TELOMERE DYNAMICS AND TELOMERASE-INDEPENDENT CELL SURVIVAL IN *Arabidopsis thaliana*

A Dissertation

by

JAMES MATTHEW WATSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Biochemistry

TELOMERE DYNAMICS AND TELOMERASE-INDEPENDENT CELL SURVIVAL IN *Arabidopsis thaliana*

A Dissertation

by

JAMES MATTHEW WATSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Dorothy E. Shippen
Committee Members,	Mary E. Bryk
	Alan E. Pepper
	Donald W. Pettigrew
Head of Department,	Gregory D. Reinhart

May 2007

Major Subject: Biochemistry

ABSTRACT

Telomere Dynamics and Telomerase Independent Cell Survival in *Arabidopsis thaliana.*

(May 2007)

James Matthew Watson, B.S., Beloit College

Chair of Advisory Committee: Dr. Dorothy E. Shippen

Telomeres are the nucleoprotein structures that protect the ends of eukaryotic chromosomes from being recognized as DNA double-strand breaks. Telomeres are recognized by the ribonucleoprotein telomerase, a reverse transcriptase that catalyzes addition of G-rich telomeric DNA repeats to the 3' overhang of the telomere. The action of telomerase allows cells to overcome the end-replication problem defined by the inability of conventional DNA polymerases to fully replicate the end of the chromosome. Telomeric DNA tracts are maintained in a species-specific size range primarily through the competition between telomerase and the end-replication problem. In many organisms, recombinational activities can function at telomeres outside of the wild type range, in some cases resulting in telomerase-independent telomere maintenance. Telomere rapid deletion (TRD) can dramatically shorten elongated telomeres. Elongation of telomeres below the normal range in the absence of telomerase is known as alternative lengthening of telomeres (ALT).

Here we demonstrate that telomeres in *Arabidopsis thaliana* are also subjected to these recombinational activities. Elongated telomeres in *ku70* mutants are shortened by TRD. In contrast to other organisms, TRD functions on telomeres of wild type length. TRD produces extra-chromosomal telomeric circles, which can serve as substrates for ALT. In *Arabidopsis,* ALT may require the byproducts of TRD, as telomerase mutants with

extremely short telomeres are unable to maintain telomeric repeats by recombination and instead secure their genome through an unknown mechanism. Finally, we follow the fate of cells with telomere-to-telomere fusions. Fusions are not propagated to viable progeny. We propose that a G1 checkpoint dependent upon the checkpoint protein ATM arrests cells following the break of a single telomere fusion. We design reporter constructs to follow the fate of individual cells with telomere fusions, and present initial characterization of their expression. We find no evidence for the propagation of telomere fusions in somatic cells, though later generation mutants will provide a better test of this hypothesis. This work begins the study of the fate of cells with telomere fusions in *Arabidopsis*. Furthermore, it sets the foundation for studying recombinational shortening and elongation of telomeres in *Arabidopsis* and the effects of these processes on telomere length regulation.

DEDICATION

To my family, for their constant love and support.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Dorothy Shippen: for taking the chance on a student based only on a phone call from an acquaintance, for encouraging me despite the craziness of most of my ideas, for helping me figure out which ideas were feasible and which were mostly just crazy, and for the advice and guidance that have made this possible.

I would like to thank Dr. Roc Ordman, for making the phone call, and along with all of my previous science teachers, for fostering my curiosity and encouraging me to explore.

I would like to thank the members of my committee, Dr. Donald Pettigrew, Dr. Alan Pepper, and Dr. Mary Bryk, for their help, guidance, and advice throughout this process.

I would like to single out Dr. Gary Kunkel, Dr. David Peterson, and Dr. Donald Pettigrew for always answering my questions on all things scientific. Also, Dr. Geoffrey Kapler whose technical expertise and constructive criticism have made everything I've done better. Special thanks to Dr. Tom McKnight who always had a good idea (and if you look through my notebooks you'll see 4 or 5 places where I wrote exactly that).

Members of the Shippen lab have made working here for the past several years a fun and enjoyable experience. Dr. Libin Wang, Dr. Karel Riha, and Dr. Eugene Shakirov provided some of the liveliest conversations, political, social, and scientific, that I have ever had. Michelle Heacock, Yulia Surovsteva, and Kalpana Kannan, who have suffered and laughed through graduate school with me.

Special thanks are owed to Dr. Karel Riha and Barbara Zwellinger for providing many mutants, as well as technical expertise, and the sharing of data prior to publication. Dr. Boris Vyskot and Petra Bulankova were coauthors on the paper reprinted in chapter II. This paper would have been impossible to publish without their help.

Finally, I would like to thank my family, especially my parents and my wife, for their never-ending love and support.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

A BRIEF HISTORY OF TELOMERE BIOLOGY

Telomere biology begins with Herman Müller in the 1920s. Working with *Drosophila* that had been mutagenized with X-rays, he realized that the terminus of the chromosome had properties distinct from the rest of the chromosome. He never found mutants where the end of the chromosome had been deleted. Because the end of the chromosome had distinct properties, he decided that it needed a special name, and called the end "telomere."

In the 1930's and 40's, Barbara McClintock was studying the fate of dicentric chromosomes in maize. During anaphase, dicentric chromosomes can be pulled to opposite spindle poles. Eventually, the force pulling the chromosome apart results in the dicentric chromosome breaking. As one cell loses terminal DNA during this process, markers on the end of the chromosome will be lost in this cell lineage. The newly broken end can then fuse again, creating a new dicentric chromosome. This process is termed the breakage-fusion-bridge (BFB) cycle. Interestingly, her work showed that while BFB cycles can occur during development of the maize endosperm, it did not appear to occur during development of the embryo. She termed this process "chromosome healing."

_____

This dissertation follows the style of Molecular and Cellular Biology.

Thirty years later, following a great deal of work on the structure of DNA and its replication, James Watson and Alexei Olovnikov independently described the “end-replication problem” FIG. 1. DNA polymerases require RNA primers to initiate replication. While the leading strand replication machinery can replicate to the terminus of the chromosome, the lagging strand machinery requires an RNA primer to be placed at the extreme terminus. Watson and Olovnikov realized that the conventional replication machinery would have no way to fill in the gap left following removal of this primer and this would result in the loss of terminal sequences after each cell division. Over several generations, the erosion of the chromosome terminus would be catastrophic for any organism that didn’t have a means to overcome this problem.

While studying DNA amplification in the ciliated protozoa *Tetrahymena*, Elizabeth Blackburn discovered that the ends of *Tetrahymena* mini-chromosomes ended in repeats of the hexa-nucleotide sequence TTGGGG (19). Several reports followed detailing similar sequences at other ciliates (138, 207). Jack Szostak then teamed up with Elizabeth Blackburn to transform *Saccharomyces cerevisiae* with linear molecules capped by *Tetrahymena* telomere repeats. Normally, linear molecules transformed into yeast recombine, but those capped by *Tetrahymena* telomeres remained stable as linear molecules (257). Using an elegant strategy, they then ligated yeast DNA to a linear molecule that had *Tetrahymena* repeats on only one end, and isolated fragments that remained stable following transformation into yeast. Using this approach, they were able to clone telomeres from budding yeast. Though an irregular repeat ($TG_{1\text{-}3}$), the yeast telomere sequence was remarkably similar to the sequences from ciliated protozoa. This sequence was added to the terminus of the *Tetrahymena* telomeres on

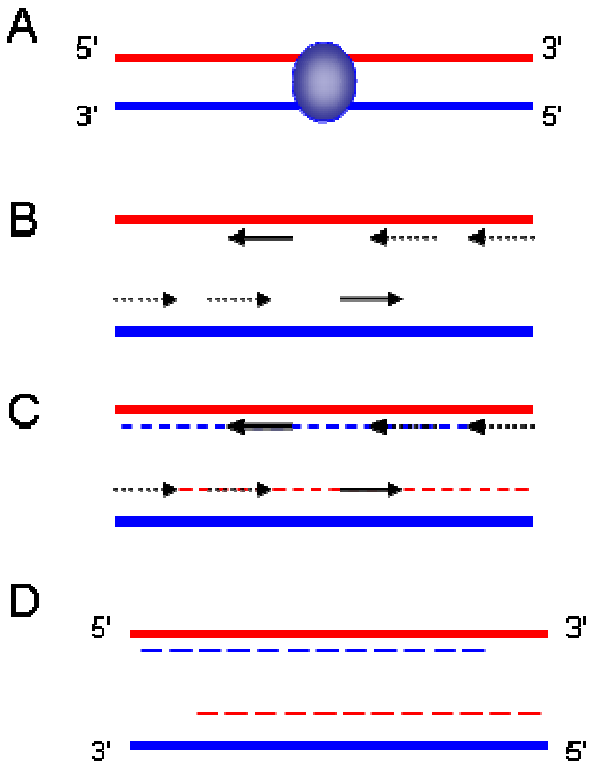


FIG. 1. The end-replication problem.

A) A mini-chromosome with a single origin of replication represented as a blue circle. B) At the beginning of replication, RNA primers (arrows) are laid down. Lagging strand primers are dotted, while leading strand primers are solid. C) Replication begins from the RNA primers and continues until it reaches a terminus or an RNA primer. D) RNA primers are removed and fill-in synthesis occurs. Removal of the terminal RNA primers results in loss of DNA from the 5' ends of the daughter strands.

the other side of the transformed DNA elongating that side of the linear DNA molecule (241).

The telomeric sequence of a higher eukaryote was first cloned from *Arabidopsis thaliana* (TTTAGGG) by Eric Richards (227). This discovery was rapidly followed by the identification of the human telomeric DNA sequence, TTAGGG (195). All mammalian telomeres examined to date possess the TTAGGG repeat. While most plants contain the *Arabidopsis* type repeat, there are exceptions (2), including some plants which have the human telomeric sequence (286).

Though it appeared that these telomeric sequences somehow provided an end-protection function, it was still unclear how they were maintained. Once again using ciliated protozoa, Carol Greider and Elizabeth Blackburn identified an enzymatic activity that would add telomeric repeats to a telomere repeat containing primer *in vitro*. This enzyme is now known as telomerase and is the primary mechanism in eukaryotes to maintain telomeres and overcome the end-replication problem.

TELOMERASE

Telomerase is an unusual ribonucleoprotein reverse transcriptase (RT) capable of extending telomeric repeats. Minimally, the enzyme consists of a catalytic reverse transcriptase subunit TERT (telomerase reverse transcriptase) and an integral RNA template TER (telomerase rna) (52).

TERT is a specialized reverse transcriptase. It contains seven RT domains common to all known RTs, as well as a telomerase-specific domain T (158, 202). While TERT is generally conserved at the amino acid level among organisms, TER contains almost no sequence conservation. Furthermore, TERs range dramatically in size between different organisms (150 nucleotides in ciliates to 1300 nucleotides in yeast) and are transcribed by different RNA polymerases (RNA pol III for ciliates and pol II for yeast and vertebrates, reviewed in (43).

In most organisms, telomerase is a processive enzyme, capable of extending DNA primers by more than 2 kb in a standard (non-PCR based) reaction *in vitro* (96). The general model for enzyme activity involves three steps: 1) binding of single strand G-rich telomeric DNA by TER and TERT; 2) TER-templated addition of nucleotides to the 3'-end of the DNA; 3) translocation and repositioning of the active site upon reaching the template boundary FIG. 2. While ciliate and human enzymes are highly processive, mouse and yeast enzymes produce much shorter products in a standard elongation assay (49, 220). In addition to regions within TERT itself (118, 164), telomerase processivity is governed by multiple factors *in vivo*. Changes in the composition of the holoenzyme (95), interactions with proteins at the chromosome terminus (149), as well as regions within TER (66, 142, 179) all affect telomerase processivity. Telomerase activity is confined to S phase, although the exact timing of telomere replication is somewhat different in humans and yeast (183, 295). Because telomerase is only able to extend one strand of the chromosome, lagging strand replication machinery is required for complete replication of telomerase-extended telomeres (65, 70, 223).

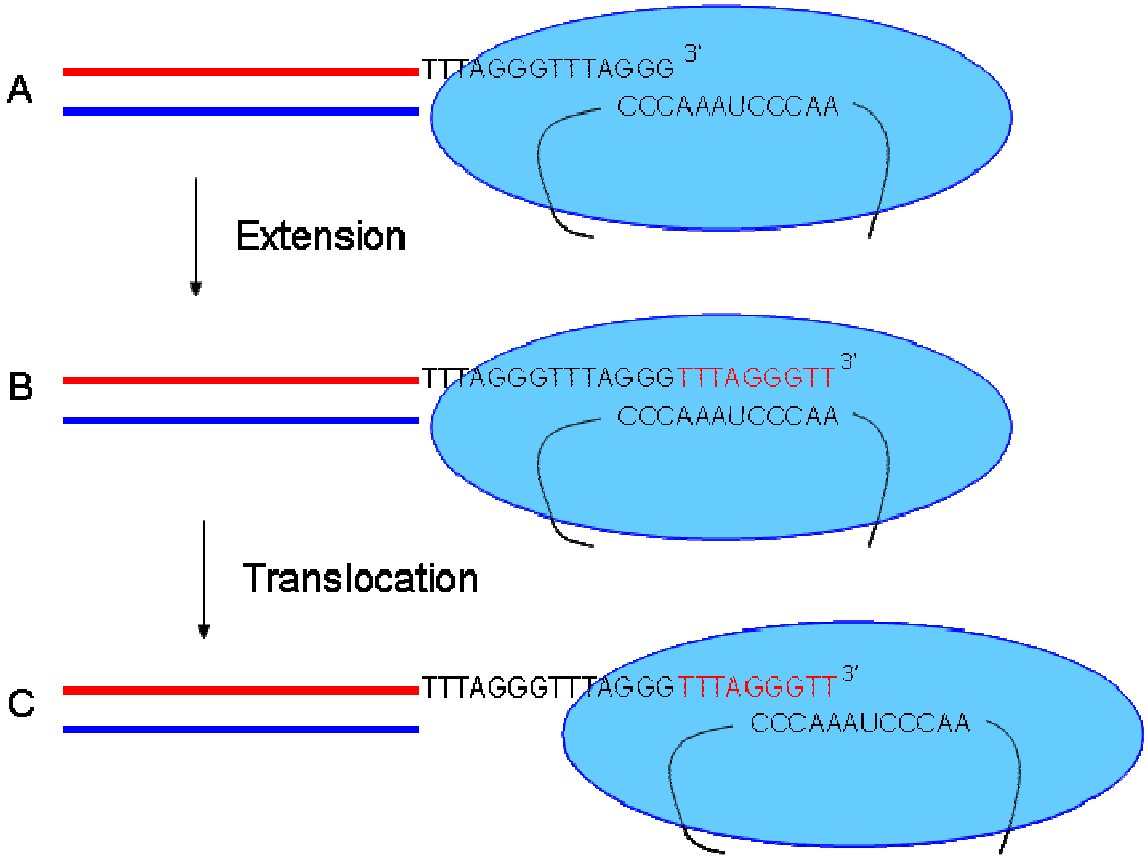


FIG. 2. The mechanism of telomerase extension.

A) The first step of telomere elongation involves binding of the telomerase holoenzyme to the 3'-overhang on the chromosome terminus. The template sequence within the telomerase RNA serves as a template and positions telomerase in the correct register for synthesis. B) Extension occurs through reverse-transcription by the catalytic subunit TERT. Synthesis continues until the end of the RNA template. C) Once synthesis reaches the end of the template, telomerase translocates, repositioning the RNA template for an additional round of synthesis. The *Arabidopsis* telomeric repeat and the predicted template sequence for the telomerase RNA are shown.

In most organisms, telomerase activity is restricted to specific stages of development. One of the most unusual examples is in the ciliate *Euplotes crassus*, where TERT is encoded by three separate genes that are expressed in a developmentally programmed fashion (124). The protein encoded by *EcTERT-2* is expressed only during the stage of development where telomeres are formed *de novo* on the organism's mini-chromosomes. Expression of *EcTERT-2* occurs concomitantly with a change in the higher-order structure and biochemical properties of the enzyme. Following this developmental stage, the *EcTERT-2* gene is degraded and TERT is synthesized from the other two genes. At this stage, *de novo* telomere addition is inhibited and short telomeres are maintained during vegetative growth (124).

Although most organisms encode only a single *TERT* gene, its expression is highly regulated. In humans, *TERT* mRNA is expressed only in highly proliferative stem cells and the germ line (135, 294). Similarly, in *Arabidopsis*, *TERT* mRNA expression is confined to cells of the germ line (flowers and root tips) and un-differentiated callus tissue (75). Although *TERT* is expressed in all tissues in the mouse, deletion of a single allele of *TERT* results in telomere shortening (161). Thus, telomerase is haplo-insufficient in mice. In addition to its regulation at the transcriptional level, TERT is negatively regulated by phosphorylation in both plants and mammals (161, 300). Furthermore, TERT mRNA displays complex splicing patterns in both rice and humans (110, 133), and at least in humans, one splice variant is capable of functioning as a dominant-negative protein, inhibiting telomerase activity (51). In contrast to this tight regulation of TERT mRNA, TER is constitutively expressed in humans and mice (21, 72).

While TERT and TER are sufficient for telomerase activity *in vitro,* several proteins associated with the telomerase holoenzyme are also necessary for its function *in vivo*. In *S. cerevisiae*, Est1p, Est3p, and Cdc13p are essential for telomerase activity *in vivo* (157). EST1 is capable of binding to TER and recruiting telomerase to the chromosome end via a direct interaction with Cdc13p (213, 305). Additionally, EST1 serves a role in either activating or stabilizing telomerase at the chromosome end (258). EST1 homologues have been identified in both humans and *Arabidopsis*. In humans, hEST1a has been associated with telomerase activity, and appears to play a role in both chromosome capping and regulating telomerase activity (224, 253). The role of two putative *Est1* genes in *Arabidopsis* remains unclear, although no telomere defects have been observed in mutants (R. Idol and D. Shippen, unpublished data). No homologues of Est3 have been identified outside of yeast, and its exact role remains a mystery. Cdc13p is discussed in more detail below.

In addition to the EST proteins in yeast, at least two chaperones, p23 and hsp90, are stably associated with telomerase in humans (80, 116), and inhibition of hsp90 results in loss of telomerase activity. The H/ACA box RNA binding protein Dyskerin is also associated with telomerase activity (37, 191). Mutations in either dyskerin or TER lead to Dyskeratosis Congenita, a human disease characterized by decreased telomerase activity and shorter telomeres (191, 279). The human protein TP1 associates with human telomerase, and antibodies to this protein can immunoprecipitate telomerase activity (105). TP1 shares sequence homology with p80, a protein associated with telomerase activity in *Tetrahymena* (53). Disruption of p80 in *Tetrahymena* does not disrupt telomerase activity, but it does lead to telomere elongation. Finally, proteins in

several organisms have been identified that associate with telomerase and contain a La motif. p43 in *Euplotes* (5), p65 in *Tetrahymena (290),* and the La antigen in human (78) have all been found in direct association with telomerase activity. While the exact function of these La motif containing proteins is unknown, depletion of p65 in *Tetrahymena* results in telomere shortening and a loss of telomerase RNA accumulation (290), suggesting a role in telomerase biogenesis. The La antigen is involved in biogenesis of RNA polymerase III transcripts (291), supporting this model for its role in telomerase biogenesis. Interestingly, *Euplotes* p43 seems to play a role in modulating the processivity of telomerase, suggesting La proteins may play additional roles beyond telomerase biogenesis (4).

This complex level of regulation prevents two deleterious outcomes. First, telomerase is generally inhibited from extending non-telomeric DNA substrates (139). DNA double-strand breaks, if healed by telomerase, would result in terminal deletions. Second, the gradual loss of telomeric DNA during somatic cell division in mammals limits replicative lifespan for cells in culture. This phenomenon is termed the Hayflick Limit. While expression of telomerase in fibroblasts allows cells to overcome this limit and divide indefinitely (24), it also provides a mechanism for cancer cells to proliferate indefinitely. Telomerase is expressed in over 95% of all primary tumors examined (135).

In the absence of telomerase, telomeres gradually shorten due to the end-replication problem. If the shortening continues unabated for several generations, telomere dysfunction occurs, resulting in the loss of cell proliferation and the appearance of chromosome fusions (FIG. 3 and refs. (22, 167, 230). In *Arabidopsis*, genomic

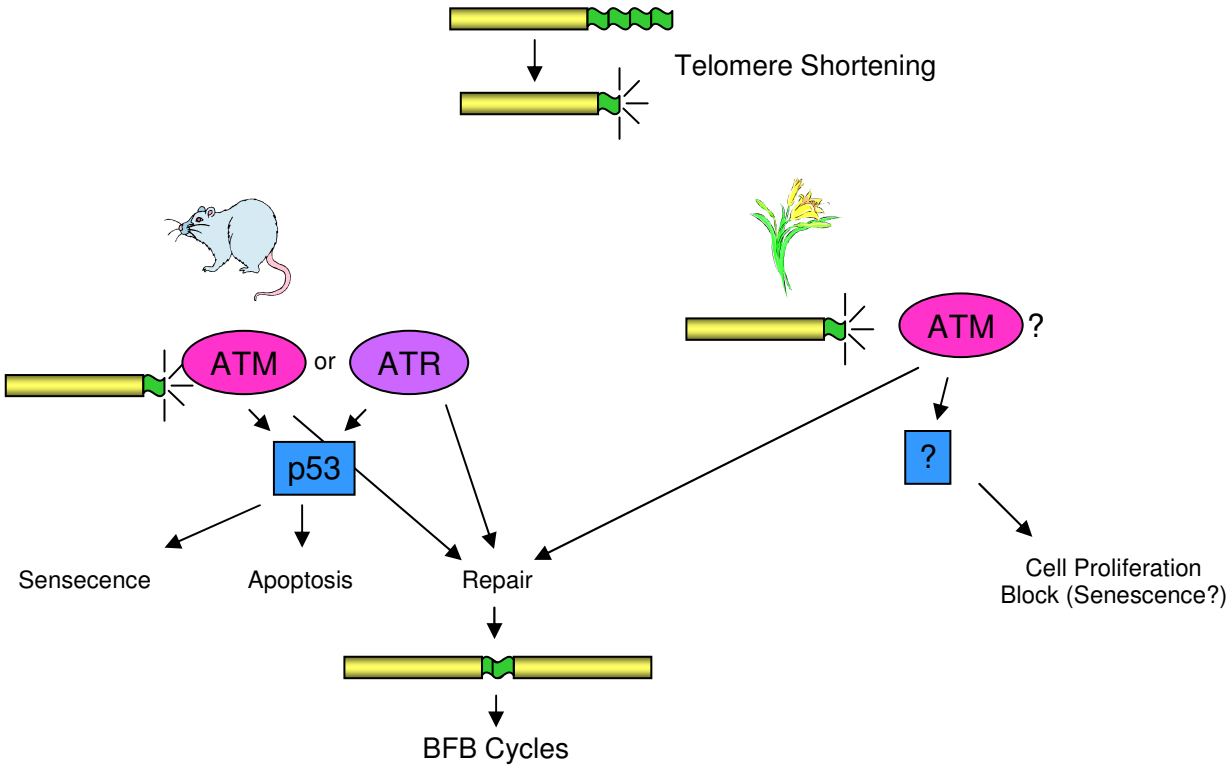


FIG. 3. The response to dysfunctional telomere in mammals and plants.

In the absence of telomerase, telomeres shorten due to the end-replication problem until they reach a critical "uncapped" length. In mammals, uncapped telomeres are recognized as DNA double strand breaks, and a signaling pathway starting from ATM or ATR results in activation of p53, leading to either senescence or apoptosis. In *Arabidopsis*, there is no p53 homolog, however recent evidence suggests that ATM can also recognize and signal a response to telomere fusions, though which protein or proteins it activates is unclear.

instability and phenotypic abnormalities begin in the sixth plant generation of a telomerase deficiency. However, plants continue to proliferate with worsening phenotypes for up to four more generations. Plants eventually arrest at a terminal vegetative state. Telomere dysfunction results in telomere-to-telomere fusions, which can be assayed in *Arabidopsis* by taking advantage of unique sub-telomeric sequences found in this organism (108). Telomeres in wild type *Arabidopsis* range from 2-5 kb. When telomeres reach approximately 1.2 kb, they become unstable and are increasingly likely to fuse to another telomere (Heacock et al. in preparation). The minimal telomere length, below which fusion is inevitable, is approximately 350 bp (108).

In contrast to the situation in *Arabidopsis*, telomerase knockouts in mice survive for a maximum of six generations with genetic defects becoming apparent as early as the second generation (22). Loss of reproductive efficiency begins in G4, with severe defects in the male germline evident by G5, and in the female germline at G6 (147). The difference in the generation of onset is likely due to an increased cell proliferation required for the male germline (67). The decrease in germ cell viability results from massive apoptosis, and this loss of germinal tissue can be rescued by deletion of p53, resulting in two additional generations of mice (46). However, the increased proliferation is associated with a substantial increase in genetic instability, eventually resulting in loss of cell proliferation. The difference in cellular response to telomere dysfunction between mice and *Arabidopsis* may be due to the absence of a p53 checkpoint protein in plants, and the continued proliferation is likely due to the differences in development; the plant germline is set aside late during development

perhaps allowing for slowly dividing cells in the somatic tissue to escape telomere loss due to cell proliferation (230).

DOUBLE-STRAND TELOMERE BINDING PROTEINS

The sequence-specific nature of telomeric DNA not only provides a substrate for telomerase, but also provides a specific chromosomal address for a host of telomeric DNA binding proteins. This interaction between telomeric DNA and its associated binding proteins is how the chromosome end acquires its protective cap; this essential capping function is why Herman Müller was unable to isolate terminal deletions in *Drosophila*.

The length of the telomere tract varies among organism (discussed below), but a conserved feature of all telomeres is a short 3', G-rich overhang at the extreme terminus (112). Thus, telomeres consist of both a double-stranded (ds) region, and a single-stranded (ss) end. Telomere binding proteins can therefore be categorized based on their affinity for either the ds or ss telomeric DNA.

The *S. cerevisiae* protein Rap1p was the first ds telomere binding protein identified (55, 163, 170). The study of temperature-sensitive alleles and over-expressing mutants led to the conclusion that Rap1p is essential for both chromosome end-protection and negative regulation of telomerase activity. The regulation of telomere length by Rap1p is mediated by two Rap1p interacting factors, Rif1p and Rif2p (103, 293). Rif1p and Rif2p are recruited to yeast telomeres via their interaction with Rap1p. Deletion of either protein results in a slight increase in telomere length, while double mutants have

dramatically elongated telomeres (293). Rap1p and Rif1p are conserved in *Schizosaccharomyces pombe* as well as in humans (123, 154, 245), although their functions are slightly different. In contrast to budding yeast, Rap1p in *S. pombe* and humans does not bind to telomeric DNA directly, but is instead recruited through ds telomere binding proteins discussed below (123, 154). The role of Rap1p as a negative regulator of telomerase is conserved (123, 169, 189). Rif1p functions as a negative regulator of telomere length in *S. pombe*, but it does not interact with Rap1p in this organism (189). RIF1 does not appear to play a role in telomere length regulation in humans, but instead is needed at dysfunctional telomeres (245, 298).

In *S. pombe* and humans, Rap1p is recruited to telomeric DNA via the homologous major ds telomere binding proteins Taz1p and TRF1, respectively. As expected based on their association with Rap1p, Taz1p and TRF1 are negative regulators of telomere length (56, 273). TRF1 is further regulated through its association with four different proteins: Tankyrase 1 and Tankyrase 2, TIN2, and PINX1. Tankyrase 1 and 2 are poly (ADP-ribose) polymerases that catalyze the ADP-ribosylation of TRF1 (248, 249). This post-translational modification of TRF1 decreases its ability to bind telomeric DNA. Consistent with this function, overexpression of tankyrase 1 leads to loss of TRF1 at the telomere and a global increase in telomere length (248). In contrast, TIN2 is a negative regulator of telomere length (136) that modulates the interaction between Tankyrase 1 and TRF1 (301). TIN2 stabilizes the interaction of TRF1 and Tankyrase, but inhibits ADP-ribosylation of TRF1. In addition, TIN2 plays a critical role in connecting TRF1 with other telomere binding proteins (reviewed in (64). The final TRF1 interacting factor, PINX1, interacts directly with telomerase as well as TRF1 and like TIN2 is a negative

regulator of telomere length (306). In contrast to the other TRF1 associated factors, PINX1 regulates telomerase activity by modulating the interaction of TERT with TER, a function that is conserved between yeast and humans (11, 155).

The *S. pombe* ds telomere binding protein is Taz1p. It shares a high degree of sequence conservation with TRF1 and similarly functions as a negative regulator of telomerase (56). In addition to its role in telomere length regulation, Taz1p is critically important for the proper localization of telomeres in *S. pombe* during meiosis; *taz1* mutants have dramatically reduced spore viability (57). Furthermore, Taz1p plays a direct role in assisting DNA replication machinery during telomere replication (190).

The final well characterized ds telomere binding protein is the human protein TRF2. In contrast to the roles of Rap1p, Taz1p, and TRF1 in regulating telomere length, TRF2's primary function is telomere end-protection (130). Most work with TRF2 has been performed using a dominant-negative version of the protein that forms a heterodimer with the endogenous protein. This heterodimer is unable to bind telomeric DNA, resulting in depletion of TRF2 from the telomere and a concomitant increase in telomere fusions (274). Physiologically, this de-protection of the chromosome terminus results in either p53-dependent apoptosis or cellular senescence (127). Such cellular outcomes are similar to what is observed when telomeres become critically shortened in the absence of telomerase (46), indicating that the senescence induced by telomere shortening may be due to loss TRF2 from the telomere.

SINGLE-STRAND TELOMERE BINDING PROTEINS

Although ds telomere binding proteins are critical for the proper function of telomeres, any activity that affects telomeres, be it telomerase elongation, nuclease attack, or fusion to a DNA double-strand break, happens at the extreme terminus. As previously mentioned, the terminus consists of an evolutionarily conserved 3'-overhang (reviewed in (284). The length of the overhang varies from 3-5 nucleotides in yeast, to hundreds of nucleotides in humans (284). Single-strand telomere binding proteins associate with this 3'-overhang, and the functions of the ds telomere binding proteins must ultimately be transduced to the ss telomere binding proteins located at the terminus.

The *S. cerevisiae* ss telomere binding protein is Cdc13p. As judged by its name, it was originally isolated as a cell-cycle defective mutant (285). A genetic screen to identify mutants in telomerase extension independently uncovered a temperature-sensitive (ts) allele of *CDC13* (named *cdc-13-2*^{est} (206)) that was defective in telomere maintenance. Cdc13p binds ss yeast telomeric DNA both *in vivo* and *in vitro* (156, 206), and functions in end-protection (89) and positive- and negative-regulation of telomere length through the coordinate regulation of leading and lagging strand telomere synthesis (41).

At the non-permissive temperature, ts alleles of *Cdc13* display extremely long 3'-overhangs due to exonucleolytic digestion of the C-rich strand (175). Single-stranded DNA is detected more than 8 kb away from the telomere, indicating a highly processive attack in the absence of Cdc13p. The 3'-overhang is normally generated in a cell cycle regulated manner, even in the absence of telomerase, indicating that the normal overhang is also a product of exonuclease digestion (287, 288). Cdc13p is therefore

believed to limit the amount of ssDNA generated by exonucleases to an appropriate length.

The capping role of Cdc13p is mediated by an associated protein, Stn1 (92). Like Δ*cdc13*, loss of Stn1 function leads to cell-cycle arrest and the generation of long ss DNA (92). While deletion of Δ*cdc13* is lethal, this phenotype can be overcome by fusing Stn1 to the minimal telomere binding domain of Cdc13p (213). The Cdc13p binding domain alone is insufficient to rescue Δ*cdc13* lethality, indicating that the primary function of Cdc13p in end-protection is the recruitment of Stn1 to the telomere (213). An additional protein, Ten1, is recruited to the telomere via an interaction with Stn1 and is additionally required for telomere end-protection (91). Indeed, the essential role of *Cdc13* can be completely bypassed by overexpressing a truncated form of Stn1 along with Ten1 (217).

Cdc13p is additionally involved in telomere-length regulation, by both positively and negatively regulating telomerase's access to the telomere. Cdc13p recruits telomerase to the chromosome end via a direct protein-protein interaction with Est1, a component of the telomerase RNP (213). Negative regulation of telomerase is mediated by Stn1 and Ten1 (41, 91, 92). Interestingly, the same region of Cdc13p responsible for recruiting Est1 is also responsible for the recruitment of Stn1 (91, 213), suggesting that there is a direct competition between Est1 and Stn1 for binding to Cdc13p and the end of the chromosome. Current models posit a multi-step reaction, wherein Cdc13p initially recruits Est1 to the telomere, resulting in telomere extension. The Stn1-Ten1

complex displaces Est1p ands allows for lagging-strand synthesis to fill in the extended telomere (reviewed in (168).

The ss telomere binding protein in other eukaryotes is Pot1. It was originally identified in *S. pombe* based on sequence similarity to the ciliate end-binding protein TEBPα (14). TEBPα and Pot1 share reasonable sequence similarity over an N-terminal DNA binding domain, but neither protein shares any primary sequence similarity to Cdc13p. Despite this, structural studies have shown that all three proteins interact with telomeric DNA via an oligonucleotide/oligosaccharide binding (OB) fold (117, 148, 192, 212).

Deletion of Pot1 in *S. pombe* results in immediate cell death, with a few cells surviving by circularizing their three chromosomes (14). Overexpression of a dominant-negative form of Pot2 in *Arabidopsis* similarly results in catastrophic genome instability (240). Work by several labs suggests that Pot1 is critical for chromosome end-protection in mammals as well (107, 115, 275, 296, 299), but the exact function of Pot1 in protecting mammalian telomeres remains unclear (reviewed in (13)). Pot1 plays an important role in regulating telomere length. Similar to Cdc13p, Pot1 both positively and negatively regulates telomere length (50, 132, 149, 162).

Work in *S. pombe* suggested that the amount of Pot1 bound to the chromosome end was key for deciding whether telomeres would be elongated or shortened (33). Pot1 bound to the extreme 3'-overhang of a telomeric substrate *in vitro* leads to inhibition of telomerase activity, while moving Pot1 one repeat inward results in an increase in telomerase processivity (149), thus Pot1 can function as a negative or positive regulator

depending on its exact binding location. Interestingly, Pot1 can localize to telomeres via an interaction with TRF1, completely independently of the DNA binding domain of Pot1 (162). However, in the absence of the DNA binding domain, Pot1 is unable to properly regulate telomerase activity at chromosome ends, and telomeres become extensively elongated. Thus, Pot1 is likely to transduce the negative regulatory signal of TRF1 to the extreme terminus of the chromosome.

DNA REPAIR PROTEINS

One of the major functions of telomeres is to prevent the natural ends of the chromosomes from being recognized as DNA double-strand breaks (DSBs). It was somewhat paradoxical that many proteins involved in the recognition and repair of DSBs are actually required for the proper function of telomeres (reviewed in (178, 289). The non-homologous end-joining (NHEJ) pathway for DNA repair is the primary mechanism responsible for fusion of dysfunctional telomeres (40, 108, 251). Major components of this pathway include the KU70/KU80 heterodimer, which recognizes DSBs, and Ligase IV, which catalyzes the ligation of broken ends. Surprisingly, the KU heterodimer plays an important role at telomeres in many organisms.

In yeast, KU is localized to telomeres and rapidly transits from this repository when DSBs are detected elsewhere in the genome (177). Deletion of KU results in short, but stable telomeres (27), and its loss increases nuclease and recombinase activities at telomeres (26, 94, 219). In yeast, KU additionally associates with the TER subunit Tlc1, and this interaction is important for localization of telomerase to the telomere in a cell-cycle dependent manner (74, 216).

The role of KU in higher eukaryotes is less clear. In *Arabidopsis*, KU is a negative regulator of telomerase; its deletion results in extensive telomerase-dependent telomere elongation (85, 232). Similar to yeast, it also appears to play a role in protecting the C-strand from degradation, as loss of both TERT and KU results in much faster telomere shortening and extended 3'-overhangs (231).

The role of KU in mammals is more confusing. In mice, some groups have reported little change in telomere length (36, 235), while others have shown telomeres shorten in the absence of KU (61, 235). The situation is similarly muddy in humans, where groups have reported either extensive shortening of telomeres (121, 199), or no change (269). Similar to yeast, human KU also appears to physically interact with telomerase (264). Although its role in regulating telomere length is unclear, KU clearly inhibits telomere-telomere fusions (61, 121, 199, 235).

Additional interesting components of the NHEJ machinery are the site-specific nucleases Artemis and Apollo. Mutation of Artemis results in a form of severe combined immunodeficiency (SCID) in native Alaskans (194). This disease results from the inability of Artemis to properly open DNA hairpins formed during V(D)J recombination (171). Artemis is a site- and structure-specific nuclease, changing its biochemical properties based on protein-protein and DNA-protein interactions (171). Cells deficient in Artemis display a radiation-sensitive phenotype as well as a low level of telomere-telomere fusions (234). While Artemis has not yet been directly localized to the telomere, a related protein, termed Apollo, has been recently identified as a TRF2/RAP1 interacting protein in human cells that localized directly to telomeres (82,

151, 272). Knockdown of Apollo results in DNA damage responses and telomere fusion, a phenotype that is exacerbated when combined with dominant-negative alleles of TRF2 (151, 272). Purified Apollo protein possesses 5'-to-3' exonuclease activity, and may therefore be involved in properly forming 3'-overhangs (151).

Two other proteins involved in telomere biology as well as DNA damage recognition are the protein kinases ATM and ATR (Tel1 and Mec1, respectively, in *S. cerevisiae*). ATM and ATR are master regulators of DNA damage checkpoint; activation of either proteins leads to cell cycle arrest in all phases of the cell cycle, and in response to a diverse range of DNA damage signals (1). While there is significant overlap in the types of damage that triggers signaling by these two proteins, ATM can be considered a first responder to DSBs, while ATR signals damage during replication or damage that inhibits replication.

In yeast, Tel1 and Mec1 are required for efficient telomerase activity; telomeres in either single mutant are short but stable, while the double mutant is completely incapable of maintaining telomeres and they gradually shorten due to the end-replication problem (233). A dominant negative form of ATM in human cells also displays telomere shortening (247), and mice doubly deficient for ATM and TER display enhanced proliferation defects that result in early death, though the effect of ATM deficiency on telomere length is unclear (221, 292). ATR mutants in mammals are lethal, prohibiting functional studies (28, 29).

Tel1 and Mec1 are recruited reciprocally to telomeres during specific stages of the cell cycle (259). Consistent with its predominant role in responding to replication damage, Mec1 is recruited to yeast telomeres in late S-phase, while Tel1 is localized throughout the rest of the cell cycle. In humans, the association of ATM with telomeres following replication is actually necessary for telomere capping; inhibition of ATM activity during this stage of the cell cycle results in telomere fusions (276).

Null mutants of ATM and ATR are viable in *Arabidopsis* (88), and their deletion does not result in changes in telomere length (277). The double mutants are completely sterile, and exhibit a low level of anaphase bridges, but still maintain proper telomere length (60, 277). However, when combined with a *tert* mutant, *atr* mutants exhibit an increased rate of telomere loss, and *atm* mutants display an early onset of chromosome instabilities, despite no change in the loss of telomeric DNA relative to a the single *tert* mutants (277). Thus, in *Arabidopsis*, ATR is required for telomere maintenance while ATM is important for protecting critically shortened telomeres from fusions.

T-LOOPS

How telomeres mask themselves from being recognized as DSBs is still unclear. Indeed, it appears that following replication, human telomeres *must* be transiently recognized as a DSB for their proper protection (276). A likely mechanism for this protective aspect is the formation of a higher-order nucleoprotein structure termed the t-loop (98). In this structure, the 3'-overhang folds back upon and invades duplex DNA, forming a displaced-loop at the junction FIG. 4. T-loops are a conserved structure, and

have been observed in mammals, plants, ciliates, fission yeast, and trypanosomes (39, 98, 196, 198, 266). While no direct evidence for this structure has been obtained in budding yeast, experimental evidence shows that the terminus is associated with internal sequences, suggesting a fold-back structure is possible (62, 99).

Formation of t-loops *in vitro* requires the presence of TRF2 and a 3'-overhang (98). T-loop structures are stable following DNA crosslinking and protein removal, suggesting that the structure involves specific DNA-DNA interactions that are initially formed with the help of TRF2. These structures can be obtained by crosslinking DNA *in vivo* prior to DNA extraction, demonstrating that they are formed naturally in the cell. Finally, single-strand binding protein bound specifically to the loop junction, indicating the presence of ss DNA at this location, and consistent with the formation of a displaced-loop (98). The amount of DNA located in the looped region varies by organism, but is generally proportional to the total size of the telomere tract (reviewed in (284).

TRF2 lacks an apparent helicase activity, which would presumably be necessary for strand-invasion of the 3'-overhang. TRF2 associates with the DNA repair complex MRE11/RAD50/NBS1 (MRN), specifically during S phase, when a t-loop would have to be unwound for replication. The t-loop must be re-formed to provide chromosome end-protection (307). This suggests that TRF2 can mediate t-loop formation via interactions with associated proteins. Though not required for T-loop formation *in vitro*, TRF1 has been shown to bend telomeric DNA, perhaps assisting in the folding of ds telomeric DNA for T-loop formation (16, 17, 97).

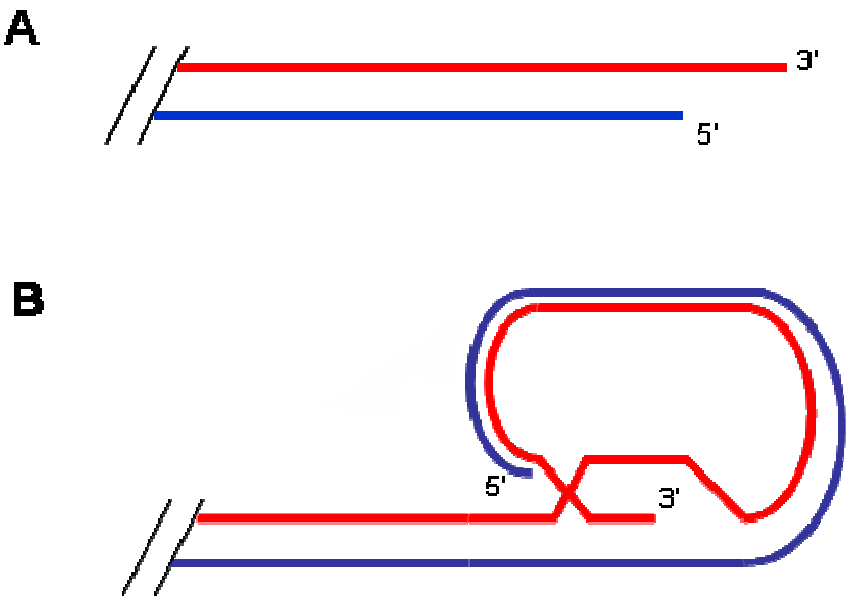


FIG. 4. The t-loop structure of telomeres.

A) A linear model of a telomere with the 3'-overhang is shown. The G-rich strand of the telomere is shown in red and the C-rich strand in blue. B) The t-loop structure. The 3'-overhang has folded back upon and invaded the duplex DNA, displacing G-rich DNA.

How T-loops mediate end-protection is not entirely clear. Sequestration of the 3'-overhang inside a duplex structure essentially removes the end, presumably masking it from NHEJ activities (64). Additionally, TRF2 interacts with ATM at a conserved serine residue that is auto-phosphorylated in response to DNA damage (10). TRF2 binding can inhibit this auto-phosphorylation (128), providing a specific anti-damage signal at the telomere. Confusingly, ATM phosphorylates TRF2 in response to DNA damage, and phosphorylated TRF2 localizes transiently to DSBs (260). Thus, the interaction between ATM and TRF2 is complex, and likely mediated by additional factors.

TELOMERE LENGTH HOMEOSTASIS

Different species maintain their telomeres at widely different lengths. For example, some ciliate telomeres are less than 50 nucleotides, *S. cerevisiae* telomeres are tightly maintained at approximately 350 bp (261), *Arabidopsis* telomeres range from 2-8 kb (239), human telomeres range between 5 and 15 kb (143), mice telomeres vary by breed but range between 10 and greater than 60 kb (308), and telomeres in tobacco range between 20 and 166 kb (256). Telomere length homeostasis is thought to be maintained primarily through a competition between the end-replication problem and the action of telomerase, with telomerase acting preferentially on the shortest telomeres in the population (reviewed in (119). As mentioned previously, several of the ds telomere binding proteins (TRF1, Rap1p, and Taz1p) function as negative regulators of telomere length, likely through a "protein-counting" mechanism.

The protein counting mechanism was originally proposed based on work done by Marcand, Gilson, and Shore (174). The authors inserted a *URA3* marker into the *S.*

cerevisiae genome, which was flanked on the centromere distal side by telomeric repeats. The construct integrated by homologous recombination and the distal telomere tract was extended by telomerase until it became wild type length (300bp). The authors inserted additional telomeric DNA on the centromere proximal side of the *URA3* gene, and the length of the distal telomere decreased by approximately the same length. This indicated that the telomeric DNA centromere proximal to the *URA3* cassette was counted as bona fide telomeric DNA. The authors extended this work by fusing Rap1p to the Gal4 DNA binding domain and replacing the centromere proximal telomeric DNA with Gal4 binding sites. Targeting of Rap1p in this way was sufficient to reduce telomere length. Thus, the amount of telomeric DNA is "counted" by the binding of ds telomere binding proteins. Large numbers of ds telomere binding proteins result in telomerase inhibition, and this inhibition is relieved as telomeres shorten and the number of ds binding proteins bound to the telomere decreases. The protein counting mechanism has been recapitulated at human telomeres by work with TRF1 (7). Furthermore, telomerase has been demonstrated to preferentially extend shorter telomeres in *Arabidopsis* (239) and mice (160), suggesting that this is a conserved mechanism for regulating telomere length.

This work was recently extended by Teixeira et al. (261), who demonstrated that in yeast, regulation of telomere length is achieved via a switch between telomerase-extendible and -nonextendible states. The authors crossed a clonal line of telomerase-deficient yeast carrying a marked telomere with a wild-type strain. Telomerase adds imperfect repeats (TG_{1-3}) in yeast, so by sequencing telomeres from individual progeny, the authors were able to measure how many nucleotides were added to a given

telomere. They found that the amount of telomeric DNA added to a given telomere was highly variable, and did not correlate well with initial telomere length. In contrast, the probability of being extended by telomerase increased markedly as telomeres shortened. The authors then deleted the Rap1p interacting proteins Rif1p and Rif2p, negative regulators of telomerase and telomere length. In this background, telomeres were extended by approximately similar amounts as in the wild type situation, but the frequency of extension was increased at all telomere lengths. Thus, the negative regulation of telomerase by Rap1p and its associated factors in yeast is mediated by whether or not telomerase is allowed to extend a given telomere.

One of the major remaining questions is how the negative regulatory signal is sent from the ds binding proteins along the duplex telomeric DNA to the ss binding proteins located at the terminus of the chromosome. In humans, Pot1 is recruited to the telomere via a bridging protein, TPP1 (159, 302). TPP1 binds both POT1 and TRF1, and TPP1 knockdown results in telomere elongation. Furthermore, POT1 mutants lacking the DNA binding domain lose the ability to negatively regulate telomere length via TRF1 (162). POT1 can function as either a negative or positive regulator of telomerase activity *in vitro* (149), so how the association with TPP1 and TRF1 results in specifically negative regulation remains unclear.

ALTERNATIVE LENGTHENING OF TELOMERES

Telomeres must maintain a minimal length to protect the end of the chromosome from fusion via NHEJ, apoptosis, or senescence (reviewed in (18). Not surprisingly, chronic telomere dysfunction due to loss of telomerase selects for cells that are capable of

overcoming the end-replication problem through alternate means, thus restoring proper chromosome end-protection.

In yeast, the absence of any of the EST proteins results in gradual loss of telomeric DNA, and eventually the cells senesce. However, a small number of cells are able to escape this fate and continue growing despite the absence of telomerase. This renewed growth potential is accompanied by dramatic structural changes to their telomeres (166). These survivors were dependent upon RAD52, a protein required for essentially all homologous recombination in *S. cerevisiae.* Two classes of survivors were identified. In the first class, a repetitive sub-telomeric element normally located at approximately two-thirds of the telomeres became dramatically amplified. These Y' elements spread to most chromosome ends, and formed tandem arrays. Between these elements were short stretches of telomeric DNA, and the extreme terminus also consisted of a short stretch of telomeric repeats. In these Type I Survivors, the terminal telomeric DNA along with the short stretches between the Y' could account for as much as 4% of the total genomic DNA.

In contrast, Type II survivors did not amplify Y' elements. Instead, telomeres in these cells ended in extremely heterogeneous tracts of telomeric repeats. These telomeres slowly shorten due to the end-replication problem, and rare elongation events result in extension of the telomere (262, 263). The Type II survivors, once formed, are stably maintained. In contrast, Type I survivors have a high tendency to convert to Type II, and if the two survivor types are grown in culture together, Type II survivors out compete Type I survivors (263). While both Type I and Type II survivors are dependent

upon Rad52p, they can be distinguished by other genetic requirements. Type I survivors require Rad51p, while Type II survivors require Rad50p.

The genetic requirements for these survival pathways suggest that breakage-induced-replication (BIR) could be the mechanism of survival in these cells. In BIR, a broken end invades a homologous region, and replicates from the homologue to the end of the chromosome, resulting in non-reciprocal transfer of DNA sequences (140). The genetic requirements of BIR include *RAD52*, and either *RAD50* or *RAD51* (172, 244). Further experiments with terminal deletions derived from telomerase dysfunction demonstrated that these terminal deletions could be repaired by BIR (100).

One problem with BIR as the mechanism for survival is that telomeres become elongated very quickly, much faster than could be accounted for by BIR off other short telomeres in the cell. One possible explanation is that a single telomere is rapidly elongated through a different mechanism, and this telomere then serves as a substrate for elongation by other telomeres. Rolling-circle amplification from extra-chromosomal telomeric circles (ECTCs) would provide the cell with a single elongated telomere. Evidence for ECTCs was first discovered in the mitochondria of some yeast, where the genome is not circular, but linear (265). Later work demonstrated that the telomeres of these linear mitochondrial genomes were likely maintained via rolling-circle amplification, with the ECTCs serving as the substrate (205).

Further support for ECTCs serving as rolling-circle amplification substrates has come from work in *Kluveromyces lactis*. Similar to budding yeast, *K. lactis* mutants deficient for telomerase are able to form survivors. *K. lactis* lacks sub-telomeric Y' elements, so

all survivors are similar to Type II survivors of *S. cerevisiae*, they contain long, heterogeneous stretches of telomeric DNA. These survivors are also strongly dependent upon Rad52p, demonstrating that the mechanism of survival is recombinational (186). In yeast cells with two distinct types of telomeric repeats, survivors generated specific patterns of elongated telomeres. These patterns were distinct between cells, but within the same cell, the telomeric pattern at each chromosome was the same (203). Similar results could be obtained by transforming cells with a circular plasmid containing a *URA3* marker. If two different circular molecules were transformed into cells, elongated telomeres within a given cell would contain DNA from only one of the two circles (203). This mechanism of survival was termed the Roll and Spread Mechanism. In this model, a single telomere becomes dramatically elongated via rolling circle amplification from ECTCs. The elongated telomere then serves as a substrate for BIR by the remaining telomeres in the cell. Indeed, when a single telomere containing a specific repeat type is elongated, the sequence of the elongated telomere spreads to all other telomeres in 90-95% of cells examined. In contrast, when the marked telomere is the same size as other telomeres in the cell, it spreads to all other chromosomes in only about 10% of all cells, consistent with random selection (268). Thus, an elongated telomere will preferentially serve as a BIR substrate for other telomeres in the cell.

Telomerase-independent telomere elongation has also been described in telomerase negative immortalized mammalian cells, where it is termed ALT, for alternative lengthening of telomeres (30). ALT cells have very heterogeneous telomeres that range from both longer (>20 kb) to shorter (<1 kb) than normal human telomeres

(reviewed in (113), and are therefore similar to Type II survivors and survivors from *K. lactis*. ALT cells are also distinguished by the presence of ALT-associated PML bodies (APBs) (303). APBs contain telomeric DNA, the ds telomere binding proteins TRF1 and TRF2, as well as a large number of proteins associated with recombinational activities, including Rad50p, Rad51p, and Rad52p (reviewed in (113). Telomeres in ALT cells shorten slowly due to the end-replication problem and then are dramatically extended, with documented increases of more than 23 kb (197).

The molecular mechanism of ALT has been difficult to determine, although some evidence suggests it functions in a similar manner to Type II recombination and the Roll and Spread mechanism in *K. lactis*. In ALT cells where a single telomere had been tagged with a specific DNA sequence, the DNA tag spread to other chromosome ends over several population doublings (68), consistent with a BIR mechanism. Additionally, recent data have demonstrated the presence of ECTCs in ALT cells (38). Finally, over-expression of a PML protein, Sp100, resulted in loss of APBs and inactivation of the ALT mechanism. The highly over-expressed Sp100 sequestered MRE11/RAD50/NBS1 (the MRN complex) into Sp100 aggregates (122). Thus, ALT in human cells may also occur through a roll and spread mechanism.

TELOMERE RAPID DELETION

As proper telomere function is essential for chromosome stability, selection for telomerase-independent mechanisms of telomere maintenance was not entirely unexpected. More surprising, however, has been the discovery that there are mechanisms in several organisms that function to shorten elongated telomeres.

The end-replication problems is thought to shorten telomeres at the same rate, regardless of the length of the telomere (173). It was surprising then, that in a C-terminal deletion of Rap1p, telomeres became very heterogeneous. Following a marked telomere showed that telomeres were both getting longer, as well as undergoing dramatic, stochastic shortening events termed TRD for telomere rapid deletion (153). Further work used a telomere where a single *Hae*III site had been inserted into the telomere tract. When telomeres containing this restriction site underwent TRD, the restriction site was never lost or rearranged. This demonstrated that DNA lost during TRD was lost from the extreme terminus of the telomere (32). Genetic requirements for this process include *RAD52* and at least some components of the *MRE11/RAD50/XRS2* complex (32, 153). Deletion of either component of KU results in an increase in the frequency of TRD (219), as does deletion of HPR1, a protein responsible for repression of intra-chromosomal recombination (153). The authors propose a mechanism whereby a t-loop structure is formed at elongated telomeres. Rad52p and the MRX complex promote branch migration of the t-Loop structure, forming a Holliday junction that can then be resolved, resulting in a shortened telomere and an ECTC (FIG. 5 and ref. (169).

Support for such a model has been recently obtained in two studies in human cells. In the first study, EM examination of telomeric DNA from ALT cells demonstrated large numbers of ECTCs. The size of the ECTCs observed in these cells was roughly equal to the size of the t-loops (38). In the second study, cells over-expressing a dominant-negative allele of TRF2 displayed dramatic loss of telomeric DNA, losing 20% of the

.

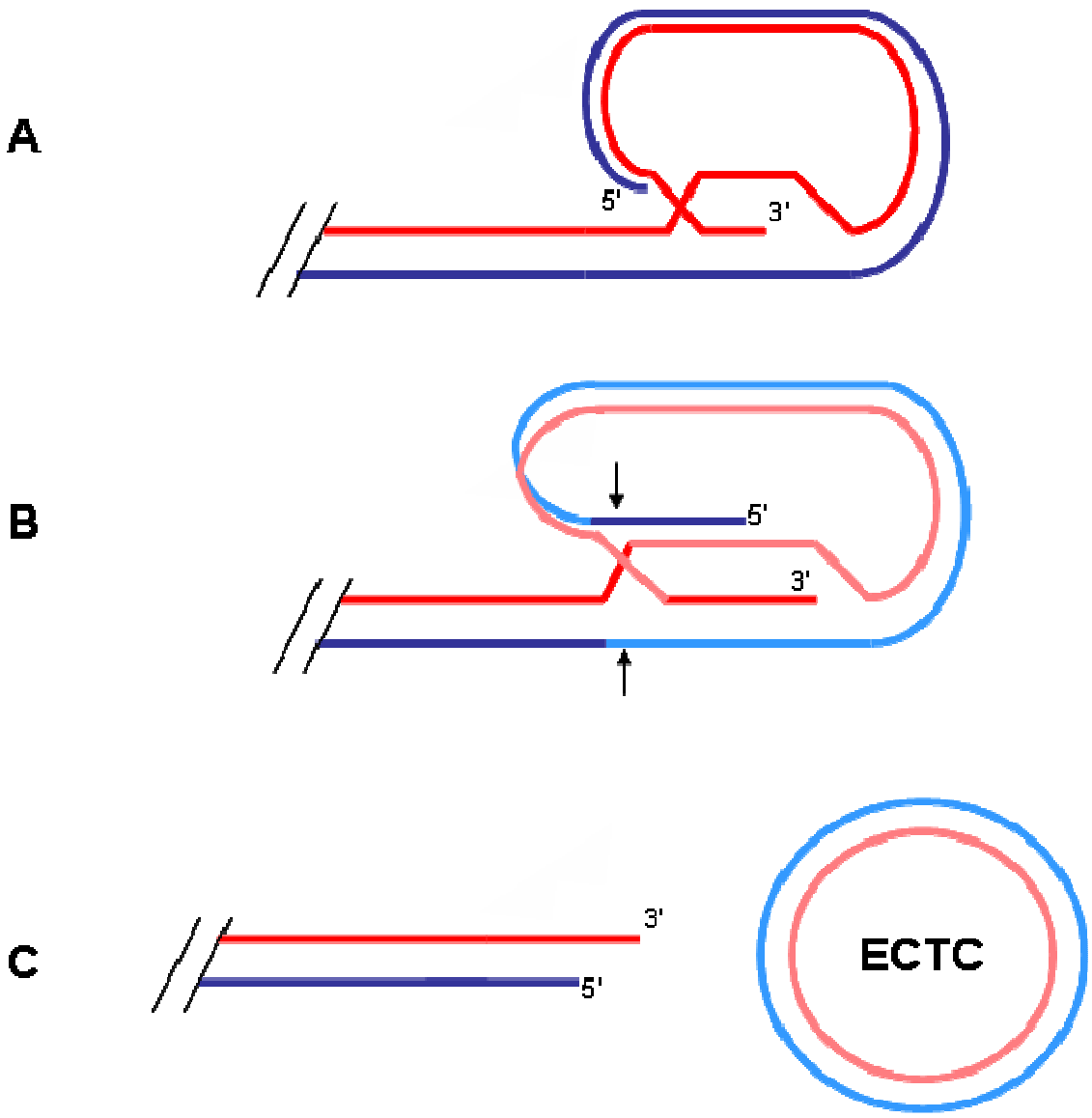


FIG. 5. Telomere rapid deletion.

A) Telomere in the t-loop conformation. B) Branch migration of the displaced-loop, resulting on formation of a Holliday junction. Strands that will be exchanged are colored in a lighter shade, and the plane of cleavage is indicated by arrows. C) Holliday junction resolution results in a shortened telomere and an extra-chromosomal telomeric circle (ECTC).

duplex tract. These deletion events occurred post-replicatively, and preferentially shortened telomeres replicated by leading strand machinery. Deletions were dependent upon NBS1 (a member of the MRN complex) and XRCC3, a putative Holliday-junction resolvase. Finally, expression of the TRF2 mutant resulted in the appearance of ECTCs (280).

Work on an STN1 mutant in *K. lactis* suggests that ALT and TRD may in fact be regulated by similar proteins. In *stn1-M1* mutants, telomeres become dramatically elongated and heterogeneous. This elongation does not require functional telomerase, but does require Rad52p, similar to telomerase-independent survivors in many organisms. Restoration of a wild type allele of STN1 in the mutant background returned telomeres to their wild-type length, suggesting that TRD functions in this background to restore appropriate telomere length (120). Thus, STN1 may function as a regulator of both TRD and ALT. However, STN1 homologues have yet to be identified outside of yeast.

ARABIDOPSIS **AS A MODEL FOR TELOMERE BIOLOGY**

The flowering plant *Arabidopsis thaliana* has proven to be a useful system for the study of telomere biology. A multi-cellular higher eukaryote, *Arabidopsis* has a small, sequenced genome (8), and is extremely genetically tractable. Several stock centers contain different mutant lines, including T-DNA insertion (6) and EMS mutagenized (54) lines, allowing one to quickly screen a gene of interest for mutations. Furthermore, *Agrobacterium* mediated transformation is extremely easy and efficient (47). Due to its relatively short life-cycle, several generations of mutants can be analyzed over a short

period of time. Furthermore, this short life-cycle along with ease of crossing and classical genetics allows for the generation of compound mutants, with septuple mutants being currently reported (150)

With regard to telomere biology, *Arabidopsis* has several advantageous features. Telomeres range from 2-5 kb in many ecotypes (239). This small telomere size facilitates length analysis as conventional agarose gels can be used to accurately measure telomere length. This is in contrast to humans and mice, where pulsed field gel electrophoresis (PFGE) or fluorescent *in situ* hybridization (FISH) are needed to measure telomere length due to the larger telomere size in these organisms. *Arabidopsis* contains only 5 chromosomes, for a 2n content of 10 and 20 telomeres. This is the second lowest number of telomeres in a model system, with *S. pombe* being the smallest with only 6 telomeres arms. Additionally, unique subtelomeric sequences are located on eight of the ten chromosome ends, allowing analysis of individual telomeres and facilitating the development of unique methods for measuring telomere length and studying telomere-to-telomere fusions (108).

Somewhat surprisingly, telomerase activity is regulated similarly in *Arabidopsis* and humans, with only highly proliferative tissue and the germ line displaying activity (75). Mutants in telomerase expression in *Arabidopsis* may have functional homologues in humans, making it a useful model for understanding telomerase expression and activation (226).

One of the most important features of *Arabidopsis* is its extreme tolerance to genomic instability (230). *Arabidopsis tert* plants survive for ten generations, with over 50% of anaphases in terminal plants displaying dicentric chromosomes. Furthermore, terminal *tert* cells do not undergo apoptosis in *Arabidopsis*, a striking contrast to cells in mice (147). The lack of an apoptotic response allows the study of cells that would otherwise apoptose in humans. Many proteins involved in telomere biology in humans are lethal, including all three members of the MRN complex (reviewed in (270), ATR (28) and TRF1 (129). While a homologue for TRF1 has yet to be identified in *Arabidopsis*, *atr* (60), *mre11* (34), and *rad50* (86) are all viable mutants in *Arabidopsis*, facilitating the functional analysis of these proteins in telomere biology.

OVERVIEW

In this dissertation, I explore the role of recombinational activities at telomeres in *Arabidopsis*. In Chapter II, I discuss the results of experiments designed to generate *Arabidopsis* cells that could continue proliferation in the absence of telomerase. Interestingly, in two different cell lines examined, an alternative form of telomere maintenance was not observed. Instead, these cells lost all telomeric DNA. Despite this loss, cell growth remained relatively unperturbed and these cells were able to proliferate for greater than three years in culture. Possible explanations for how these cells were able to continue proliferating are discussed.

In Chapter III, I investigate whether a TRD mechanism is capable of functioning at *Arabidopsis* telomeres. By restoring proper telomerase regulation in *ku70* mutants with elongated telomeres, I observe frequent TRD events as telomeres return to wild type

length. Interestingly, these TRD events are not dependent upon proteins known to be important for this process in other organisms, namely MRE11 and XRCC3. Finally, I demonstrate that TRD functions at telomeres even within the wild type range. While there is no obvious relationship between TRD and telomere length above 2 kb, the frequency of TRD drops precipitously when telomeres fall below the 2kb length. As 2 kb is lower end of wild type telomere lengths, this finding suggests that TRD may regulate telomere length in wild type plants.

In Chapter IV, I explore the fate of cells within the plant that have undergone telomere fusions. I propose that chromosomes broken after a telomere-to-telomere fusion are not subjected to further fusion events, but are instead arrested in G, likely in an ATM dependent manner. By crossing late-generation telomerase mutants with plants heterozygous for *tert*, I demonstrate that telomeric fusions are not propagated to the next generation when telomerase is present. In contrast, both pollen and embryos derived from plants deficient in telomerase display telomere fusions. In an attempt to distinguish whether these fusions arise *de novo* or are propagated to progeny when telomerase is absent, I design constructs to assay for the presence of fusions, and provide initial characterizations of their expression.

CHAPTER II

TELOMERASE-INDEPENDENT CELL SURVIVAL IN *Arabidopsis thaliana*[*]

SUMMARY

Telomerase is the reverse transcriptase responsible for the maintenance of telomeric repeat sequences in most species studied. Inactivation of telomerase causes telomere shortening and results in the loss of the telomere's protective function, which in mammals leads to cell cycle arrest and apoptosis. Experiments performed on *Arabidopsis thaliana* mutants lacking telomerase activity revealed their unusually high tolerance for genome instability. Here we present molecular and cytogenetic analysis of two cell lines (A and B) derived from seeds of late generation telomerase-deficient *A. thaliana*. These cultures have survived for about three years and are still viable. However, neither culture has adapted mechanisms to maintain terminal telomeric repeats. One of the cultures (B) suffers from severe growth irregularities and a high degree of mortality. Karyological analysis revealed dramatic genomic rearrangements, a large variation in ploidy, and an extremely high percentage of anaphase bridges. The second cell line (A) survived an apparent crisis and phenotypically appears wild-type with respect to growth and morphology. Despite these indications of genome stabilization, a high percentage of anaphase bridges was observed in the A line. We

---

*Reprinted with permission from **Watson, J. M., P. Bulankova, K. Riha, D. E. Shippen, and B. Vyskot.** 2005. Telomerase-independent cell survival in Arabidopsis thaliana. Plant J **43:**662-674. Copyright © 2005 by Blackwell Publishing Ltd. on behalf of the Society for Experimental Biology. (282).

conclude that the restructured chromosome termini provide the A line a partial protection from end-joining repair activities, thus allowing normal growth.

INTRODUCTION

Maintenance of genome stability is essential for cellular survival. The integrity of chromosomes is assaulted by both endogenous and exogenous forces, with the most damaging lesion being DNA double-strand breaks (DSB) (271). In budding yeast, a single DSB is sufficient to arrest the cell cycle or cause cell death (15). Improper repair of DSBs is a primary cause of genome rearrangement and can result in both gain and loss of DNA.

Telomeres are the nucleoprotein complexes at the ends of eukaryotic chromosomes that distinguish natural ends from DSBs (18). In most eukaryotes telomeres are composed of long repetitive arrays of short G-rich sequences that are extended by the telomerase reverse transcriptase (250). In the absence of telomerase, telomeres shorten with each cell division due to the end-replication problem (208, 281). When telomeres shorten to a critical length, cells cease division and enter senescence (167). If forced to continue dividing, telomere ends become recognized as DSBs and a period of crisis ensues, resulting in genomic instability and, in mammals, apoptosis (59).

In most human somatic cells, telomerase activity is undetectable, and telomeres shorten with each cell division (104, 135). However, the majority of human tumours are telomerase positive (242), and telomerase activity is important for tumour survival (101), particularly tumours with short average telomere lengths (304). Telomerase deficient

mice survive until the sixth generation (G6), but begin showing genomic instability in G4 (22). While the terminal phenotype of telomerase knockout mice is delayed in p53 telomerase double knockout lines (46), late generation mutants show an increase in epithelial cancers resulting from breakage-fusion-bridge cycles (9). Dysfunctional telomeres fuse to one another, producing dicentric chromosomes that break during anaphase to produce ends that will fuse again, only to be broken in the next mitosis.

Telomerase is not the only means of capping chromosomes and overcoming the end-replication problem. Several alternative processes have been described, the most well studied being in *Drosophila melanogaster. Drosophila* lacks telomerase and canonical G-rich telomeric repeats. Instead, chromosome ends are composed of retrotransposons (152) and are bound by the highly conserved protein HP1 (215), which protects chromosomes from being recognized as DSBs (71). HP1 is associated with heterochromatin (12), suggesting that in the absence of canonical telomeres, *Drosophila* has adapted unique chromatin structures to cap telomeres.

Even in organisms that normally use telomerase for telomere maintenance, telomerase-independent mechanisms are revealed when telomerase activity is disrupted. In *Saccharomyces cerevisiae* mutants deficient for components of telomerase, at least two distinct mechanisms of telomerase independent telomere maintenance allow cells to survive (166, 263). Both are recombinational in nature and require genes in the *RAD52* epistasis group. Type I survivors dramatically amplify the copy number of sub-telomeric Y' elements and stabilize chromosomes through short telomeric tracts (166). In contrast, Type II survivors fail to amplify Y' elements and instead have extremely

heterogeneous tracts of telomeric repeats, stretching much longer than telomeres in wild-type cells (263). Telomerase-independent survivors have also been identified in some immortalized human cell lines, including a small percentage of cancer cell lines that lack detectable telomerase activity (31). The cells that undergo Alternative Lengthening of Telomeres (ALT) possess extremely heterogeneous telomeres. Several lines of evidence suggest that they are maintained through recombination (68, 122).

Experiments in *Arabidopsis thaliana* lacking telomerase demonstrate that this plant is much more tolerant to genomic instability than mammals (231). *Arabidopsis* telomeres consist of 2-8 kb tracts of TTTAGGG repeats that are maintained by telomerase (239). Like humans, telomerase activity in *Arabidopsis* is confined to highly proliferating cells and the germ line (75). The gene encoding the *Arabidopsis* telomerase catalytic subunit (*AtTERT*) has been cloned, and *AtTERT* mRNA expression mirrors enzyme activity (76). Remarkably, plants harbouring a T-DNA insertion in *AtTERT* survive for ten generations, although their telomeres shorten at a rate of 250-500 bp/generation (76, 230). Beginning in G6, defects in vegetative and reproductive tissues appear and are accompanied by genome instability as seen by an increased incidence of anaphase bridges. Telomerase mutants survive for up to ten generations despite increasing cytogenetic and phenotypic abnormalities (230, 246). Cytogenetic analysis of sequences at the junctions of fused chromosomes in G8 telomerase mutants show that rDNA loci are over-represented approximately 10-fold more than expected for random fusion events, and are frequently translocated to ectopic positions in the genome. This finding suggests that several rounds of breakage-fusion-bridge cycles have occurred in late generation telomerase mutants (246).

In this study, we present initial characterization of two telomerase-independent callus cultures derived from progeny of G8 telomerase mutants that have survived for about four years. Molecular analysis indicates that these cells no longer maintain telomeric DNA as neither culture has detectable terminal telomeric repeats as assayed by FISH and Southern blotting. One culture, which we have named the B line, has been propagated for nearly three years. This culture suffers from severe growth irregularities, including a loss of typical morphology and a high mortality in numerous callus sectors. Karyological analysis shows that the B line has a high variation in measurable chromosome parameters (ploidy, number of FISH signals from rDNA and centromeres), as well as frequent anaphase bridges. The second line, termed A, has been cultured for more than four years and survived an apparent crisis. This line is phenotypically stable and fast growing. In contrast to the B line, the A line is homogenous with regard to measurable karyological parameters, suggesting that it has a stabilized genome. Remarkably, the A line still shows a high percentage of anaphase bridges. We speculate that the A line has restructured chromosome termini to provide partial, but not complete, protection from DSB repair activities.

MATERIALS AND METHODS

Plant material and establishment and maintenance of callus cultures. *Arabidopsis thaliana* ecotype Columbia gl1-1 wild-type and G8 *tert* mutants were used (230) to establish callus cultures. Plants were cultured in a climate chamber at 22°C at long day (16 h) light conditions. The A line culture was initiated February 2001. Seeds from G8 telomerase mutants were surface sterilized by rinsing with 70% ethanol before incubating for 7 min in 50% bleach/0.1% Triton X-100. Seeds were rinsed 3 times with

sterile water and placed in 50 mL Erlenmeyer flasks containing liquid 0.5x MS media supplemented with 3% sucrose (182). Flasks were shaken at 100 rpm at room temperature in the dark. Roots were harvested at three weeks and were finely chopped and placed in MS media supplemented with 0.5 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 2 mg/L IAA (indole-3-acetic acid), 0.5 mg/L IPAR (N6-(2-isopentenyl)adenosine riboside) and 3% sucrose. Root material was shaken at 150 rpm at 23ºC in the dark. Cultures were subcultured weekly by adding 100 mL fresh media to 50 mL culture. One mL undiluted culture was spread onto solid media of the same composition containing 2.8g/L phytagel. Calli grown on solid media were transferred to fresh media every 4 weeks. After six months, all material was transferred to MS media supplemented with 0.5 mg/L 2,4-D, 0.05mg/L kinetin, and 3% sucrose. Solid media was further supplemented with 2.8g/L phytagel (termed Callus Induction Media). The B line culture was initiated July 2002 by germinating seeds directly on CIM. Calli were maintained on this media and transferred to fresh media every 4 weeks. For DNA damage response experiments, callus was transferred to CIM media supplemented with the indicated concentration of bleomycin, menadione, or methyl-methane sulfonate (MMS) (Sigma).

DNA extraction, TRF analysis, TRAP assays, and Bal31 digestions. DNA extraction was performed as previously described (25). TRF analysis was performed by digesting approximately 1 µg genomic DNA with Tru9I for hybridization with telomeric probes, or with *Spe*I and *Pvu*II for hybridization with sub-telomeric probes to chromosome arms 2R and 5L (239). Digested DNA was separated by electrophoresis through agarose and transferred to nylon membrane. Hybridization was carried out for 16 h at 55ºC for telomeric probes, and 65ºC for subtelomeric probes. Blots were pre-

hybridized in hybridization buffer (0.25M NaPO4 pH 7.4, 7% SDS and 1 g/L BSA). Washing conditions maintained the same temperature as hybridization. Two 10 min washes with 2xSSC, 0.1%SDS were followed by two 10 min washes with 0.2xSSC, 0.1% SDS. Southern blots were scanned with a Storm Phosphoimager and analyzed with ImageQuant software. For reprobing, blots were stripped with 0.4M NaOH for 30 min at 42 ºC, followed by a 30 min wash with stripping solution (200mM TrisCl pH 7.5, 0.1xSSC, 0.1% SDS) before hybridization, again as described above. *TERT* genotyping, protein extraction, and TRAP assays were performed as previously described (Fitzgerald et al., 1996). Seven µg of genomic DNA was digested with 10 units Bal31 (NEB) in 500 µL total volume. One hundred µL was removed at time 0, and at 5 min intervals thereafter. Bal31 was then inactivated at 65ºC for 20 min in the presence of 20 mM EGTA. DNA was ethanol precipitated and digested for subtelomeric analysis as described above.

Slide preparations, analysis of anaphase bridges, and measurement of integrated DAPI fluorescence. Intact terminal inflorescences and calli were harvested and immediately fixed in ethanol: acetic acid (3:1). Pistils excised from young floral buds and calli were enzymatically macerated with 0.5% pectolyase and 0.5% cellulase and squashed in 50% acetic acid as previously described (114). To visualize chromosomes, slides were stained with DAPI (4`, 6-diamidino-2-phenylindole, 1 µg/ml) and mounted in Vectashield (Vector). Each slide was scored for anaphases and the ratio of aberrant anaphases counted. Squashed mitotic preparations from plants (wild-type and G8 *tert* mutants) and calli (wild-type, A-line and B-line) were quantitatively stained with DAPI mounted in Vectashield). The images of individual nuclei were

acquired by a CCD camera (AxioCam; Zeiss). All the images were captured using the same time interval (0.12 s). Integrated DAPI fluorescence was determined as a multiple of the area and intensity of pixels for individual nuclei using ImageSpace software (Molecular Dynamics) run on a O2 workstation (SGI). Fluorescence *in situ* hybridization on these nuclei was performed after post-fixation as described below.

***In situ* hybridization.** For bicolor FISH the probes were labelled with SpectrumGreen-dUTP (marker for the centromere, i.e., BAC F21I2 obtained from ABRC, Columbus, Ohio) and Cy3-dUTP (rDNA probe, the 2478 bp fragment of tomato 25S rDNA;(137). Squashed mitotic preparations of pistils from wild-type and G8 terminal plants and wild-type, A-line, B-line calli were digested with RNase A (Promega, 100 mg/ml, 1 h at 37°C) and pepsin (Sigma, 100 mg/ml in 0.01 N HCl, 10 min) followed by subsequent washes in 2xSSC. After postfixation in 3.7% neutral formaldehyde (10 min) and washing in 2xSSC, the slides were dehydrated in an increasing ethanol series and air-dried. The hybridization mix (30 µl per slide) consisted of 30 ng SpectrumGreen-labeled F21I2 BAC DNA, 10 ng Cy3-labeled rDNA probe, 10% dextran sulfate, and 50% formamide in 2xSSC. Hybridization mixture was applied on slides, covered with plastic coverslips, and the slides subjected to heat denaturation and gradual lowering of the temperature to 37°C using a PHC-3 thermal cycler equipped with a flat plate (Techne). After incubation at 37°C for 18 h, slides were washed under stringent conditions (42°C, 0.1xSSC) and mounted in Vectashield with DAPI (1 µg/ml). Hybridization with telomeric TTTAGGG peptide nucleic acid probe (PNA) was performed as in the case of fluorochrome-labelled probes. Hybridization mix (15 µl per slide) contained 7.5 ng Cy3-labeled telomeric PNA probe, 5% dextran sulfate, 60% formamide in 2xSSC and sterile

water. Slides were subject of heat denaturation and incubation as described above. A stringent wash was done in 0.1xSSC with 0.5 % Tween 20 at 42°C three times at room temperature for 5 min. Slides were mounted in Vectashield with DAPI (1 μg/ml). In the presented illustrations DAPI is illuminated in red, Cy-3 in yellow, and SpectrumGreen in blue for better resolution. Fluorescence was visualised using an epifluorescence microscope (Olympus AX70) and signals were captured with a CCD camera (AxioCam; Zeiss) and evaluated using ISIS software (MetaSystems, Germany).

RESULTS

Isolation and growth characteristics of telomerase-independent cell cultures. To determine whether *Arabidopsis* can activate a telomerase-independent mechanism of cell survival, we selected for telomerase-negative cells that could be maintained in a suspension culture. We initiated a suspension culture from roots of G8 *tert* mutants. G8 *tert* mutants have telomeres ranging in size from 0.5 to 2 kb, and an abundance of telomere fusions (108, 230). This culture, started in February of 2001, is referred to as the A line. A second telomerase-independent cell line (B line) was initiated in July of 2002 using a slightly different protocol. In this case, G8 *tert* seeds were plated directly onto callus-inducing media and the resulting calli were transferred to suspension culture. Cells were subcultured either weekly or bi-weekly depending on the visible density of the culture.

Initial growth of the A line in suspension was slow, with passages occurring every two weeks until the sixth passage (P6), when cultures reached high visible density on a weekly basis. To monitor the growth characteristics of cells in the culture, one mL of

suspension culture was plated onto solid media where calli could be observed. As seen in FIG. 6A, calli from P3 were large, white and fluffy, indicative of healthy growth. However, beginning in P4, massive browning occurred, and growth slowed. Calli derived from P5 were very small and by P6 no calli were obtained. While calli from P7 and P8 exhibited slow growth, they displayed a wild-type like fluffy white appearance. The calli derived from P9 were indistinguishable from wild-type in growth and morphology. During this same period in culture, wild-type suspensions consistently produced large numbers of calli, which did not undergo browning (data not shown). To date, the A line has been propagated for 49 months and continues to retain the growth characteristics and appearance of wild-type cells.

To test whether the sustained growth of the A line cells was due to a reactivation of telomerase activity, PCR was used to verify that the cells retained the T-DNA insertion in the *TERT* gene. As seen in FIG. 6B, PCR with all three primers produced a single band from wild-type cells corresponding to the wild-type allele. In contrast, DNA from the A line did not produce the wild-type band. Instead, a product corresponding to the mutant allele carrying the T-DNA insertion was produced. Confirmation that telomerase had not been activated was obtained by performing TRAP (telomere repeat amplification protocol), a PCR-based assay that detects telomerase activity. As expected, extracts prepared from wild-type leaves displayed little or no activity, while extracts prepared from highly-proliferative wild-type callus showed robust telomerase activity (FIG. 6C). Telomerase activity was undetectable in extract from A line cells, demonstrating that A line cells survive in the absence of telomerase.

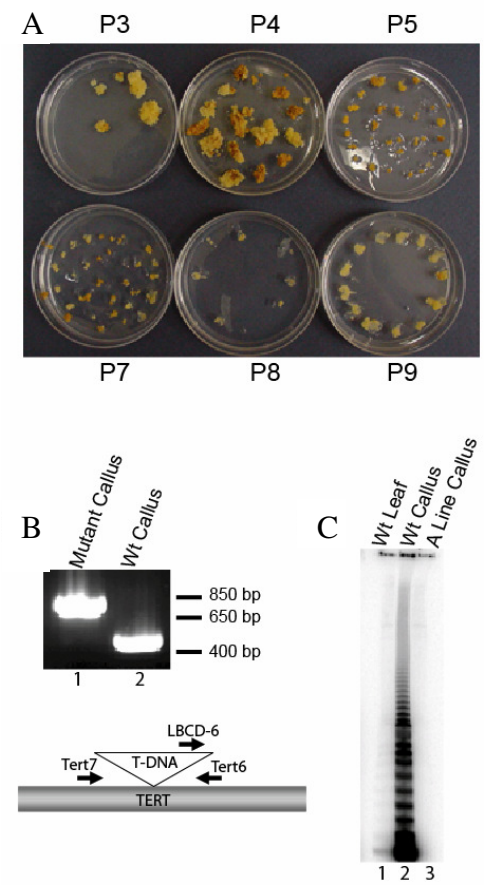


FIG. 6. Characteristics of A line cultures.

A) Callus induced from G8 *tert* mutants was analyzed for its growth properties at the indicated passages, B) for the presence of a T-DNA insertion in *TERT*, C) and for telomerase activity. PCR primers for genotyping are diagrammed in B; the wild-type reaction produced a 500 bp product from primers Tert6 and Tert7, while the mutant reaction produced a 700 bp product from Tert6 and LBCD-6. Each reaction contains all three primers.

In contrast to the A line, early growth of the B line was robust with passages occurring mostly on a weekly basis. However, beginning around P20, growth declined noticeably. Although B line cells have been maintained for 30 months, they continue to grow more slowly than either the wild-type or A line cultures, and calli derived from the B line show large and abundant sectors of browning. We hypothesize that the B line has not yet enabled survivors with wild-type growth characteristics.

The A and B lines lack terminal telomeric DNA. Terminal restriction fragment (TRF) analysis was performed on DNA extracted from P6 callus as well as subsequent passages to examine the length of the telomeric DNA tract. As diagrammed in FIG. 7A, *Tru*1I digests immediately adjacent to telomeric DNA. Hence, the length of the telomeric DNA tract can be monitored by hybridizing a Southern blot with a telomeric DNA probe.

As expected, telomeres from wild-type plants of the Columbia ecotype appeared as a smear, ranging in size from 2-5 kb (FIG. 7B, lane 1). In addition to the terminal telomeric repeats, there are several blocks of internal telomeric DNA sequences in *Arabidopsis* (228). Under our digestion conditions, interstitial sequences appeared as diffuse bands migrating below 500 bp. Disrupting telomerase led to progressive telomere shortening and telomeres in G8 mutants ranged in size from 0.8 to 1.2 kb (FIG. 7B, lane 2). Remarkably, even in the earliest passage of the A line examined, P6, the telomeric DNA smear was absent, and only interstitial repeats could be detected (FIG. 7B, lanes 3-6).

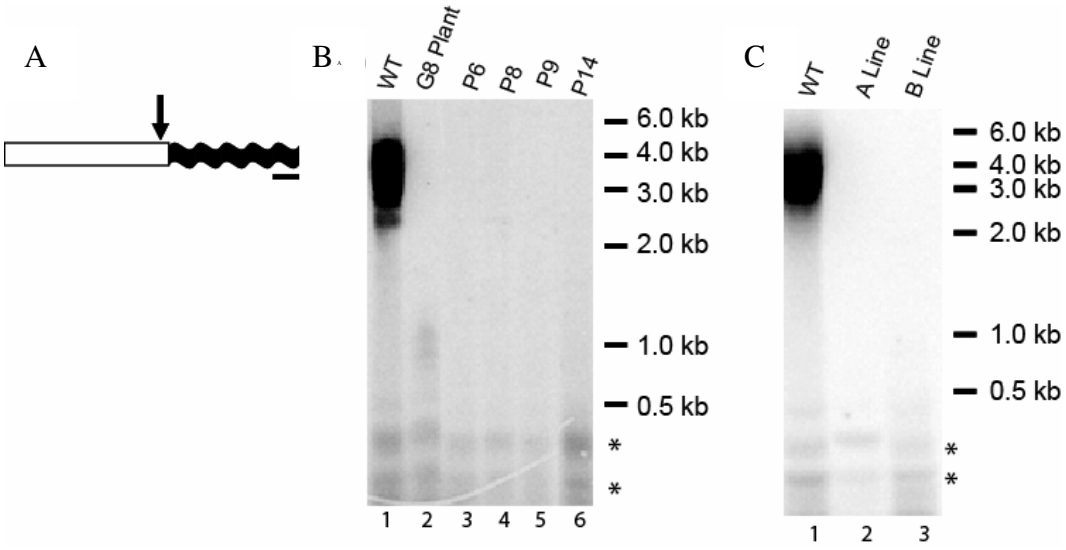


FIG. 7. Telomerase-independent calli lack telomeric DNA at chromosome ends. A) Scheme of TRF analysis. The wavy line represents telomeric DNA repeats, the rectangle subtelomeric DNA, the line the telomeric probe, and the arrow the *Tru*1I restriction site. B) TRF analysis of initial passages of the A line culture C) and a more recent TRF from A and B line calli that have survived in culture for >3 years are shown Internal telomeric DNA is indicated by the asterisks.

The minimal functional telomere length in *Arabidopsis* is approximately 350 bp (108). Since telomeres in this size range would be masked by the signal from interstitial repeats, TRF analysis was also performed with DNA digested with *Hae*III. A different restriction pattern for the interstitial repeats was produced, but no signal consistent with terminal telomeric DNA was observed (data not shown). These data argue that telomere tracts are absent from chromosome termini in A line callus. Similar results were obtained for B line cells (FIG. 7C). Taken together, these data indicate that telomerase negative-callus have not activated a Type II or ALT-like mechanism for telomere maintenance, as both of these mechanisms produce heterogeneous tracts of telomeric DNA that surpass the length of wild-type telomeres (31, 263).

***In situ* localization of telomeric DNA sequences**. To further examine architecture of chromosome ends in telomerase-negative cells, we used fluorescent *in situ* hybridization (FISH) with a peptide-nucleic acid PNA probe to assess the distribution of telomeric sequences in wild-type callus and in the telomerase deficient A and B lines. As expected, wild-type cells yielded a regular pattern with twenty telomeric signals corresponding to the number of chromosome ends (median value) (FIG. 8A). The A and B lines were remarkably similar with approximately the same median values: six telomeric signals in B line (FIG. 8B) and eight in A line (FIG. 8C). Similarly to late generation *tert* mutants, anaphase bridges were detected in A and B line cells. The distribution of telomeric sequences on anaphase figures is apparent for both the B (FIG. 8B) and A (FIG. 8C) cell lines. Some telomeric signals localized at the point of rupture of the chromatid, while other telomeric signals corresponded to internal portions of the

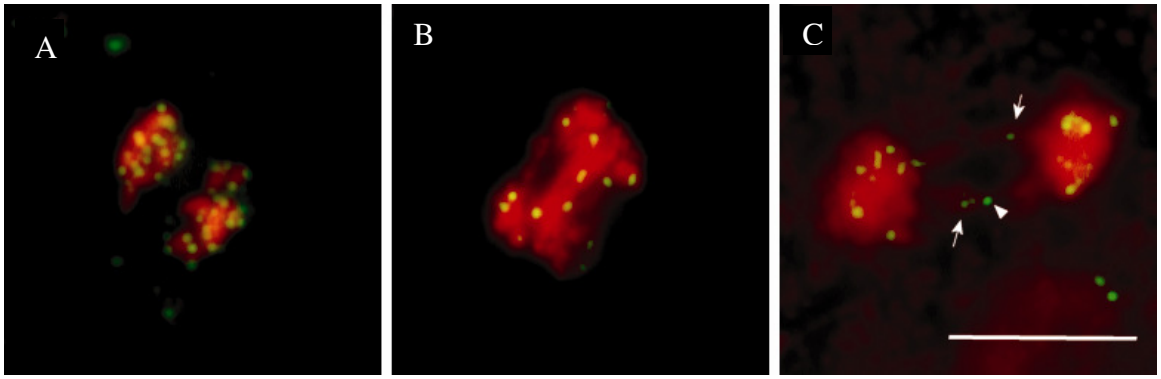


FIG. 8. Comparison of telomeric FISH pattern.

Comparison of FISH pattern with telomeric probe (yellow) on wild-type (A), B line (B) and A line (C) callus nuclei counterstained with DAPI (red). A) Wild-type nuclei yielded twenty telomeric signals. B) An anaphase figure with multiple bridges in the B line is illuminated with telomeric signals. C) Example of FISH on the A line nuclei with an anaphase with two bridges is shown. Interstitial telomeric signals are indicated by arrows, and the terminal signal by an arrowhead. Scale bar indicates 10 μm.

chromosome. The telomeric signals are likely to be remnants of interstitial repeats and telomeric DNA trapped within chromosome fusion junctions.

Rearrangement of subtelomeric sequences in A line calli. Since we failed to detect telomeric sequences in both the A and B lines, we examined the status of subtelomeric DNA sequences. Type I survivors in yeast do not have extended telomeric DNA tracts. Rather, they have dramatically amplified sub-telomeric Y' elements and very short tracts of telomeric repeats are at the terminus (166). *Arabidopsis* is unusual in that at least seven of the ten telomere tracts are abutted by unique sequences allowing us to follow the fate of individual chromosome arms (Heacock et al., 2004). To further characterize the terminal structure in A line callus, we digested DNA with *Spe*I and *Pvu*II, which release terminal restriction fragments containing both telomeric and subtelomeric DNA (FIG. 9A). Hybridization with probes directed at specific chromosome arms reveals subtelomeric DNA tracts on individual chromosome ends.

In both wild-type and *tert* mutants, hybridization with subtelomeric probes usually produced a single fragment (FIG. 9B, lanes 1, 2, 5 and 6), the size of which reflects the length of the telomere tract as well as the distance of subtelomeric sequence to the first restriction site (FIG. 9A) (230). For chromosome 5L this distance is approximately 2.5 kb, and for chromosome 2R it is 1.3 kb. The A line callus produced a distinctly different pattern from wild-type and G3 *tert* plants. Analysis of subtelomeric DNA from two passages yielded a complex digestion profile with fragments ranging in size from 2 kb to near limit mobility (FIG. 9B). The restriction fragment profile changed in subsequent passages, suggesting chromosomal rearrangement was ongoing. Analysis of later

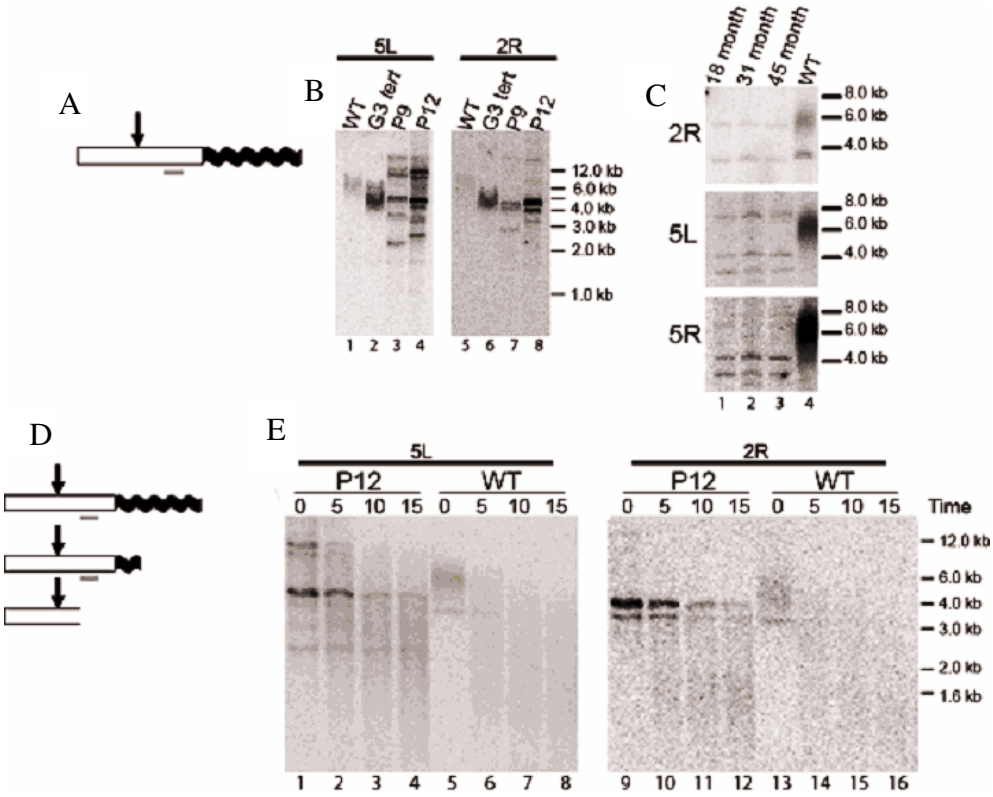


FIG. 9. DNA is rearranged in telomerase-independent callus, line A.

A) Schematic diagram of subtelomeric TRF analysis. B) Southern hybridization of a subtelomeric digestion of DNA from two different A line passages using probes for chromosome 5L and 2R is shown. Both A line passages show a dramatic increase in copy number, as well as changes in restriction profile between passages. C) Southern of subtelomeric digestion of DNA from A line cells taken at approximately one year intervals is shown. D) Schematic of *Bal*31 exonuclease digestion. E) Southern hybridization of DNA sequences digested with *Bal*31 exonuclease prior to restriction digestions. Analysis of DNA from P12 and wild-type is shown. Subtelomeric sequences from P12 callus are largely insensitive to *Bal*31 digestion while DNA from wild-type is rapidly degraded.

cultures revealed that the complexity of the restriction pattern was reduced relative to early passage cells. The profile remained consistent for more than two years, arguing that rearrangement at these loci had decreased (FIG. 9C).

To test directly whether the fragments hybridizing to the subtelomeric probe corresponded to sequences at the chromosome terminus, we performed *Bal*31 exonuclease digestion (FIG. 9D). DNA from P12 and wild-type callus was treated with *Bal*31 exonuclease for increasing times, and then the DNA was subjected to digestion with *Spe*I and *Pvu*II. Southern blot analysis was then performed with subtelomeric DNA probes for chromosomes 5L or 2R. In this assay, *Bal*31 progressively degrades terminal sequences, and hence the corresponding restriction fragments will shorten or disappear upon extended treatment with *Bal*31 nuclease (FIG. 9D). Sequences that are not located at the chromosome terminus will be refractory to *Bal*31 digestion. As expected, subtelomeric DNA sequences from wild-type plants appeared as a single heterogeneous band that diminished over time (FIG. 9E, lanes 5-8 and 13-16). In contrast, the majority of the hybridizing signals from P12 of the A line were largely insensitive to *Bal*31 treatment, suggesting that these sequences were internalized.

Both A and B line cultures display mitotic irregularities. To gauge the level of genome stability associated with telomerase-negative calli, we determined the ratio of normal to aberrant anaphases (with anaphase bridges or chromosome fragments) in each culture (Table 1). Slides were prepared from samples taken from several independent calli from each line. Samples of wild-type cultures showed a very low level of irregularities, with only five of 522 anaphases containing an anaphase bridge

Table 1. Summarized results of normal and aberrant anaphases in all three calli lines.

	Normal anaphases	Aberrant anaphases	Ratio of aberrant anaphases to all anaphases
WT callus	517	5	0.0095 (0.95%)
A line	54	45	0.45 (45%)
B line	11	55	0.83 (83%)

The number of anaphases and the ratio of aberrant anaphases to all anaphases are shown.

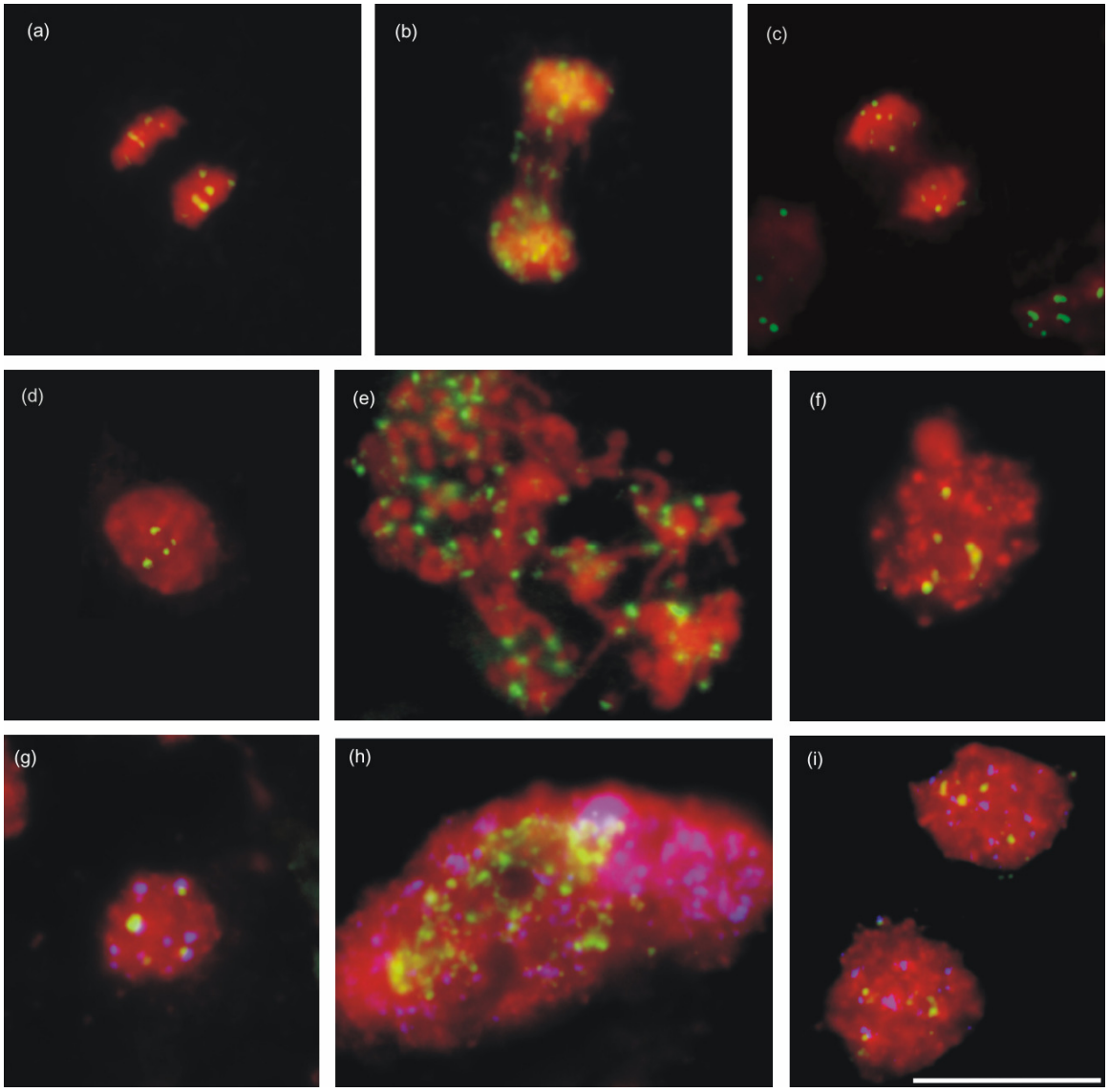
(0.95%). Typical wild-type callus nuclei are shown in FIG. 10A and D. In contrast, G8 *tert* plants, the progenitors of the A and B line, display a high proportion of anaphase bridges, up to 40% (230). In the poorly growing B line, the proportion of anaphase bridges was twice as high as in G8 *tert* mutants with 83% of anaphases displaying aberations (55 out of 66). Most anaphases contained multiple bridges (FIG. 10B) and examples of huge polyploid nuclei were found (24 out of 264 mitotic figures) (FIG. 10E).

In contrast to the B line, the nuclei of the A line were morphologically similar to wild-type nuclei (FIG. 10C and F). Since the A line is phenotypically stable and fast growing, we presumed an alternative pathway of chromosome end protection had arisen in these cells and stabilized their genome. However, 45% of anaphases displayed bridges (45 out of 99 anaphases) (Table 1). Thus, despite the stabilized phenotype and rapid proliferation of A line cells, these cells have not engaged a mechanism that allows complete protection of chromosome ends.

Genome variation in the B line and stability in the A line. The presence of anaphase bridges in both telomerase-deficient lines may result in unequal distribution of genetic material into daughter nuclei, which can lead to aneuploidy and chromosomal rearrangements. As we have previously shown, the participation of 25S rDNA loci in these fusions is ten times higher than expected for random fusion events (246). To examine aneuploidy and genome rearrangement in the telomerase-deficient cell cultures, we estimated the DNA content for each nucleus to correlate it with the number of chromosomes and number of 25S rDNA loci. In wild-type nuclei we observed a

FIG. 10. Comparison of rDNA and centromeric FISH patterns.

Comparison of FISH patterns on wild-type callus nuclei (A, D, G), B line (B, E, H), and A line (C, F, I) hybridized with 25S rDNA (yellow; A-I), and centromeric (blue; G-I) probe, counterstained with DAPI (red). Note the differences among size of huge nuclei in B line and nuclei of the other two lines. Wild-type nuclei show the standard FISH pattern with four dots of 25S rDNA (A, D, G). In the B line, anaphase bridges (B) and huge polyploid nuclei (E) were frequently found. Multiple rDNA loci are apparent in both figures. The multiplication of rDNA loci was not found in the A line (C, F, I) despite the presence of anaphase bridges (C). Wild-type interphase nuclei yielded four rDNA and ten centromeric hybridization signals (G). Huge B line nuclei showing multiplication of 25S rDNA and centromeric signals (H). Nuclei of the A line hybridized with rDNA and centromeric probes (I). Note a similarity of the A line nuclei with wild-type with the exception of increased number of centromere signals. Scale bar indicates 10 μm in all images.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

standard hybridization pattern in most cases, 10 centromeric signals and four 25S rDNA spots, two larger spots representing rDNA from chromosome 4 and two minor signals coming from loci on chromosome 2 (FIG. 10G). The 3D graph (FIG. 11A) shows the homogeneity of the wild-type cell line with respect to DNA content and number of rDNA and centromeric signals. Only a few polyploid nuclei (6 out of 68 ~ 8.8%) were observed, which may be a result of *in vitro* cultivation. We conclude that the genome of wild-type callus, under our *in vitro* conditions, is largely stable.

A very different situation was observed in B line calli (FIG. 10H). The 3D graph (FIG. 11B) shows a high dispersion of values in all parameters measured. In contrast to wild-type nuclei, the DNA content in nuclei from B line calli displayed a much greater variation with a range of C-values (lowest and highest values) spanning one order of magnitude. The B line cells frequently exhibited a large number of rDNA signals. The number of chromosomes, as indicated by a centromeric probe, was also highly variable among nuclei.

Although anaphase bridges were frequent in the A line culture (45%), this culture was much more homogeneous in all parameters than the B line (FIG. 11C). All nuclei clustered to a single region on the 3D graph, and the median values were similar to wild-type, with fourteen centromeric and six 25S rDNA signals (FIG. 10I). Thus, despite the high proportion of aberrant anaphases, A line cells did not display the variability found in the B line.

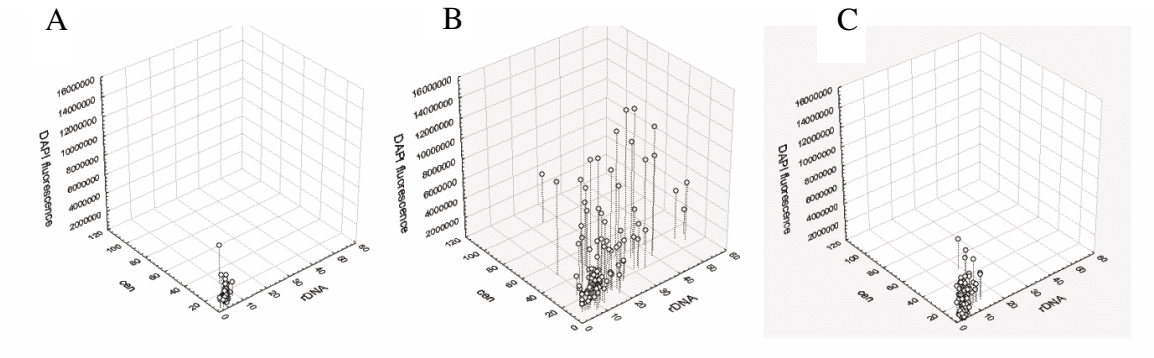


FIG. 11. 3D Graph evaluation of chromosome number, rDNA copies, and DNA content. 3D-graph evaluation of chromosome number (number of centromeric signals; X axis), number of 25S rDNA signals (Y axis), and DNA content (integrated DAPI fluorescence; Z axis) in callus cultures. A) The 3D-graph of the wild-type line shows homogeneity of nuclei with only a few irregularities likely due to *in vitro* cultivation. B) For the B line a huge dispersion of values was found in all parameters measured without any correlation between them. C) The distribution of values in the A line is rather homogeneous when compared to the B line calli and multiplication of rDNA signals correlates with the number of chromosomes. Each point represents one nucleus. 68, 95, and 98 nuclei were evaluated in the wild-type, A line, and B line, respectively. All graphs are in the same scale.

We next asked whether there was a relationship between rDNA loci amplification and the ploidy level or number of chromosomes in individual nuclei. The approach was to discriminate between 25S rDNA loci amplification caused by increased ploidy and the amplification caused by chromosomal rearrangements due to telomerase inactivation. We failed to detect a correlation between the number of chromosomes (measured as a number of centromeric signals) and the multiplication of 25S rDNA loci. Nuclei with a very high number of 25S rDNA signals and a relatively low level of chromosome number were observed as well as nuclei with the reverse situation. Hence, the genome instability and resulting aneuploidy, demonstrated by the very high level of anaphase bridges (83%), is likely to be responsible for the heterogeneity of this culture. The instability of rDNA loci is in agreement with our previous study which showed preferential involvement of rDNA bearing chromosome arms in chromosome fusions (246).

To examine the influence of *in vitro* culture, we performed the same analysis on wild-type and G8 *tert* mutant plants. Wild-type calli showed a six-fold increase in the number of polyploid nuclei (6 out of 68 ~ 8.8%) compared to wild-type plants (2 out of 158 ~ 1.3%). Nuclei from G8 *tert* plants were generally similar to those from wild-type plants, with the exception of occasional small changes in the number of centromeric (9 or 11) and 25S rDNA loci (3 or 5) and the presence of anaphase bridges (Siroky *et al.,* 2003; P. Bulankova, data not shown). Therefore, we conclude that the dramatic changes in ploidy observed in mutant calli are not due to *in vitro* cultivation *per se* but to the instability caused by continual proliferation in the presence of telomere dysfunction.

A line calli are sensitive to DNA damaging agents. Telomere dysfunction results in hypersensitivity to ionizing radiation (IR), due partly to the improper fusion of dysfunctional telomeres to the double-strand breaks resulting from IR (145). To determine if mutant calli were hypersensitive to an increase in the number of DSBs, wild-type and A line cells were transferred to CIM media containing varying concentrations of the DNA damaging agents menadione which induces oxidative stress (225), methyl-methane sulfonate (MMS), an alkylating agent (188), and bleomycin, a radiomimetic drug that induces DNA single- and double-strand breaks (188). As seen in FIG. 12, mutant calli were extremely sensitive to all three DNA damaging agents, and this sensitivity occurred at the lowest drug concentrations tested. In contrast, wild-type cells continued to grow on all three drugs at concentrations eight times higher than shown in FIG. 12 (data not presented). These data indicate that A line cells are highly sensitive to DNA damaging agents, arguing that the mechanism used to partially stabilize the genome in A line cells is unable to respond to even a small increase in DNA damage.

DISCUSSION

The importance of functional telomeres for genome stability was first elucidated by Barbara McClintock (185), and from these studies emerged the concept that telomeres cap and maintain chromosome structural integrity. Loss of telomere function results in chromosome instability through telomere-to-telomere fusion and the onset of the breakage-fusion-bridge cycle. Mechanisms to bypass the lethal consequences of telomere dysfunction in the absence of telomerase have been described. To determine what mechanisms, if any, plants would employ to proliferate in the absence of

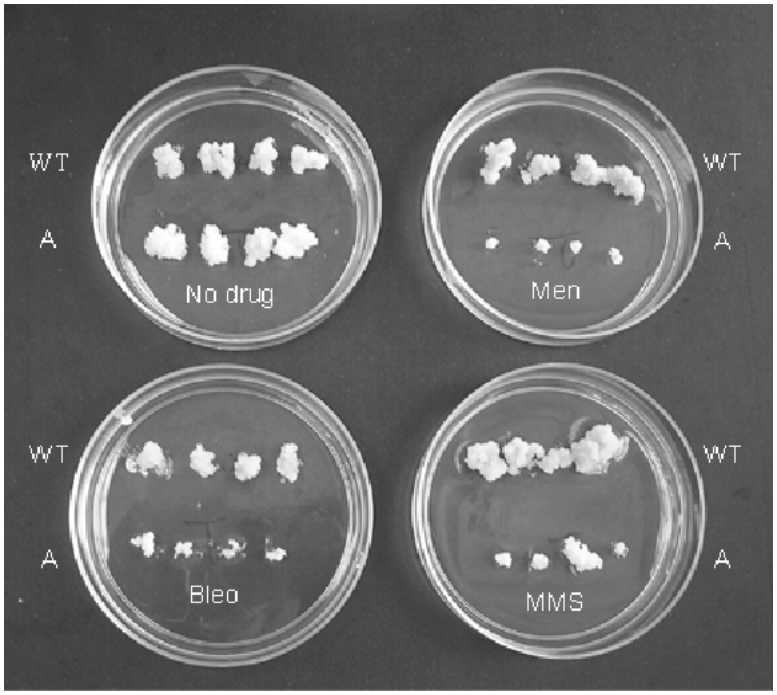


FIG. 12. Hypersensitivity of telomerase-deficient calli to DNA damaging agents. Both wild-type and telomerase-deficient calli were transferred to media containing varying concentrations of menadione (Men), bleomycin (Bleo), or methyl-methane sulfonate (MMS). Shown are results with the lowest concentrations tested (6.9 µg/ml menadione, 1.25 µg/ml bleomycin, 25 µg/ml MMS). Mutant callus A shows extreme sensitivity when compared to wild-type callus.

telomerase, we followed the fate of cells that lack telomerase for a long period in culture. Our data show that in two independent telomerase-negative cell lines, chromosome end-protection is not fully re-established, and yet both cell lines continue to proliferate.

None of the previously characterized telomerase-independent pathways for chromosome end-maintenance have been engaged in *Arabidopsis*. In telomerase-negative Type I survivors of *S. cerevisiae*, recombinational amplification of subtelomeric Y' elements produces long arrays of tandem repeats that contain short stretches of telomeric DNA (166). In contrast, we found that the subtelomeric repeats in telomerase-negative calli do not show gross amplification and are no longer associated with chromosome termini. Our data also indicate that *Arabidopsis* has not activated a Type II or ALT survivor pathway. Type II survivors in *S. cerevisiae* have extremely heterogeneous tracts of telomeric DNA (263), and this molecular phenotype is common among survivor pathways in other organisms, including *K. lactis* (186), humans (31), and perhaps mice (204). Accumulating evidence suggests that this pathway occurs through a roll-and-spread mechanism, where a single telomere becomes elongated through rolling circle replication from an extra-chromosomal telomeric circle, which then serves as a substrate for break-induced replication to lengthen the remaining telomeres in the cell (203). Since we are unable to detect terminal telomeric DNA in our mutants, recombinational amplification of telomeric DNA is not likely to be responsible for survival in our cell populations. It is conceivable that *Arabidopsis* is capable of activating a Type-II or ALT like survivor pathway, but our experimental design was not optimal to detect it.

Our callus cultures were initiated from plants that already had critically shortened telomeres. Such telomeres may have been too short to form the initiating extra-chromosomal circle. It is possible that if calli were induced from telomerase-mutant cells that had longer telomeres, a recombinational mechanism might arise. Support for this possibility comes from experiments with *rad50 Arabidopsis* mutants (87). Telomeres from *rad50* mutants are wild-type in length, but when callus is induced, telomeric DNA is rapidly lost. Survivors arising from this crisis have longer telomeres than those observed in wild-type cultures. However, it is not clear whether the observed elongation is result of telomerase activity or recombination.

Another mechanism for survival in the absence of telomerase is through chromosome circularization. The three *S. pombe* chromosomes undergo circularisation in response to telomere dysfunction (201). It has been hypothesized that the small number of chromosomes in *S. pombe* facilitates this mechanism; intra-molecular ligation becomes more likely as the number of ends available is reduced. The relatively small number of chromosomes in *Arabidopsis* suggested that chromosome circularization is possible. However, attempts to PCR amplify fusion junctions between the left and right arms of individual chromosomes in A line cells were unsuccessful (J. Watson and D. Shippen, unpublished data). This observation does not rule out the possibility that circular chromosomes form by ligation of novel ends created by breakage-fusion-bridge cycles. *Arabidopsis* chromosomes are generally too small to definitively determine whether they are circular.

Recently, Maringele and Lydall generated yeast cells that overcome the end-replication problem through a palindrome-mediated replication pathway (176). Acquisition of this mechanism required the deletion of the telomerase RNA (*TLC1*) as well as *RAD52*, *EXO1*, and *MRE11*. Interestingly, Exo1p and Mre11p contribute to signal DNA damage to the checkpoint kinase Mec1 (200). Loss of this signaling may be required for continued cell division, which then allows for selection of cells that can overcome the end-replication problem. In light of this, it is interesting that the A line callus is extremely sensitive to DNA damaging agents, and this sensitivity may be due to loss of checkpoint machinery.

Cell culture is thought to promote genome instability due to the dedifferentiation that occurs as a consequence of removing cells from their normal organismal location to culture (184). In *Arabidopsis* callus cultures, genome instability is mainly manifested by changes in chromosome number and polyploidization and the level of this instability is dependent on the ecotype and type of tissue (81). However, the frequency of polyploidization in wild-type callus in our experiment was considerably lower than in the B culture established from telomerase-deficient plants with ongoing genomic instability. In contrast to these observations, the A line retained a relatively stable amount of DNA content per nucleus.

Arabidopsis has a small amount of repetitive sequences, clustered mainly within the pericentromeric heterochromatin and the nucleolar organizing regions (NORs). It was recently shown that interphase chromosomes in *Arabidopsis* are randomly associated, with the exception of chromosomes 2 and 4, which contain the NORs (211).

Remarkably, NORs participate in chromosome fusion events with a ten fold higher frequency than expected in late generations of *Arabidopsis tert* plants (Siroky *et al*., 2003). Consistent with this observation, continuous rearrangement and rDNA amplification, which did not correlate with the number of chromosomes, was found in our B callus line. This supports the idea that genome in this cell line is destabilised by ongoing BFB cycles. In contrast, the constant number of rDNA loci and their correlation with other nuclear parameters (number of chromosomes and DNA content) in the A line argues that genome is to a large extent stabilised.

Although both telomerase-deficient cultures proliferate continuously, they display a high frequency of anaphase bridges, presumably as a result of the fusions of uncapped chromosome ends. This fact together with the lack of telomeric DNA and internalization of subtelomeric sequences suggests that both callus cultures lack functional telomeres. Interestingly, A line calli are hypersensitive to DNA damage agents. We hypothesize that when genotoxic drugs introduce additional DNA breaks, the mechanism responsible for the partial capping of chromosome ends becomes unable to cope with the increased damage. The already high percentage of aberrant anaphases in mutant cultures argues that mechanisms for joining broken ends are functional. However, by increasing the number of ends available for repair and joining, the genomic instability is likely to increase dramatically, resulting in the observed sensitivity. It will be interesting to determine how these cells are capable of withstanding the large amount of genomic instability present even in the absence of exogenous damaging agents.

We propose a model whereby chromosome uncapping due to loss of telomeric DNA results in continuous breakage-fusion-bridge cycles. The partial stabilization of the genome in the A line cells likely arose from a stochastic event, as an attempt to recapitulate this phenotype in another culture (the B line) was unsuccessful. Despite the inability of cells in the B line to achieve even partial genome stability, they proliferate at a low level. The apparent absence of checkpoint pathways that respond to telomere dysfunction in *Arabidopsis* could provide the B line with a mechanism to overcome the end-replication problem: survival through aneuploidy. As long as a cell can maintain a genome that includes all genes essential for cell division, proliferation can continue. Moreover, acquisition of multiple copies of essential genes on different chromosomes through breakage-fusion-bridge cycles could facilitate cell survival.

One mechanism that could offer partial end-protection in A line cells is through a heterochromatic barrier to DNA damage repair. In *Drosophila* chromosome end-protection is sequence non-specific, due at least in part to the localization of the protein HP1 to chromosome ends (71). Perhaps a special heterochromatic structure partially protects the telomere ends in the A line cells. When the end-replication problem results in loss of an entire heterochromatic region, the now unprotected end is fused. Alternatively, this heterochromatic barrier may be not as effective as the natural telomere machinery in disguising a chromosome end from the double-strand break repair machinery.

CHAPTER III

TELOMERE RAPID DELETION REGULATES TELOMERE LENGTH IN *Arabidopsis thaliana*[*]

SUMMARY

Telomere length is maintained in species-specific equilibrium primarily through a competition between telomerase-mediated elongation and the loss of terminal DNA through the end-replication problem. Recombinational activities are also capable of both lengthening and shortening telomeres. Here we demonstrate that elongated telomeres in *Arabidopsis ku70* mutants reach a new length set point after three generations. Restoration of wild type *KU70* in these mutants leads to discrete telomere shortening events consistent with telomere rapid deletion (TRD). These findings imply that the longer telomere length set-point is achieved through competition between over-active telomerase and TRD. Surprisingly, in the absence of telomerase, a subset of elongated telomeres was further lengthened, suggesting that in this background telomerase-independent mechanisms operate. Unexpectedly, we found that plants possessing wild type length telomeres exhibit TRD when telomerase is inactivated. TRD is stochastic and all chromosome ends appear to be equally susceptible. The frequency of TRD decreases as telomeres shorten; telomeres less than 2 kb in length are rarely subject to TRD. We conclude that TRD functions as a potent force to regulate telomere length in *Arabidopsis*.

---

[*] Reprinted with permission from **Watson, J.M., and D. Shippen.** 2006**.** Telomere rapid deletion regulates telomere length in *Arabidopsis thaliana*. Mol Cell Biol In Press.

INTRODUCTION

Telomeres are dynamic nucleoprotein complexes at the end of eukaryotic chromosomes that consist of long stretches of a simple G-rich repeat and sequence-specific DNA binding proteins. The primary function of telomeres is to protect chromosome termini from being recognized as a double-strand break. The extreme 3' terminus of the chromosome is single-stranded and can undergo a protein-assisted conformational change, folding back upon and invading the duplex region to form a structure termed the t-loop (98). The t-loop is thought to physically sequester the chromosome end, masking the telomere from DNA repair machinery (63).

Telomeric DNA is maintained through a variety of mechanisms that compensate for loss of terminal DNA sequences that occurs as a consequence of nucleolytic processing or the end-replication problem (104, 175). Slow loss of telomeric sequences during DNA replication can be offset by the action of telomerase, a ribonucleoprotein reverse transcriptase that extends the 3' overhang through reiterative copying of its internal RNA template (reviewed in ref. (11). Telomerase is subjected to both positive and negative regulation in *cis* on the chromosome terminus, mitigating its ability to extend any given telomere (7, 162, 174, 213, 252, 261, 273)

The protein counting model posits that the primary means of telomere length regulation is through an ability to "count" the number of telomeric binding proteins (174). If too many proteins are bound, the telomere will be recalcitrant to extension by telomerase, while if too few proteins are bound, the telomere will be in a more open conformation and will be accessible to telomerase activity. Accordingly, telomerase extension results

in an increase in the number of binding sites for telomere proteins and hence an increase in protein occupancy. On the other hand, telomere loss due to the end-replication problem or nuclease attack results in a decrease in the number of sites and fewer proteins bound. This model is strongly supported by studies in yeast (261), mammals (7, 111), and *Arabidopsis* (239), where telomerase has been shown to act preferentially on the shortest telomeres in the population. Competition between the end-replication problem and telomerase results in a range of telomere lengths that fluctuate between species-specific boundaries. For example, telomeres in *S. cerevisiae* are approximately 300 bp (167), in *Arabidopsis* from 2-8 kb (239), and in mice from 10-60 kb (308).

Positive and negative regulators of telomere length include the double-strand telomere binding proteins TRF1 (252), Rap1p (170), Taz1p (56) and the single strand-telomere binding protein POT1 (162). Additionally, telomere length is influenced by KU, a heterodimer of 70 and 80 kda subunits that is an integral component of the non-homologous end joining (NHEJ) DNA double-strand break repair pathway (229). KU is a strong negative regulator of telomerase in *Arabidopsis* (34, 85, 232); its deletion results in rapid telomerase-dependent extension of telomere tracts (85, 231). Interestingly, in *S. cerevisiae* and humans, deletion of KU leads to telomere shortening (26, 199).

In the absence of telomerase, telomeres progressively shorten until they reach a critical length that elicits a DNA damage checkpoint response (73). If cells are forced to continue dividing, telomeres will become uncapped and fuse together. The resulting

dicentric chromosomes may then break during the next mitosis only to fuse in the next cell cycle. The resulting breakage-fusion-bridge cycle leads to genomic instability (187). Strong selective pressure against genome instability results in the formation of different types of survivors in yeast (166), whose chromosome ends are maintained through alternate means (146, 262). Several different types of survival have been identified, including recombinational elongation and rolling circle amplification (113, 165). In humans, this form of telomere maintenance is termed alternative lengthening of telomeres (ALT).

Cells with elongated telomeres do not face the same selective pressure as cells with extremely short telomeres. Indeed, *Arabidopsis KU70* mutants maintain telomeres much longer than wild type, with no apparent affect on growth, development, or genome stability (232). However, studies in yeast indicate that elongated telomeres are quickly returned to wild type length in a single-step event termed telomere rapid deletion (TRD) (153). These deletion events are intra-chromosomal, and result in loss of the most terminal sequences (32). A similar phenomenon has been described in humans and *K. lactis.* Human cells expressing a mutant form of the telomere double-strand binding protein TRF2 undergo catastrophic telomere deletions, concomitant with the formation of extra-chromosomal telomere circles (ECTCs) the size of t-loops (280). Similarly, in *K. lactis* mutants with elongated telomeres due to a mutation in Stn1p, reintroduction of Stn1p results in rapid loss of the elongated telomeres and a return to wild type length (120).

It has been proposed that branch migration of the displacement loop formed by the invading G-overhang within the t-loop structure results in a Holliday junction. This structure is then resolved, leading to the formation of a shortened telomere and an extra-chromosomal telomeric DNA fragment (169), which in mammals is a circle (ECTC). In *S. cerevisiae*, TRD and the two major types of survivors are dependent upon Rad52p, indicating both processes are recombinational in nature (153, 166). Telomere lengthening in *stn1 K. lactis* mutants is similarly dependent upon Rad52p (120). Sequestration of the MRX complex in human ALT cells results in slow loss of telomeric DNA and repression of the ALT mechanism of elongation (122). Additionally, the RAD51 paralogue XRCC3, which may be a mammalian Holliday junction resolvase (159), is required for the TRD events observed in TRF2 mutants (280).

Arabidopsis is a genetically tractable model that has been exploited for studies of telomere dynamics (187). One important feature of this organism is that eight of the ten chromosome arms are abutted by unique subtelomeric sequences, making it possible to study the fate of individual telomeres in different genetic backgrounds. Here we examine the fate of ultra-long telomeres in *Arabidopsis ku70* mutants. We demonstrate that elongated telomeres in this background can be rapidly shortened by TRD, either upon reintroduction of *KU70* or through loss of telomerase. In addition, we provide evidence for ALT in plants with elongated telomeres. Finally, we show that wild type length telomeres are subject to both TRD and ALT, arguing that recombinational mechanisms play a role in regulating telomere length in wild type plants.

MATERIALS AND METHODS

Plant growth conditions and mutants. Plants were grown in EGC growth chambers (Chagrin Falls, Ohio) with a 16 hr photoperiod at 22℃. Generation of *ku70* and *tert* mutants was previously described (230, 232). The k*u70/rad51* paralogue double mutants were generous gifts from Dr. Karel Riha. Characterization of the *rad51* mutants was described before (23). Primers and genotyping conditions for *tert, ku70, mre11*, and the *rad51* paralogs are as described (23, 108, 230, 232).

TRF and FIGE analysis. Genomic DNA was extracted using a CTAB based method (25). For bulk telomere analysis, approximately 1µg of genomic DNA was digested with 20 units of *Tru1*I overnight in 200 µL at 65℃. For subtelomere analysis, approximately 1.5 µg of genomic DNA was digested overnight with 10 units of *Spe*I and *Pvu*II in 200 µL at 37℃. Digested DNA was precipitated and subjected to electrophoresis through 0.7% agarose. Field inversion gel electrophoresis (FIGE) was performed with a CHEF Mapper XA (BioRad Hercules, CA). DNA was separated through 1% agarose in 0.5X TBE at 14℃. Conditions were determined using the auto-algorithm function to separate 4-50 kb molecules. Conditions were 9 V/cm forward 6 V/cm reverse with a linear ramp from 0.08s s to 0.92 s with a total run time of 19 hr 2 min. Transfer to nylon membranes and hybridization were performed as previously described (239). Average telomere length was measured using telometric (93).

Constructs and transformation. T-DNA constructs were previously described (232). Briefly, the over-expression construct pCBK21 consists of the CaMV35S promoter driving the cDNA of *KU70*. The genomic construct, pCBK22, consists of 6.7 kb of the

KU70 gene along with 1.6 kb of putative promoter sequence. Primary transformants were designated T1, with successive generations being numbered sequentially.

PETRA analysis. PETRA was performed as discussed in (108) with slight modifications. CTAB extracted DNA (25) from a single flower or leaf was resuspended in 30 µL of water. Primer extension was carried out in a 20 µL reaction containing 8 µL DNA, 1X *Ex-Taq* Buffer (TAKARA), 125 µM dNTPs, 1 µM PETRA-T, 2 U *Ex-Taq* polymerase. This reaction was incubated at 65°C for 5 min, 55°C for 1 min, and 72°C for 10 min. 1 µL of the reaction was used in a 20 µL reaction containing 1X *Ex-Taq* buffer, 200 µM dNTPs, 0.25 µM PETRA-A, 0.25 µM telomere-specific primer, 0.5U *Ex-Taq*. These samples were incubated at 96°C for 2 min followed by 16-18 cycles of 96°C 30 s, 60°C 30 s, 72°C 2 min 30 s, with a final incubation at 72°C for 5 min. PCR products were subjected to Southern blotting and hybridization with a [^{32}P] 5' end-labeled $(T_3AG_3)_4$ probe. Signals were visualized using a STORM phosphorimager (GE Healthcare) and were quantified using Imagequant (Molecular Dynamics). To measure telomere length, a 6th order polynomial equation was fit using Excel to the distance migrated of a 1 kb$^+$ DNA ladder (Invitrogen) and the length of a given PETRA signal was then converted to DNA size using this equation. Finally, the distance of the PETRA primer to the telomere was subtracted from the total length measured by PETRA to give the actual length of the telomere tract.

RESULTS

Telomeres establish a new set point length in the absence of KU70. We previously showed that telomeres in KU70-deficient mutants become rapidly elongated, and within

two generations run near limit mobility in regular agarose gels (232). To more accurately determine the size of telomeres in these mutants, we performed TRF (terminal restriction fragment) analysis on successive generations of *ku70*. Genomic DNA was digested with *Tru1*I, which cuts DNA in the subtelomere region and releases the terminal telomere tract. The products were separated using field-inversion gel electrophoresis (FIGE) and hybridized with a telomere specific probe. As expected, telomeric DNA from wild type plants migrated near the bottom of the gel consistent with its known size of 2-5 kb (FIG. 13A lane 1). In contrast, first generation (G1) *ku70* mutants displayed elongated telomeres reaching 9 kb (FIG. 13A lane 2), while in G2 bulk telomeres ranged from approximately 10 to 25 kb (FIG. 13A lane 3). No further dramatic lengthening was observed in subsequent generations, and the average length of telomeres in G2-G7 mutants was 15.9 kb±2.1 kb (FIG. 13A lanes 4-8). While bulk telomeres in *ku70* ran as a heterogeneous smear, a smaller discrete telomeric fragment was visible at approximately 7 kb in the G7 *ku70* DNA sample (FIG. 13A lane 8). Of 48 plants analyzed individually by TRD, 21 had similar hybridizing products (data not shown). We were unable to determine the exact nature of these products, although they appeared to be inherited in a Mendelian fashion (data not shown). One possibility is that they reflect point mutations in the elongated telomere tracts that produce novel *Tru1*I restriction sites. A mutation of the wild type repeat, TTTAGGG to either TTTAAGG or TTAAGGG would allow cleavage by *Tru1*I.

Restoration of KU70 leads to TRD. We have previously shown that telomere elongation in the absence of *KU70* is telomerase-dependent (231). Thus, our data indicate that telomerase is capable of extending telomeres by as much as 15 kb within a

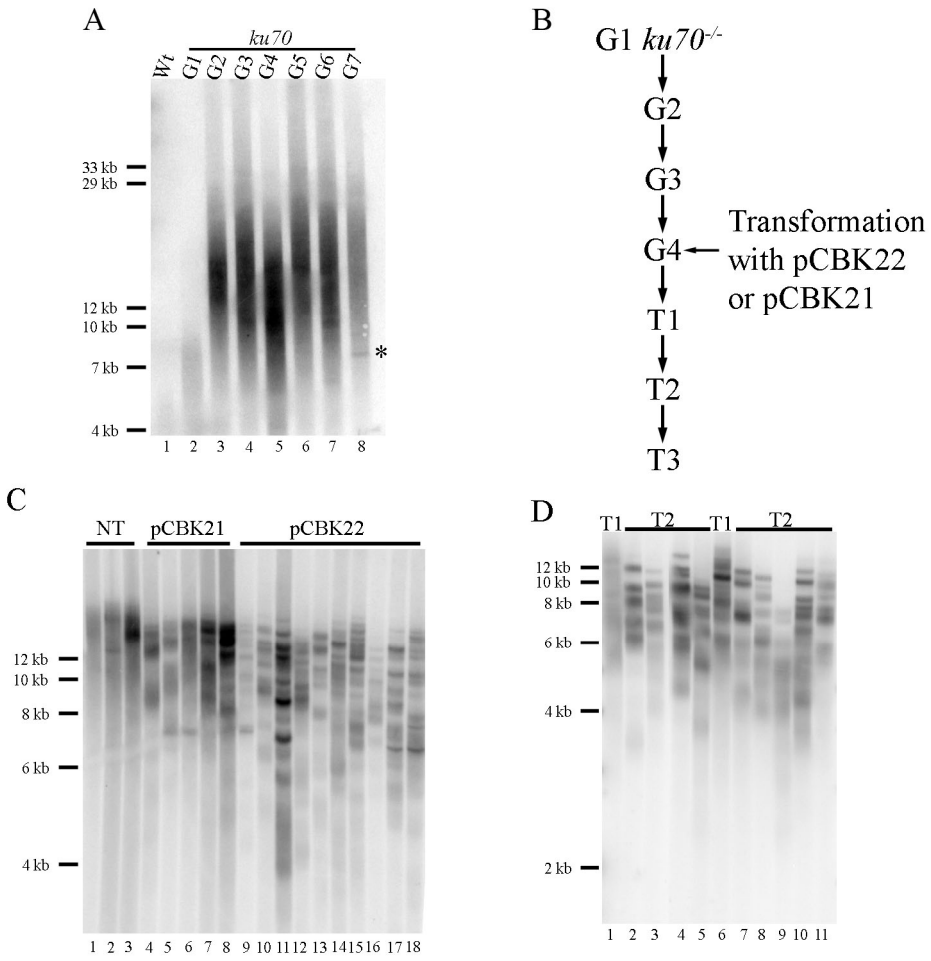


FIG. 13. Telomere length homeostasis and TRD in *ku70* mutants and rescues. A) FIGE of wild type and successive generations of *ku70* mutants. TRF analysis was carried out on DNA extracted from ~50 seedlings. Asterisk denotes a specific hybridizing signal in the G7 line. B) Scheme for creating *KU70* rescues. G4 *ku70* mutant plants were transformed with either pCBK21 or pCBK22. Plants selected in the next generation correspond to T1. C) TRF analysis of non-transformed (NT) and selected plants. D) Parent-progeny TRF analysis of two independent T1 transformants.

single generation (FIG. 13A compare lane 2 to 3). Telomeres reach a maximum size of approximately 25 kb and do not continue to elongate. This new set point could be established through telomerase inhibition at elongated telomeres. Alternatively, ultra-long telomeres may reach homeostasis through competition between telomerase and TRD. We reasoned that reintroduction of *KU70* would ultimately restore telomeres to their wild type length, allowing us to examine the dynamics of re-establishing the wild type telomere length set point. If length equilibrium is achieved through telomerase inhibition, telomeres should slowly drift back down to the wild type length, losing 200-500 bp per generation (76) as a consequence of the end-replication problem. Alternatively, if TRD was operational, telomeres should shorten much more rapidly. We define TRD as any telomere shortening event that leads to a loss of more than 500 bp in a single plant generation.

To examine the fate of elongated telomeres, we transformed G4 *ku70* with either a construct over-expressing *KU70* cDNA (35S::*KU70*, pCBK21) or a genomic copy of the *KU70* gene (pCBK22), and then selected for T1 transformants (FIG. 13B). As expected, telomeres from non-selected siblings migrated near limit mobility as a heterogeneous smear (FIG. 13C lanes 1-3). In contrast, plants transformed with pCBK21 (FIG. 13C lanes 4-8) produced a TRF pattern with shortened telomeres. Bulk telomeres in these plants shortened by an average of 0.5±0.3 kb. Strikingly, all of the T1 plants transformed with pCBK22 displayed shortened telomeres. In contrast to the pCBK21 transformants, telomeres in these plants showed a discrete banding profile reminiscent of telomerase mutants (76). The shortest telomeres in the pCBK22 transformants approached the wild type length of 4kb in a single generation, with an

average loss of 2.3±0.8 kb of telomeric DNA (FIG. 13C lanes 9-18). The appearance of discrete hybridizing bands in the pCBK22 transformants implies that they were resistant to telomerase-mediated elongation. Furthermore, this sharp banding pattern is not consistent with the action of exonucleases, which would likely produce a much more heterogeneous profile. Notably, several telomeric fragments were not shortened in the pCBK22 transformants, and instead migrated near the length of the telomeres in their mutant siblings. This observation indicates that individual telomeres are differentially processed.

We conclude that a subpopulation of elongated telomeres shorten much more rapidly than can be accounted for by the end-replication problem, implying that they have been subjected to TRD. Since most of the pCBK22 transformants displayed evidence for TRD all subsequent work was carried out on these lines.

To further examine the dynamics of telomeres shortening, TRF analysis was carried out on T1 plants and their T2 progeny. From T1 to T2, the longest telomeres continued to shorten in a stochastic manner (FIG. 13D). Some T2 plants exhibited dramatic telomere shortening relative to their parent (FIG. 13D, compare lane 5 with lane 1, and lane 9 with lane 6), while other telomeres remained relatively unchanged (FIG. 13D compare lane 2 with lane 1). On average, telomeres in T2 shortened by 1.9±1.2 kb. This stochastic shortening continued for the two subsequent generations that were analyzed. Strikingly, the frequency of obvious TRD events decreased as telomeres returned to the wild type length. The average rate of shortening also declined, with a loss of 0.45±0.36 kb from the T2 to T3 generation (data not shown). Telomeres in T3

generation plants average 7±0.6 kb, within the wild type range of this ecotype of *Arabidopsis.* Thus, over three generations, telomeres in plants where *KU70* had been restored lost almost 9 kb of telomeric DNA (15.9 kb in G2-G7 *ku70* to 7 kb in T3 transformants).

TRD is not dependent upon KU70. We asked whether TRD was dependent upon reintroduction of *KU70*. If TRD functions to limit telomere size in *ku70* mutants, any telomere shortened by TRD would likely be re-extended by telomerase, thus masking TRD. We therefore examined the fate of elongated telomeres in the absence of both *TERT* and *KU70*. To accomplish this, plants heterozygous for *TERT*, the gene encoding the catalytic subunit of telomerase, and homozygous for *ku70,* were propagated for three generations to elongate telomeres. Plants were transformed with pCBK22, and then segregated for *tert* in G4, generating a population of $TERT^{+/+}ku70^{-/-}$ and $tert^{-/-}ku70^{-/-}$ progeny, with or without the *KU70* transgene (FIG. 14A).

Telomeres in both telomerase-positive and telomerase-negative T1 plants shortened to similar lengths, though telomeres in *tert* mutants appeared as somewhat more sharp bands than in telomerase-positive plants (FIG. 14B lanes 5 and 6). The average telomere length in pCBK22 transformed $TERT^{+/+}$ plants was 12.3±0.2 kb and in $tert^{-/-}$ was 12±0.3 kb. Telomerase-positive non-selected plants (genotype $ku70^{-/-}tert^{+/+}$) displayed the elongated telomeres of *ku70* mutants, and telomeres averaged 13.4±0.3 kb, several kb less than the average measured by FIGE. This discrepancy is likely due to the poor resolution at high MW under these gel conditions (FIG. 14B lanes 7 and 8). Non-selected plants that were homozygous null for *tert* also showed TRD (FIG. 14B

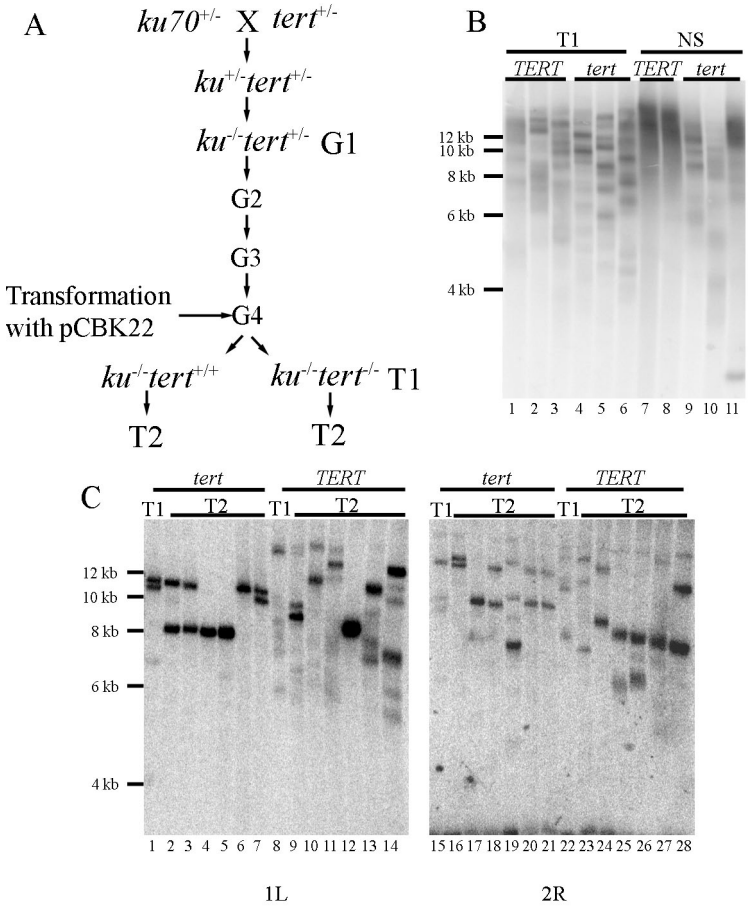


FIG. 14. *KU70* is not required for TRD.

A) Schematic diagram for generating *ku70 tert* double mutants. A plant heterozygous for *ku70* was crossed to a plant heterozygous for *tert*. Double heterozygotes for both genes were genotyped in the F1. Selfed progeny of the F1 were genotyped to identify *ku70*$^{-/-}$*tert*$^{+/-}$ (designated G1). These plants were selfed, and progeny were maintained as *ku70*$^{-/-}$*tert*$^{+/-}$ until G4. G4 plants were transformed with pCBK22 prior to segregation for *tert*. B) TRF analysis of T1 and non-selected (NS) progeny of a G4 plant transformed with pCBK22. C) Subtelomere analysis of T1 parents and their T2 progeny. The subtelomere probe used for the experiments is indicated below each blot. The panels represent sequential hybridization of a single membrane.

lanes 9 and 10), although one plant of this genotype retained most of its telomeres at an elongated length (FIG. 14B lane 11). The average size of telomeres in these *ku70 tert* double mutants was 11.9±1.4 kb. These data indicate that TRD does not require *KU70* and can occur in the absence of telomerase.

TRD is a stochastic process. To more accurately gauge the rate of telomere shortening, we followed the fate of individual telomeres in the T2 progeny of these transformants (FIG. 14A, both $tert^{-/-}$ and $tert^{+/+}$ plants were followed). Genomic DNA was digested with restriction enzymes that cut several kb internal to the telomere, and then hybridized with probes directed to specific chromosomes arms. As seen in FIG. 14C, dramatic, stochastic changes in telomere length were detected between the parent and their progeny in both genetic backgrounds. Again, telomerase-positive plants showed more heterogeneous signals at shorter lengths, consistent with telomerase acting on these shorter telomeres.

The clearest example of telomere shortening was seen with the 1L telomere in *tert* mutant plants. The parental plant had two prominent hybridizing signals at approximately 10.5 and 11 kb (FIG. 14C lane 1). Additionally, a much less intense signal of approximately 7 kb was observed. Four of the six progeny of this plant displayed a telomere of approximately 8 kb (FIG. 14C lanes 2 through 5), and two plants had completely lost the 10.5 and 11 kb signals (FIG. 14C lanes 4 and 5). We can envision two ways in which the 1L telomere shown in lanes 4 and 5 arose. First, TRD could shorten one of the prominent hybridizing signals in the parent, leading to a decrease of 2.5 kb. Alternatively, telomeres in the progeny could arise through a

telomerase-independent lengthening of the weakly hybridizing 7 kb signal in the parent. We consider the latter possibility less likely; the very weak hybridization of the 7 kb telomere in the parent is more consistent with a somatic TRD event that occurred during plant development. If this is true, the 7 kb fragment arose from a TRD event that shortened the 10.5 kb telomere by 3.5 kb, implying that TRD is capable of shortening an individual telomere by several kb in a single generation.

The subtelomeric analysis also revealed a surprisingly complex array of products. A plant can inherit a maximum of two different length telomeres (on the homologous chromosomes) from its parents. Thus, the presence of more than two hybridizing bands for an individual subtelomere arm argues that shortened telomeres in the progeny are not simply due to the inheritance of an undetectable subset of shorter telomeres from the parent. Rather, these telomeres must be derived from discrete telomere processing events in the progeny.

Plants displaying multiple signals for one chromosome arm do not necessarily have multiple signals at other chromosome ends. The *tert* mutant plant analyzed in FIG. 14C, lanes 5 and 19, has a single hybridizing signal for the 1L telomere, and four hybridizing signals for the 2R telomeres. Similarly, the telomerase-positive plant analyzed in FIG. 14C, lanes 14 and 28 gives rise to six hybridizing signals for chromosome 1L and only three signals for chromosome 2R. Thus, the number of TRD events that occur upon restoration of *KU70* is relatively small. The presence of six hybridizing signals indicates that only four or five TRD events occurred at that telomere throughout the lifespan of this plant. We conclude that TRD functions stochastically on different telomeres, and can shorten telomeres by at least 2.5 kb in a single generation.

TRD proceeds in *Arabidopsis* in the absence of genes required for TRD in other organisms. We tested whether *MRE11* and the available *RAD51* paralogs are required for TRD in *Arabidopsis*. Plants homozygous null for *ku70* and heterozygous for an additional mutation in *MRE11*, *RAD51B, RAD51C, XRCC2,* or *XRCC3* were propagated for several generations before transformation with pCBK22 (FIG. 15A). The selected T1 plants were then genotyped for the presence of the additional mutant allele. FIG. 15B shows the TRF profile of T1 plants mutant for *xrcc2*, *xrcc3*, *rad51B*, and *rad51C*. Deletion of any of these four genes did not inhibit TRD upon reintroduction of *KU70*. This finding was verified through subtelomere analysis, with all four mutants showing multiple signals for at least one of two tested subtelomeric probes (data not shown).

In three independent experiments, and in contrast to the other mutants tested, we were unable to select plants that were null for *MRE11* in the T1 population (we recovered a total of 14 $mre11^{+/+}$ and 22 $mre11^{+/-}$ in the three separate transformations). Therefore, we genotyped T2 progeny of a T1 plant heterozygous for *MRE11* and isolated individuals mutant for *MRE11* (FIG. 15C). Of the two plants mutant for *MRE11*, one showed four hybridizing signals for chromosome 2R (FIG. 15C lane 5), suggesting that *MRE11* is also not required for observed TRD events. From this data we conclude that genes previously shown to be required for TRD in other organisms are not necessary for TRD in *Arabidopsis*.

ALT occurs at elongated *Arabidopsis* telomeres. To date, no evidence for telomerase-independent telomere lengthening has been observed in *Arabidopsis*

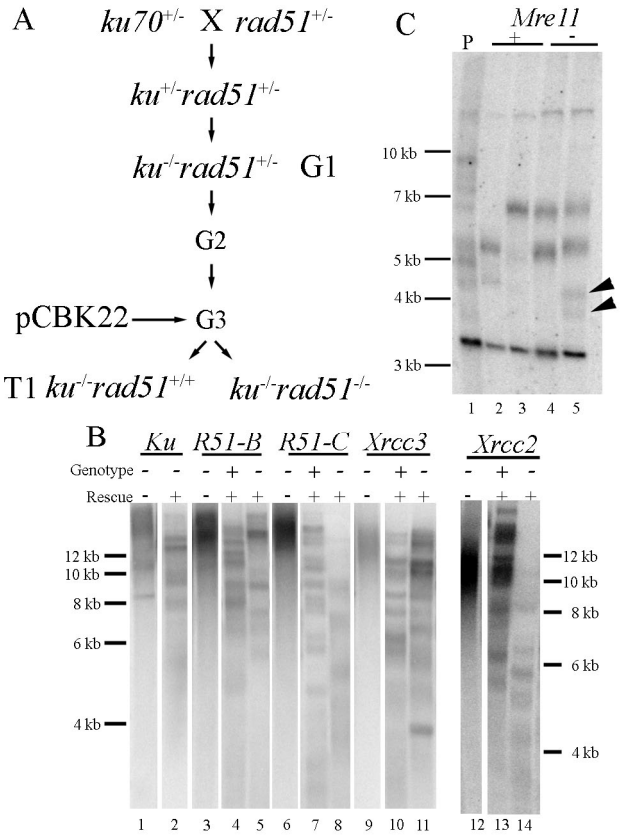


FIG. 15. TRD in *Arabidopsis* is not dependent upon known recombinases.

A) Genetic scheme for obtaining *rad51* mutants with elongated telomeres. Plants null for *ku70* and heterozygous for the indicated genotypes were transformed with pCBK22 and the transformed progeny were genotyped to identify transformants homozygous null for the indicated genotype. B) TRF analysis of T1 progeny of the indicated genotypes. Transformants (+) and non-transformants (-) are indicated. C) Parent progeny subtelomere analysis of a single T1 *MRE11* heterozygous plants. Selfed progeny of this plant were genotyped for *MRE11* and for the presence of the pCBK22 derived T-DNA. Arrowheads denote additional products in one of the *mre11* mutants. Probe is 2R.

mutants with critically shortened telomeres (282). However, recent data from humans and *K. lactis* indicates that ALT can be driven by ECTCs generated as a byproduct of TRD (38, 120, 280). Although we found no evidence for ECTCs by 2D gel analysis of pCBK22 transformants (data not shown), this could simply reflect the low frequency of TRD events. Therefore, we looked for ALT in plants with evidence of TRD using the genetic approach described in FIG. 14A. Specifically, we performed parent-progeny subtelomere analysis on T1 and T2 pCBK22 transformants that were mutant for *ku70* and *tert*.

In one of three lines examined the 1L telomere of several progeny plants was 5-10 kb longer than the longest telomere in its parent (FIG. 16A lanes 3, 4, 9, and 11). A trivial explanation for this finding is that subtelomeric DNA was rearranged changing the restriction profile of this telomere to make it appear elongated. Several observations are inconsistent with this conclusion. First, the elongated products hybridized with a telomeric probe (data not shown), suggesting they are in fact terminal. Second, digestion of the DNA with other restriction enzymes that cleave in the subtelomeric region generated products of expected sizes (data not shown). Third, other subtelomere arms were elongated (see below). Taken together, these data argue that the subtelomeric sequence of 1L has not been grossly rearranged.

Further analysis of individual telomeres in these plants conducted with three different subtelomere specific probes showed that telomeres behaved independently (FIG. 16B). For example, telomeres from the plants analyzed in FIG. 16B, lanes 5 and 6 showed no lengthening relative to their parent for any of the arms examined. In contrast, telomeres

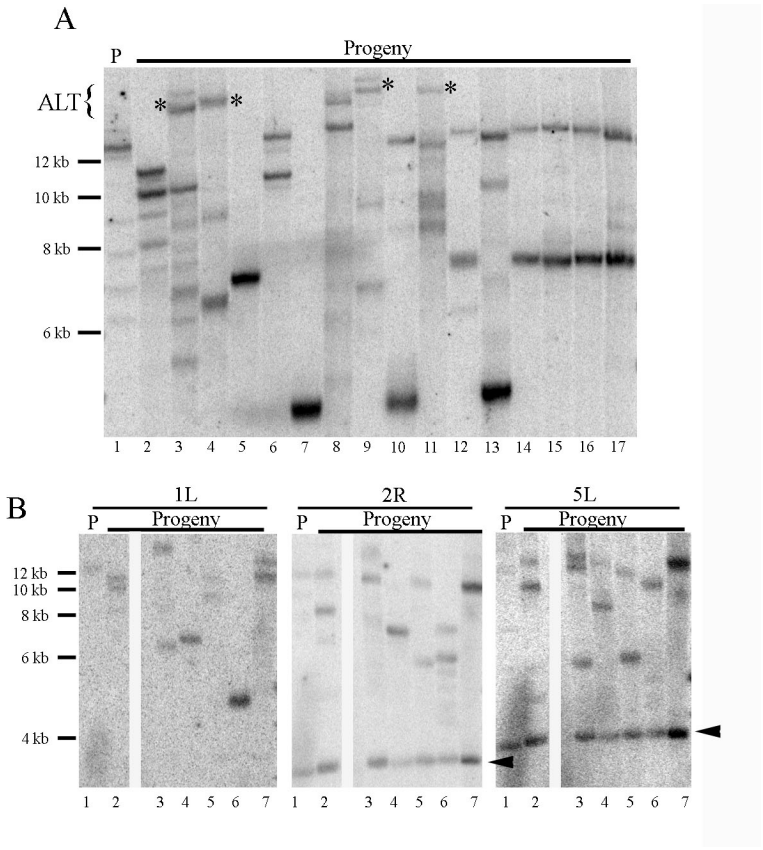


FIG. 16. ALT in *tert* mutants with elongated telomeres.

A) Parent progeny subtelomere analysis of a T1 pCBK22 transformant homozygous for *tert*. Mutants were generated as described in FIG. 14A. Asterisks denote telomeres that were elongated relative to the parent. The hybridizing probe is 1L. B) Sequential hybridization of three probes to a blot containing a subset of samples from panel A. Arrowheads denote interstitial hybridizing signals.

from the plant analyzed in lane 3 were all extended relative to their parent, while some telomeres from the plants in lanes 2, 4, and 7 were elongated, and others were shortened. Rare elongation events in other lines were also observed (FIG. 14C lane 16). These data indicate that ALT like TRD is a stochastic event.

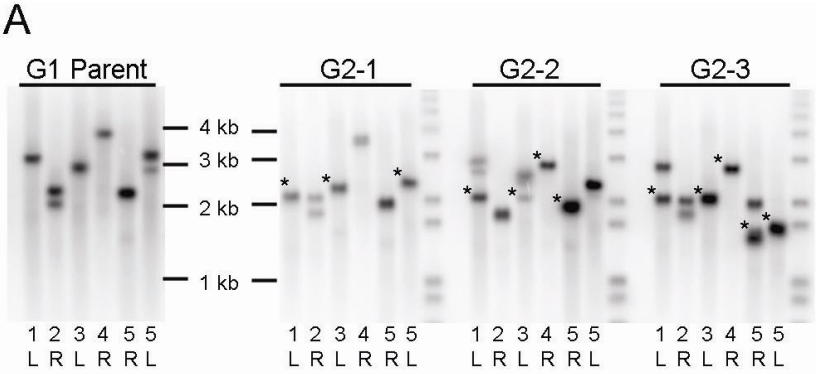
TRD functions at telomeres with lengths in the wild type range. We next asked whether telomeres in the wild type range are subjected to TRD. For this analysis, we employed primer-extension telomere repeat amplification (PETRA), a sensitive method for accurately measuring telomere length at individual chromosome arms (108). Although the ultra-long telomeres in *ku70* mutants are not good substrates for PETRA, this is the preferred method for single telomere analysis in plants with wild type length telomeres as minimal quantities of DNA (a single *Arabidopsis* leaf is sufficient) are required, and seven chromosome arms can be measured at the same time. If TRD occurs in telomerase-positive tissues, the newly shortened telomere is likely to be efficiently elongated by telomerase. To avoid this confounding factor, we examined the rate of telomere shortening in G1, G2, and G6 *tert* mutants.

A representative gel with PETRA products is shown in FIG. 17A. The parent is a G1 *tert* mutant (FIG. 17A, left panel). Notably, in the heterozygous parent of this G1 progeny, only the 2R telomere had undergone TRD (data not shown). However, in the three G2 progeny shown (FIG. 17A, left panel), several examples of TRD were detected, as noted by asterisks.

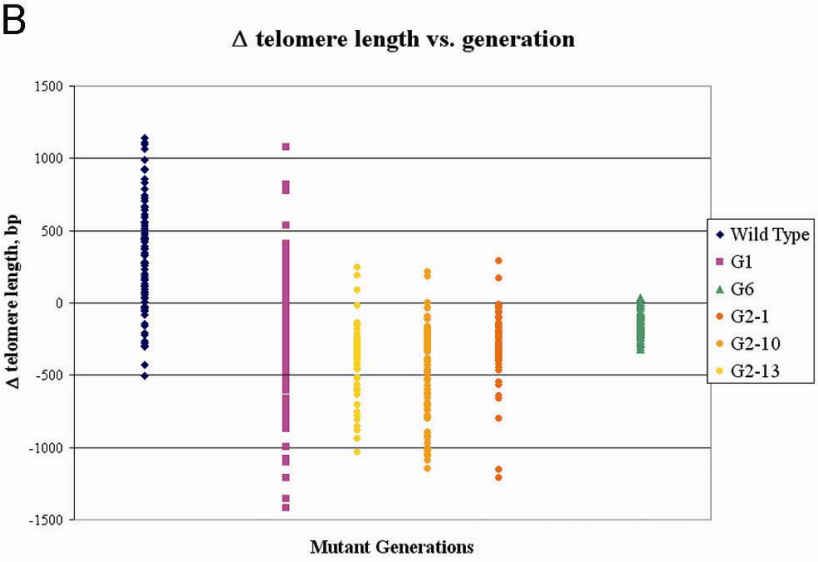
FIG. 17. TRD occurs in telomeres within the wild type range.

A) Representative PETRA samples. Changes defined as TRD are indicated by asterisks. The telomere that was monitored is indicated below each lane. B) Graph depicting the change in telomere length versus generation for different genotypes and their progeny. B) Graph depicting the change in telomere length from parent to progeny relative to the length of the telomere in the parent.

A


G1 Parent
G2-1
G2-2
G2-3
4 kb
3 kb
2 kb
1 kb
1 2 3 4 5 5
L R L R R L

B


Δ telomere length vs. generation
Δ telomere length, bp
Mutant Generations
Wild Type
G1
G6
G2-1
G2-10
G2-13

C

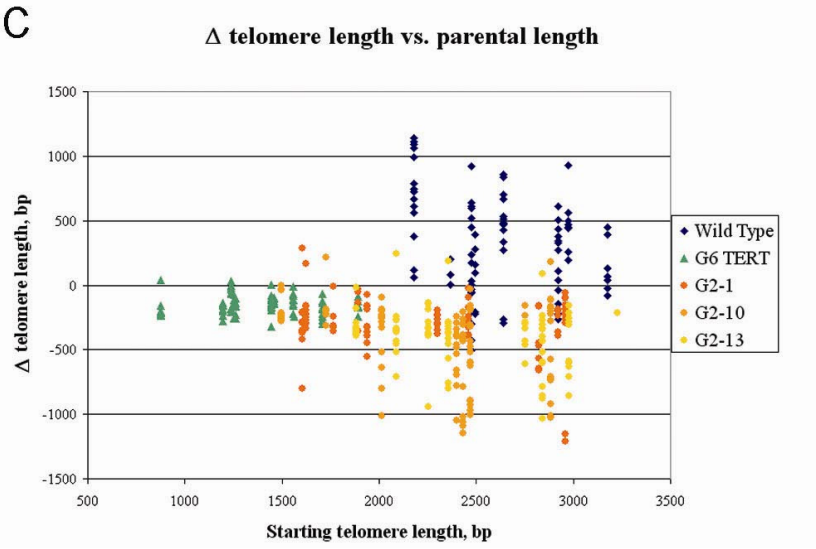

Δ telomere length vs. parental length
Δ telomere length, bp
Starting telomere length, bp
Wild Type
G6 TERT
G2-1
G2-10
G2-13

Table 2 shows the mean rate of telomere length change for all generations analyzed. Individual data points are displayed graphically in FIG. 17B. In wild type plants, there is a broad distribution of the change in telomere length, with most telomeres showing a net increase relative to their parent. Strikingly, of 88 telomere changes measured in telomerase-positive plants, only a single telomere shortened by more than 500 bp. A similar degree of stochastic telomere length changes are observed in G1 and G2 *tert* mutants. However, as expected for telomerase mutants, the net change in telomere length is negative. In contrast, there is very little change in telomere length for G6 *tert* mutants; the maximal shortening observed in the G6 population was 254 bp. Telomeres in both G1 and G2 *tert* mutants shortened by well over 1 kb in a single generation. In a total of 355 telomere length changes measured in G1 and G2 *tert* mutants, 19 telomeres shortened by over 1 kb, and 66 shortened by more than 500 bp, twice the maximal shortening (254 bp) observed in the G6 *tert* mutants. These data imply that telomeres in the G6 mutants shorten only via the end-replication problem, while telomeres in G1 and G2 *tert* mutants are also substrates for TRD. The dramatic increase in TRD frequency in G1 and G2 *tert* mutants relative to wild type could indicate that *TERT* protects against TRD. Alternatively TRD products may simply be rapidly re-extended by telomerase. Taken together, these data support the conclusion the telomeres in early generation *tert* mutants, despite being within the wild type length range overall, are subjected to TRD.

Interestingly, there are several examples of putative ALT events in *tert* mutants (FIG. 17B). In G6 *tert* mutants only a very small degree of elongation was observed (<73 bp), which may be due to errors in the accuracy of measurement. Telomere elongation in

Table 2. Mean changes in telomere length from parents.

Genotype	N	Mean	StDev
Wild type	88	301	382
G1 *tert*	88	-246	476
G2-1 *tert*	85	-285	212
G2-10 *tert*	96	-450	322
G2-13 *tert*	86	-391	222
G6 *tert*	82	-154	77

.

G1 mutants is difficult to assess as telomeres could have been extended by telomerase in the previous generation. In contrast, 5 of 265 telomeres in G2 *tert* mutants were lengthened from 90 to 288 bp; the greatest differential likely represents ALT events. Notably, the G2 *tert* mutants had a much higher frequency of these elongation events relative to the G6 *tert* mutant, supporting the notion of a mechanistic link between TRD and ALT.

Finally, to specifically address whether TRD is length-dependent, we plotted the change in telomere length versus the parental telomere length (FIG. 17C). In telomerase-positive plants, and consistent with previous reports (239), the shortest telomeres were more likely to be elongated than the longer telomeres (compare the shortest wild type telomere to the longest). Moreover, the shortest telomeres were elongated to a greater extent than the longest telomeres. In G6 *tert* mutants, all telomeres shortened by the same amount, regardless of the initial telomere length. Strikingly, telomeres in G2 *tert* mutants shortened by a much larger amount if the parental telomere was longer than approximately 2 kb. Telomeres below 2 kb displayed a rate of shortening similar to G6 *tert* mutants. The frequency of TRD by initial telomere length is shown in Table 3. When queried by chromosome arm, all telomeres underwent TRD with approximately equal frequencies (between 16% and 34%, data not shown). While there is no clear relationship between telomere lengths above 2 kb and the incidence of TRD, the frequency of TRD drops dramatically for telomeres that are less than 2 kb, which is the minimal size of telomeres in wild type plants.

Table 3. Frequency of telomere length changes in G1 and G2 *tert.*

Initial Length	Total	Gain[a]	0 to 500 bp loss	500 to 1000 bp loss	>1000 bp loss	Frequency of TRD[b]
>3 kb	2	0	1	1	0	0.5
2.5-3 kb	116	14	67	27	8	0.30
2-2.5 kb	167	13	107	36	11	0.27
<2 kb	70	3	65	2	0	0.03
Total	355	30	240	66	19	

[a]Number of progeny telomeres that were elongated relative to the parent.

[b]The number of telomeres that lost more than 500 bp of telomeric DNA divided by the total number examined.

DISCUSSION

Telomere dynamics in *ku70*. Cells must maintain a minimal telomere length to provide full protection for chromosome ends and to distinguish them from double-strand breaks. Regulation of a maximal telomere length is also likely to be important to inhibit recombination and reduce the total amount of DNA synthesis. In the absence of *KU70*, telomeres in *Arabidopsis* are dramatically elongated (34, 85, 232). This feature, along with the unique subtelomeric sequences on most *Arabidopsis* chromosome arms, allowed us to examine the dynamics of ultra-long telomeres. We find that a new, longer telomere length set point is established, and this set point is maintained primarily by a competition between a highly active telomerase and a TRD-like mechanism that shortens grossly elongated telomeres. Re-introduction of *KU70* results in dramatic telomere shortening, at a rate much greater than can be accounted for by the end-replication problem. A loss of at least 2.5kb is readily apparent in FIG. 14C, and several telomeres in the T2 generation appear to have shortened by up to 6 kb over two generations. This rate of telomere attrition is not sufficient to offset 15 kb of telomerase-dependent elongation observed in G1 to G2 *ku* mutants, suggesting either that telomerase is inhibited at extremely long telomeres, or that TRD can shorten telomeres by even larger amounts than those we observed. We further demonstrated that TRD is not dependent upon reintroduction of *KU70* itself, as segregating *tert* from *ku70* mutants with long telomeres is sufficient to dramatically shorten telomeres.

Our data are consistent with the dynamics of elongated telomeres in other organisms (48, 134, 144, 153), and argue that TRD is a highly conserved mechanism for telomere size control. The role of the KU heterodimer in *Arabidopsis* TRD is unclear. In yeast

(219), and perhaps humans (169), KU inhibits TRD. In contrast, TRD events in *ku70* mutants rescued with pCBK22 were indistinguishable from plants doubly deficient for *ku70* and *tert*. Current models of the mechanism of TRD posit that the 3' G-overhang invades the duplex telomeric DNA, forming a displaced loop at the site of invasion (38, 169, 280). Branch migration would then convert this structure into a replication intermediate resembling a Holliday-Junction (HJ), which could be resolved into a shortened telomere and an extra-chromosomal telomeric circle. The genetic requirements for TRD in *Arabidopsis* are not clear. Surprisingly, deletion of either *MRE11* or *XRCC3*, which are shown to be required for TRD in mammals and yeast, does not result in loss of TRD in *Arabidopsis*. Several explanations for these findings can be considered. First, there is extensive redundancy in the *Arabidopsis* genome (as much as 60% of the genome is present in duplications), and thus there may be another enzyme capable of resolving HJs in *Arabidopsis* (20). Alternatively, the terminal structure formed at *Arabidopsis* telomeres may be slightly different than that formed in yeast or mammals. Interestingly, Mus81p, an enzyme that resolves HJ-like structures in *S. pombe* has two homologues in *Arabidopsis*. A third consideration is that our assays can not fully distinguish between TRD events that occur in meiosis or mitosis. It may be necessary to disrupt both processes to observe inhibition of TRD.

ECTCs and ALT. ECTCs have been shown to drive telomerase-independent lengthening of telomeres in *K. lactis* (203), and have been associated with ALT in mammals (38, 280). In previous experiments designed to select for telomerase-negative *Arabidopsis* cells using ALT, no telomere lengthening was observed (282). However, the cells used in these experiments had extremely short telomeres, and thus

would be unlikely substrates for TRD. As a consequence, we speculate there would not be an accumulation of ECTCs to serve as substrates for telomere elongation and ALT through a roll-and-spread mechanism. Although we failed to detect ECTCs in plants with elongated telomeres where we had restored *KU70*, we did find evidence for stochastic ALT events in the absence of *tert.* Thus, our data support a model in which TRD and ALT are mechanistically linked.

TRD as a means to regulate telomere length. One important observation from our study is that the frequency of TRD is proportional the length of telomeres, arguing that TRD can function as a form of length regulation. Although a role for TRD has been established in budding yeast (153), here we show that TRD not only shortens grossly elongated *Arabidopsis* telomeres, but also acts on telomeres within the wild type size range. Notably, the extent of telomere shortening in G1 and G2 *tert* mutants is much greater than in G6 *tert* mutants. Furthermore, the amount of DNA lost varies dramatically between different telomeres within the same cellular population. These two findings indicate that TRD can function stochastically at wild type length telomeres in early generation *tert* mutants. We found that the frequency of TRD decreases as telomere length declines, with a very sharp decrease when telomeres drop below 2 kb in length. Intriguingly, the lower range of telomeres in wild type *Arabidopsis* is 2 kb. Thus, TRD might play a role in determining the minimal telomere length.

Our findings argue that TRD acts in concert with telomerase and the end-replication problem as a potent force for controlling telomere length in *Arabidopsis*. How could TRD regulate telomere length? A protein counting model similar to one that regulates

telomerase activity is attractive, but what protein is counted? KU is an interesting possibility. KU is associated with telomeres in all organisms studied where it contributes to telomere length regulation as well as chromosome end-protection. It is possible that KU serves as a roadblock to branch migration, and that as more KU binds to the telomere tract, roadblocks are more frequent. One prediction of this model is that an increase in the amount of KU can prevent TRD. While this model will require more extensive study, we note that over expressing *KU70* in *ku70* mutant plants results in a telomere profile distinct from restoration of the wild type construct (FIG. 13C).

TRD events at telomeres in wild type plants place tremendous pressure on telomerase to extend the truncated telomeres. An unlucky TRD event could result in a telomere that falls below the critical length, leading to telomere dysfunction. Studies in mammalian cells reveal that even a single short telomere is sufficient to trigger genomic instability (111). It is possible that telomerase actively inhibits TRD. Such a model is supported by work in *Caenorhabditis elegans*, where loss of *mrt-2* results in an ever-shorter-telomere phenotype, while loss of telomerase results in sudden telomere shortening events (45). Thus, telomerase appears to be critical either for extending telomeres subjected to TRD or for protecting them from TRD in the first place.

CHAPTER IV

THE FATE OF CELLS WITH TELOMERE FUSIONS

SUMMARY

In mice, shortened telomeres eventually lead to cell-cycle arrest via a p53 dependent cell-cycle checkpoint pathway. In *Arabidopsis*, late generation *tert* plants eventually arrest as miniature, sterile, but metabolically active plants due to the inability of their cells to continue proliferating. Although there is no p53 orthologue in *Arabidopsis*, deletion of the checkpoint kinase ATM in middle-generation *tert* plants leads to an earlier onset of developmental phenotypes. One explanation for these data is that disruption of ATM releases a checkpoint response, allowing cells with dysfunctional telomeres to continue proliferating, leading to ongoing breakage-fusion-bridge cycles. In this study, we examine the fate of cells with dysfunctional telomeres. In crosses between late-generation *tert* and wild type plants, telomeres are not fully restored to a wild type length. However, telomere fusions in the *tert* parent are not propagated to the progeny, suggesting that cells with telomere fusions are not able to propagate for extended periods of time. To monitor the fate of cells with dysfunctional telomeres that display a DNA damage response, we generated GFP-GUS reporter constructs that are driven by the promoters of two genes highly upregulated in response to DNA double-strand breaks (DSBs). Initial characterization of these lines is presented, and we believe they will be useful tools in studying the fate of cells with telomere fusions.

INTRODUCTION

Telomeres are the nucleoprotein structures that protect the ends of eukaryotic chromosomes from being recognized as DNA double-strand breaks (DSBs, reviewed in (18). Telomeric DNA is composed of multiple repeats of a specific, short sequence referred to as the telomere repeat. In *Arabidopsis*, the telomere repeat consists of the hepta-nucleotide sequence TTTAGGG (227), repeated between 300 and 1000 times, producing a telomere tract of between 2 and 7 kb (239). In addition to the long ds telomeric DNA, a short 3' overhang is a conserved characteristic of telomeres (reviewed in (284). Telomeric DNA tracts are maintained through the action of telomerase, a specialized reverse-transcriptase that catalyzes the reiterative addition of telomeric DNA onto the 3' end of existing telomere tracts (reviewed in (52).

The absence of telomerase in mice causes gradual shortening of telomeres which ultimately elicits a cell-cycle checkpoint response, resulting in replicative senescence or apoptosis (24). If cells are forced to continue dividing, telomeres shorten until they reach a critical length, below which they are recognized as DSBs and repaired, usually through telomere-to-telomere fusions (108). In both mice and *Arabidopsis*, loss of telomerase is well-tolerated for several generations, until growth begins to be impaired. In mice, telomere dysfunction results in cellular apoptosis and senescence, most notable in the haematopoietic system and the male germline (147). These lines are eventually unable to continue proliferation due to failure of the male and female germline. This phenotype can be briefly rescued by inactivating the major DNA damage response protein p53 (46), allowing telomerase-deficient mice to propagate for two additional generations. However, telomere dysfunction ultimately results in massive

genomic instability and an increase in the incidence of cancers. Loss of p53 completely abrogates the G1 checkpoint response to DNA damage, and p53 activation during this phase of the cell cycle is partially dependent upon the action of ATM and ATR (1). Mice lacking both ATM and *TERT* display increased apoptosis, demonstrating that ATM is not absolutely necessary for the apoptotic response to dysfunctional telomeres. These results further demonstrate that loss of ATM exacerbates the cellular response to dysfunctional telomeres, suggesting that ATM plays a role in protecting critically shortened telomeres (221, 292).

In contrast to mice, *Arabidopsis* mutants lacking telomerase eventually arrest during vegetative development, without undergoing apoptosis (230). In these plants, loss of telomeric DNA results in the generation of telomere-to-telomere chromosome fusions (108). The number of fusions increases with successive generations and the increase of cytogenetic abnormalities due to telomere fusions correlates well with the loss of proliferative potential in both vegetative and reproductive tissue (230). Plants do not contain an obvious homolog of p53, the major protein required for eliciting checkpoint responses in mice. However, they do contain homologs to ATM and ATR (60, 88). Interestingly, in contrast to mice where p53 deficiency results in partial relief from proliferation controls, deletion of the checkpoint kinase ATM in *tert* plants leads to a sudden onset of severe growth abnormalities in generation five, several generations before similar phenotypes are observed in *tert* plants (277). This correlates well with data demonstrating increased genomic instability in mice deficient for both ATM and *TERT* (221, 292). Two non-exclusive possibilities for this finding are that the loss of

ATM relieves a cell-cycle checkpoint, allowing continuous breakage-fusion-bridge cycles to occur, or that loss of ATM somehow protects shortened telomeres from fusion.

In this study, we investigate the fate of *Arabidopsis* cells that contain a telomere-to-telomere fusion, specifically determining whether that cell is capable of dividing, and if so, whether genome instability is increased in daughter cells. To this end, we began by crossing late generation *tert* mutants with wild type plants to determine whether cells with telomere fusions were capable of progressing through meiosis and generating viable progeny. We found that one generation in the presence of active telomerase is not sufficient to restore telomere length. However, telomere fusions are not propagated in large numbers to F1 progeny, though abundant fusions are detected in pollen. Finally, we developed a DNA damage reporter system to directly monitor, *in planta*, the fate of cells with telomere fusions.

MATERIALS AND METHODS

DNA extraction, fusion PCR, and PETRA. DNA was extracted from whole plants or the indicated tissue using a CTAB based protocol (25). Fusion PCR and PETRA assays were performed as previously described (108).

Generation of PARP-2 and BRCA-1 GFP-GUS reporter constructs. 1.4 kb of the BRCA-1 promoter and 1.2 kb of the PARP-2 promoter were PCR amplified using Phusion High-Fidelity DNA polymerase (NEB) and primers 12attB1BRCA1 (AAA AAG CAG GCT CGA GCT TTA GCG CTT TAT ACT G) with 12attB2BRCA1 (AGA AAG CTG GGT ATT TCG ATC TTC ACT CAG AG) and 12attB1PARP2(AAA AAG CAG GCT

CGG GAC AGG CTG TAA TGT TAG) with 12attB2PARP2 (AGA AAG CTG GGT ATT TCG TCT TCT TCT TCA GAA G). Two-step PCR was performed, with gene specific primers to amplify product from 1 µL of total genomic DNA in a 50 µL reaction with ten cycles of PCR. 10 µL of this reaction was then amplified with universal adapter primers attB1 UA (GGG GAC AAG TTT GTA CAA AAA AGC AGG CT) and attB2UA (GGG GAC CAC TTT GTA CAA GAA AGC TGG GT) with 10 cycles at 45°C followed by 20 cycles at 55°C. PCR products were then cloned into pDONR201 (Invitrogen) using BP Clonase II (Invitrogen) following the manufacturer's instructions. The promoters were then Gateway cloned into pBGWFS7, which expresses a GFP-GUS fusion from the transformed promoters (126). Constructs were transformed into GV3101 and then into G4 *tert* mutant plants as previously described (232).

GFP-GUS analysis. GFP analysis was performed in the lab of Dr. Keerti Rathore in the Borlaug Center. The microscope used was a Zeiss M^2BIO Fluorescence Combination Zoom Stereo/Compound microscope fitted with GFP filters exciting at BP470/40 nm, a dichromatic beam splitter at 495 nm, a barrier filter at LP 500 nm, and a band-pass interference filter at 525/50 nm. The light source is an HBO 100W mercury lamp, and photographs were taken with a Zeiss AxioCam color digital camera using Zeiss AxioVision 3.0.6 software. GUS assays were performed in 1 ml total volume containing 100 µL 1M Na(PO_4) pH 7.0, 50 µL 1 mg/ml X-Gluc, 5 µL Triton X-100, 10 µL 0.5M EDTA, and 835 µL water . Tissue was vacuum infiltrated with GUS staining solution for 10 min and then incubated for 24 hr at 37°C. Tissue was de-stained twice with 70% EtOH for 12 hr.

RESULTS

Telomerase expression in plants with short telomeres restores genome stability. Unpublished data from R. Idol and D. Shippen demonstrated when G5-G7 *tert* mutants are crossed to wild type, telomere length in the F1 progeny is intermediate relative to either parent. The shortest telomeres in the F1 progeny were extended by approximately 250 bp, while the longest telomeres lost approximately 500 bp. F2 segregants from the F1 progeny were not examined. Nevertheless, the data are consistent with work in *Arabidopsis* and yeast that demonstrates that not all telomeres are acted upon by telomerase, and telomerase preferentially extends the shortest telomeres (239, 261). The Idol study further suggested that even G7 *tert* mutants were able to produce viable gametes for propagation.

Our first experiment was designed to test whether telomere fusions can be stabilized by the presence of telomerase. Telomerase can stabilize broken chromosomes and disrupt the breakage-fusion-bridge cycle through a process termed “chromosome healing” (83, 95, 187). In tissues with high levels of telomerase activity, new telomeres can be added to a broken end, resulting in stabilization of the chromosome and blocking further end-joining reactions. We crossed generation 8 (G8) *tert* plants to wild type plants, generating plants heterozygous for *TERT* in the F1. The F1 plants were then selfed to generate an F2 population containing wild type, heterozygous, and mutant plants. The F1 progeny inherit a population of long telomeres from the wild type parent and a population of short telomeres from the G8 *tert* plant. Analysis of telomere length in the F2 allows us to examine how telomeres behave when both long and short telomeres are present in the same cell.

The G8 *tert* plants were very sick, corresponding to a Type II phenotype (230). The F1 population of plants containing a single wild type copy of the *TERT* gene were phenotypically indistinguishable from wild type (data not shown), indicating that the decreased proliferation phenotype of *tert* mutants is rescued either by the presence of telomerase, or by an increase in the average telomere length. The latter possibility is unlikely, as the shortest telomere, and not average telomere length, is critical for viability in mice (111). Direct testing of this hypothesis will, however, require a cross between G8 *tert* and plants heterozygous for *TERT* with wild type length telomeres.

We examined telomere length in several of the F1 and F2 plants using PETRA. PETRA is a PCR based assay that utilizes primer extension to extend a tagged primer which has been annealed to the 3'-overhang of a functional telomere. The primer extended product is PCR amplified with a primer directed at a unique subtelomere and a primer specific for the tagged primer used in the first step. PCR amplified products are then hybridized to a telomeric probe following Southern blotting. We arbitrarily measured the length of the 3L telomere to determine whether the length of this telomere correlated with the presence or absence of fusions.

As seen in FIG. 18**,** lane 1, the 3L telomere in the mutant parent was extremely short, measuring approximately 750 bp, very close to the absolute minimal telomere length of 350 bp (108). In the F1, this telomere was extended by only 200 nucleotides (FIG. 18 lanes 3 and 4) to just under 1 kb. This extent of elongation is similar to that observed previously (R. Idol and D. Shippen, unpublished data). The amount of telomeric DNA

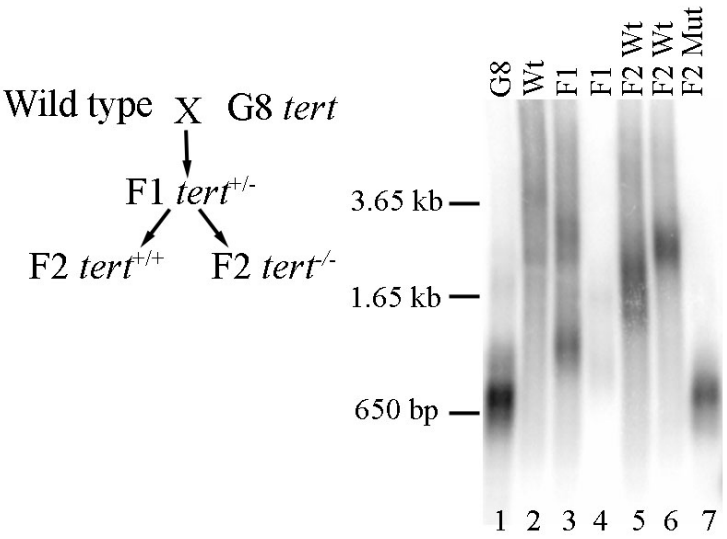


FIG. 18. Single telomere analysis of the F1 and F2 progeny of crosses between G8 *tert* and wild type plants.

In the left panel, the crossing scheme for generating *tert* mutants with long telomeres is diagrammed. Heterozygous F1 progeny have inherited long telomeres from the wild type parent, and critically short telomeres from the G8 parent. The F1 is heterozygous for *TERT*, and this gene can be segregated in the F2, producing wild type plants with short telomeres, or *tert* mutant plants with long and short telomeres. The right panel is PETRA analysis on parents and F1 and F2 progeny performed using a primer directed at chromosome 3L. Molecular weight markers have been adjusted to reflect the size of the telomere tract.

lost due to the end-replication problem is approximately 200-500 bp/generation (76). Interestingly, the gain in telomere length in the F1 is completely lost due to the end-replication problem in F2 *tert* plants, as seen in FIG. 18 lane 7. Thus, the net result of this gain and loss is that the 3L telomere in the F2 *tert* is the same length as in the G8 *tert* parent. Notably, telomeres in wild type F2 progeny were an intermediate length between the original parental plants. This demonstrates that the shorter telomere was extended by telomerase while the longer telomere from the wild type parent was shortened due to the end-replication problem. This finding provides further evidence for the presence of a mechanism that equalizes telomere length between homologous chromosomes (239).

To evaluate the stability of the genome in F1 plants and their F2 progeny, we performed telomere fusion PCR (108). This method takes advantage of the unique subtelomeric sequences at *Arabidopsis* subtelomeres. PCR primers are directed towards the telomere, and PCR is performed with primers from two different subtelomeres. If the two telomeres assayed are covalently joined, PCR products will be generated that hybridize to a telomeric probe after Southern blotting. As seen in FIG. 19, telomere fusions are readily detectable in the G8 *tert* parental plant but no fusions products are generated in reactions with DNA from wild-type parents. Surprisingly, only a very small number of fusions were detected in the F1 plants (FIG. 19 lane 4). It is impossible to determine from this experiment whether the fusions present in this F1 plant were generated *de novo* after embryogenesis, or were inherited from the G8 *tert* parent. It is worth noting that the F1 plant that had fusions was derived from a cross where the G8 *tert* was the mother. In contrast, no fusions were identified from the F1 where the G8

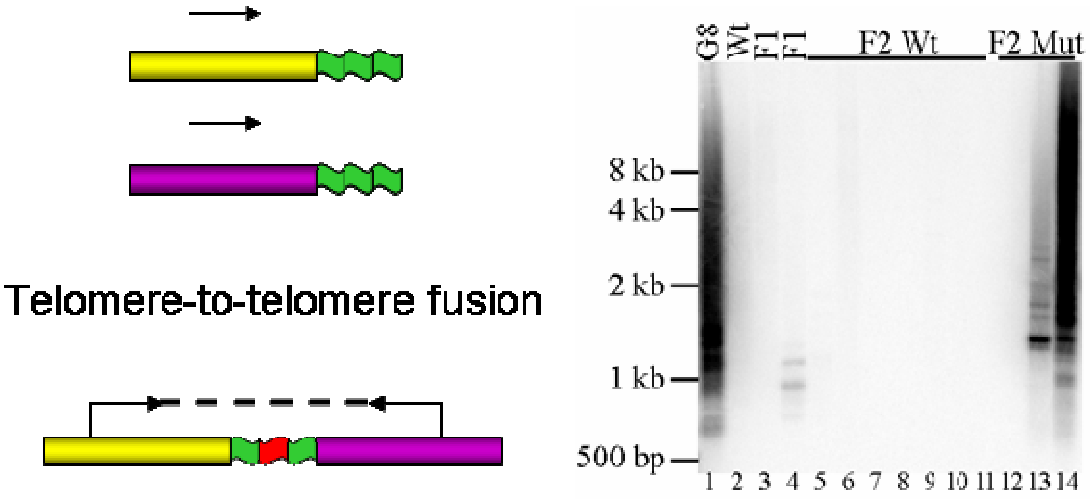


FIG. 19. Telomere fusions in crosses between late generation *tert* and wild type plants. A diagram of the outline of telomere fusion PCR is shown on the left. Following telomere-to-telomere fusion, primers directed towards the telomere will generate a PCR product. Telomere fusion PCR was performed (right panel) using primers directed at chromosome 3L and 4R. PCR products were subjected to agarose electrophoresis followed by Southern blotting and hybridization with a telomeric probe.

plants served as the father. In mice, the male germline fails prior to the female, possibly due to the increased number of cell divisions required (147). Thus the female gametes of *Arabidopsis* may have undergone fewer cell divisions with telomere fusions, resulting in less genomic instability and allowing for propagation of the telomere fusions to the progeny.

Fusion PCR products were not detectable in any wild type F2 progeny. The simplest explanation is that the telomere fusions present in the F1 were not stabilized through chromosome healing, and cells containing these fusion products were lost. Interestingly, a large number of fusions were detected in two of three F2 *tert* plants. The fusions in the F2 plants likely represent fusions generated *de novo* in these plants.

To determine whether fusions were present in germline tissue that could be propagated to the progeny, we performed fusion PCR on pollen and embryos derived from G7 and G8 *tert* plants. As seen in FIG. 20 fusions PCR products are evident in pollen, as well as in individual embryos from both generations. The individual embryos represent a relatively small population of cells, yet the fusion PCR profile suggests multiple independent fusions are present. While this finding does not rule out the possibility that fusions are inherited from parental gametes, it strongly suggests that a large number of fusions are derived *de novo* in the embryo.

Development of reporter constructs to follow the fate of broken chromosomes. In order to specifically determine the fate of cells with telomere-to-telomere fusions, we developed a novel reporter assay. This assay takes advantage of the fact that the

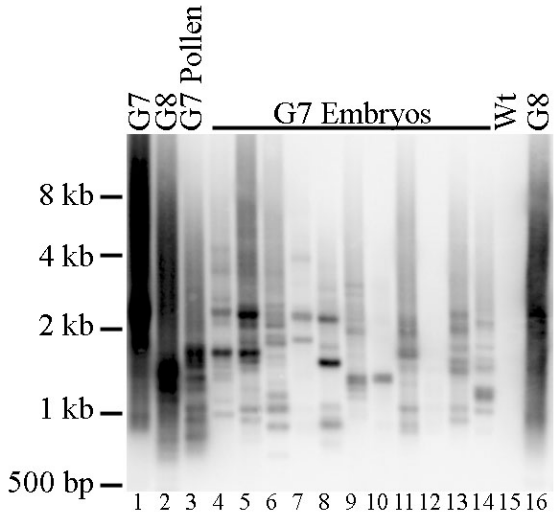


FIG. 20. Telomere fusion PCR in pollen and embryos.

Fusion PCR was performed on tissues (whole plant, individual embryos, bulk pollen) from the indicated generations of *tert* mutants. Primers were directed at chromosomes 3L and 4R. G7 embryos refers to the progeny of G7 *tert* plants.

resolution of anaphase bridges will result in DSBs in the daughter cell. It is known that ionizing radiation induces DSBs and dramatic changes in the transcription profile to upregulate genes required for DSB repair. Two well-characterized *Arabidopsis* genes that are dramatically upregulated under these conditions are PARP2 and BRCA1 (42, 141). Therefore, the DSBs that arise from resolution of anaphase bridges in mitosis are likely to result in a transcriptional up-regulation of either PARP-2 or BRCA-1 during the G1 phase. By driving a GFP-GUS fusion construct from these promoters, DSBs should be visible at a cellular level.

To follow the fate of cells showing a DNA damage response, the reporter constructs were generated by Gateway-cloning approximately 1.2 kb of the putative promoter sequence of either PARP-2 or BRCA-1 into a promoter-less vector containing the coding sequence for a GFP-GUS fusion protein. The combination of GFP and GUS allows for live cell monitoring as well as sensitive histochemical staining (222). These constructs were transformed into wild type, G4 *tert*, G2 *ku/tert*, G2 *atr/tert*, and *Atm*$^{+/-}$ */tert*.

To test whether the PARP2 and BRCA1 promoters were effectively inducing expression in response to DNA damage, wild type transformants were grown on plates containing MMS, which induces post-replicative DSBs (188). Both constructs properly expressed the GFP-GUS fusion in replicating root tips (FIG. 21), and there was some fluorescence in leaf tissue. GUS staining loosely matched GFP expression, although the strongest staining in the PARP2 construct was evident in roots, while the BRCA1 construct resulted in the most intense staining in the leaf (data not shown). Thus, it appears that

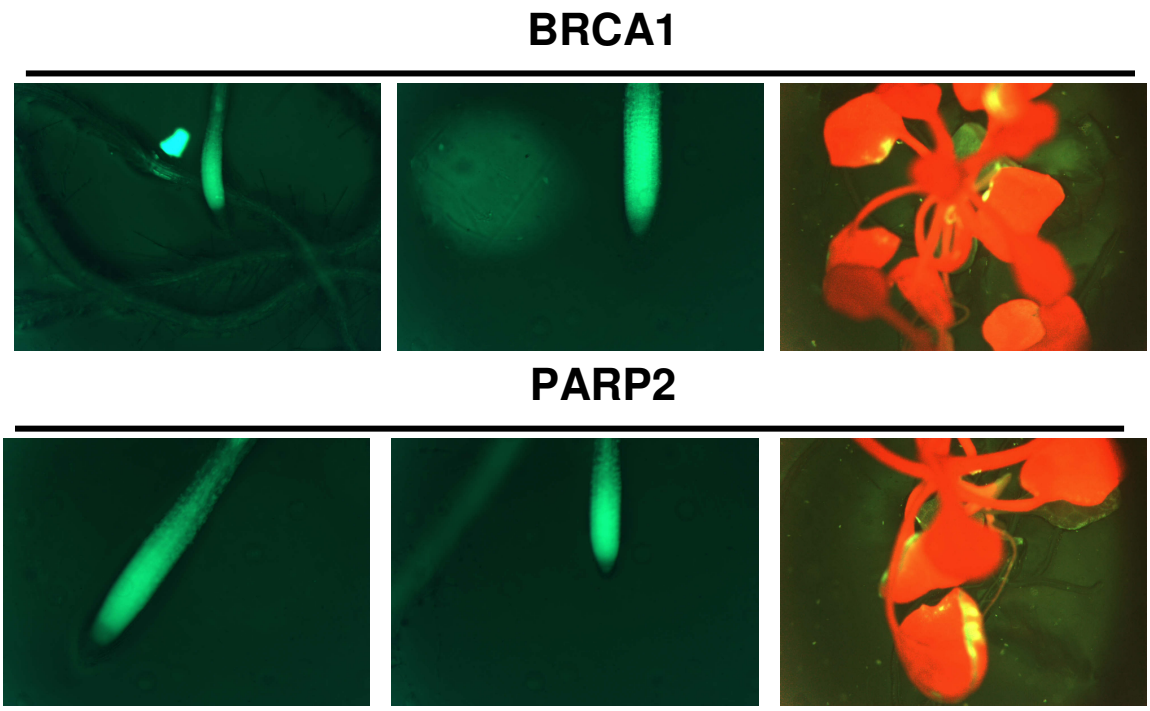


FIG. 21. Response of GFP-GUS reporter constructs to DNA damage induced by MMS treatment.

GFP fluorescence of two individual root tips and leaf fluorescence is presented. The red signal in leaves is due to auto-fluorescence from chlorophyll, the yellowish signals represent GFP fluorescence.

both constructs effectively transcribe the GFP-GUS fusion in response to DNA damage, although the promoters may be more efficient in different types of tissue. Non-treated controls did not display fluorescence in root tips, though some fluorescence was still observed in leaves (data not shown).

We next examined GFP staining in leaves from G6 *tert* mutants that were T2 for the PARP2 construct. Unfortunately, both pollen and necrotic tissue auto-fluoresce quite strongly, making the initial analysis of these plants difficult to interpret. In G6 *tert* plants, leaves displayed large areas in which several individual fluorescing cells were seen FIG. 22. However, wild type cells also displayed similar patterns of GFP fluorescence FIG. 22. Thus, at this early stage of telomere dysfunction, it is unclear whether these reporter constructs will accurately report the presence of telomere fusions. As phenotypic abnormalities and telomere fusions increase with generational age, analysis of subsequent generations of transformants should prove useful in determining whether distinct patterns are evident between wild type and *tert* plants.

DISCUSSION

Telomere dynamics in plants with restored telomerase activity. Although this study was not designed specifically to examine the changes in telomere length when telomerase activity is restored, several interesting points can be noted. First, in the heterozygous F1 background, even very short telomeres are not dramatically extended. The 3L telomere in the G8 *tert* was only 750 bp long, just 400 bp above the absolute minimal telomere length of 350 bp (108). Being so short, this telomere was elongated by only 200 bp in the F1 progeny. In wild type backgrounds, telomerase can extend

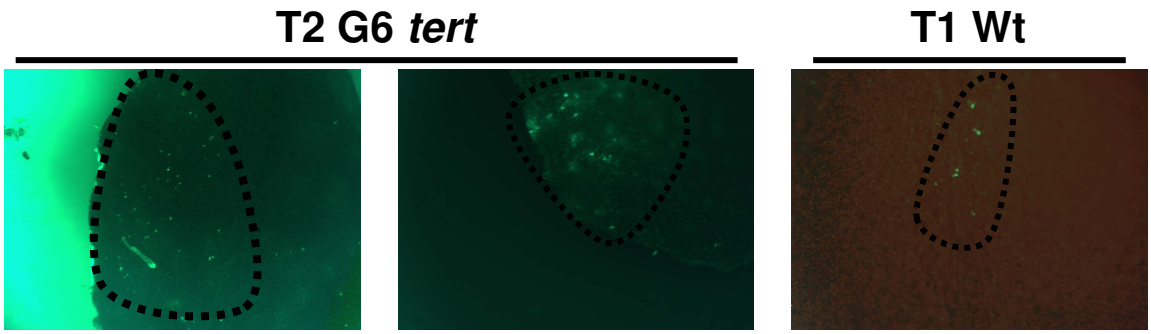


FIG. 22. GFP fluorescence of T1 wild type and T2 G6 *tert* plants.

Two independent G6 *tert* leaves are shown. Several individual cells, usually clustered in a small area are observed. The area of clustering is indicated by a black, dashed line. In wild type leaves, a similar situation is observed, though generally less fluorescent cells are present.

telomeres by 1 kb or more (Chapter III). This very low rate of extension suggests that in the heterozygous F1 background, telomerase may be haploinsufficient, or may be incapable of long extensions when so many telomeres are critically short. Telomerase is haploinsufficient in mammals, though short telomeres can be maintained (69). This haploinsufficiency is evident when either *TERT* or *TER* are heterozygous (180).

The other interesting point is that the longer telomere derived from the wild type parent shortens quite dramatically. In wild type F2 plants, chromosome 3L is composed of a single broad smear, between the lengths of the two parental telomeres, suggesting the cell has a mechanism to bring the telomeres of homologous chromosomes to approximately the same length. While it is possible that a single telomeric smear arose from simple segregation of the parental telomeres, a single signal was observed for two different arms in three different plants. The probability of six telomeres segregating in this manner is quite low (1 in 64). While unique subtelomeric sequences allow us to distinguish specific chromosomes from one another, further direct analysis of this mechanism will require generation of ecotype specific subtelomeric markers to distinguish homologous chromosomes from different parents.

Unpublished data from R. Idol and D. Shippen similarly demonstrate that bulk telomeres in F1 progeny reach a length intermediate between the two parental telomeres. However, those F1 plants were not propagated to the F2 generation. It will be interesting to examine the changes in telomere length in crosses from different generation *tert* plants to wild type or *TERT* $^{+/-}$. One question that remains to be addressed is whether the average telomere length or the shortest telomere is critical for

cell proliferation in *Arabidopsis* as it is in mice (111). Testing this will require a cross between *tert* and *TERT*$^{+/-}$ plants to generate *tert* plant with an average telomere length much higher than the *tert* parent.

While late generation *tert* mutants contain a large number of telomere-to-telomere fusions, the evidence presented here suggests that these fusions are not propagated through the germline. Restoration of *TERT* by crossing G8 *tert* plants with wild type plants results in F1 progeny with a very low abundance of telomere fusions. If telomeric fusions were propagated through meiosis, the dicentric chromosomes produced would give rise to DSBs due to BFB cycles. Broken maize chromosomes undergo chromosome healing in embryos, likely due to an increased activity of telomerase in embryonic tissue (246). We would therefore expect that chromosome healing would occur in F1 telomerase positive embryos, trapping the telomere fusions in the genome. The nearly complete absence of telomere fusions demonstrates that chromosome healing did not occur. Many more F1 progeny will need to be examined to determine if chromosome healing can occur at a low frequency.

Evidence for the absence of a breakage-fusion-bridge cycle in *Arabidopsis tert* plants. The absence of telomere fusions in the F1 progeny of G8 *tert* and wild type plants prompted us to re-examine the data describing telomere fusions in *Arabidopsis* (230, 246). Ongoing breakage-fusion-bridge cycles should result in at least some telomere fusions propagating through meiosis, which should be recovered by fusion PCR in F1 plants that have telomerase activity. The absence of an abundance of fusion

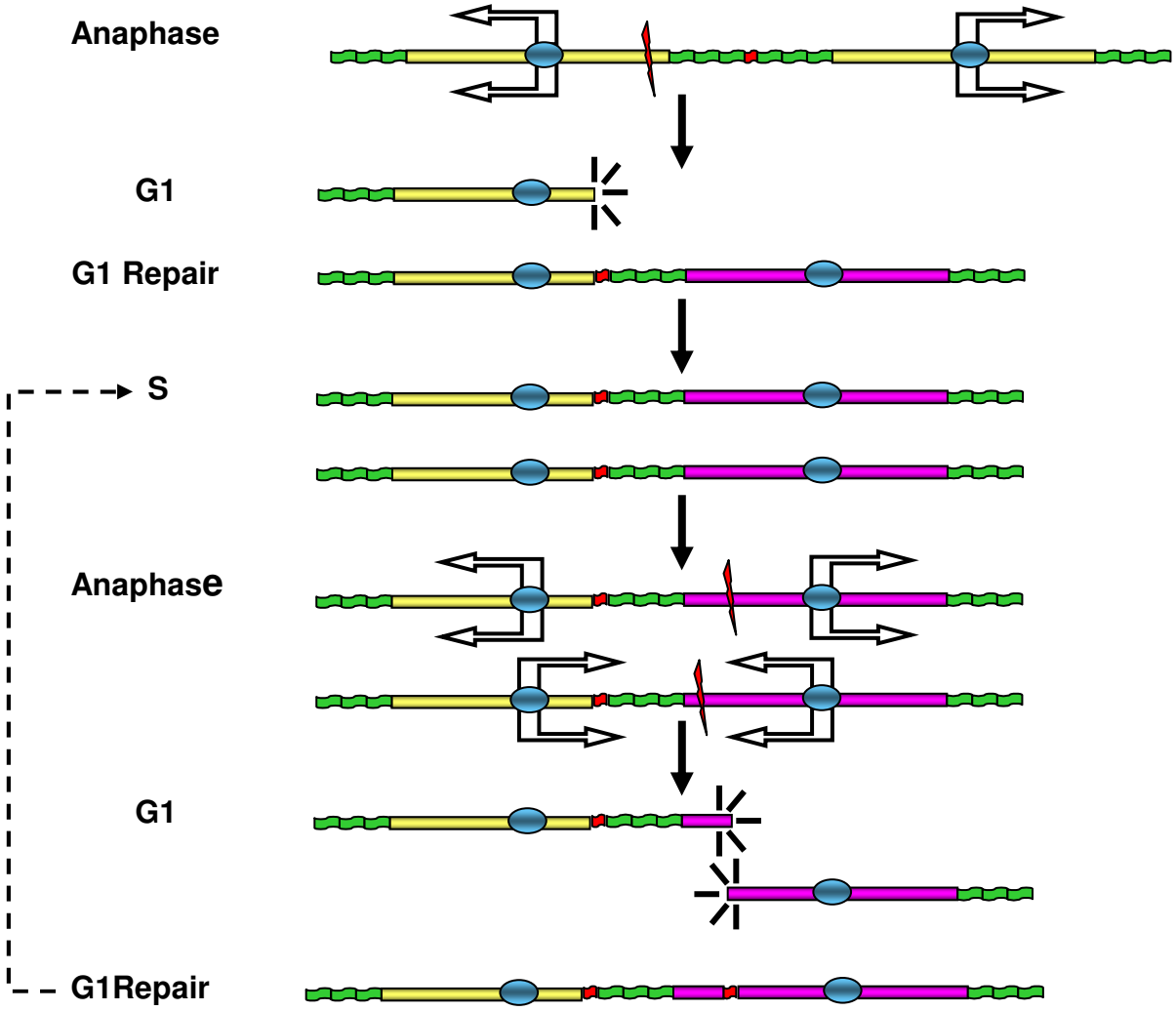
PCR products suggested that perhaps breakage-fusion-bridge cycles do not commonly occur in *tert* plants.

The proportion of cells in anaphase and metaphase is essentially the same in wild type and late generation *tert* plants (230), despite the large number of chromosome fusions present in mutant plants. Furthermore, anaphase bridges are readily detectable beginning in G6, and the number of bridges and their frequency increases each generation (230). These data indicate that telomere-to-telomere fusions are efficiently propagated through at least one mitosis. Cells that progress through mitosis with a single anaphase bridge result in daughter cells that each obtain a single DSB. In yeast, a single un-repaired DSB, even in a non-essential plasmid, usually results in cell cycle arrest, both in G1 and S/G2 (15). Though similar experiments have yet to be carried out in plants, the conservation of checkpoint machinery suggests a similar outcome in plants with un-repaired breaks (238, 278). The meristematic tissue of heavily irradiated plants does in fact result in a G1 arrest, though the exact cause of this arrest is unclear (109).

If the single DSBs generated following mitosis were repaired and initiated breakage-fusion-bridge cycles, the predicted outcome might likely be similar to that detailed in FIG. 23. First, the newly broken end would likely not contain telomeric DNA if there is an equal probability of breakage anywhere on the chromosome arm. If this DSB fuses with another chromosome, it would have to be a telomere, as there are no other DNA ends present in the cell. This fusion would then be replicated during S phase, producing two dicentric chromosomes. These two dicentrics could then be pulled to

FIG. 23. The fate of a telomere fusion following repair in G1.

An anaphase bridge is generated following formation of a dicentric chromosome. Chromosomes are represented as colored rectangles, centromeres as blue circles, telomeres as green wavy rectangles, and the fusion junction is represented as a red wavy rectangle. The white arrows indicate direction of centromere movement and the red lightning bolt represents the location of the DNA break. Black arrows indicate progression through the cell cycle. If a single broken end is present during G1, repair will require fusion of the broken end to a telomere. The dicentric chromosome will then be replicated during S phase, and in 50% of cases, the dicentric sister chromatids will be pulled to opposite pulls. Breakage will occur randomly during mitosis, but each daughter cell will inherit two broken chromosomes. These ends can join to one another, resulting in a dicentric chromosome that will continue through the cell cycle. This model predicts a prevalence of two anaphase bridges being formed during mitosis, and an increase in non-telomeric DNA in the fusion junction.


Anaphase
G1
G1 Repair
S
Anaphase
G1
G1Repair

separate poles during anaphase, producing an anaphase figure with two bridges before breaking in random positions.

The daughter cells of this cell containing two dicentric chromosomes could then easily repair, as there are two broken ends present in the cell. The fusion site in this case would be predominantly between internal DNA, devoid of telomeric or subtelomeric sequences. Following replication, these daughter cells would once again have two dicentric chromosomes producing two anaphase bridges.

An alternative possibility is detailed in FIG. 24. In this case, the single DSB produced following formation of an anaphase bridge would not elicit a checkpoint response, and would instead proceed through S phase, likely resulting in fusions of the sister chromatids. Such a breakage-fusion-bridge cycle would produce only a single anaphase bridge.

Following telomere-to-telomere fusion, an average of only 250 bp of telomeric DNA remains present in the fusion junction (108). Therefore, the likely location of the DSB is in internal regions of the fused chromosome. However, this assumes that the fusion junction and telomeric DNA in general is no more prone to breakage than the rest of the chromosome. Based on these models and this assumption, a critical outcome of the breakage-fusion-bridge cycle would be incorporation of non-telomere associated DNA in the fusion junction.

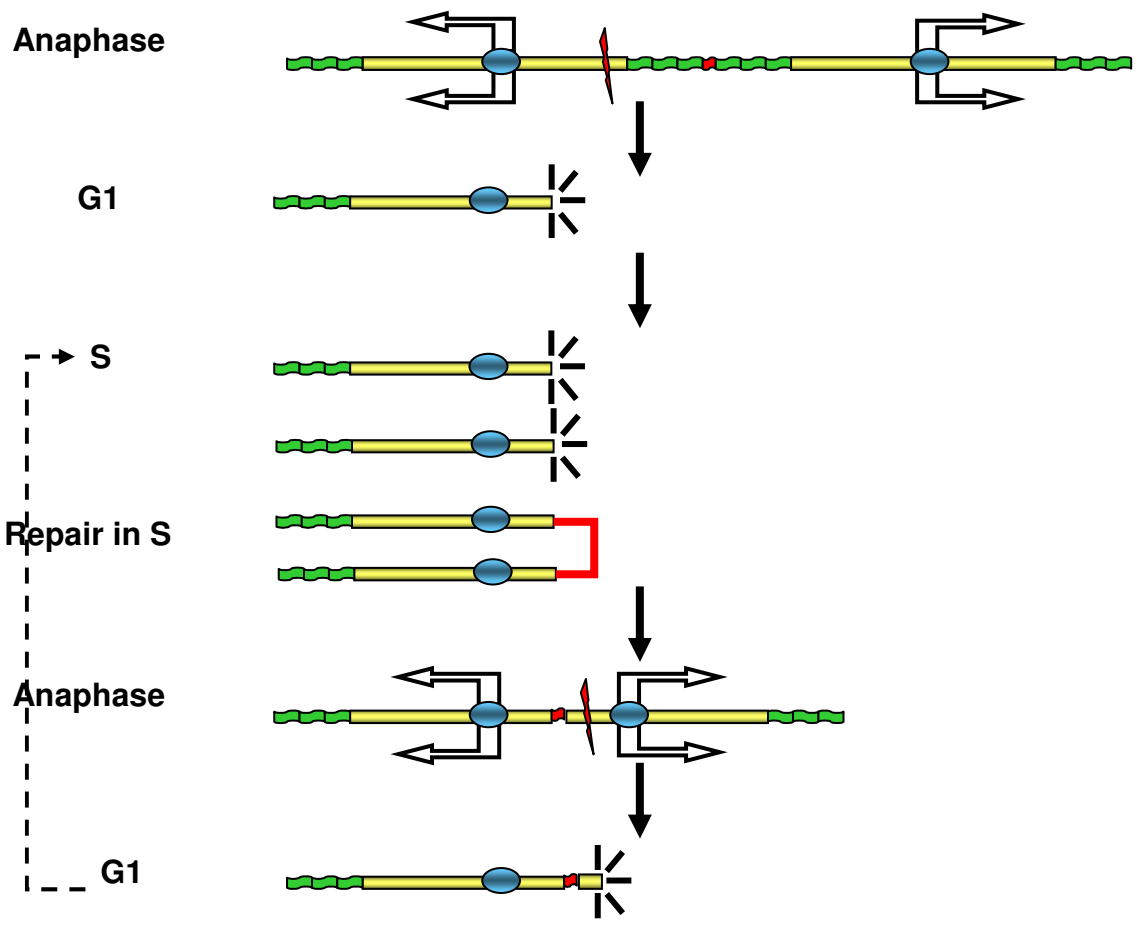


FIG. 24. The fate of a telomere fusion following repair in S.

If a single broken end is present in G1, and is not repaired, but allowed to proceed through the cell cycle, fusion will likely occur between the broken end and its sister chromatid. This dicentric chromosome will then be pulled to opposite poles, resulting in chromosome breakage, and a repeat of this cycle. This model predicts a prevalence of single bridges in anaphase figures, and an increase in non-telomeric DNA at the junction.

Analysis of anaphase bridges in late generation *tert* mutants provides the following data. First, the proportion of cells with a single anaphase bridge is always greater than the number of cells with two bridges (230), suggesting that the breakage of a single anaphase bridge does not result in chromosome fusion during G1. Second, in FISH analysis of late generation *tert* mutants, the number of fusions containing telomeres derived from chromosome ends with rDNA clusters was significantly higher than expected. This demonstrates that the type of DNA commonly found at fusion junctions is subtelomeric, and not internal as would be expected from continual breakage-fusion-bridge cycles (246). Thus, the current data argue against a continued breakage-fusion-bridge cycle in *tert* mutants.

The G1 checkpoint limits breakage-fusion-bridge cycles. We suggest that breakage-fusion-bridge cycles are not an outcome of single telomere-to-telomere fusion events. In cases where two dicentric chromosomes are generated, breakage-fusion-bridge cycles should be able to proceed due to the presence of two DSBs in the G1 cell. However, in the presence of a single DSB, such as those following resolution of a single anaphase bridge, a G1 checkpoint should arrest the cell, as there are no other broken ends to fuse with (15).

A likely candidate for regulation of the G1 checkpoint is the DNA damage response kinase ATM. ATM plays roles in many different checkpoint responses, including the G1 response to DSBs (131). Plants doubly deficient for *Atm* and *TERT* display no synergistic defect for four generations (277). However, G5 double mutants display a rapid onset of telomere fusions and developmental abnormalities resulting in a terminal

phenotype in this generation. This finding is in stark contrast to *tert* mutants which develop progressively worse phenotypes and genomic instability over several generations (277). One possible explanation is that in the absence of ATM, breakage-fusion-bridge cycles occur unimpeded, resulting in an early onset of extreme aneuploidy and cessation of cell proliferation due to the loss of essential genetic information.

As described above, one predicted outcome of continued breakage-fusion-bridge cycles is the presence of non-telomeric DNA in the fusion junction. Indeed, fluorescent *in situ* hybridization with subtelomeric and telomeric DNA probes in *tert* and *atm/tert* mutants indicate that *atm/tert* mutants have a higher proportion of non-telomeric DNA in the fusion junctions (J. Siroky, L. Vespa, B. Vyskot, and D. Shippen, unpublished data).

An alternate way to examine this proposed checkpoint response is to follow the fate of cells with telomere-to-telomere fusions. We designed constructs that express a GFP-GUS fusion protein in response to the presence of DSBs. Our preliminary data indicate that the pattern of expression is indistinguishable between wild type and G6 *tert* plants. DSBs are expected to occur at some level naturally so it is not entirely surprising that wild type plants also express the reporter. Examination of later generation *tert* plants will determine whether these reporter constructs will prove useful in following the fate of cells with telomere fusions. Additional strategies to identify telomere fusions should also be examined, as these constructs may not prove effective in following fusions, and transcriptional activation of BRCA1 and PARP2 is likely to be dependent upon the checkpoint activity of ATM.

If cells with telomere-to-telomere fusions can be identified *in planta*, a large number of experiments can be performed. Telomere length in the sector with fusions can be compared to sectors without fusions to determine which telomeres are the shortest and whether there are large changes in telomere length in the cells with fusions. Telomere fusion PCR can be performed to determine which telomeres were joined. By comparing these data with results from PETRA, we can determine whether the shortest telomere in a cell fuses to the next shortest telomere or if there other types of fusion events. A system of this sort would not only provide a powerful tool in understanding the fate of cells with dysfunctional telomeres at the cellular level, but would also provide insight into the fate of cells with genome instability within the developmental context of an entire organism.

CHAPTER V

SUMMARY AND CONCLUSIONS

TELOMERE BIOLOGY IN *ARABIDOPSIS*

Telomere biology has become a major area of interest over the past two decades. The discovery that telomerase is inactive in most somatic tissues but is expressed in >95% of all human tumors makes it an attractive target for cancer therapy (243). The role of telomeres as “molecular clocks” was advanced following the discovery that telomerase expression could immortalize human cells (24). Thus, telomerase may hold the key to an immortal, cancer-free life. To understand telomere biology means understanding the complex protein-nucleic acid interactions that prevent recognition of the natural chromosome end as a double-strand break.

While it is unlikely that *Arabidopsis* holds either the key to curing cancer or the map to the fountain of youth, studying telomere structure, function, and synthesis in this organism is providing unique tools and insights. Several experiments that are difficult or impossible to perform in humans can be done with relative ease in *Arabidopsis*.

The small sequenced genome, ease of genetic transformation, and relatively short generational time has made *Arabidopsis* the model plant system. Vast germplasm libraries containing everything from point mutants to T-DNA insertion lines make the identification and characterization of mutants in novel genes a straightforward process. In addition to its general usefulness as a model organism, *Arabidopsis* has several key traits that make it especially suitable for the analysis of telomere biology. Foremost is

the extremely high tolerance this plant has to DNA damage and genome instability. Whether this feature is dependent upon the developmental organization of plants or an evolutionary adaptation to genotoxic insult, or both, is unclear. What is clear is that this trait allows us to study the effects of gene deletions that are lethal in mammals. One of the prime examples is the checkpoint kinase ATR, which is absolutely essential in mammals, but completely dispensable for survival in *Arabidopsis* (277).

Another unique aspect of *Arabidopsis* that has made it amenable to the study of telomere length dynamics is the small size of its terminal telomere tract relative to mammals. At only 2-5 kb in the most useful ecotypes (239), relatively minor changes to telomere length can be easily measured with standard Southern blotting techniques. Further facilitating the measurement of telomere length are the unique subtelomeric sequences located on at least seven of ten chromosome arms. Directing PCR primers and hybridization probes to these sequences has allowed us to measure the dynamics of individual telomere tracts without modifying the normal subtelomeric structure. The discovery that changes in subtelomeric DNA methylation dramatically effect telomere length makes the study of completely unmodified chromosome ends even more important (90).

ALTERNATIVE LENGTHENING OF TELOMERES IN *ARABIDOPSIS*

In every organism studied to date, disruption of telomerase activity leads to gradual telomere loss followed by a period of crisis and cell death. While most cells cease to proliferate, a small population is able to overcome telomerase deficiency and continue proliferation. Most organisms find some way to re-extend their shortened telomeres.

Budding yeast (166, 203), fission yeast (201), humans (31) and mice (102) are all able to activate some form of recombination, the end result of which is the extension of telomeric DNA.

Non-recombinational mechanisms have also been identified in several organisms. Dipteran insects evolved a transposon-based mechanism of telomere elongation, likely due to a deleterious mutation in the normal telomere machinery (210). *S. pombe* is able to circularize its three chromosomes in response to telomere dysfunction, removing the end-replication problem all together (201). Circular chromosomes have their own limitations, however, and *S. pombe* survivors are severely deficient in sexual reproduction. The choice of chromosome circularization may be at least partly influenced by the total number of telomere ends present in a cell. With only six telomeres, the probability that a chromosome end will randomly fuse to the end on the opposite side of its chromosome is high.

In order to determine whether *Arabidopsis* could also activate mechanisms to maintain telomeres in the absence of telomerase, we generated a cell suspension culture from late generation *tert* mutants (Chapter II). As expected, we were able to identify phenotypic changes in the cell culture consistent with crisis, and cells that were able to continue proliferating were identified. Unexpectedly, such cells did not appear to use recombination to maintain telomeric DNA. Telomere length analysis demonstrated that there were no telomeres longer than the size of interstitial repeats of approximately 400-500 bp. Using the unique subtelomeric sequences of *Arabidopsis*, we examined the structure of several individual chromosome arms. The subtelomeric sequences were

recalcitrant to BAL31 exonuclease digestion, demonstrating that they were no longer located on the physical end of the chromosome. This data suggested that telomere dysfunction had resulted in telomere-to-telomere fusions. This was corroborated by the fact that the restriction profile of subtelomeric sequences remained unchanged for several years in culture.

In contrast to the stability of the subtelomeric sequences, cytogenetic analysis of these cells showed that the genome was highly unstable. Cells examined during the crisis period showed unprecedented levels of anaphase bridges and gross aneuploidy. When individual cells were examined for three independent cytogenetic parameters, there was essentially no correlation between different cells, further demonstrating the severe genomic instability produced by crisis. In contrast, cells that had survived this period were relatively homogenous for the three cytogenetic parameters. Despite this fact, nearly half of the cells examined had anaphase bridges. This finding demonstrated that how ever these cells were proliferating, they had not found a way to completely stabilize their genome.

While the exact mechanism these cells employed to overcome the end-replication problem is not clear, several likely conclusions can be drawn. First, and similar to human cells that lack telomerase, these survivors had probably lost several critical checkpoint proteins, possibly including ATM and/or ATR. It would be interesting to examine the expression profile and post-translational modifications of these two kinases. Second, these cells are unlikely to contain functional telomeres as they lack sufficient lengths of telomeric DNA. While transposon activation similar to that

observed in *Drosophila* is a distinct possibility, examination of chromosomal DNA content did not demonstrate a significant increase in any single locus, which would be consistent with a transposon-based mechanism.

Perhaps the most likely explanation is that these cells formed circular chromosomes. A diploid *Arabidopsis* genome provides twenty distinct chromosomes ends, a much larger number than the six in *S. pombe*. However, in the absence of telomerase, telomeres are expected to become dysfunctional over time, and not all together. This could result in only a few chromosome ends being capable of fusion at any given point. If these ends were located on the same chromosome, it could facilitate formation of circular chromosomes. Pulsed-field gel electrophoresis examination of rare-cutting restriction profiles could answer whether this was in fact the mechanism of survival in these cells.

A CONSERVED LINK BETWEEN TELOMERE RAPID DELETION AND ALT

The absence of recombinational telomere elongation in late generation *tert* cells prompted us to examine whether recombinational shortening, also termed telomere rapid deletion (TRD), occurred in *Arabidopsis*. Much of the early work by Arthur Lustig and coworkers in yeast demonstrated that TRD was a recombinational process where only the most terminal telomeric DNA was lost (32, 153, 169, 219). Work in humans in 2004 (38, 280), and in *K. lactis* in 2005 demonstrated that TRD (120) is in fact a conserved mechanism. Recombinational deletion of telomeric DNA in these organisms produced as a by-product an extra-chromosomal telomeric circle (ECTC). In all cases, TRD occurred at elongated telomeres.

To determine whether TRD could operate in *Arabidopsis*, we took advantage of the elongated telomeres in *ku70* plants (Chapter III). Loss of *KU70* leads to rapid, telomerase-dependent telomere elongation. Interestingly, we found that telomere elongation in these mutants appears to occur in two steps. In G1 *ku70* plants, telomeres become approximately twice as long as wild type plants, an extension of 4-5 kb. However, in G2, the longest telomeres were extended by as much as 15 kb. This extension did not continue however. The longest telomeres in G2 *ku70* were approximately as long as those in the G3 plants, and no further extension was apparent for four more generations. Thus, telomeres in *ku70* mutants reach homeostasis, albeit at a much longer length than wild type telomeres.

At least three distinct but not mutually exclusive mechanisms can be hypothesized for the new telomere length homeostasis. First, telomerase is regulated by ds telomere binding proteins in other organisms. Though homologous proteins have yet to be definitively identified in *Arabidopsis*, our lab has identified several strong candidates (125). In this model, the elongated telomere tracts in *ku70* plants would recruit more of the ds telomere binding proteins, and the combined negative regulatory effect of these proteins would overcome the loss of regulation by *ku70*. This would result in telomerase not acting upon the longest telomeres. If this were the only mechanism controlling telomere length, however, we might expect that most telomeres would reach homeostasis at a long length, but the distribution of lengths would likely be relatively small. Overactive telomerase would extend all telomeres until they reached a length capable of recruiting enough ds telomere binding proteins to halt the action of telomerase.

A second mechanism could be an increase in the amount of telomeric DNA lost due to the end-replication problem. KU is known to play a role in regulating origin firing (58), and one explanation for elongated 3'-overhangs in *ku70/tert* plants is that KU is involved in the proper positioning of RNA primers. KU is associated with DNA Polymerase α, which is responsible for production of the RNA primers used during DNA replication (181). Therefore, it is not inconceivable that the rate of nucleotide loss due to the end-replication problem in *ku70* plants may be higher than that observed in wild type. However, for this difference to contribute to telomere length regulation, it would need to affect long telomeres more severely. There is no evidence to date for any length dependence in the rate of nucleotide loss due to the end-replication problem. Alternatively, an increased rate of telomere shortening could result from increased sensitivity to exonuclease digestion at long telomeres. Again, there is no evidence for a length-dependent recruitment of exonucleases. In the absence of telomerase, either of these mechanisms should produce heterogeneous smears for individual chromosome arms.

The final possible mechanism is TRD. TRD has been previously shown to be length dependent in budding yeast (153) and functions primarily at deregulated telomeres in humans that are elongated relative to wild type cells (38, 280). In the absence of telomerase, TRD should be evident as dramatic telomere shortening events producing discrete products.

We hypothesized that restoration of *KU70* would restore proper telomerase regulation, allowing us to examine telomere dynamics as the elongated telomeres returned to a

wild type length. We therefore transformed G4 *ku70* plants with a construct overexpressing the cDNA of *KU70* or a construct containing *KU70* along with 1.5 kb of putative promoter sequence.

Restoration of *KU70* through either means resulted in a dramatic shortening of telomere length, demonstrating that homeostasis was achieved not solely through telomerase inhibition at elongated telomeres, but also through a mechanism that increased the rate of telomere loss. The telomere profile in plants where *KU70* was restored is consistent with TRD, as telomeres appeared as discrete products and not heterogeneous smears in TRF analysis. We further demonstrated that TRD is not dependent upon a functional *KU70* gene, and can instead be observed by disrupting telomerase function. These findings demonstrate that TRD functions at elongated telomeres in *Arabidopsis*.

TRD in other organisms produces a shortened telomere as well as an extra-chromosomal telomeric circle (ECTC). We used 2-dimensional gel electrophoresis to assay for the presence of circular molecules in plants undergoing TRD. We were unable to detect ECTCs using this conventional assay. In *K. lactis*, ECTCs can serve as substrates for rolling-circle amplification (203), resulting in telomerase-independent telomere elongation. While we could not detect ECTCs by 2D-gel analysis, we hypothesized that if they were present they could serve as rolling circle substrates for telomere elongation. This elongation would be evident in a *tert* background. Therefore, we assayed several *ku70/tert* plants with elongated telomeres, and found in one line a large number of plants displaying telomeres that were elongated relative to their parent, indicating the presence of an ALT like mechanism in these plants.

It is somewhat surprising that ALT was discovered in this background, where telomeres, though long, are fully functional in capping. In other organisms, ALT is preceded first by telomere dysfunction due to loss of telomerase (30) or by severely disrupting chromosome capping (120, 280). Phenotypically, *ku70/tert* mutants are indistinguishable from wild type in early generations, suggesting that there is no severe defect in capping, and telomeres in the background we assayed were still extremely long relative to wild type. This suggests that perhaps KU inhibits inter-chromosomal telomere recombination in *Arabidopsis*. This is a known function of KU in both yeast and humans (36, 219). Another possibility is that a low number of ECTCs formed during TRD can initiate telomere elongation in *Arabidopsis*, similar to the situation observed in *K. lactis* (120, 203). Whether ECTCs function to promote ALT in humans is unclear, but they are present in cells utilizing this mechanism to maintain telomeres (38, 280). Thus, TRD events may in fact be a pre-requisite for ALT, forming a telomeric circle that can elongate shortened telomeres.

The use of G8 *tert* mutants in the initial selection for telomerase-independent cell survivors may have severely biased our findings away from recombinational mechanisms of telomere elongation. The extremely short telomeres present in these cells are unlikely substrates for ALT. In yeast, breakage-induced replication (BIR) mechanisms of telomere elongation happen very early in response to telomere dysfunction (261). However, at these early stages, the amount of DNA gained through these mechanisms is not enough to counteract the loss due to the end-replication problem. Michael McEachern has proposed that formation of a telomeric circle, and subsequent elongation of a single telomere, is necessary to provide the long templates

needed for survival through these BIR mechanisms (268). It may be possible then that rare elongation events due to BIR did in fact occur during early proliferation of the G8 *tert* cells, but the lack of TRD at this shortened stage precluded the formation of the single elongated telomere necessary for survival.

These data, combined with the data from other organisms, strongly suggest that recombination at telomeres is a conserved process. Formation of an ECTC in a cellular context proficient for telomere elongation with the ECTC as a rolling-circle substrate may be the critical event necessary for survival in the absence of telomerase. Once a single elongated telomere is generated, this telomere can serve as a BIR substrate for other chromosome ends. Alternatively, this newly elongated telomere may undergo TRD, producing another ECTC that can serve as a rolling-circle substrate for other telomeres. Analysis of individual chromosome ends from different organisms strongly supports a model in which stochastic TRD, ALT, and BIR events contribute to maintain telomeres within a very broad length range (68, 120, 262, 280).

In yeast, deletion of *RAD52* results in almost complete loss of survivor formation (44, 166, 262) as well as dramatically curtailing of TRD (153). Rad52p is a ss DNA binding protein that appears to facilitate the loading of Rad51p (254), which then promotes strand exchange (255), the first step of homologous recombination. In humans, the Holliday junction resolvase XRCC3 and the MRE11/RAD50/NBS1 complex have been implicated in both ALT and TRD (122, 280). The *Arabidopsis* genome does not contain a sequence-conserved homologue of *RAD52* although homologues of *XRCC3*, *RAD51*, and *MRE11* are easily identified. Surprisingly, deletion of any of these three proteins in

Arabidopsis does not appear to negatively affect TRD. The well-conserved nature of TRD and ALT in other organisms suggests that the deleted proteins may play a role in TRD, but are functionally redundant, either with other *RAD51* homologues or other non-related proteins. Several candidates are discussed in more detail below.

TRD AS A MEANS OF TELOMERE LENGTH REGULATION

In *S. cerevisiae*, TRD appears to be a mechanism of preventing telomeres from being dramatically elongated. While short telomeres must be maintained in order for cells to continue proliferating, the evolutionary advantage of limiting telomere elongation is not understood. The most striking case is Type I survivors in yeast, where recombination results in up to 4% of the genome being comprised of telomeric and subtelomeric DNA (166). The increase in the length of the cell cycle due to having to replicate through so much more additional DNA likely accounts for the loss of this survivor type when assayed in competition experiments with Type II survivors, which do not amplify their telomeric DNA so dramatically (263). While this selective disadvantage is not likely to affect multi-cellular organisms so dramatically, replication through telomeres may generally be difficult, as *S. pombe* requires Taz1 for efficient semi-conservative replication through telomeres (190).

Limiting telomere length may also be important if there is a limited pool of ds telomere binding proteins. Extremely long telomeric DNA may bind all available ds telomere binding proteins. If length regulation is dependent not only upon the number of ds telomere binding proteins associated with a telomere, but also on the relative distribution of the telomere binding proteins, a point could be reached where telomeres

become so long that there are not enough proteins available to negatively regulate telomeres. In humans, TRF1 binds to approximately 20% of the telomeric repeats, regardless of the length of the telomere, suggesting if there is a length beyond which the pool of ds telomere binding proteins is limiting, it has not been reached (250).

Finally, limiting telomere length may be important for inhibiting TRD, and thereby inhibiting ALT. Elongated telomeres appear to be more susceptible to TRD, indicating that this mechanism has some length dependence. Longer telomeres would be more susceptible to TRD, producing more ECTCs, and possible leading to loss of telomere length control due to an increase in ALT.

Surprisingly, we demonstrated that TRD happens in *Arabidopsis* with a frequency of almost 30% in telomeres near the low end of the wild type range (2-3 kb). Furthermore, the frequency of TRD dropped to below 5% when telomeres fell below the minimal wild type length of 2 kb. This finding suggests that TRD serves to regulate telomere length not only in dramatically elongated telomeres, but also at telomeres within the wild type range. The sharp cut-off at 2 kb further suggests that TRD may serve to define the minimal wild type length. Two examples were noted where TRD resulted in telomeres that were under 1.2 kb, a limit below which telomeres become increasingly dysfunctional (108). This places tremendous pressure on telomerase to extend these shortened telomeres, or face telomere-to-telomere fusions.

This length dependence of TRD accounts for the apparent decrease in the amount of telomeric DNA lost due to the end-replication problem in later generation *tert* mutants

(230). In this study, the end-replication problem is estimated to result in the loss of only 150 bp of telomeric DNA per sexual generation, a number similar to that observed in late generation *tert* plants.

Interestingly, TRD was not observed in plants with active telomerase. Three models can be envisioned for this finding. First, as we know telomerase preferentially extends the shortest telomeres in a population, TRD may simply provide a good substrate for telomerase due to its length. Second, telomerase may actively prevent TRD, even when it is not extending a given telomere. The continued debate on whether ALT and telomerase activity can co-exist in the same cell (79, 214), along with a growing body of work demonstrating additional roles for telomerase (77, 236), suggest this may be a possible explanation. Finally, TRD may produce a substrate that is preferentially recognized by telomerase, not because of its length, but due in part to the end-structure formed by TRD. Work in *C. elegans* has demonstrated that deletion of the checkpoint protein MRT-2 results in an inability to elongate telomeres by telomerase (3). However, mutations in the catalytic subunit TRT-1 result in not only telomere loss due to the end-replication problem, but also in shortened telomeres consistent with products of TRD (45). This finding suggests that even in the absence of its ability to extend telomeres, TRT-1 is somehow capable of either preventing TRD from occurring or can extend the shortened products of TRD.

THE FATE OF CELLS WITH TELOMERE FUSIONS

In the absence of telomerase, telomeres continue to shorten until they become dysfunctional and are recruited into fusions with other telomeres. In *Arabidopsis*, the

primary pathway for the formation of fusions is through the non-homologous end-joining mechanism (108). Late generation *tert* mutants display an abundance of fusions, with as many as 50% of all anaphases having one or more cytologically visible bridges (230).

To examine the fate of cells with telomere fusions, we crossed G8 *tert* plants displaying abundant fusions to wild type plants, restoring telomerase activity (Chapter IV). The telomeric fusions present in the *tert* parents are not propagated to the F1 progeny. If the fusions were propagated, the breakage-fusion-bridge process would likely be arrested due to chromosome healing in the embryo. We further demonstrated that there are abundant fusions in the pollen of late generation *tert* mutants, ruling out the possibility that all gametes are selected to be free of telomere fusions. This is in contrast to mice, where dramatic apoptosis results in loss of dysfunctional germinal tissue (147). Furthermore, the distinct fusion PCR profile observed in sibling embryos from late generation *tert* mutants suggests that fusions arise predominantly *de novo*, and are not transmitted through the germline.

Interestingly, restoration of telomerase for a single generation is not sufficient to elongate all telomeres more than several hundred bp. In one instance, the elongated telomeric DNA in the F1 was completely lost due to the end-replication problem in the F2 *tert* plants. This may suggest that *TERT is* haplo-insufficient or that in this cross, where half of the telomeres are critically short, telomerase is simply incapable of extending all the short telomeres by appreciable amounts. Another interesting observation is apparent coordinate regulation of telomeres on homologous

chromosomes. While two homologous telomeres of different lengths are present in the F1, only a single telomeric signal is present in the F2 plants, and the length is intermediate between the two parents. This had previously been observed in *Arabidopsis*, as subtelomeric analysis of wild type plants generally produces a single hybridizing band (239). While the mechanism regulating this process is unknown, *tert* plants generally have several hybridizing signals for individual chromosome arms, suggesting that telomerase may be required for this process.

There is strong evidence for the lack of continuous breakage-fusion-bridge cycles in *Arabidopsis* with telomere fusions. The strongest evidence for this is the prevalence of telomeric and subtelomeric DNA present in the fusion junctions of anaphase bridges (246). ATM is a likely candidate for a checkpoint protein that limits cell proliferation in the presence of un-repaired DSBs, due to the rapid onset of developmental abnormalities and chromosome fusions in the G5 *atm/tert* background (277).

In order to examine this putative checkpoint response, we constructed two reporter constructs to visualize cells with telomere fusions. These constructs are based on the hypothesis that DNA breaks produced after dicentric chromosomes are pulled to opposite poles will elicit a DNA damage response. Ionizing radiation, which produces large numbers of DSBs, results in the transcriptional upregulation of PARP2 and BRCA1 mRNA (42). The constructs consist of either the BRCA1 or PARP2 promoter driving expression of a GFP-GUS fusion construct.

Wild type plants containing these constructs produce the reporter protein in response to treatment with MMS, which induces DSBs following DNA replication. As expected, the highly proliferative cells in the root tip displayed GFP fluorescence and showed intense GUS staining. The PARP2 promoter showed the strongest GUS signal in root tips, while BRCA1 had the strongest staining in leaf tissue. This suggests that the two constructs may be more suited to different studies, depending on which tissue is to be examined.

Only G6 *tert* mutants containing these constructs have been examined to date. In these plants, which display minor developmental abnormalities and show a low incidence of telomere fusion, small clusters of individual fluorescing cells were observed. The pattern of fluorescence was not consistent with a BFB cycle producing large sectors of cells. Instead the pattern was more consistent with several independent cells in a small region of the leaf undergoing telomere fusions independently. However, a similar pattern was observed in wild type leaf tissue, though generally fewer cells were found to be fluorescing. Examination of the G7 *tert* progeny, which will have a much higher frequency of telomere fusions, will determine whether these constructs will be useful for following the fate of cells with telomere fusions.

FUTURE EXPERIMENTS

The results presented here suggest several obvious experiments. If ALT does indeed require formation of a telomeric circle by TRD, generation of a *tert* suspension culture from earlier generation mutants (G2 or G3) may allow for ALT to occur. A more useful approach may be the generation of *ku70/tert* plants, where *tert* is segregated following

telomere elongation in the absence of *ku70* (similar to those derived in FIG. 14A, but with only a single generation of *ku70* deficiency, to keep telomeres from becoming too elongated). If ALT is able to effectively compensate for the loss of telomerase in these plants, it would be the first case of ALT functioning at the organism level. Such plants would be useful for ascertaining the evolutionary advantage conferred by telomerase over recombination.

Such a line would also be extremely useful in determining which genes are required for ALT and TRD in *Arabidopsis*. EMS mutagenesis or T-DNA integration could be used to randomly mutagenize these ALT plants, and then screen for those that are no longer able to maintain telomeres. While laborious, this may be the only way of identifying these genes. ALT should result in a heterogeneous smear of telomeric DNA when visualized by Southern blotting. Loss of this mechanism should result in the re-appearance of distinct telomere bands.

Without undertaking a large screen of this sort, we are left with a candidate gene approach. The sterility phenotypes of *xrcc3* and *rad51* indicate they are essential for meiotic progression. Based on the known function of these two proteins, as well as the sterility phenotype associated with the T-DNA mutants of *xrcc3* and *rad51*, deletion of either severely impairs homologous recombination. BLAST searches are unable to identify any obvious duplication of either *XRCC3* or *RAD51*. XRCC3 is required for resolution of Holliday junctions, and RAD51 is generally required for strand exchange. However, TRF2 is capable of forming t-loop structures *in vitro*, suggesting that the

RAD51 function may not be essential for TRD. While t-loops may be able to form in the absence of RAD51, a Holliday junction resolvase is absolutely essential for TRD.

While XRCC3 is apparently the mammalian Holliday junction resolvase, the nuclease required for resolution of meiotic crossover in *S. pombe* is Mus81p/Eme1p (84, 209). Differing requirements for Mus81p in *S. pombe and S. cerevisiae* led to the proposal that Mus81p cleaves cross-over DNA prior to formation of a double Holliday junction (209). This structure may be more applicable to the single Holliday junction found in Lustig's TRD model (169). Two *MUS81* homologues are present in *Arabidopsis*, At4g30870 and At5g39770. At4g30870 has been recently characterized, and the authors provide evidence that At5g39770 is in fact a non-functional pseudo-gene (106). Crosses should be made to generate *tert/mus81* mutants to determine whether this protein is required for TRD. While *mus81* plants are viable, it is possible that TRD can function through either MUS81 or XRCC3. If this is indeed the case, a triple mutant would need to be generated, and the sterility of *xrcc3* would provide only a single generation for analysis.

The role of TRD in regulating telomere length is intriguing; however a direct test of its role will require identification of proteins required for its function. If TRD mutants are not sterile, it will be extremely interesting to determine whether telomeres elongate. Such experiments would provide direct evidence of length regulation by TRD. Another interesting feature is the apparent absence of TRD in telomerase-positive plants. One intriguing experiment would be to create a catalytically inactive *tert* gene and follow

TRD in this background. This experiment would address whether *TERT* in fact protects against TRD.

Finally, the role of ATM in telomere dysfunction is intriguing. The GFP-GUS constructs generated to follow telomere fusions may prove useful in elucidating the fate of cells with dysfunctional telomeres in *tert* plants. However, up-regulation of both PARP2 and BRCA1 may be ATM dependent, which would make these constructs worthless in examining the role of ATM in the proposed G1 checkpoint. Alternate means of identifying and following telomere fusions *in planta* should be explored, in case these constructs are unable to be used in ATM deficient plants.

REFERENCES

1. **Abraham, R. T.** 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev **15:**2177-2196.

2. **Adams, S. P., T. P. Hartman, K. Y. Lim, M. W. Chase, M. D. Bennett, I. J. Leitch, and A. R. Leitch.** 2001. Loss and recovery of Arabidopsis-type telomere repeat sequences 5'-(TTTAGGG)(n)-3' in the evolution of a major radiation of flowering plants. Proc Biol Sci **268:**1541-1546.

3. **Ahmed, S., and J. Hodgkin.** 2000. MRT-2 checkpoint protein is required for germline immortality and telomere replication in C. elegans. Nature **403:**159-164.

4. **Aigner, S., and T. R. Cech.** 2004. The Euplotes telomerase subunit p43 stimulates enzymatic activity and processivity in vitro. Rna **10:**1108-1118.

5. **Aigner, S., J. Lingner, K. J. Goodrich, C. A. Grosshans, A. Shevchenko, M. Mann, and T. R. Cech.** 2000. Euplotes telomerase contains an La motif protein produced by apparent translational frameshifting. Embo J **19:**6230-6239.

6. **Alonso, J. M., A. N. Stepanova, T. J. Leisse, C. J. Kim, H. Chen, P. Shinn, D. K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C. C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D. E. Carter, T. Marchand, E. Risseeuw, D. Brogden, A. Zeko, W. L. Crosby, C. C. Berry, and J. R. Ecker.** 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:**653-657.

7. **Ancelin, K., M. Brunori, S. Bauwens, C. E. Koering, C. Brun, M. Ricoul, J. P. Pommier, L. Sabatier, and E. Gilson.** 2002. Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. Mol Cell Biol **22:**3474-3487.

8. **Arabidopsis Genome Initiative.** 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408:**796-815.

9. **Artandi, S. E., S. Chang, S. L. Lee, S. Alson, G. J. Gottlieb, L. Chin, and R. A. DePinho.** 2000. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature **406:**641-645.

10. **Bakkenist, C. J., and M. B. Kastan.** 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature **421:**499-506.

11. **Banik, S. S., and C. M. Counter.** 2004. Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. J Biol Chem **279:**51745-51748.

12. **Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides.** 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:**120-124.

13. **Baumann, P.** 2006. Are mouse telomeres going to pot? Cell **126:**33-36.

14. **Baumann, P., and T. R. Cech.** 2001. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science **292:**1171-1175.

15. **Bennett, C. B., A. L. Lewis, K. K. Baldwin, and M. A. Resnick.** 1993. Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. Proc Natl Acad Sci U S A **90:**5613-5617.

16. **Bianchi, A., S. Smith, L. Chong, P. Elias, and T. de Lange.** 1997. TRF1 is a dimer and bends telomeric DNA. EMBO J **16:**1785-1794.

17. **Bianchi, A., R. M. Stansel, L. Fairall, J. D. Griffith, D. Rhodes, and T. de Lange.** 1999. TRF1 binds a bipartite telomeric site with extreme spatial flexibility. EMBO J **18:**5735-5744.

18. **Blackburn, E. H.** 2001. Switching and signaling at the telomere. Cell **106:**661-673.

19. **Blackburn, E. H., and J. G. Gall.** 1978. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. J Mol Biol **120:**33-53.

20. **Blanc, G., A. Barakat, R. Guyot, R. Cooke, and M. Delseny.** 2000. Extensive duplication and reshuffling in the Arabidopsis genome. Plant Cell **12:**1093-1101.

21. **Blasco, M. A., W. Funk, B. Villeponteau, and C. W. Greider.** 1995. Functional characterization and developmental regulation of mouse telomerase RNA. Science **269:**1267-1270.

22. **Blasco, M. A., H. W. Lee, M. P. Hande, E. Samper, P. M. Lansdorp, R. A. DePinho, and C. W. Greider.** 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell **91:**25-34.

23. **Bleuyard, J. Y., M. E. Gallego, F. Savigny, and C. I. White.** 2005. Differing requirements for the Arabidopsis Rad51 paralogs in meiosis and DNA repair. Plant J **41:**533-545.

24. **Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright.** 1998. Extension of

life-span by introduction of telomerase into normal human cells. Science **279:**349-352.

25. **Borevitz, J. O., D. Liang, D. Plouffe, H. S. Chang, T. Zhu, D. Weigel, C. C. Berry, E. Winzeler, and J. Chory.** 2003. Large-scale identification of single-feature polymorphisms in complex genomes. Genome Res **13:**513-523.

26. **Boulton, S. J., and S. P. Jackson.** 1998. Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. EMBO J **17:**1819-1828.

27. **Boulton, S. J., and S. P. Jackson.** 1996. Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res **24:**4639-4648.

28. **Brown, E. J., and D. Baltimore.** 2000. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev **14:**397-402.

29. **Brown, E. J., and D. Baltimore.** 2003. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev **17:**615-628.

30. **Bryan, T. M., A. Englezou, L. Dalla-Pozza, M. A. Dunham, and R. R. Reddel.** 1997. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med **3:**1271-1274.

31. **Bryan, T. M., A. Englezou, J. Gupta, S. Bacchetti, and R. R. Reddel.** 1995. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J **14:**4240-4248.

32. **Bucholc, M., Y. Park, and A. J. Lustig.** 2001. Intrachromatid excision of telomeric DNA as a mechanism for telomere size control in Saccharomyces cerevisiae. Mol Cell Biol **21:**6559-6573.

33. **Bunch, J. T., N. S. Bae, J. Leonardi, and P. Baumann.** 2005. Distinct requirements for Pot1 in limiting telomere length and maintaining chromosome stability. Mol Cell Biol **25:**5567-5578.

34. **Bundock, P., and P. Hooykaas.** 2002. Severe developmental defects, hypersensitivity to DNA-damaging agents, and lengthened telomeres in Arabidopsis MRE11 mutants. Plant Cell **14:**2451-2462.

35. **Callebaut, I., D. Moshous, J. P. Mornon, and J. P. de Villartay.** 2002. Metallo-beta-lactamase fold within nucleic acids processing enzymes: the beta-CASP family. Nucleic Acids Res **30:**3592-3601.

36. **Celli, G. B., E. L. Denchi, and T. de Lange.** 2006. Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. Nat Cell Biol **8:**885-890.

37. **Cerone, M. A., R. J. Ward, J. A. Londono-Vallejo, and C. Autexier.** 2005. Telomerase RNA mutated in autosomal dyskeratosis congenita reconstitutes a weakly active telomerase enzyme defective in telomere elongation. Cell Cycle **4:**585-589.

38. **Cesare, A. J., and J. D. Griffith.** 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol Cell Biol **24:**9948-9957.

39. **Cesare, A. J., N. Quinney, S. Willcox, D. Subramanian, and J. D. Griffith.** 2003. Telomere looping in P. sativum (common garden pea). Plant J **36:**271-279.

40. **Chan, S. W., and E. H. Blackburn.** 2003. Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol Cell **11:**1379-1387.

41. **Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad.** 2001. Cdc13 both positively and negatively regulates telomere replication. Genes Dev **15:**404-414.

42. **Chen, I. P., U. Haehnel, L. Altschmied, I. Schubert, and H. Puchta.** 2003. The transcriptional response of Arabidopsis to genotoxic stress - a high-density colony array study (HDCA). Plant J **35:**771-786.

43. **Chen, J. L., and C. W. Greider.** 2004. Telomerase RNA structure and function: implications for dyskeratosis congenita. Trends Biochem Sci **29:**183-192.

44. **Chen, Q., A. Ijpma, and C. W. Greider.** 2001. Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. Mol Cell Biol **21:**1819-1827.

45. **Cheung, I., M. Schertzer, A. Rose, and P. M. Lansdorp.** 2006. High incidence of rapid telomere loss in telomerase-deficient Caenorhabditis elegans. Nucleic Acids Res **34:**96-103.

46. **Chin, L., S. E. Artandi, Q. Shen, A. Tam, S. L. Lee, G. J. Gottlieb, C. W. Greider, and R. A. DePinho.** 1999. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell **97:**527-538.

47. **Clough, S. J., and A. F. Bent.** 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:**735-743.

48. **Cohen, S., and M. Mechali.** 2002. Formation of extrachromosomal circles from telomeric DNA in Xenopus laevis. EMBO Rep **3:**1168-1174.

49. **Cohn, M., and E. H. Blackburn.** 1995. Telomerase in yeast. Science **269:**396-400.

50. **Colgin, L. M., K. Baran, P. Baumann, T. R. Cech, and R. R. Reddel.** 2003. Human POT1 facilitates telomere elongation by telomerase. Curr Biol **13:**942-946.

51. **Colgin, L. M., C. Wilkinson, A. Englezou, A. Kilian, M. O. Robinson, and R. R. Reddel.** 2000. The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity. Neoplasia **2:**426-432.

52. **Collins, K.** 2006. The biogenesis and regulation of telomerase holoenzymes. Nat Rev Mol Cell Biol **7:**484-494.

53. **Collins, K., R. Kobayashi, and C. W. Greider.** 1995. Purification of Tetrahymena telomerase and cloning of genes encoding the two protein components of the enzyme. Cell **81:**677-686.

54. **Comai, L., and S. Henikoff.** 2006. TILLING: practical single-nucleotide mutation discovery. Plant J **45:**684-694.

55. **Conrad, M. N., J. H. Wright, A. J. Wolf, and V. A. Zakian.** 1990. RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell **63:**739-750.

56. **Cooper, J. P., E. R. Nimmo, R. C. Allshire, and T. R. Cech.** 1997. Regulation of telomere length and function by a Myb-domain protein in fission yeast. Nature **385:**744-747.

57. **Cooper, J. P., Y. Watanabe, and P. Nurse.** 1998. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination. Nature **392:**828-831.

58. **Cosgrove, A. J., C. A. Nieduszynski, and A. D. Donaldson.** 2002. Ku complex controls the replication time of DNA in telomere regions. Genes Dev **16:**2485-2490.

59. **Counter, C. M., A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti.** 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J **11:**1921-1929.

60. **Culligan, K., A. Tissier, and A. Britt.** 2004. ATR regulates a G2-phase cell-cycle checkpoint in Arabidopsis thaliana. Plant Cell **16:**1091-1104.

61. **d'Adda di Fagagna, F., M. P. Hande, W. M. Tong, D. Roth, P. M. Lansdorp, Z. Q. Wang, and S. P. Jackson.** 2001. Effects of DNA nonhomologous end-joining factors on telomere length and chromosomal stability in mammalian cells. Curr Biol **11:**1192-1196.

62. **de Bruin, D., Z. Zaman, R. A. Liberatore, and M. Ptashne.** 2001. Telomere looping permits gene activation by a downstream UAS in yeast. Nature **409:**109-113.

63. **de Lange, T.** 2002. Protection of mammalian telomeres. Oncogene **21:**532-540.

64. **de Lange, T.** 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev **19:**2100-2110.

65. **Diede, S. J., and D. E. Gottschling.** 1999. Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. Cell **99:**723-733.

66. **Drosopoulos, W. C., R. Direnzo, and V. R. Prasad.** 2005. Human telomerase RNA template sequence is a determinant of telomere repeat extension rate. J Biol Chem **280:**32801-32810.

67. **Drost, J. B., and W. R. Lee.** 1995. Biological basis of germline mutation: comparisons of spontaneous germline mutation rates among drosophila, mouse, and human. Environ Mol Mutagen **25 Suppl 26:**48-64.

68. **Dunham, M. A., A. A. Neumann, C. L. Fasching, and R. R. Reddel.** 2000. Telomere maintenance by recombination in human cells. Nat Genet **26:**447-450.

69. **Erdmann, N., Y. Liu, and L. Harrington.** 2004. Distinct dosage requirements for the maintenance of long and short telomeres in mTert heterozygous mice. Proc Natl Acad Sci U S A **101:**6080-6085.

70. **Fan, X., and C. M. Price.** 1997. Coordinate regulation of G- and C strand length during new telomere synthesis. Mol Biol Cell **8:**2145-2155.

71. **Fanti, L., G. Giovinazzo, M. Berloco, and S. Pimpinelli.** 1998. The heterochromatin protein 1 prevents telomere fusions in Drosophila. Mol Cell **2:**527-538.

72. **Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, and et al.** 1995. The RNA component of human telomerase. Science **269:**1236-1241.

73. **Ferreira, M. G., K. M. Miller, and J. P. Cooper.** 2004. Indecent exposure: when telomeres become uncapped. Mol Cell **13:**7-18.

74. **Fisher, T. S., A. K. Taggart, and V. A. Zakian.** 2004. Cell cycle-dependent regulation of yeast telomerase by Ku. Nat Struct Mol Biol **11:**1198-1205.

75. **Fitzgerald, M. S., T. D. McKnight, and D. E. Shippen.** 1996. Characterization and developmental patterns of telomerase expression in plants. Proc Natl Acad Sci U S A **93:**14422-14427.

76. **Fitzgerald, M. S., K. Riha, F. Gao, S. Ren, T. D. McKnight, and D. E. Shippen.** 1999. Disruption of the telomerase catalytic subunit gene from Arabidopsis inactivates telomerase and leads to a slow loss of telomeric DNA. Proc Natl Acad Sci U S A **96:**14813-14818.

77. **Flores, I., M. L. Cayuela, and M. A. Blasco.** 2005. Effects of telomerase and telomere length on epidermal stem cell behavior. Science **309:**1253-1256.

78. **Ford, L. P., J. W. Shay, and W. E. Wright.** 2001. The La antigen associates with the human telomerase ribonucleoprotein and influences telomere length in vivo. Rna **7:**1068-1075.

79. **Ford, L. P., Y. Zou, K. Pongracz, S. M. Gryaznov, J. W. Shay, and W. E. Wright.** 2001. Telomerase can inhibit the recombination-based pathway of telomere maintenance in human cells. J Biol Chem **276:**32198-32203.

80. **Forsythe, H. L., J. L. Jarvis, J. W. Turner, L. W. Elmore, and S. E. Holt.** 2001. Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. J Biol Chem **276:**15571-15574.

81. **Fras, A., and J. Maluszynska.** 2004. The correlation between the chromosome variation in callus and genotype of explants of Arabidopsis thaliana. Genetica **121:**145-154.

82. **Freibaum, B. D., and C. M. Counter.** 2006. hSnm1B is a novel telomere-associated protein. J Biol Chem **281:**15033-15036.

83. **Friebe, B., R. G. Kynast, P. Zhang, L. Qi, M. Dhar, and B. S. Gill.** 2001. Chromosome healing by addition of telomeric repeats in wheat occurs during the first mitotic divisions of the sporophyte and is a gradual process. Chromosome Res **9:**137-146.

84. **Gaillard, P. H., E. Noguchi, P. Shanahan, and P. Russell.** 2003. The endogenous Mus81-Eme1 complex resolves Holliday junctions by a nick and counternick mechanism. Mol Cell **12:**747-759.

85. **Gallego, M. E., N. Jalut, and C. I. White.** 2003. Telomerase dependence of telomere lengthening in Ku80 mutant Arabidopsis. Plant Cell **15:**782-789.

86. **Gallego, M. E., M. Jeanneau, F. Granier, D. Bouchez, N. Bechtold, and C. I. White.** 2001. Disruption of the Arabidopsis RAD50 gene leads to plant sterility and MMS sensitivity. Plant J **25:**31-41.

87. **Gallego, M. E., and C. I. White.** 2001. RAD50 function is essential for telomere maintenance in Arabidopsis. Proc Natl Acad Sci U S A **98:**1711-1716.

88. **Garcia, V., H. Bruchet, D. Camescasse, F. Granier, D. Bouchez, and A. Tissier.** 2003. AtATM is essential for meiosis and the somatic response to DNA damage in plants. Plant Cell **15:**119-132.

89. **Garvik, B., M. Carson, and L. Hartwell.** 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol **15:**6128-6138.

90. **Gonzalo, S., I. Jaco, M. F. Fraga, T. Chen, E. Li, M. Esteller, and M. A. Blasco.** 2006. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol **8:**416-424.

91. **Grandin, N., C. Damon, and M. Charbonneau.** 2001. Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. EMBO J **20:**1173-1183.

92. **Grandin, N., S. I. Reed, and M. Charbonneau.** 1997. Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. Genes Dev **11:**512-527.

93. **Grant, J. D., D. Broccoli, M. Muquit, F. J. Manion, J. Tisdall, and M. F. Ochs.** 2001. Telometric: a tool providing simplified, reproducible measurements of telomeric DNA from constant field agarose gels. Biotechniques **31:**1314-1316, 1318.

94. **Gravel, S., M. Larrivee, P. Labrecque, and R. J. Wellinger.** 1998. Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:**741-744.

95. **Greene, E. C., and D. E. Shippen.** 1998. Developmentally programmed assembly of higher order telomerase complexes with distinct biochemical and structural properties. Genes Dev **12:**2921-2931.

96. **Greider, C. W.** 1991. Telomerase is processive. Mol Cell Biol **11:**4572-4580.

97. **Griffith, J., A. Bianchi, and T. de Lange.** 1998. TRF1 promotes parallel pairing of telomeric tracts in vitro. J Mol Biol **278:**79-88.

98. **Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange.** 1999. Mammalian telomeres end in a large duplex loop. Cell **97:**503-514.

99. **Grunstein, M.** 1997. Molecular model for telomeric heterochromatin in yeast. Curr Opin Cell Biol **9:**383-387.

100. **Hackett, J. A., D. M. Feldser, and C. W. Greider.** 2001. Telomere dysfunction increases mutation rate and genomic instability. Cell **106:**275-286.

101. **Hahn, W. C., S. A. Stewart, M. W. Brooks, S. G. York, E. Eaton, A. Kurachi, R. L. Beijersbergen, J. H. Knoll, M. Meyerson, and R. A. Weinberg.** 1999. Inhibition of telomerase limits the growth of human cancer cells. Nat Med **5:**1164-1170.

102. **Hande, M. P., E. Samper, P. Lansdorp, and M. A. Blasco.** 1999. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. J Cell Biol **144:**589-601.

103. **Hardy, C. F., L. Sussel, and D. Shore.** 1992. A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. Genes Dev **6:**801-814.

104. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature **345:**458-460.

105. **Harrington, L., T. McPhail, V. Mar, W. Zhou, R. Oulton, M. B. Bass, I. Arruda, and M. O. Robinson.** 1997. A mammalian telomerase-associated protein. Science **275:**973-977.

106. **Hartung, F., S. Suer, T. Bergmann, and H. Puchta.** 2006. The role of AtMUS81 in DNA repair and its genetic interaction with the helicase AtRecQ4A. Nucleic Acids Res **34:**4438-4448.

107. **He, H., A. S. Multani, W. Cosme-Blanco, H. Tahara, J. Ma, S. Pathak, Y. Deng, and S. Chang.** 2006. POT1b protects telomeres from end-to-end chromosomal fusions and aberrant homologous recombination. EMBO J **25:**5180-5190.

108. **Heacock, M., E. Spangler, K. Riha, J. Puizina, and D. E. Shippen.** 2004. Molecular analysis of telomere fusions in Arabidopsis: multiple pathways for chromosome end-joining. EMBO J **23:**2304-2313.

109. **Hefner, E., N. Huefner, and A. B. Britt.** 2006. Tissue-specific regulation of cell-cycle responses to DNA damage in Arabidopsis seedlings. DNA Repair (Amst) **5:**102-110.

110. **Heller-Uszynska, K., W. Schnippenkoetter, and A. Kilian.** 2002. Cloning and characterization of rice (Oryza sativa L) telomerase reverse transcriptase, which reveals complex splicing patterns. Plant J **31:**75-86.

111. **Hemann, M. T., M. A. Strong, L. Y. Hao, and C. W. Greider.** 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell **107:**67-77.

112. **Henderson, E. R., and E. H. Blackburn.** 1989. An overhanging 3' terminus is a conserved feature of telomeres. Mol Cell Biol **9:**345-348.

113. **Henson, J. D., A. A. Neumann, T. R. Yeager, and R. R. Reddel.** 2002. Alternative lengthening of telomeres in mammalian cells. Oncogene **21:**598-610.

114. **Heslop-Harrison, J. S.** 1998. Cytogenetic analysis of Arabidopsis. Methods Mol Biol **82:**119-127.

115. **Hockemeyer, D., J. P. Daniels, H. Takai, and T. de Lange.** 2006. Recent expansion of the telomeric complex in rodents: two distinct POT1 proteins protect mouse telomeres. Cell **126:**63-77.

116. **Holt, S. E., D. L. Aisner, J. Baur, V. M. Tesmer, M. Dy, M. Ouellette, J. B. Trager, G. B. Morin, D. O. Toft, J. W. Shay, W. E. Wright, and M. A. White.** 1999. Functional requirement of p23 and Hsp90 in telomerase complexes. Genes Dev **13:**817-826.

117. **Horvath, M. P., V. L. Schweiker, J. M. Bevilacqua, J. A. Ruggles, and S. C. Schultz.** 1998. Crystal structure of the Oxytricha nova telomere end binding protein complexed with single strand DNA. Cell **95:**963-974.

118. **Huard, S., T. J. Moriarty, and C. Autexier.** 2003. The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity. Nucleic Acids Res **31:**4059-4070.

119. **Hug, N., and J. Lingner.** 2006. Telomere length homeostasis. Chromosoma **115:**413-425.

120. **Iyer, S., A. D. Chadha, and M. J. McEachern.** 2005. A mutation in the STN1 gene triggers an alternative lengthening of telomere-like runaway recombinational telomere elongation and rapid deletion in yeast. Mol Cell Biol **25:**8064-8073.

121. **Jaco, I., P. Munoz, and M. A. Blasco.** 2004. Role of human Ku86 in telomere length maintenance and telomere capping. Cancer Res **64:**7271-7278.

122. **Jiang, W. Q., Z. H. Zhong, J. D. Henson, A. A. Neumann, A. C. Chang, and R. R. Reddel.** 2005. Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. Mol Cell Biol **25:**2708-2721.

123. **Kanoh, J., and F. Ishikawa.** 2001. spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast. Curr Biol **11:**1624-1630.

124. **Karamysheva, Z., L. Wang, T. Shrode, J. Bednenko, L. A. Hurley, and D. E. Shippen.** 2003. Developmentally programmed gene elimination in Euplotes crassus facilitates a switch in the telomerase catalytic subunit. Cell **113:**565-576.

125. **Karamysheva, Z. N., Y. V. Surovtseva, L. Vespa, E. V. Shakirov, and D. E. Shippen.** 2004. A C-terminal Myb extension domain defines a novel family of double-strand telomeric DNA-binding proteins in Arabidopsis. J Biol Chem **279:**47799-47807.

126. **Karimi, M., D. Inze, and A. Depicker.** 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7:**193-195.

127. **Karlseder, J., D. Broccoli, Y. Dai, S. Hardy, and T. de Lange.** 1999. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science **283:**1321-1325.

128. **Karlseder, J., K. Hoke, O. K. Mirzoeva, C. Bakkenist, M. B. Kastan, J. H. Petrini, and T. de Lange.** 2004. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol **2:**E240.

129. **Karlseder, J., L. Kachatrian, H. Takai, K. Mercer, S. Hingorani, T. Jacks, and T. de Lange.** 2003. Targeted deletion reveals an essential function for the telomere length regulator Trf1. Mol Cell Biol **23:**6533-6541.

130. **Karlseder, J., A. Smogorzewska, and T. de Lange.** 2002. Senescence induced by altered telomere state, not telomere loss. Science **295:**2446-2449.

131. **Kastan, M. B., and D. S. Lim.** 2000. The many substrates and functions of ATM. Nat Rev Mol Cell Biol **1:**179-186.

132. **Kelleher, C., I. Kurth, and J. Lingner.** 2005. Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. Mol Cell Biol **25:**808-818.

133. **Kilian, A., D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel, and R. A. Jefferson.** 1997. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet **6:**2011-2019.

134. **Kilian, A., C. Stiff, and A. Kleinhofs.** 1995. Barley telomeres shorten during differentiation but grow in callus culture. Proc Natl Acad Sci U S A **92:**9555-9559.

135. **Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay.** 1994. Specific association of human telomerase activity with immortal cells and cancer. Science **266:**2011-2015.

136. **Kim, S. H., P. Kaminker, and J. Campisi.** 1999. TIN2, a new regulator of telomere length in human cells. Nat Genet **23:**405-412.

137. **Kiss, T., M. Kis, and F. Solymosy.** 1989. Nucleotide sequence of a 25S rRNA gene from tomato. Nucleic Acids Res **17:**796-796.

138. **Klobutcher, L. A., M. T. Swanton, P. Donini, and D. M. Prescott.** 1981. All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus. Proc Natl Acad Sci U S A **78:**3015-3019.

139. **Kramer, K. M., and J. E. Haber.** 1993. New telomeres in yeast are initiated with a highly selected subset of TG1-3 repeats. Genes Dev **7:**2345-2356.

140. **Kraus, E., W. Y. Leung, and J. E. Haber.** 2001. Break-induced replication: a review and an example in budding yeast. Proc Natl Acad Sci U S A **98:**8255-8262.

141. **Lafarge, S., and M. H. Montane.** 2003. Characterization of Arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1, strongly induced by gamma rays. Nucleic Acids Res **31:**1148-1155.

142. **Lai, C. K., M. C. Miller, and K. Collins.** 2003. Roles for RNA in telomerase nucleotide and repeat addition processivity. Mol Cell **11:**1673-1683.

143. **Lansdorp, P. M., N. P. Verwoerd, F. M. van de Rijke, V. Dragowska, M. T. Little, R. W. Dirks, A. K. Raap, and H. J. Tanke.** 1996. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet **5:**685-691.

144. **Larson, D. D., E. A. Spangler, and E. H. Blackburn.** 1987. Dynamics of telomere length variation in Tetrahymena thermophila. Cell **50:**477-483.

145. **Latre, L., L. Tusell, M. Martin, R. Miro, J. Egozcue, M. A. Blasco, and A. Genesca.** 2003. Shortened telomeres join to DNA breaks interfering with their correct repair. Exp Cell Res **287:**282-288.

146. **Le, S., J. K. Moore, J. E. Haber, and C. W. Greider.** 1999. RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics **152:**143-152.

147. **Lee, H. W., M. A. Blasco, G. J. Gottlieb, J. W. Horner, 2nd, C. W. Greider, and R. A. DePinho.** 1998. Essential role of mouse telomerase in highly proliferative organs. Nature **392:**569-574.

148. **Lei, M., E. R. Podell, and T. R. Cech.** 2004. Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. Nat Struct Mol Biol **11:**1223-1229.

149. **Lei, M., A. J. Zaug, E. R. Podell, and T. R. Cech.** 2005. Switching human telomerase on and off with hPOT1 protein in vitro. J Biol Chem **280:**20449-20456.

150. **Leibfried, A., J. P. To, W. Busch, S. Stehling, A. Kehle, M. Demar, J. J. Kieber, and J. U. Lohmann.** 2005. WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438:**1172-1175.

151. **Lenain, C., S. Bauwens, S. Amiard, M. Brunori, M. J. Giraud-Panis, and E. Gilson.** 2006. The Apollo 5' exonuclease functions together with TRF2 to protect telomeres from DNA repair. Curr Biol **16:**1303-1310.

152. **Levis, R. W., R. Ganesan, K. Houtchens, L. A. Tolar, and F. M. Sheen.** 1993. Transposons in place of telomeric repeats at a Drosophila telomere. Cell **75:**1083-1093.

153. **Li, B., and A. J. Lustig.** 1996. A novel mechanism for telomere size control in Saccharomyces cerevisiae. Genes Dev **10:**1310-1326.

154. **Li, B., S. Oestreich, and T. de Lange.** 2000. Identification of human Rap1: implications for telomere evolution. Cell **101:**471-483.

155. **Lin, J., and E. H. Blackburn.** 2004. Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. Genes Dev **18:**387-396.

156. **Lin, J. J., and V. A. Zakian.** 1996. The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc Natl Acad Sci U S A **93:**13760-13765.

157. **Lingner, J., T. R. Cech, T. R. Hughes, and V. Lundblad.** 1997. Three Ever Shorter Telomere (EST) genes are dispensable for in vitro yeast telomerase activity. Proc Natl Acad Sci U S A **94:**11190-11195.

158. **Lingner, J., T. R. Hughes, A. Shevchenko, M. Mann, V. Lundblad, and T. R. Cech.** 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science **276:**561-567.

159. **Liu, D., A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin, and Z. Songyang.** 2004. PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol **6:**673-680.

160. **Liu, Y., H. Kha, M. Ungrin, M. O. Robinson, and L. Harrington.** 2002. Preferential maintenance of critically short telomeres in mammalian cells heterozygous for mTert. Proc Natl Acad Sci U S A **99:**3597-3602.

161. **Liu, Y., B. E. Snow, M. P. Hande, D. Yeung, N. J. Erdmann, A. Wakeham, A. Itie, D. P. Siderovski, P. M. Lansdorp, M. O. Robinson, and L. Harrington.** 2000. The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo. Curr Biol **10:**1459-1462.

162. **Loayza, D., and T. De Lange.** 2003. POT1 as a terminal transducer of TRF1 telomere length control. Nature **423:**1013-1018.

163. **Longtine, M. S., N. M. Wilson, M. E. Petracek, and J. Berman.** 1989. A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. Curr Genet **16:**225-239.

164. **Lue, N. F., Y. C. Lin, and I. S. Mian.** 2003. A conserved telomerase motif within the catalytic domain of telomerase reverse transcriptase is specifically required for repeat addition processivity. Mol Cell Biol **23:**8440-8449.

165. **Lundblad, V.** 2002. Telomere maintenance without telomerase. Oncogene **21:**522-531.

166. **Lundblad, V., and E. H. Blackburn.** 1993. An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell **73:**347-360.

167. **Lundblad, V., and J. W. Szostak.** 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. Cell **57:**633-643.

168. **Lustig, A. J.** 2001. Cdc13 subcomplexes regulate multiple telomere functions. Nat Struct Biol **8:**297-299.

169. **Lustig, A. J.** 2003. Clues to catastrophic telomere loss in mammals from yeast telomere rapid deletion. Nat Rev Genet **4:**916-923.

170. **Lustig, A. J., S. Kurtz, and D. Shore.** 1990. Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. Science **250:**549-553.

171. **Ma, Y., U. Pannicke, K. Schwarz, and M. R. Lieber.** 2002. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell **108:**781-794.

172. **Malkova, A., E. L. Ivanov, and J. E. Haber.** 1996. Double-strand break repair in the absence of RAD51 in yeast: a possible role for break-induced DNA replication. Proc Natl Acad Sci U S A **93:**7131-7136.

173. **Marcand, S., V. Brevet, and E. Gilson.** 1999. Progressive cis-inhibition of telomerase upon telomere elongation. EMBO J **18:**3509-3519.

174. **Marcand, S., E. Gilson, and D. Shore.** 1997. A protein-counting mechanism for telomere length regulation in yeast. Science **275:**986-990.

175. **Maringele, L., and D. Lydall.** 2002. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. Genes Dev **16:**1919-1933.

176. **Maringele, L., and D. Lydall.** 2004. Telomerase- and recombination-independent immortalization of budding yeast. Genes Dev **18:**2663-2675.

177. **Martin, S. G., T. Laroche, N. Suka, M. Grunstein, and S. M. Gasser.** 1999. Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell **97:**621-633.

178. **Maser, R. S., and R. A. DePinho.** 2004. Telomeres and the DNA damage response: why the fox is guarding the henhouse. DNA Repair (Amst) **3:**979-988.

179. **Mason, D. X., E. Goneska, and C. W. Greider.** 2003. Stem-loop IV of tetrahymena telomerase RNA stimulates processivity in trans. Mol Cell Biol **23:**5606-5613.

180. **Mason, P. J., and M. Bessler.** 2004. Heterozygous telomerase deficiency in mouse and man: when less is definitely not more. Cell Cycle **3:**1127-1129.

181. **Matheos, D., M. T. Ruiz, G. B. Price, and M. Zannis-Hadjopoulos.** 2002. Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication. Biochim Biophys Acta **1578:**59-72.

182. **Mathur, J., and C. Koncz.** 1998. Callus culture and regeneration. Methods Mol Biol **82:**31-34.

183. **McCarroll, R. M., and W. L. Fangman.** 1988. Time of replication of yeast centromeres and telomeres. Cell **54:**505-513.

184. **McClintock, B.** 1984. The significance of responses of the genome to challenge. Science **226:**792-801.

185. **McClintock, B.** 1941. The stability of broken ends of chromosomes in Zea mays. . Genetics **26:**234-282.

186. **McEachern, M. J., and E. H. Blackburn.** 1996. Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in Kluyveromyces lactis lacking telomerase. Genes Dev **10:**1822-1834.

187. **McKnight, T. D., and D. E. Shippen.** 2004. Plant telomere biology. Plant Cell **16:**794-803.

188. **Menke, M., I. Chen, K. J. Angelis, and I. Schubert.** 2001. DNA damage and repair in Arabidopsis thaliana as measured by the comet assay after treatment with different classes of genotoxins. Mutat Res **493:**87-93.

189. **Miller, K. M., M. G. Ferreira, and J. P. Cooper.** 2005. Taz1, Rap1 and Rif1 act both interdependently and independently to maintain telomeres. EMBO J **24:**3128-3135.

190. **Miller, K. M., O. Rog, and J. P. Cooper.** 2006. Semi-conservative DNA replication through telomeres requires Taz1. Nature **440:**824-828.

191. **Mitchell, J. R., E. Wood, and K. Collins.** 1999. A telomerase component is defective in the human disease dyskeratosis congenita. Nature **402:**551-555.

192. **Mitton-Fry, R. M., E. M. Anderson, D. L. Theobald, L. W. Glustrom, and D. S. Wuttke.** 2004. Structural basis for telomeric single-stranded DNA recognition by yeast Cdc13. J Mol Biol **338:**241-255.

193. **Molinier, J., M. E. Stamm, and B. Hohn.** 2004. SNM-dependent recombinational repair of oxidatively induced DNA damage in Arabidopsis thaliana. EMBO Rep **5:**994-999.

194. **Moshous, D., I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, A. Fischer, and J. P. de Villartay.** 2001. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. Cell **105:**177-186.

195. **Moyzis, R. K., J. M. Buckingham, L. S. Cram, M. Dani, L. L. Deaven, M. D. Jones, J. Meyne, R. L. Ratliff, and J. R. Wu.** 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A **85:**6622-6626.

196. **Munoz-Jordan, J. L., G. A. Cross, T. de Lange, and J. D. Griffith.** 2001. t-loops at trypanosome telomeres. EMBO J **20:**579-588.

197. **Murnane, J. P., L. Sabatier, B. A. Marder, and W. F. Morgan.** 1994. Telomere dynamics in an immortal human cell line. EMBO J **13:**4953-4962.

198. **Murti, K. G., and D. M. Prescott.** 1999. Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops. Proc Natl Acad Sci U S A **96:**14436-14439.

199. **Myung, K., G. Ghosh, F. J. Fattah, G. Li, H. Kim, A. Dutia, E. Pak, S. Smith, and E. A. Hendrickson.** 2004. Regulation of telomere length and suppression of genomic instability in human somatic cells by Ku86. Mol Cell Biol **24:**5050-5059.

200. **Nakada, D., Y. Hirano, and K. Sugimoto.** 2004. Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. Mol Cell Biol **24:**10016-10025.

201. **Nakamura, T. M., J. P. Cooper, and T. R. Cech.** 1998. Two modes of survival of fission yeast without telomerase. Science **282:**493-496.

202. **Nakamura, T. M., G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews, J. Lingner, C. B. Harley, and T. R. Cech.** 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science **277:**955-959.

203. **Natarajan, S., and M. J. McEachern.** 2002. Recombinational telomere elongation promoted by DNA circles. Mol Cell Biol **22:**4512-4521.

204. **Niida, H., Y. Shinkai, M. P. Hande, T. Matsumoto, S. Takehara, M. Tachibana, M. Oshimura, P. M. Lansdorp, and Y. Furuichi.** 2000. Telomere maintenance in telomerase-deficient mouse embryonic stem cells: characterization of an amplified telomeric DNA. Mol Cell Biol **20:**4115-4127.

205. **Nosek, J., A. Rycovska, A. M. Makhov, J. D. Griffith, and L. Tomaska.** 2005. Amplification of telomeric arrays via rolling-circle mechanism. J Biol Chem **280:**10840-10845.

206. **Nugent, C. I., T. R. Hughes, N. F. Lue, and V. Lundblad.** 1996. Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science **274:**249-252.

207. **Oka, Y., S. Shiota, S. Nakai, Y. Nishida, and S. Okubo.** 1980. Inverted terminal repeat sequence in the macronuclear DNA of Stylonychia pustulata. Gene **10:**301-306.

208. **Olovnikov, A. M.** 1973. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol **41:**181-190.

209. **Osman, F., J. Dixon, C. L. Doe, and M. C. Whitby.** 2003. Generating crossovers by resolution of nicked Holliday junctions: a role for Mus81-Eme1 in meiosis. Mol Cell **12:**761-774.

210. **Pardue, M. L., and P. G. DeBaryshe.** 2003. Retrotransposons provide an evolutionarily robust non-telomerase mechanism to maintain telomeres. Annu Rev Genet **37:**485-511.

211. **Pecinka, A., V. Schubert, A. Meister, G. Kreth, M. Klatte, M. A. Lysak, J. Fuchs, and I. Schubert.** 2004. Chromosome territory arrangement and homologous pairing in nuclei of Arabidopsis thaliana are predominantly random except for NOR-bearing chromosomes. Chromosoma **113:**258-269.

212. **Peersen, O. B., J. A. Ruggles, and S. C. Schultz.** 2002. Dimeric structure of the Oxytricha nova telomere end-binding protein alpha-subunit bound to ssDNA. Nat Struct Biol **9:**182-187.

213. **Pennock, E., K. Buckley, and V. Lundblad.** 2001. Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell **104:**387-396.

214. **Perrem, K., L. M. Colgin, A. A. Neumann, T. R. Yeager, and R. R. Reddel.** 2001. Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. Mol Cell Biol **21:**3862-3875.

215. **Perrini, B., L. Piacentini, L. Fanti, F. Altieri, S. Chichiarelli, M. Berloco, C. Turano, A. Ferraro, and S. Pimpinelli.** 2004. HP1 controls telomere capping, telomere elongation, and telomere silencing by two different mechanisms in Drosophila. Mol Cell **15:**467-476.

216. **Peterson, S. E., A. E. Stellwagen, S. J. Diede, M. S. Singer, Z. W. Haimberger, C. O. Johnson, M. Tzoneva, and D. E. Gottschling.** 2001. The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. Nat Genet **27:**64-67.

217. **Petreaca, R. C., H. C. Chiu, H. A. Eckelhoefer, C. Chuang, L. Xu, and C. I. Nugent.** 2006. Chromosome end protection plasticity revealed by Stn1p and Ten1p bypass of Cdc13p. Nat Cell Biol **8:**748-755.

218. **Poinsignon, C., D. Moshous, I. Callebaut, R. de Chasseval, I. Villey, and J. P. de Villartay.** 2004. The metallo-beta-lactamase/beta-CASP domain of Artemis constitutes the catalytic core for V(D)J recombination. J Exp Med **199:**315-321.

219. **Polotnianka, R. M., J. Li, and A. J. Lustig.** 1998. The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. Curr Biol **8:**831-834.

220. **Prowse, K. R., A. A. Avilion, and C. W. Greider.** 1993. Identification of a nonprocessive telomerase activity from mouse cells. Proc Natl Acad Sci U S A **90:**1493-1497.

221. **Qi, L., M. A. Strong, B. O. Karim, M. Armanios, D. L. Huso, and C. W. Greider.** 2003. Short telomeres and ataxia-telangiectasia mutated deficiency cooperatively increase telomere dysfunction and suppress tumorigenesis. Cancer Res **63:**8188-8196.

222. **Quaedvlieg, N. E., H. R. Schlaman, P. C. Admiraal, S. E. Wijting, J. Stougaard, and H. P. Spaink.** 1998. Fusions between green fluorescent protein and beta-glucuronidase as sensitive and vital bifunctional reporters in plants. Plant Mol Biol **38:**861-873.

223. **Ray, S., Z. Karamysheva, L. Wang, D. E. Shippen, and C. M. Price.** 2002. Interactions between telomerase and primase physically link the telomere and chromosome replication machinery. Mol Cell Biol **22:**5859-5868.

224. **Reichenbach, P., M. Hoss, C. M. Azzalin, M. Nabholz, P. Bucher, and J. Lingner.** 2003. A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. Curr Biol **13:**568-574.

225. **Reichheld, J. P., T. Vernoux, F. Lardon, M. Van Montagu, and D. Inze.** 1999. Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. **17:**647-656.

226. **Ren, S., J. S. Johnston, D. E. Shippen, and T. D. McKnight.** 2004. TELOMERASE ACTIVATOR1 induces telomerase activity and potentiates responses to auxin in Arabidopsis. Plant Cell **16:**2910-2922.

227. **Richards, E. J., and F. M. Ausubel.** 1988. Isolation of a higher eukaryotic telomere from Arabidopsis thaliana. Cell **53:**127-136.

228. **Richards, E. J., H. M. Goodman, and F. M. Ausubel.** 1991. The centromere region of Arabidopsis thaliana chromosome 1 contains telomere-similar sequences. Nucleic Acids Res **19:**3351-3357.

229. **Riha, K., M. L. Heacock, and D. E. Shippen.** 2006. The role of the nonhomologous end-joining DNA double-strand break repair pathway in telomere biology. Annu Rev Genet **40:**237-277.

230. **Riha, K., T. D. McKnight, L. R. Griffing, and D. E. Shippen.** 2001. Living with genome instability: plant responses to telomere dysfunction. Science **291:**1797-1800.

231. **Riha, K., and D. E. Shippen.** 2003. Ku is required for telomeric C-rich strand maintenance but not for end-to-end chromosome fusions in Arabidopsis. Proc Natl Acad Sci U S A **100:**611-615.

232. **Riha, K., J. M. Watson, J. Parkey, and D. E. Shippen.** 2002. Telomere length deregulation and enhanced sensitivity to genotoxic stress in Arabidopsis mutants deficient in Ku70. EMBO J **21:**2819-2826.

233. **Ritchie, K. B., J. C. Mallory, and T. D. Petes.** 1999. Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast Saccharomyces cerevisiae. Mol Cell Biol **19:**6065-6075.

234. **Rooney, S., F. W. Alt, D. Lombard, S. Whitlow, M. Eckersdorff, J. Fleming, S. Fugmann, D. O. Ferguson, D. G. Schatz, and J. Sekiguchi.** 2003. Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. J Exp Med **197:**553-565.

235. **Samper, E., F. A. Goytisolo, P. Slijepcevic, P. P. van Buul, and M. A. Blasco.** 2000. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. EMBO Rep **1:**244-252.

236. **Sarin, K. Y., P. Cheung, D. Gilison, E. Lee, R. I. Tennen, E. Wang, M. K. Artandi, A. E. Oro, and S. E. Artandi.** 2005. Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature **436:**1048-1052.

237. **Schlissel, M. S.** 2002. Does artemis end the hunt for the hairpin-opening activity in V(D)J recombination? Cell **109:**1-4.

238. **Schuermann, D., J. Molinier, O. Fritsch, and B. Hohn.** 2005. The dual nature of homologous recombination in plants. Trends Genet **21:**172-181.

239. **Shakirov, E. V., and D. E. Shippen.** 2004. Length regulation and dynamics of individual telomere tracts in wild-type Arabidopsis. Plant Cell **16:**1959-1967.

240. **Shakirov, E. V., Y. V. Surovtseva, N. Osbun, and D. E. Shippen.** 2005. The Arabidopsis Pot1 and Pot2 proteins function in telomere length homeostasis and chromosome end protection. Mol Cell Biol **25:**7725-7733.

241. **Shampay, J., J. W. Szostak, and E. H. Blackburn.** 1984. DNA sequences of telomeres maintained in yeast. Nature **310:**154-157.

242. **Shay, J. W., and S. Bacchetti.** 1997. A survey of telomerase activity in human cancer. Eur J Cancer **33:**787-791.

243. **Shay, J. W., and W. E. Wright.** 2006. Telomerase therapeutics for cancer: challenges and new directions. Nat Rev Drug Discov **5:**577-584.

244. **Signon, L., A. Malkova, M. L. Naylor, H. Klein, and J. E. Haber.** 2001. Genetic requirements for RAD51- and RAD54-independent break-induced replication repair of a chromosomal double-strand break. Mol Cell Biol **21:**2048-2056.

245. **Silverman, J., H. Takai, S. B. Buonomo, F. Eisenhaber, and T. de Lange.** 2004. Human Rif1, ortholog of a yeast telomeric protein, is regulated by ATM and 53BP1 and functions in the S-phase checkpoint. Genes Dev **18:**2108-2119.

246. **Siroky, J., J. Zluvova, K. Riha, D. E. Shippen, and B. Vyskot.** 2003. Rearrangements of ribosomal DNA clusters in late generation telomerase-deficient Arabidopsis. Chromosoma **112:**116-123.

247. **Smilenov, L. B., S. E. Morgan, W. Mellado, S. G. Sawant, M. B. Kastan, and T. K. Pandita.** 1997. Influence of ATM function on telomere metabolism. Oncogene **15:**2659-2665.

248. **Smith, S., and T. de Lange.** 2000. Tankyrase promotes telomere elongation in human cells. Curr Biol **10:**1299-1302.

249. **Smith, S., I. Giriat, A. Schmitt, and T. de Lange.** 1998. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. Science **282:**1484-1487.

250. **Smogorzewska, A., and T. de Lange.** 2004. Regulation of telomerase by telomeric proteins. Annu Rev Biochem **73:**177-208.

251. **Smogorzewska, A., J. Karlseder, H. Holtgreve-Grez, A. Jauch, and T. de Lange.** 2002. DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. Curr Biol **12:**1635-1644.

252. **Smogorzewska, A., B. van Steensel, A. Bianchi, S. Oelmann, M. R. Schaefer, G. Schnapp, and T. de Lange.** 2000. Control of human telomere length by TRF1 and TRF2. Mol Cell Biol **20:**1659-1668.

253. **Snow, B. E., N. Erdmann, J. Cruickshank, H. Goldman, R. M. Gill, M. O. Robinson, and L. Harrington.** 2003. Functional conservation of the telomerase protein Est1p in humans. Curr Biol **13:**698-704.

254. **Song, B., and P. Sung.** 2000. Functional interactions among yeast Rad51 recombinase, Rad52 mediator, and replication protein A in DNA strand exchange. J Biol Chem **275:**15895-15904.

255. **Sung, P., and D. L. Robberson.** 1995. DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. Cell **82:**453-461.

256. **Suzuki, K., Y. Yamagiwa, T. Matsui, and K. Yoshida.** 1994. Restriction enzyme-resistant high molecular weight telomeric DNA fragments in tobacco. DNA Res **1:**129-138.

257. **Szostak, J. W., and E. H. Blackburn.** 1982. Cloning yeast telomeres on linear plasmid vectors. Cell **29:**245-255.

258. **Taggart, A. K., S. C. Teng, and V. A. Zakian.** 2002. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science **297:**1023-1026.

259. **Takata, H., Y. Kanoh, N. Gunge, K. Shirahige, and A. Matsuura.** 2004. Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres in vivo. Mol Cell **14:**515-522.

260. **Tanaka, H., M. S. Mendonca, P. S. Bradshaw, D. J. Hoelz, L. H. Malkas, M. S. Meyn, and D. Gilley.** 2005. DNA damage-induced phosphorylation of the human telomere-associated protein TRF2. Proc Natl Acad Sci U S A **102:**15539-15544.

261. **Teixeira, M. T., M. Arneric, P. Sperisen, and J. Lingner.** 2004. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell **117:**323-335.

262. **Teng, S. C., J. Chang, B. McCowan, and V. A. Zakian.** 2000. Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. Mol Cell **6:**947-952.

263. **Teng, S. C., and V. A. Zakian.** 1999. Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae. Mol Cell Biol **19:**8083-8093.

264. **Ting, N. S., Y. Yu, B. Pohorelic, S. P. Lees-Miller, and T. L. Beattie.** 2005. Human Ku70/80 interacts directly with hTR, the RNA component of human telomerase. Nucleic Acids Res **33:**2090-2098.

265. **Tomaska, L., J. Nosek, A. M. Makhov, A. Pastorakova, and J. D. Griffith.** 2000. Extragenomic double-stranded DNA circles in yeast with linear mitochondrial genomes: potential involvement in telomere maintenance. Nucleic Acids Res **28:**4479-4487.

266. **Tomaska, L., S. Willcox, J. Slezakova, J. Nosek, and J. D. Griffith.** 2004. Taz1 binding to a fission yeast model telomere: formation of telomeric loops and higher order structures. J Biol Chem **279:**50764-50772.

267. **Tomkinson, A. E., and D. S. Levin.** 1997. Mammalian DNA ligases. Bioessays **19:**893-901.

268. **Topcu, Z., K. Nickles, C. Davis, and M. J. McEachern.** 2005. Abrupt disruption of capping and a single source for recombinationally elongated telomeres in Kluyveromyces lactis. Proc Natl Acad Sci U S A **102:**3348-3353.

269. **Uegaki, K., N. Adachi, S. So, S. Iiizumi, and H. Koyama.** 2006. Heterozygous inactivation of human Ku70/Ku86 heterodimer does not affect cell growth, double-strand break repair, or genome integrity. DNA Repair (Amst) **5:**303-311.

270. **van den Bosch, M., R. T. Bree, and N. F. Lowndes.** 2003. The MRN complex: coordinating and mediating the response to broken chromosomes. EMBO Rep **4:**844-849.

271. **van Gent, D. C., J. H. Hoeijmakers, and R. Kanaar.** 2001. Chromosomal stability and the DNA double-stranded break connection. Nat Rev Genet **2:**196-206.

272. **van Overbeek, M., and T. de Lange.** 2006. Apollo, an Artemis-related nuclease, interacts with TRF2 and protects human telomeres in S phase. Curr Biol **16:**1295-1302.

273. **van Steensel, B., and T. de Lange.** 1997. Control of telomere length by the human telomeric protein TRF1. Nature **385:**740-743.

274. **van Steensel, B., A. Smogorzewska, and T. de Lange.** 1998. TRF2 protects human telomeres from end-to-end fusions. Cell **92:**401-413.

275. **Veldman, T., K. T. Etheridge, and C. M. Counter.** 2004. Loss of hPot1 function leads to telomere instability and a cut-like phenotype. Curr Biol **14:**2264-2270.

276. **Verdun, R. E., L. Crabbe, C. Haggblom, and J. Karlseder.** 2005. Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. Mol Cell **20:**551-561.

277. **Vespa, L., M. Couvillion, E. Spangler, and D. E. Shippen.** 2005. ATM and ATR make distinct contributions to chromosome end protection and the maintenance of telomeric DNA in Arabidopsis. Genes Dev **19:**2111-2115.

278. **Vizir, I. Y., and B. J. Mulligan.** 1999. Genetics of gamma-irradiation-induced mutations in Arabidopsis thaliana: large chromosomal deletions can be rescued through the fertilization of diploid eggs. J Hered **90:**412-417.

279. **Vulliamy, T., A. Marrone, F. Goldman, A. Dearlove, M. Bessler, P. J. Mason, and I. Dokal.** 2001. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature **413:**432-435.

280. **Wang, R. C., A. Smogorzewska, and T. de Lange.** 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell **119:**355-368.

281. **Watson, J. D.** 1972. Origin of concatemeric T7 DNA. Nat New Biol **239:**197-201.

282. **Watson, J. M., P. Bulankova, K. Riha, D. E. Shippen, and B. Vyskot.** 2005. Telomerase-independent cell survival in Arabidopsis thaliana. Plant J **43:**662-674.

283. **Watson, J. M., and D. E. Shippen.** 2006. Telomere rapid deletion regulats telomere length in Arabidopsis thaliana. Mol Cell Biol **:**In Press.

284. **Wei, C., and M. Price.** 2003. Protecting the terminus: t-loops and telomere end-binding proteins. Cell Mol Life Sci **60:**2283-2294.

285. **Weinert, T. A., and L. H. Hartwell.** 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science **241:**317-322.

286. **Weiss-Schneeweiss, H., K. Riha, C. G. Jang, J. Puizina, H. Scherthan, and D. Schweizer.** 2004. Chromosome termini of the monocot plant Othocallis

siberica are maintained by telomerase, which specifically synthesises vertebrate-type telomere sequences. Plant J **37:**484-493.

287. **Wellinger, R. J., K. Ethier, P. Labrecque, and V. A. Zakian.** 1996. Evidence for a new step in telomere maintenance. Cell **85:**423-433.

288. **Wellinger, R. J., A. J. Wolf, and V. A. Zakian.** 1993. Saccharomyces telomeres acquire single-strand TG1-3 tails late in S phase. Cell **72:**51-60.

289. **Williams, B., and A. J. Lustig.** 2003. The paradoxical relationship between NHEJ and telomeric fusion. Mol Cell **11:**1125-1126.

290. **Witkin, K. L., and K. Collins.** 2004. Holoenzyme proteins required for the physiological assembly and activity of telomerase. Genes Dev **18:**1107-1118.

291. **Wolin, S. L., and T. Cedervall.** 2002. The La protein. Annu Rev Biochem **71:**375-403.

292. **Wong, K. K., R. S. Maser, R. M. Bachoo, J. Menon, D. R. Carrasco, Y. Gu, F. W. Alt, and R. A. DePinho.** 2003. Telomere dysfunction and Atm deficiency compromises organ homeostasis and accelerates ageing. Nature **421:**643-648.

293. **Wotton, D., and D. Shore.** 1997. A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae. Genes Dev **11:**748-760.

294. **Wright, W. E., M. A. Piatyszek, W. E. Rainey, W. Byrd, and J. W. Shay.** 1996. Telomerase activity in human germline and embryonic tissues and cells. Dev Genet **18:**173-179.

295. **Wright, W. E., V. M. Tesmer, M. L. Liao, and J. W. Shay.** 1999. Normal human telomeres are not late replicating. Exp Cell Res **251:**492-499.

296. **Wu, L., A. S. Multani, H. He, W. Cosme-Blanco, Y. Deng, J. M. Deng, O. Bachilo, S. Pathak, H. Tahara, S. M. Bailey, Y. Deng, R. R. Behringer, and S. Chang.** 2006. Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell **126:**49-62.

297. **Xin, Z., J. P. Velten, M. J. Oliver, and J. J. Burke.** 2003. High-throughput DNA extraction method suitable for PCR. Biotechniques **34:**820-824, 826.

298. **Xu, L., and E. H. Blackburn.** 2004. Human Rif1 protein binds aberrant telomeres and aligns along anaphase midzone microtubules. J Cell Biol **167:**819-830.

299. **Yang, Q., Y. L. Zheng, and C. C. Harris.** 2005. POT1 and TRF2 cooperate to maintain telomeric integrity. Mol Cell Biol **25:**1070-1080.

300. **Yang, S. W., E. Jin, I. K. Chung, and W. T. Kim.** 2002. Cell cycle-dependent regulation of telomerase activity by auxin, abscisic acid and protein phosphorylation in tobacco BY-2 suspension culture cells. Plant J **29:**617-626.

301. **Ye, J. Z., and T. de Lange.** 2004. TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. Nat Genet **36:**618-623.

302. **Ye, J. Z., D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait, and T. de Lange.** 2004. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev **18:**1649-1654.

303. **Yeager, T. R., A. A. Neumann, A. Englezou, L. I. Huschtscha, J. R. Noble, and R. R. Reddel.** 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res **59:**4175-4179.

304. **Zhang, X., V. Mar, W. Zhou, L. Harrington, and M. O. Robinson.** 1999. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev **13:**2388-2399.

305. **Zhou, J., K. Hidaka, and B. Futcher.** 2000. The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. Mol Cell Biol **20:**1947-1955.

306. **Zhou, X. Z., and K. P. Lu.** 2001. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. Cell **107:**347-359.

307. **Zhu, X. D., B. Kuster, M. Mann, J. H. Petrini, and T. de Lange.** 2000. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. Nat Genet **25:**347-352.

308. **Zijlmans, J. M., U. M. Martens, S. S. Poon, A. K. Raap, H. J. Tanke, R. K. Ward, and P. M. Lansdorp.** 1997. Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. Proc Natl Acad Sci U S A **94:**7423-7428.

APPENDIX I

ARTEMIS

INTRODUCTION

Artemis, an additional member of the non-homologous end-joining (NHEJ) DNA double-strand break (DSB) pathway, was recently identified as the gene mutated in a radiation sensitive form of severe combined immunodeficiency (RS-SCID) that results from an inability to produce antibody diversity via V(D)J recombination (194). During V(D)J recombination, RAG1 and RAG2 cleave at the VD and DJ junction sites, releasing the D region from the chromosome and leaving hairpin junctions at the V and J regions (237). To properly ligate the VJ region, the hairpins must be opened and further processed prior to ligation. Work by two independent labs demonstrated that Artemis was the protein responsible for this hairpin-opening activity (171, 194).

The radiation sensitivity of Artemis patients indicated general defects in NHEJ beyond V(D)J recombination. It was therefore not surprising that Artemis was found to associate directly with DNA-PK(cs) (171), an integral member of the NHEJ machinery. Artemis demonstrates single-strand 5'-3' exonuclease activity. Following association with DNA-PK(cs), Artemis acquires overhang endonuclease activity as well as hairpin opening activity (171). Thus, the nuclease activity of Artemis changes, based on its association with other factors.

Artemis contains a metallo-β-lactamase fold that contains the catalytic activity (218). Artemis has been classified as a member of the β-CASP family of metallo-β-lactamases

(35). Members of the β-CASP family are found in both prokaryotes and eukaryotes and display a wide range of nuclease activities, including different preferences for DNA or RNA and roles in cellular metabolism ranging from DNA repair to mRNA processing (35). Five proteins in Arabidopsis were found to have amino acid sequences that suggest they function on DNA and are partially related to Artemis.

Several proteins involved in the NHEJ pathway play critically important roles at telomeres (229). Interestingly, Artemis knockout mice display an increase in genomic instability, including a large number of telomere-to-telomere fusions (234). More recently, a human homolog of Artemis was identified and named Apollo (151, 272). Apollo is localized to telomeres through a direct interaction with TRF2, and knockdown studies demonstrated that cells deficient in Apollo show telomere dysfunction specifically during S phase, indicating a role in telomere replication (151, 272). Intriguingly, purified Artemis demonstrates 5'-3' exonuclease activity (151). Formation of the protective t-loop structure requires a 3'-overhang (98), and while this overhang can be generated naturally during replication of the lagging strand, an overhang must be actively created through endo- or exo-nucleolytic processing on the leading strand. Further support for a role of Artemis/Apollo-like proteins at telomeres comes from *C. elegans*, where one Artemis related protein possesses an N-terminal domain with high sequence similarity to POT1 (35), the primary ss telomere binding protein in many organisms (250).

In this study, we examine mutants in two Artemis-like genes in *Arabidopsis*. Mutations in either *ARTB* or *ARTE* do not result in changes in telomere length. As human Artemis

associates with DNA-PK(cs), we examined the association of four Artemis-like proteins from *Arabidopsis* with the related protein kinase, ATR. ARTE interacted weakly and non-reproducibly with ATR, while none of the other three tested proteins showed interaction. Further experiments to study the function of the remaining genes are suggested.

MATERIALS AND METHODS

Identification of Artemis mutants. The five predicted *Arabidopsis* proteins related to Artemis (35) are encoded by At1g19025 (*ARTA*), At1g27410 (*ARTC*), At1g66730 (*ARTB*), At3g26680 (*ARTD*), and At2g45700 (*ARTE*). TDNA insertion lines have been identified and ordered for all but At1g27410 and At3g26680. Characterization of At3g26680 was recently reported, and this gene has been named *AtSNM1* (193).

cDNA synthesis, cloning, translation, and co-immunoprecipitation. PCR products representing the full length cDNA of the different Artemis genes was performed as described (240). First-strand Poly-A cDNA was PCR amplified with the following primer pairs:

ArtAFLCF-2 TAC CCG GGG TCG ATA GAG ATG CCA AGA and

ArtAFLCR TAG AGC TCC TAG AAA CTC AAG TGA TTC CG

ArtBFLCF TAC CCG GGA TGG CCT CTG ATT CCG CC and

ArtBFLCR TAG AGC TGT TAA TGT TGA GAA GTG ATG TTC AT

ArtCFLCF-2 TAC CCG GGG GAG AGT GGT CTG ATA TCA and

ArtCFLCR TAG AGC TCC TAA TCA ACC TCT AAA ATC TTT GC

ArtDFLCF-2 TAC CCG GGG GAT TTT TCT GAT GAA GAC and

ArtDFLCR TAG AGC TCT CAG CGT CTG AGC CAT TA

ArtEFLCF-1 TAG AAT TCT GTC GAA CAC CGT CGA AGA T and

ArtEFLCR TAG AGC TCC TAC GTA ACC AAC AAA GAC

No PCR products could be generated from *ARTB* using primers that should amplify the entire cDNA or primers designed to amplify the 5' or 3' halves of the gene. Full-length cDNA were cloned into Pet28(a) using *Xma*I-*Sac*I for *ARTA*, *ARTC* and *ARTD* and *EcoR*I-*Sac*I for *ARTE*. Translation and co-immunoprecipitation experiments were performed as described (125).

Genotyping of T-DNA insertion mutants. For *ARTB*, At1g66730, three independent Salk lines were examined, SALK_079512, SALK_065305, and SALK_079499. Primers used to genotype these lines are:

For SALK_065307 ArtBLP ATC GC TGC CTT TAT TGC TGC and ArtBRP GAA AAC TAC AAA AAT GAC AAT GGA C.

For SALK_079512 and SALK_079499 ArtCDLP CAA TGC AGG CTG TTG GCT CTT and ArtCDRP GGC ATC AGA AAA ACC AGA CAT GA.

For *ARTE*, SALK_020527 was genotyped using ArtE1 ATG GTC ACA AGT TTT TGC CG and ArtE2 GCA GTC AAT CAA TGG CGT AAC. In each case, the mutant product was amplified using the second primer in the set and LBb1 GCG TGG ACC GCT TGC TGC AAC T.

DNA extraction and TRF analysis. PCR genotyping was performed using a high-throughput method (297) with the primers indicated above. DNA extraction for TRF and TRF analysis were performed as described in Chapter III.

RESULTS

A bioinformatics approach identified five predicted *Arabidopsis* proteins with sequence similarity to Artemis (35). Three putative proteins were classified as SNM1C-like (a classification which includes both Artemis and Apollo), while two were classified as SNM1/PSO2-like. Interestingly, an SNM1C-like protein from *C. elegans* also contains an N-terminal domain that shares high sequence homology to the second OB fold of POT1 proteins. The three proteins classified as SNM1C-like were named ARTA, ARTB, and ARTC and correspond to genes At1g19025, At1g66730, and At1g27410, respectively. The structure of these three proteins, as well as Artemis and *C. elegans* protein F39H2 are shown in FIG. 25. The two predicted proteins classified as SNM1/PSO2-like are ARTD and ARTE, At3g26680 and At2g45700, respectively.

Ligase I is involved in ligation of Okazaki fragments during DNA replication, suggesting that this protein may play a role in DNA replication (267). Search of the SALK database identified three T-DNA insertion lines in putative exons near the middle and 3'-terminus of the gene. Mutants were identified by PCR genotyping and TRF analysis was performed on wild type and mutant plants. None of the three lines displayed a telomere length defect (FIG. 26).

RT-PCR analysis failed to amplify a full length PCR product of *ARTB*, suggesting either that the gene prediction is incorrect, or that *ARTB* is expressed at very low levels. The human Artemis protein interacts with DNA-PK(cs), however, no clear homolog of DNA-PK(cs) has been identified in *Arabidopsis*. ATR in *Arabidopsis* may share some

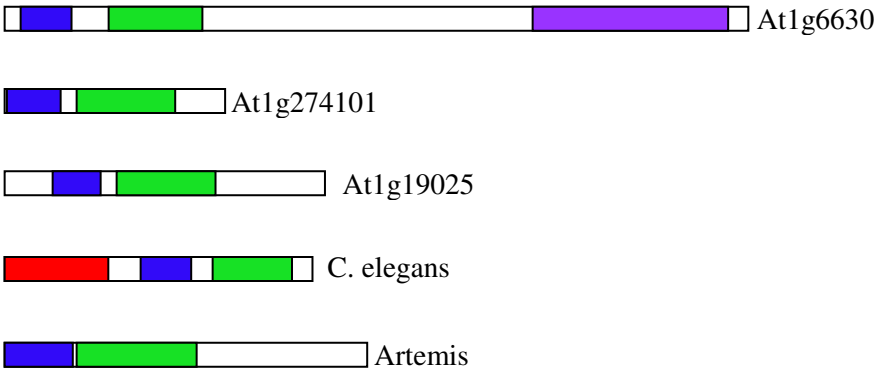


FIG. 25. Structure of Artemis homologs.

The structure of three putative Artemis homologs, as well as Artemis and a *C. elegans* protein are indicated. The blue box represents the metallo-β-lactamase domain, the green box represents the β-CASP domain. The purple box corresponds to a DNA Ligase I domain, and the red box represents the second OB fold of Pot1.

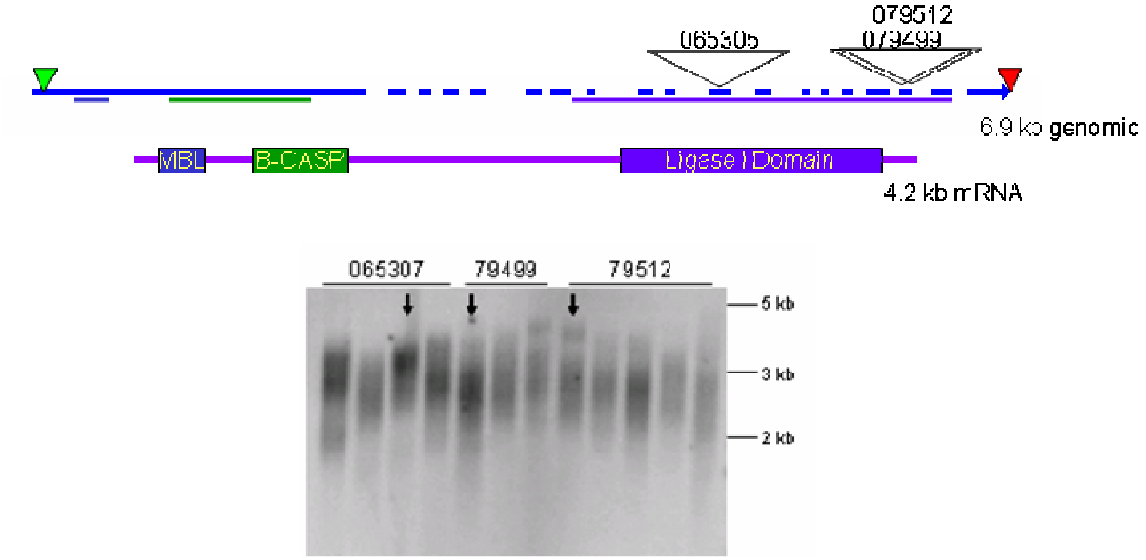


FIG. 26. TRF analysis of three *artb* mutant alleles.

The top figure represents the location of the three T-DNA insertions examined in this study (green arrow represents initiating ATG, and red arrow indicates stop). Lines below the genomic construct display location of domains within the gene. The predicted mRNA structure with associated domains is shown below (MBL is metallo-β-lactamase, B-CASP is β-CASP) . Bottom, TRF analysis of three homozygous T-DNA mutants in At1g66730 (*ARTB*). Wild type and mutant telomeres are in the wild type range of 2-5 kb. The SALK line number for each mutant line is indicated at the top. Arrows indicate wild type samples and all other lanes show results for individual mutant plants.

functional relationship with DNA-PK(cs) (L. Vespa and D. Shippen, unpublished data), so we examined the interaction of ATR and the remaining Artemis homologs by co-immunoprecipitation experiments.

Full length cDNA corresponding to the predicted mRNA was generated by RT-PCR (FIG. 27A), and these products were cloned into a pET28 expression vector for *in vitro* translation. Most cDNA products were translated well (FIG. 27B), though ARTD ran as a tightly spaced doublet, indicating either proteolytic cleavage or poor translation of this protein.

To test for interactions between ATR and the four ART proteins, the ART proteins were expressed with N-terminal T7 tags, and ATR was radiolabeled with ^{35}S-Met. Translation was terminated with cycloheximide and the reactions were mixed together and then immunoprecipitated with T7-tag antibody beads. ARTE demonstrated a weak interaction with the N-terminus of ATR (FIG. 28). However, reciprocal experiments where ARTE was radiolabeled failed to detect an interaction (data not shown), indicating the interaction may be weak, or that a T7 tag on ATR interferes with binding.

To examine whether ARTE played a role at telomeres, a line (SALK_020527) containing a T-DNA insertion in the second exon was examined by TRF analysis. As seen in FIG. 29, no change in telomere length was observed in the mutant.

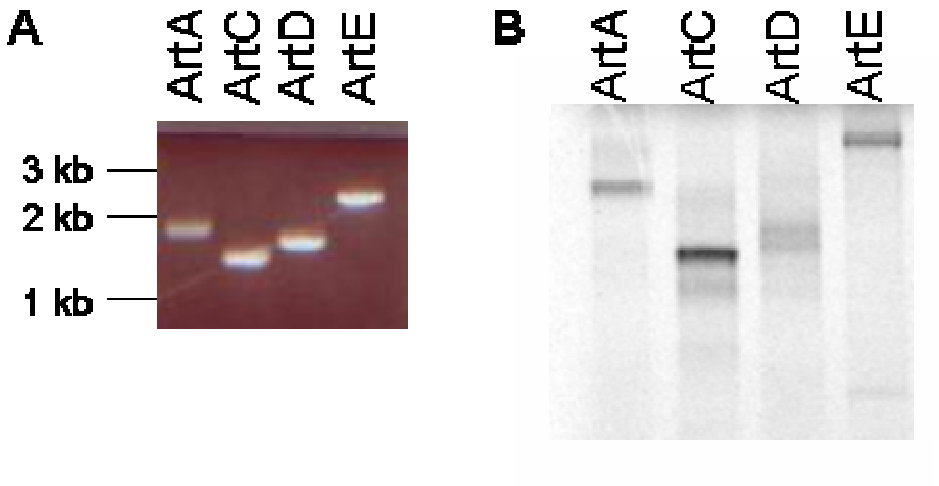


FIG. 27. cDNA amplification and translation of putative Artemis genes.

A) Full length cDNA products corresponding to the predicted mRNA are shown. B) Coupled *in vitro* transcription/translation products. Proteins were translated in the presence of ^{35}S-Met for visualization. The predicted MW of the proteins are: 62 kDa ARTA, 47 kDa for ARTC, 55kDa for ARTD, and 80 kDa for ARTE.

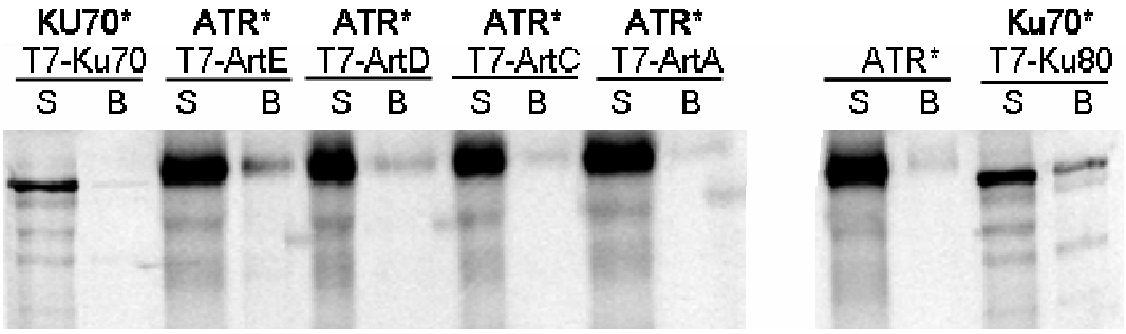


FIG. 28. Co-IP of ATR and ART proteins.

ATR proteins were *in vitro* translated as T7-tag fusions and mixed with the ^{35}S-Met labeled N-terminus of ATR. Radiolabeled proteins are indicated by asterisks. The strong interaction between KU70 and KU80 serves as a positive control and the negative control is homo-dimerization of KU70. S represents the supernatant input and B represents beads. Signals in B lanes indicated interaction between the two tested proteins.

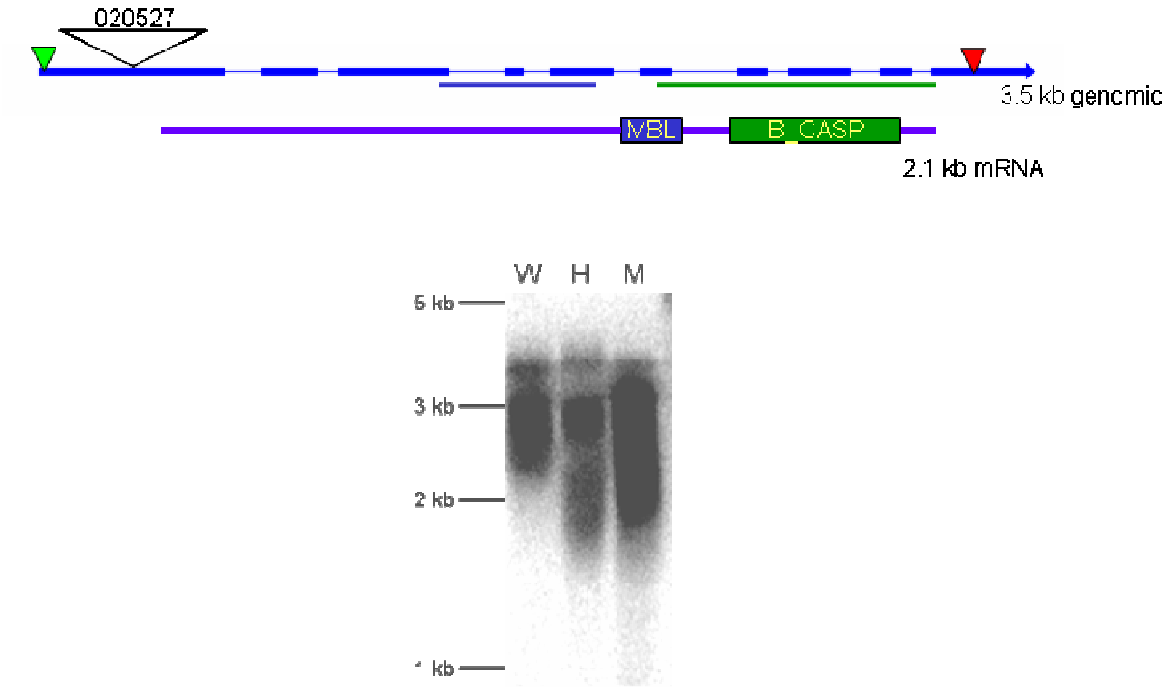


FIG. 29. TRF analysis of an *arte* mutant allele.

Top panel represents the T-DNA insertion line in *ARTE*. The bottom panel is TRF analysis of pooled plants either wild type (W) heterozygous (H) or mutant (M) for the T-DNA insertion in *ARTE*.

DISCUSSION

Artemis was originally identified as a protein involved in V(D)J recombination (194), though it was later shown to also play a role in general NHEJ DNA repair (171). The recent discovery that an Artemis homolog directly interacts with TRF2 (151, 272) warrants the study of homologous proteins in other organisms. The identification of 3'-overhang generating exonuclease activity (151) and prediction of an Artemis/Apollo-like protein with an N-terminal motif corresponding to Pot1 (35) further justifies study of these proteins.

Five predicted *Arabidopsis* proteins with similarity to Artemis were identified through a bioinformatics study (35). Characterization of one gene has recently been reported (193). *AtSNM1* (*ARTD* in my nomenclature) was shown to be involved in the repair of DNA oxidative damage. The remaining four proteins have yet to be studied.

Our data suggest that ARTB, an Artemis protein with a Ligase I domain at the extreme C-terminus, is likely mis-annotated as no mRNA products corresponding to this gene were amplified. Furthermore, three independent T-DNA lines failed to display changes in telomere length. Additional work should be performed to fully characterize *ARTB*, with the focus of determining whether the N terminal Artemis-like region is actually expressed.

ARTE showed a weak and non-reproducible interaction with ATR. Furthermore, T-DNA insertions in the second exon of this gene did not results in changes in telomere length. However, RT-PCR analysis should be performed to verify this insertion results in loss of

gene expression. ARTE is more closely related to the SNM1/PSO2 family of DNA repair proteins, and is less closely related to Artemis.

The two genes remaining to be characterized are *ARTA* and *ARTC*. These two genes are the most closely related to human Artemis, and are both viable targets for study. A T-DNA insertion in *ARTA*, SAIL_1175_F01, has been ordered but has not yet been genotyped. The T-DNA is located in the fourth exon, approximately in the middle of the predicted mRNA. *ARTC* has several GABI-Kat T-DNA lines as well as two lines from Cold Spring Harbor, all of which are located in predicted exons. CSHL_ET9929 and CSHL_GT9350 are the two T-DNA lines in *ARTC*, and both are located in the first exon.

While characterization of these proteins is in its infancy, they are viable targets for further study. Exo- or endo-nucleases that produce 3' overhangs on leading strand synthesized DNA must be present in all organisms, but direct demonstration of any nuclease responsible for this activity has yet to be presented. Apollo makes an attractive target for such a nuclease due to its association with TRF2 as well as the modulation of nuclease activity by protein interaction seen in the related protein Artemis. Apollo has so far been studied only by knockdown experiments in humans, making analysis of null mutants in *Arabidopsis* an attractive target. In addition to T-DNA lines, I have generated full length cDNA clones that could prove useful for yeast two-hybrid analysis. Apollo's interaction with TRF2 makes these proteins good candidates for bait in a large scale screen for putative TRF like proteins in *Arabidopsis*, or in direct interaction studies with the putative ds telomere binding proteins (125). Thus, analysis

of the remaining Artemis-like proteins in *Arabidopsis* may provide fundamental information on telomere biology, not only in *Arabidopsis*, but in other organisms as well.

VITA

Name:	James Matthew Watson
Address:	Gregor Mendel Institute GMI GmbH
	Dr. Bohr-Gasse 3
	A-1030 Vienna, Austria
Education:	Ph.D., Biochemistry, Texas A&M University 2007
	B.S., Biochemistry, Beloit College 2000