows) or premature separation of a bivalent. Bars = 10 μm.

mutants, with chromatid fusions visible at anaphase as chromatin bridges, chromosome abnormalities and death in somatic cells. In that work they observed no bridges in wild-type plants or in G1–G4; in G5, bridges were detected in 0.7% of the anaphases, and by G6 the number rose to 6%. The frequency of bridges per anaphase dramatically increased in successive generations in direct proportion to the severity of the phenotype. Ultimately, catastrophic genome rearrangements occurred and in G10 the plants were arrested in a terminal vegetative state, and some dedifferentiated into a callusoid mass (Riha et al., 2001).

Fertility decline has been observed in all other organisms in which the influence of telomere erosion was studied. Telomere dysfunction in late-generation $TR^{-/-}$ mice leads to various pathological conditions including defects in development, growth and immune function, and it influences tumorigenesis (Lee et al., 1998; Herrera et al., 1999). Similar effects of loss of telomerase activity were observed in *C. elegans* (Meier et al., 2006).

MEIOTIC ABNORMALITIES

The major meiotic abnormalities we found in G6, G7 and G8 *tert* mutants involved extensive chromosome fusion/fragmentation visible during anaphase/telophase as chromatin bridges. The first drop in fertility, which occurred in G6, coincided with chromosome fusions (found in 5.45% of meiocytes), suggesting that a subset of telomeres had become dysfunctional. Such a result is consistent with Heacock et al.'s (2004) finding that in *Arabidopsis* the first telomeric fusions became detectable when the shortest telomere within a plant dipped below 1 kb. Analyzing chromosome terminal restriction fragments (TRFs), Riha et al. (2001) confirmed that telomere length in G6 varied from 2 kb to only a few hundred base pairs.

The frequency of chromosome fusions increased in the meiocytes of G7 and G8 and was directly correlated with shortening of telomeres. Using fluorescence in situ hybridization (FISH) with ribosomal DNA (rDNA) and specific bacterial artificial chromosome (BAC) probes, Siroky et al. (2003) analyzed the structure of chromosome fusions in somatic tissue of G8 *tert* mutants of *Arabidopsis*. They demonstrated that both homologous and non-homologous chromosomes formed transient anaphase bridges whose breakage during anaphase movement and unequal separation, (i.e., the breakage-fusion-bridge (BFB) cycle) led to genome rearrangements, including nonreciprocal translocations and aneuploidy. Particularly interesting was that the ends of chromosomes that contained the ribosomal genes were up to ten times more likely to be involved in chromosome bridges, probably because of the functional association of nucleolar rDNA sequences at nucleoli. It is reasonable to speculate that the chromosome pairing and homologous recombination during meiotic prophase may additionally stimulate chromosome fusions at late generations of *tert* mutants. This would explain the find-

ing that even 81% of the meiocytes at G8 showed chromosomal fusion (this study), as opposed to 40–50% of somatic cells (Riha et al., 2001; Siroky et al., 2003).

In this study we observed unpaired and misaligned chromosomes in a subset of *Arabidopsis* meiocytes in G7 and G8. Similarly, chromosome misalignment and/or spindle disruption were recorded in telomerase-deficient mouse metaphases in G4 (Liu et al., 2002). In further work those mutants exhibited a disrupted perinuclear distribution of telomeres and a lack of synaptonemal complex protein 3 (SPC3) (Liu et al., 2004). Mammalian ring chromosomes without telomeric repeats failed to localize to the nuclear periphery of spermatocytes (Voet et al., 2003). In maize the telomeres of newly created telocentric chromosomes and the interstitial telomeres on a ring chromosome are both recruited to the bouquet, suggesting that telomeres act autonomously from the rest of the chromosome (Carlton and Cande, 2002). All these studies confirm that telomeres are structural and functional components of chromosomes which are required for meiosis and fertility in both mammals and plants. The underlying mechanism by which telomeres affect chromosome pairing and recombination remains to be explained.

DIFFERENCES BETWEEN PLANT AND ANIMAL MEIOSIS

Our study also points to some interesting differences in plant and animal responses to dysfunctional telomeres during meiosis. Chromosomal fusions and bridges in meiocytes of *Arabidopsis tert* mutants, which are the major chromosomal abnormalities we observed, were not reported in meiocytes of telomerase-deficient mice (Hemann et al., 2001; Liu et al., 2002), in contrast to the abundance of such aberrations in somatic telomerase-deficient cells (Siderakis and Tarsounas, 2007). Instead, extensive apoptosis has been reported in mouse male germ cells shortly before or upon entering meiosis, and misalignment and unpaired chromosomes were observed in mouse oocytes (Heman et al., 2001; Liu et al., 2002). Unlike their mammalian counterparts, late-generation telomerase-deficient plants continue both mitotic and meiotic cycling in the face of massive cytogenetic aberrations until total genomic catastrophe is reached. This suggests that *Arabidopsis* lacks a checkpoint mechanism that would prevent cells with dysfunctional telomeres from entering and progressing through meiosis. The idea finds support in findings from *Arabidopsis* mutants deficient in meiotic double-strand break repair such as *mre11*, *rad51* and *com1*, which complete meiosis and produce aberrant spores despite massive chromosome fragmentation (for review see Jones et al., 2003; Caryl et al., 2003; Puizina et al., 2004; Li et al., 2004). Programmed cell death frequently is associated with telomere dysfunction in metazoans, but this is not the case with plants, in which no functional homologue of p53 has been identified (Klosterman et al., 2000). All these differences confirm the more plastic nature of plant development and genome organization.

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