

**CHARACTERIZATION OF TELOMERASE RNP IN *Arabidopsis thaliana***

A Dissertation

by

KALPANA KANNAN

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

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Biochemistry

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## ABSTRACT

Characterization of Telomerase RNP in *Arabidopsis thaliana*.

(December 2008)

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Telomeres are critical for the integrity of eukaryotic genomes. They function to protect chromosome ends from DNA damage surveillance and inappropriate repair. Telomeres are maintained by the specialized ribonucleoprotein complex telomerase. Without telomerase, telomere shortening would ultimately lead to compromised genome stability and cellular senescence. Therefore, telomerase function is necessary for extension of the proliferative capacity of the cell.

In this dissertation, we describe the characterization of core components of telomerase ribonucleoprotein complex in the flowering plant, *Arabidopsis thaliana*. We find that dyskerin, one of the core telomerase components in humans is also conserved in *Arabidopsis* telomerase. *Arabidopsis* dyskerin associates with the telomerase RNP in an RNA-dependent manner and is required for telomere length maintenance in this organism. We also describe the characterization of another core telomerase component, the telomerase RNA subunits (TERs). Unexpectedly, we uncovered two distinct TER subunits that share a region of high identity. The two TERs named TER<sub>1G7</sub> and TER<sub>5G2</sub>, based on their chromosomal positions, display differences in their expression levels and their association with telomere-related proteins. Both TERs can serve as templates for telomerase *in vitro*. Through genetic analyses, we show a templating function for TER<sub>1G7</sub>

*in vivo* and a novel role for TER<sub>5G2</sub> as a negative regulator of telomerase. Finally, the presence of TER genes in other plant species was investigated and evidence for duplication of TER genes in plants closely related to *Arabidopsis* was obtained. We also show evidence for a template mutation in Asparagus TER that could lead to variant repeats in this organism.

In summary, the studies presented in this dissertation reveal that *Arabidopsis* telomerase shares both similarities and differences with other telomerase RNPs, making it an exciting model system for study of telomere biology.

## DEDICATION

This dissertation is dedicated

To my parents, for their endless love and support at all times and for being wonderful role models for me.

To my little nephew, the thought of whom has lifted my spirits many times. You make me smile always.

And finally to my husband, for being the best friend I could ask for. Thank you so much.

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**NOMENCLATURE**

TERT	Telomerase reverse transcriptase
TER	Telomerase RNA
EST	Ever shorter telomere
TRAP	Telomere repeat amplification protocol
DDR	DNA damage response
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 related
Rap1	Repressor activator protein 1
OB-fold	Oligonucleotide/ oligosaccharide binding fold
RPA	Replication protein A
TRF1	Telomeric repeat factor binding 1
POT1	Protection of telomeres 1
TIN2	TRF-interacting protein 2
RNAi	RNA interference
RID	RNA interacting domain
IFD	In fingers domain
TLC1	Yeast telomerase RNA
TBE	Template boundary element
hTR	Human telomerase RNA
CAB	Cajal body motif
snRNA	Small nuclear RNA
TMG	Trimethylguanosine
snoRNA	Small nucleolar RNA

DC	Dyskeratosis congenita
RNP	Ribonucleoprotein
PUA	Pseudouridine synthase and archeosine transglycosylase
rRNA	Ribosomal RNA
TRF	Terminal restriction fragment
PETRA	Primer extension telomere repeat amplification
ncRNA	Non coding RNA
oRNA	orphan RNA

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## CHAPTER I

### INTRODUCTION

#### History of Telomeres

Telomeres are the physical ends of chromosomes. The idea that the ends of chromosomes had special characteristics was established through the work of Hermann Muller and Barbara McClintock, who were studying the breakage and fusion of broken chromosomes in *Drosophila* and maize, respectively (215). They independently observed that a broken chromosome end was never attached to an intact chromosome end, suggesting that the natural ends of chromosomes were in some way protected from rearrangements. Muller coined the term 'telomeres' for these special ends of chromosomes (28). We now know that this property of telomeres is necessary for maintaining chromosomal stability from DNA repair activities and nucleolytic degradation.

Later, when the structure of DNA and the mechanism of DNA replication was understood (276), it was predicted that the very ends of chromosomes could not be fully replicated. Lagging strand DNA synthesis requires an RNA primer for synthesis of DNA and after the last RNA primer is removed, a gap would remain. This is known as the 'end-replication problem' as proposed by Alexey Olovnikov and James Watson independently (243, 327). Over time, this loss would be detrimental to any organism as sequences from chromosome termini would be lost and this could lead to cellular senescence. Seminal work by Elizabeth Blackburn in the ciliate *Tetrahymena* showed that eukaryotic organisms had found a way to avert the end-replication problem. She

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This dissertation follows the style and format of Molecular and Cellular Biology.

observed that rDNA molecules in *Tetrahymena* had tandem repeats of a hexanucleotide sequence repeat TTGGGG (26). This was the first telomere repeat identified and soon similar G-rich repetitive sequences were also identified in other organisms. However, the mechanism of addition of these telomeric repeats was still unknown.

Elizabeth Blackburn and Jack Szostak asked if *Tetrahymena* telomeres could function in yeast. They introduced a linear yeast plasmid with *Tetrahymena* termini into yeast and observed that the plasmid could be maintained (299). Moreover, sequencing of the plasmid revealed that yeast specific TG<sub>1-3</sub> repeats were added onto the *Tetrahymena* telomeres (285). This indicated that telomeres are functionally conserved across evolutionarily different organisms and that there was a mechanism to add these repeats on to the chromosome ends *de novo*.

### **Discovery of Telomerase**

The question then arose as to what generated these repeats. Two ideas were presented: one was that these sequences were transposed or recombined onto the end and the other was that an enzyme might be responsible for the addition of these repeats (27). To address this question, the Blackburn lab took a biochemical approach and purified the activity that was responsible for addition of repeats from the ciliate *Tetrahymena thermophila* (116, 117). They set up an assay in which they supplied a G-rich single-strand DNA primer (similar to the telomeric sequence) and allowed the extracts to incorporate radioactive nucleotides in the generation of telomeric repeats (116, 117). When the products were resolved on a sequencing gel, the result was a ladder of bands that had a six base periodicity corresponding to the addition of the six base TTGGGG repeat found at *Tetrahymena* telomeres (116). Ultimately, they were able to show that this activity was specific to a telomeric primer and interestingly, even an

oligonucleotide that ended in a yeast telomere could be extended by the *Tetrahymena* extract. They called this activity telomere terminal transferase or “telomerase” (117).

The Blackburn lab then investigated the mechanism that specifies the addition of specific telomere repeats. They found that the activity depended on a nucleic acid component. Subsequent work led to the identification of the telomerase RNA subunit (TER) which was found to have a sequence complementary to 1.5 repeats of the *Tetrahymena* telomere sequence. This sequence was shown by mutagenesis to act as a template for the addition of *Tetrahymena* telomere repeat (118). Thus, the telomerase complex was defined as a reverse transcriptase with an integral RNA subunit.

Several years later, the catalytic protein subunit, also known as telomerase reverse transcriptase or TERT, was identified by purification and mass spectrometry from the ciliate *Euplotes aediculatus* (191). Simultaneously, yeast mutants in TERT (Est2) were identified that had unmistakable telomere defects such as telomere shortening with every generation and an early onset of senescence. These mutants were termed EST for “ever shorter telomeres” (200). The first yeast mutants also indicated the importance of proper functioning of telomeres and telomerase for all organisms. Based on the sequence of the first TERT and the presence of canonical reverse transcriptase motifs, similar proteins from other eukaryotes were identified (238).

The sequence of human telomeres was revealed to be TTAGGG similar to *Tetrahymena* telomeres (234). Human telomerase was also identified soon thereafter (233). This was followed by the development of a sensitive PCR assay termed telomere repeat amplification protocol (TRAP) that revealed that telomerase was not expressed in all human cells but was active in many cancer cells (162). Also, telomere shortening was found to occur with human cells in culture (123). The above data fit with the prediction that the end replication problem results in telomere loss and might be the cause of the

senescence observed in human cell culture (244). An exciting finding resulted when telomerase was expressed in these cells: telomere loss was not observed anymore and the cells became immortal (31). This result pointed to a connection between telomeres and cellular aging and suggested that telomere loss is a tumor suppressor mechanism (123). Telomeres are typically shorter in cancer cells than in normal tissue because cancer cells undergo more cell divisions (71, 127). In order to survive, cancer cells must activate a telomere maintenance mechanism usually telomerase (127). Telomerase alone is not sufficient to cause the transformation of cancer cells. Work from the Weinberg lab has shown that ectopic expression of telomerase catalytic subunit as well as co-expression of two oncogenes (ras and SV40 large T-antigen), is needed for the tumorigenic conversion of normal human cells (120). Thus, the implications of telomeres and telomerase in cancer and cellular aging have fueled subsequent research to understand their composition and regulation.

The above experiments established the essential nature of telomeres for genome stability of eukaryotes. However, telomerase and telomeric proteins have diverged considerably and our knowledge of telomerase particularly in the plant kingdom is limited. Therefore, the focus of this dissertation is on the composition and function of the telomerase RNP in the model plant *Arabidopsis thaliana*.

### **Structure of Telomeres**

Telomeric DNA consists of an array of G-rich repeats: *Tetrahymena* contains about 50 copies of the repeat TTGGGG (26), while the related TTAGGG repeat is common to vertebrates (234). Although telomeres in most organisms consist of perfect repeat arrays, the telomere repeat is degenerate in some organisms including *S. cerevisiae*. In this case, telomeres consist of TG<sub>1-3</sub> repeats. It is thought that degenerate

repeats are the consequence of inappropriate translocation and stuttering along the RNA template during nucleotide addition (87).

*Arabidopsis* and most other plant species have a seven-base telomeric repeat, TTTAGGG (263). Interestingly, some plants in the monocot order of Asparagales (for example *Asparagus*) contain human-type TTAGGG repeats (297). The change in the *Asparagus* telomere repeat is most probably due to a mutation in template of the telomerase RNA subunit. Some members of the Alliaceae species (which includes onion), have lost the human-type repeat and their telomere sequence still remains to be determined (251). Some organisms like *Drosophila* have no simple telomere repeat at the end of their chromosomes. Instead, chromosomes terminate in retrotransposon sequences which are periodically transferred to the terminus to solve the end replication problem (22).

Telomeres have both a double-stranded repeat region as well as a 3' single-stranded portion known as the G-overhang (Fig. 1A). Adjacent to the double-stranded telomeric repeats is the subtelomeric region (Fig. 1A). This region has been implicated in negative regulation of telomere length, prevention of recombination, telomeric silencing and nuclear telomere positioning (16, 88, 107, 132). In budding yeast, the subtelomeric region contains repetitive elements that vary among chromosome arms (257). These include the Y' element and a core X element that is present on all chromosome arms (257). In *Arabidopsis thaliana*, 8 of the 10 chromosome arms have unique subtelomeric regions that do not contain any repetitive elements (130). This feature of *Arabidopsis* subtelomeres can be exploited to study telomere length dynamics of individual chromosome arms (130).

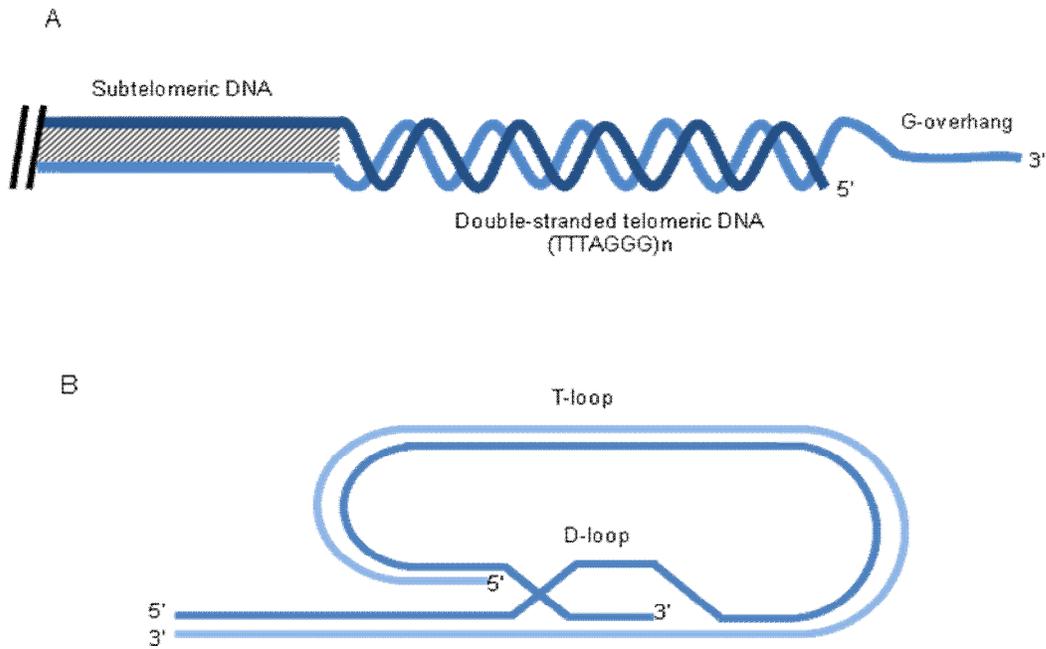


FIG. 1. Telomere structure. (A) Telomeres are composed of a double-stranded region of multiple G-rich repeats and a short single-stranded region known as the G-overhang. Adjacent to the double stranded telomeric DNA is the sub-telomeric region (B) Telomeres form a t-loop structure. The t-loop is formed by the invasion of the 3' G-overhang into the double-stranded portion of the telomere. The 3' overhang displaces one strand and anneals to other strand of telomeric DNA forming a D-loop. The 3' overhang is thus protected from deleterious activities.

The G-overhang is located at the end of the telomere. The length of the G-overhang is small when compared to the double-stranded region and it varies from 20 nts in *Tetrahymena* to 250 nts in vertebrates (167, 205). In *Arabidopsis*, the length of the overhang is around 20 nt (267). The G-overhang is essential for telomere maintenance as it is the substrate for telomerase and it is required for proper packaging of the chromosome terminus into a structure that is resistant to nucleolytic attack or recombination. When exposed, the G-overhang can trigger deleterious end-to-end chromosome fusion events (358).

The G-overhang is generated by a combination of telomerase action and nuclease events (reviewed in (104)). After DNA replication, leading strand synthesis results in synthesis of a blunt ended product; while the lagging strand telomere has a 3' overhang from the removal of the last RNA primer. However, since both ends need to be similar, the blunt ends are thought to be subject to the action of nucleases so that 3' overhangs result. The exact mechanism of G-overhang generation is currently under investigation. The overhangs are bound by specific binding proteins and are converted into a structure known as the T-loop (330) (see below). Recently, it was discovered that in *C. elegans* both G- and C-overhangs are present at telomeres and are bound by distinct proteins (259). This finding suggests that mechanisms exist to maintain both G- and C-overhangs in this organism and raises questions about the prevalence of C-overhangs in other organisms.

The formation of T-loops involves the invasion of the 3' G-overhang into the double-stranded telomeric DNA (119). The invading strand then base pairs with the C-strand and displaces the G-strand. Since, the invasion occurs at a distance from the very end of chromosome, a structure resembling a duplex lariat is formed. This is called the T-loop (Fig. 1B). Internal to the T-loop is the D-loop or displacement loop. T-loops have

not been observed *in vivo*, but through *in vitro* electron microscopy studies, t-loops have been identified in a variety of species such as humans, trypanosomes and in the pea plant (41, 119, 236). T-loops have not been observed in *S. cerevisiae* where telomeres are only 300 bp long (274). However, a fold-back structure has been proposed in yeast and in this way the telomeric ends are thought to be protected (69). Formation of these protective structures provides an architectural solution to protect telomeres from deleterious activities (such as recombination and chromosomal fusions) and at the same time control telomerase access to the ends (70). During S-phase of the cell cycle, the t-loops are thought to unfold and allow telomerase access to the chromosome end.

The G-overhang is a unique feature of telomeres, but its single-stranded nature can be a trigger for DNA damage response (DDR) pathways in the cell. The formation of a t-loop structure at telomeres, and the association of telomeric proteins with the t-loop are proposed to inhibit DDR at telomeres (67, 70) (see below). Interestingly, proteins involved in DDR, physically associate with telomeres and are in fact, required for normal telomere function (67). However, when telomeres become critically short or dysfunctional, a DDR is triggered (reviewed in (65)). The damage response involves the accumulation of several proteins including 53BP1, gamma-H2AX, Mre11 complex and phosphorylated ATM and ATR at telomeres. These proteins can be observed cytologically and result in the formation of telomere dysfunction induced foci (TIFs) (301). Surprisingly, even at functional telomeres, a transient DDR is observed (316). The sequential recruitment of ATR and ATM to telomeres during S and G2 phases of the cell cycle is necessary for the complete replication of telomeres and the formation of the protective t-loop structure (316). This transient activation may also be an effective method of targeting telomerase to the shorter telomeres in a cell population (67). Thus, the action of DDR machinery at telomeres occurs at multiple levels.

## **Telomere Associated Proteins**

Telomeres are nucleoprotein complexes and are associated with a variety of proteins that bind specifically to either the double-stranded telomeric DNA or the single-stranded G-overhang. Another class of telomeric proteins are those that do not contact DNA directly but rather act at telomeres through protein-protein interactions. This highly ordered structure of telomeric DNA and proteins is necessary for maintaining species-specific telomere length. Telomeric proteins regulate the length of telomere tracts by controlling the accessibility of DNA to telomerase extension. Moreover, these proteins are also involved in chromosome end protection and loss of these proteins can trigger genome instability.

### *Telomere protein composition in yeast*

In *S.cerevisiae*, double-strand telomeric DNA is bound by repressor activator protein 1 (Rap1) which negatively regulates telomere length (208). Rap1 is associated with two other proteins, Rif1 and Rif2 (rap1-interacting factors) that also contribute to regulation of telomere length (183). When Rap1/Rif1/Rif2 was fused to a GAL4 binding domain and targeted to a specific site in the subtelomere, the observed telomere length shortening was proportional to the number of binding sites for the proteins (183, 208). Therefore, a protein counting mechanism is proposed for the control of telomere length in budding yeast where loss of telomere bound Rap1 results in loss of telomerase inhibition and leads to telomerase-mediated telomere elongation (183, 208).

The protein responsible for binding the G-overhang in yeast is Cdc13 (239). Cdc13 contains an oligonucleotide/ oligosaccharide binding fold (OB-fold). This domain is common to single-strand telomere binding proteins in all organisms (see below). Loss of Cdc13 results in chromosome end de-protection and extension of the G-overhang

(102) . The *cdc13-2* allele results in the EST phenotype; however telomerase activity is unaffected in these mutants (189, 239). This mutation can be rescued by overexpression of Est1, a component of yeast telomerase RNP (239, 249). Therefore, Cdc13 is thought to recruit telomerase holoenzyme to telomere ends in S-phase via its interaction with Est1 and thus provide telomerase access to the telomere. Alternately, telomerase components are thought to be present at telomeres at all times and are activated by the Cdc13-Est1 interaction to synthesize telomeres at S-phase (300).

Cdc13 also forms a heterotrimeric complex with the proteins Stn1 (suppressor of *cdc* thirteen) and Ten1 (telomeric pathways in association with Stn1 number 1). This complex is involved in both telomere length control and telomere capping (44, 111). Mutants in all three genes result in long G-overhangs, C-strand resection and cell cycle arrest (110, 111). It is noteworthy that all three proteins, Cdc13, Stn1 and Ten1, bear OB-folds and are essential genes in budding yeast (100, 110, 111). The trimer of Cdc13, Stn1 and Ten1 are proposed to act in a manner similar to the RPA proteins but with specificity for telomeric DNA (100).

#### *Telomere associated proteins in humans*

Work in recent years has helped define the composition of proteins present at human telomeres. Mammalian telomeres are protected by a complex of six proteins called shelterin (70). Shelterin includes TRF1 (telomeric repeat factor binding), TRF2, TIN2 (TRF-interacting protein 2), POT1 (protection of telomeres 1), TPP1, and Rap1 (70, 192) (Fig. 2). Shelterin performs many essential functions such as generation of t-loops, control of telomere length and protection of the chromosome terminus (70).

In humans, telomere length regulation is primarily accomplished by the double-strand telomere binding proteins, TRF1 and TRF2 (23, 35). TRF1 and TRF2 can form

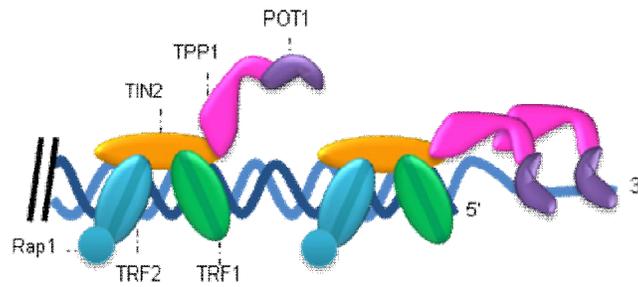


FIG. 2. The six member shelterin complex at human telomeres. Homodimers of TRF1 and TRF2 bind to the double-strand telomeric DNA. Rap1 accumulates at telomeres via its binding to TRF2. TIN2 is a protein that bridges both TRF1 and TRF2 and also contacts the TPP1-POT1 complex. TPP1 recruits POT1 which accumulates on the G-overhang and provides chromosome end protection.

homodimers and oligomers and can thus coat telomeric DNA (21, 35). A DNA-binding deficient mutant of TRF1, when overexpressed, results in longer telomeres, implicating TRF1 in negative regulation of telomere length (313). TRF2 is an essential gene that is required for chromosome end protection. Overexpression of a TRF2 mutant lacking both its DNA-binding domain and N-terminal basic domain results in severe telomere defects, including loss of 3' G-overhangs and chromosomal fusions (155, 314).

TIN2 connects TRF1 and TRF2 to the TPP1/POT1 complex at the chromosome end (163, 164, 242). TIN2 mutants that prevent its binding to either TRF1 or TRF2 cause removal of these proteins from telomeres and trigger telomere lengthening and DNA damage responses (163, 346, 347). Human Rap1 is a highly diverged ortholog of *S.cerevisiae* Rap1 that does not bind DNA directly, but rather associates with telomeres by binding to TRF2 (184). Loss of hRap1 result in mild telomere lengthening implicating hRap1 in negative telomere length regulation (184, 241).

Protection of telomeres 1 (POT1) is the most conserved component of the shelterin complex and it is the factor that binds the G-overhang. POT1 was identified as a protein similar to the  $\alpha$ -subunit of the telomere end binding protein (TEBP) from the ciliate *Oxytricha nova* (18). Interestingly, another shelterin component, TPP1, shows similarity to the  $\beta$ -subunit of TEBP, arguing that this ancient protein complex is conserved in all eukaryotes (324, 344).

POT1 accumulates both on the single-strand overhang and on the duplex region of the telomere through its interaction with TRF1 (195). This interaction is mediated by TPP1 (193, 348). DNA-binding of POT1 is through its two oligonucleotide / oligosaccharide-binding (OB) folds (178). RNAi-mediated depletion of POT1 results in

loss of G-overhangs, chromosomal fusions and apoptosis (52, 140). Studies have shown that POT1 binding to the telomeric terminus inhibits telomerase (157, 179). In contrast, POT1 mutants that lack DNA binding activity lack telomere length regulation and therefore longer telomeres result (195). Hence, POT1 is both a positive and negative regulator of telomerase.

On its own, TPP1 does not have any DNA-binding activity. However, TPP1 increases the affinity of POT1 for the 3' end of telomeric DNA (324, 344). Depletion of TPP1 by RNAi results in the loss of POT1 signal at chromosome ends and, subsequently, telomere lengthening and DNA-damage responses at the telomere (344). Interestingly, the OB fold in TPP1 was shown to physically interact with telomerase (324, 344). It is hypothesized that the binding of multiple TPP1-POT1 complexes at the 3' end results in a non-extendible state of the telomere (64). When telomeres become short, the TPP1-POT1 complex is lost and this results in an extendible state of the telomere. At this point, telomerase is enriched at the telomere via its interaction with TPP1 and is stimulated to add repeats by TPP1 (64). Thus, shelterin components mediate the access of telomerase to the telomere and function in length regulation.

#### *Telomere protein composition in Arabidopsis*

Several shelterin components, Rap1, TIN2 and TPP1, cannot be detected in the sequenced plant genome. However, *Arabidopsis* is unique in that it contains numerous proteins that resemble TRF1 and TRF2. These proteins are categorized in two families. The members of the first family contain a myb-like domain at the C-terminus, whereas the members of the second family contain the myb domain as well as an additional domain termed the myb-extension domain (154). The members of the second family of proteins termed TRF-like (TRFL) can form both homodimers and heterodimers, and bind

double-strand telomeric DNA *in vitro* (154). Functional redundancy has complicated *in vivo* analysis of mutants for the TRFL proteins. None of the single mutants show a strong telomere phenotype (L. Vespa & D. Shippen, unpublished data). However, a TRF1 homolog, AtTBP1 was identified and like TRF1, mutants in this gene show gradual telomere lengthening (148). The contributions of the rest of the proteins still remain to be assessed.

*Arabidopsis* contains three divergent POT1-like proteins. AtPOT1a and AtPOT1b are predicted to encode two OB-folds (284), while AtPOT1c has only one (A. Nelson, Y. Surovtseva, and D. Shippen, manuscript in preparation). AtPOT1a is necessary for telomere length maintenance; *pot1a* mutants show progressive telomere loss every generation, like *tert* mutants (296). Moreover, immunoprecipitation experiments indicate that AtPOT1a is physically associated with the telomerase RNP.

Overexpression of N-terminus of AtPOT1b leads to chromosomal fusions which implicates it in a capping role (284). Finally, AtPOT1c represents a recent partial duplication of AtPOT1a. Overexpression of this protein leads to longer G-overhangs, while preliminary RNA interference studies suggest that AtPOT1c is involved in protecting telomeres from recombination (A. Nelson, Y. Surovtseva, and D. Shippen, manuscript in preparation). *Arabidopsis* also harbors a STN1 homolog. Loss of AtSTN1 leads to immediate and profound telomere erosion, longer G-overhangs and multiple chromosomal fusions, indicating a chromosome capping role for this protein (Song et al., submitted).

#### *DNA damage response proteins*

Although, telomeres must be disguised from being recognized as a double-strand break and have therefore evolved mechanisms to inhibit inappropriate DNA damage

response, DNA damage response proteins are critical for telomere replication (reviewed in (196)). The telomere specific roles of some of the DDR proteins in telomere biology are discussed below.

#### *MRX/MRN complex*

The MRX or MRN complex comprises Mre11, Rad50 and Xrs2 in *S. cerevisiae* or NBS1 in mammals. These proteins act as DSB sensors which recruit and activate the checkpoint kinase ATM (in mammals)/Tel1 (in *S. cerevisiae*). MRX/N is responsible for resection of the DSB and generation of 3' single-stranded tails that are coated by a non-specific single-stranded DNA-binding complex RPA (reviewed in (266)). RPA bound to single-stranded DNA then recruits and activates ATR/Mec1. The activation of the checkpoint kinases leads to a cascade of events resulting in cell cycle arrest and repair of the DSB (196).

Mutations in human NBS1 result in the disease Nijmegen Breakage Syndrome (NBS) (74). These patients have short telomeres and show increased genome instability and predisposition to cancer. Mutation of any one of the proteins of the MRX complex in yeast also results in telomere shortening (reviewed in (289)). This telomere defect is thought to be the result of impaired processing of the G-overhang and impaired recruitment of telomerase components Est1 and Cdc13 to telomeres at S-phase in MRX mutants (303) (see below).

#### *ATM/ATR*

ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3 related) are protein kinases that are the key players in checkpoint activation in response to DNA damage (reviewed in (196)). Checkpoint activation allows the cell to delay cell

cycle progression so that damage can be repaired. ATM is involved in the response to double-strand breaks (DSB), while ATR responds to single-strand DNA that is bound by the protein RPA which can originate from stalled replication forks.

In addition to their role as checkpoint proteins, ATM and ATR are also involved in telomere biology. In humans, ATM is localized to telomeres during most of the cell cycle, but it is replaced by ATR in S phase when telomere synthesis occurs (302). The presence of ATM and ATR at telomeres argues that they are necessary for proper telomere function, despite the natural response of telomeric proteins to suppress them. In humans, TRF2 inhibits ATM at normal telomeres and POT1 binding to the G-overhang represses the activation of ATR (Fig. 3) (72). However, when telomeres become short, loading of the shelterin decreases and this leads to derepression of both ATM and ATR (72). Hence, at short dysfunctional telomeres, activation of both ATM and ATR occurs and ultimately leads to cell-cycle arrest.

ATM and ATR are also involved in telomere length maintenance. Inactivation of ATM in humans and yeast causes telomere shortening (114, 247, 266). Yeast *mec1Δ* strains do not show a significant telomere length defect, but *mec1Δ tel1Δ* and *mec1Δ mrxΔ* double mutants are unable to maintain telomeres and display an increased loss of viability (270). The telomere length effects of Tel1 and Mec1 are dependent on their kinase activity, while their association to telomeres does not require kinase activity (207). Telomerase activity is also not affected in *mec1Δ tel1Δ* cells (43), and the telomere shortening in these mutants can be bypassed by overexpression of an Est1-Cdc13 fusion protein (310). The data suggest that these kinases function in regulation of telomerase recruitment.

As mentioned above, both kinases require Mre11 for recruitment to telomeres (109). Tel1 binds preferentially to short telomeres (275) and is needed to recruit

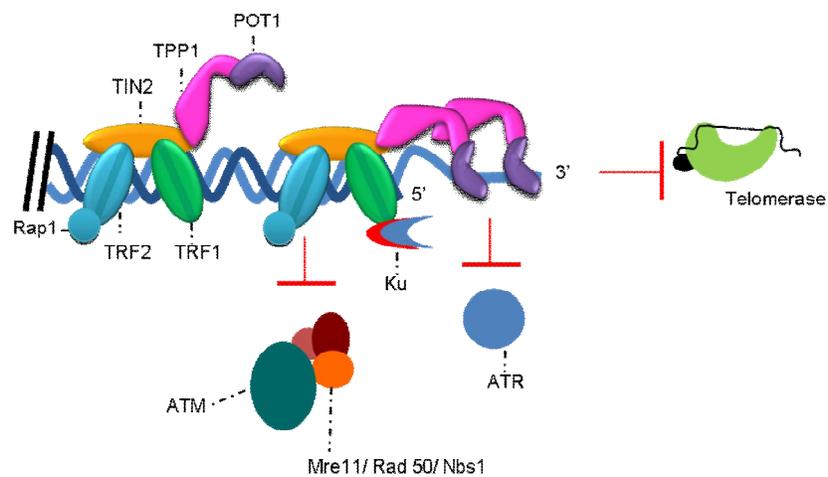


FIG. 3. Telomeric proteins protect against inappropriate DNA damage responses. Telomeres are bound by the six member shelterin complex. The double-strand telomere binding protein, TRF2, inhibits the activation of the checkpoint kinase ATM and the action of the Mre 11/Rad 50/Nbs 1(MRN) complex at telomeres. The single-strand protein POT1 is thought to inhibit action of both telomerase and ATR at telomeres (196). However, at S-phase, DDR proteins are recruited to telomeres and function in telomere regulation.

telomerase to short telomeres. Mec1 is also observed at short telomeres in conjunction with Mre11 (303). Both Tel1 and Mec1 are known to phosphorylate the G-overhang binding protein, Cdc13 (309). The phosphorylation of Cdc13 is necessary for its association with Est1 and subsequently telomerase in late S–G2 phase (309). Furthermore, in *mec1* mutants, both Est1 loading and telomere localization of Cdc13 are affected (303). In humans, ATM and MRN complex are implicated in phosphorylation of TRF1 on short telomeres which leads to its dissociation from telomeres (166, 341). Thus, telomerase is provided access to short telomeres. The above data support a model in which a short telomere, the preferred substrate for telomerase, may induce a transient cell-cycle regulated DNA damage checkpoint response that is required for telomerase binding.

ATM and ATR are also conserved in *Arabidopsis* and are implicated in both chromosome end protection and telomere length homeostasis. *Arabidopsis atm*, *atr* and *atm atr* double mutants do not show any telomere length defects (317). However, studies of *atm tert* double mutants suggest that *Arabidopsis* ATM is required for protection of short telomeres and for telomere length maintenance on homologous chromosomes (317, 318). Interestingly, telomeres in *atr tert* mutants shorten faster than in *tert* mutants alone implicating *Arabidopsis* ATR in prevention of nuclease attack as well as telomere length maintenance (317).

#### *Ku heterodimer*

Ku is a sequence-independent DNA-binding protein complex that is very abundant in cells (266). It exists as a heterodimer of Ku70 and Ku80. The Ku heterodimer is an important component of the non-homologous end joining (NHEJ) pathway for repairing DSBs (reviewed in (266)). However, Ku is also required for

telomere maintenance and chromosome end protection in various organisms and these roles of Ku are distinct from its role in NHEJ.

Ku plays multiple roles in telomere biology. Ku is required for the establishment of transcriptionally silent telomeric chromatin and for telomere maintenance in budding yeast (34, 108, 112, 253). By ChIP assays, Ku was shown to be physically associated with telomeres, but it is not known whether this association is through direct DNA binding or through protein interaction partners (112, 173). In *S. cerevisiae*, loss of Ku leads to telomere shortening to one third of wild-type telomere length and long G-overhangs (253). Ku also interacts with yeast TER, TLC1, and this interaction is essential for TERT (Est2) telomere association in G1. The above data suggest that Ku is involved in positive regulation of telomere length (82, 250).

In humans, Ku binds both hTERT and hTR (42, 307). In a situation similar to budding yeast, Ku binds to a stem loop structure in hTR and this interaction can occur in the absence of TERT (307). Furthermore, loss of Ku in both mice and human cells leads to telomere shortening (66, 237). All the above data suggest that Ku may function in recruitment of telomerase through hTR or stabilization of telomerase at the telomere (307). In addition to contacting DNA directly, Ku also interacts with both TRF1 and TRF2 (143, 291). Loss of Ku80 or Ku70 in mice can lead to massive chromosomal fusions (77). Thus, binding of Ku to double-strand telomere binding proteins may be necessary for its chromosome end protection function.

In contrast to its function in other species, in *Arabidopsis*, Ku functions as a negative regulator of telomerase. *Arabidopsis ku70* mutants display telomerase-dependent hyper elongated telomeres (97, 269). *ku70* mutants also display longer G-overhangs. The switch from positive regulatory role of Ku in other organisms to the role of a negative regulator in *Arabidopsis* may be a result of acquiring new binding partners.

The mechanism of negative regulation of telomerase by *Arabidopsis* Ku is currently unknown. When plants are deficient for both AtKu70 and AtTERT, accelerated telomere shortening and increased number of chromosomal fusions are observed in contrast to *tert* mutants alone (130, 265). The data implicate *Arabidopsis* Ku in chromosome end protection and indicate the possibility of interactions between telomere binding proteins and Ku at the chromosome terminus.

### **Telomerase**

The core components of the telomerase enzyme are its reverse transcriptase subunit (TERT) and its RNA subunit (TER). In most unicellular organisms, core telomerase components are expressed at all times. In humans, active telomerase is detected only in early embryogenesis, germline and epithelial and lymphoid progenitors and is low or undetectable in somatic cells (339). Telomerase is active early in development so that the telomere loss encountered by rapidly dividing cells is not inhibitory for proliferation. While the human telomerase RNA (hTR) is expressed in all tissues (80) and is estimated to be present at about 23,000 molecules per cell in telomerase positive cells, hTERT mRNA is estimated to be present at about 5 molecules per cell suggesting that this is the limiting component (349, 350). In this section, the focus will be on the structural features of the TERT proteins and their functions.

TERT proteins are closely related to reverse transcriptases of non-LTR (long terminal repeat) retrotransposons and group II introns (118, 289). As detailed below, TERT proteins share many features of reverse transcriptases but have notable exceptions too. The most important difference in TERT is the requirement for an intrinsic RNA that provides the template for extension, and cycles of translocation to allow the RNP to add multiple repeats.

### *Telomerase mechanism*

For telomere addition, the telomerase RNA must bind to its substrate, the G-overhang (Fig. 4). The synthesis of telomeric DNA by telomerase follows three steps: alignment, nucleotide addition, and translocation (58, 115, 197). First, the telomerase RNP binds to the telomeric DNA. This involves the formation of a hybrid between the template of the telomerase RNA and the 3' end of the DNA. Interactions between the 5' end of the DNA and the anchor site of TERT (see below) also enable alignment. Next, nucleotides are added by reverse transcription of the template sequence of the RNA by TERT. Secondary structure elements in the RNA known as template boundary elements inhibit synthesis beyond the template (311). When the boundary element is reached, the enzyme translocates and repositions itself so that the RNA template region is again re-aligned with the G-overhang. This process allows for multiple repeats to be added to the DNA end in a single binding event. In yeast, repeat synthesis only extends partially through the template resulting in the degenerate repeat TG<sub>1-3</sub> sequence (54).

### *Structural features of TERT*

All TERT proteins have seven highly conserved RT motifs (1, 2, A, B', C, D and E) present in the central region of TERT (Fig. 5). Additionally, TERT proteins also possess a large insertion between the conserved motifs A and B' called IFD (in fingers domain), a large N-terminal extension and a short C-terminal extension (Fig. 5). The identification of TERT proteins from a number of organisms has also revealed the presence of telomerase-specific motifs (GQ, CP, QPF and T) (39, 57, 63, 159, 211). TERT proteins from different organisms are not all alike; for example TERT from *Caenorhabditis elegans* has no C-terminal region (206). Another interesting variation is

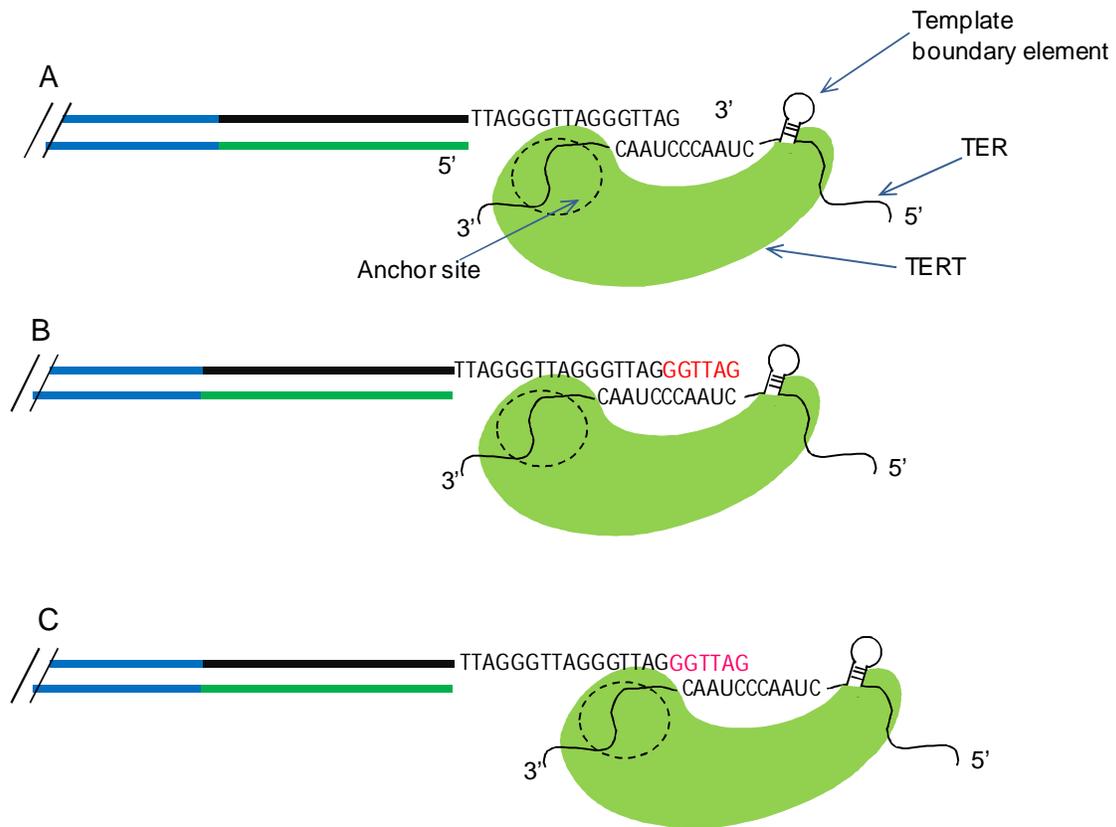


FIG. 4. Model for processive elongation of human telomeres by telomerase. (A) Telomeric DNA is recognized by the telomerase RNP consisting of TERT (green shape), TER (black line with template nucleotides indicated). The 3' end of the telomeric DNA forms a hybrid with the 3' end of the TER template while the 5' end of the DNA is in contact with the anchor site of TERT. (B) Nucleotides are added to the 3' end until the TER template boundary element is reached. (C) The RNP then translocates in order to reposition itself for nucleotide addition again.

## Human TERT

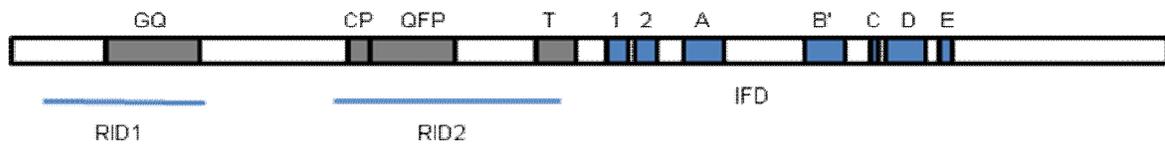


FIG. 5. Features of human TERT. hTERT contains the classical RT motifs (1, 2, A, B', C, D and E), telomerase-specific motifs (GQ, CP, QFP and T), and N and C-terminal domains. The RNA-interacting domains (RID) 1 and 2 are indicated by blue lines. A conserved feature of all TERT proteins is the insertion in between the RT motifs A and B' called the 'in fingers domain' (IFD).

in the size of the linker region present between the GQ motif and the rest of the protein in the N-terminus. This linker region can extend from 20 aa to about 500 aa (in *Plasmodium* species) making *Plasmodium* TERTs larger than TERTs from other species (reviewed in (12)). The above motifs have been implicated in various aspects of telomerase function such as repeat addition processivity, catalysis and RNA binding (see below).

The conservation of RT motifs between TERT and other reverse transcriptases suggests that TERT proteins rely on a mechanism of catalysis similar to RTs, which involves two metal binding residues that are involved in nucleotide transfer. Based on sequence similarity to HIV-1 RT, three invariant aspartate residues found in motifs A and C are considered to be important for catalysis (191). Indeed, mutation of these residues results in loss of telomerase activity *in vitro* and telomere maintenance *in vivo* (191, 331). Recent studies suggest that the N-terminus of *Tetrahymena* TERT is also involved in catalysis. Crystallization of TERT proteins has been difficult because of their large size, poor expression yields and insolubility but through high-throughput screening of GFP-tagged fragments of *Tetrahymena* TERT, one soluble domain was identified (150). The crystal structure of this domain called TERT essential N-terminal (TEN) domain reveals a novel protein fold (149). The core structure is composed of a  $\beta$ -sheet surrounded by seven  $\alpha$ -helices and a short  $\beta$ -hairpin (Fig. 6). Alignment of this region of TERT from different species, reveals the presence of three invariant residues which are required for proper folding of this domain and for DNA-protein interactions (149). Furthermore, single residues essential for telomerase activity as well as RNA-binding were also identified in the TEN domain, making this region all important for catalysis (see below).

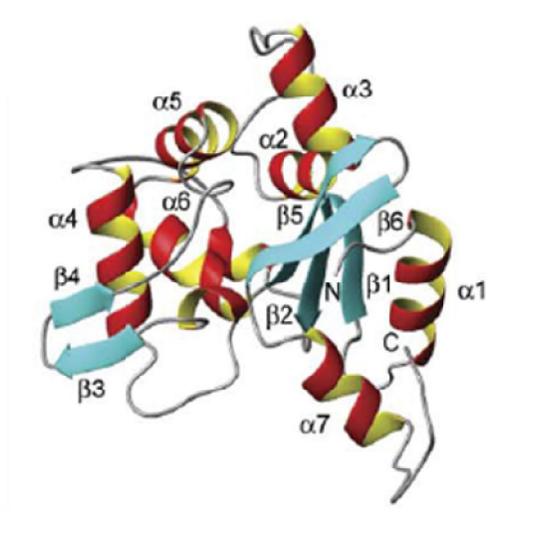


FIG. 6. Crystal structure of *Tetrahymena* TERT essential TEN domain.  $\beta$ -sheets are indicated in blue and  $\alpha$ -helices in red and yellow. Taken from (305).

### *TERT motifs involved in telomerase processivity*

The conserved motifs 1, 2 and E in TERT are implicated in nucleotide addition processivity. Point mutations in these motifs in both yeast and *Tetrahymena* TERTs result in impaired processivity (32, 225, 248). These motifs are thought to contact either the nucleotide triphosphates or the primer for extension. *In vitro*, telomerase from *Tetrahymena* and human can synthesize long products efficiently whereas mouse and yeast telomerases are non-processive and generate only short products (54, 115, 256). Interestingly, mutation of two residues present in motif E of yeast Est2 to that present in HIV RT at the same positions result in increased processivity (248). Mutations in the IFD region between conserved regions A and B' in Est2 also lead to defects in repeat addition (199). Two other regions that contribute to processivity include: motif C and the C-terminal domain of both yeast and human TERT (142, 145, 248). Deletion mutants in the C-terminus of hTERT affect both nucleotide and repeat addition processivity (145). The last region implicated in repeat addition processivity is the GQ domain of yeast TERT. Mutations in this domain result in reduced processivity as well as reduced DNA binding (198). Finally, processivity can also be influenced by proteins that contact the telomerase RNP. These include human TPP1 and *Euplotes aediculatus* p43 (1, 324, 344).

### *Anchor site of telomerase*

The influence of the 5' end of DNA primer sequences on processivity and binding affinity led to the idea that telomerase contains a template-independent DNA binding site (58, 220, 254). This site is known as the anchor site and can be divided into a template-proximal and a template-distal site. Cross-linking experiments support the existence of contacts between TERT and the 5' region of the DNA primer (122, 254). The RID1

domain which encompasses the GQ motif (Fig. 5) has been suggested to act as the template proximal anchor site. Mutations in this domain affect repeat addition processivity as well as DNA binding (19, 176, 231, 232, 342). However, more research is required to identify the contribution of TERT and TER in anchor site function.

#### *TERT motifs involved in telomerase recruitment*

Certain mutants in the N and C-terminus of TERT are capable of telomerase activity *in vitro* but are unable to support telomere maintenance *in vivo* (6, 62, 90, 342). These mutants are referred to as DAT (dissociates activities of telomerase) mutants. Telomere maintenance defects of DAT mutants may be due to compromised recruitment of telomerase to telomeres. This is the case in yeast DAT mutants, where binding to Est3, a telomerase holoenzyme component is compromised (91). The telomere maintenance defects of hTERT DAT mutants were restored when these constructs were fused to either TRF2 or POT1, thus obviating the need for recruitment (7, 8). DAT mutants also revealed defects in processivity which could contribute to telomere length defects (176, 232).

#### *RNA binding domains of TERT*

RNA-protein interactions are essential for the function of telomerase. Two domains of TERT are involved in interactions with the telomerase RNA. The N-terminal part of TERT which includes the motifs CP, QFP and T binds telomerase RNA with high affinity in yeast, humans and *Tetrahymena* (13, 19, 37, 90, 172). This domain recognizes different telomerase RNA targets in different species (Fig. 5). In humans, this domain is called RID2 (RNA-interaction domain) and it contacts the conserved region CR4-CR5 region of hTR (Fig. 7) (13, 172, 227, 231). In *Tetrahymena*, the targets of TERT on the

RNA appear to be stem II and the template boundary element (172, 186) and in yeast, TERT binds to the putative pseudoknot structure (Fig. 7) (46, 68, 188, 352). A second lower affinity interaction is observed between TERT and the RNA subunit. RID1 of hTERT (which encompasses the GQ motif) is also a site of RNA interaction and contacts the pseudoknot-template region of hTR (231). Similarly, the N-terminus of *Tetrahymena* TERT binds to helix IV of TER (240). In addition, the RNA binding domains of *Tetrahymena* TERT are also necessary for template boundary definition (170, 225). It is not known whether yeast TERT interacts with its RNA subunit at another site.

#### *Nuclease activity of telomerase*

Telomerase is associated with nuclease activity that co-purifies with elongation over several chromatographic steps (54, 58, 144, 221). Cleaved products are also observed when telomerase is reconstituted *in vitro* in rabbit reticulocyte lysate (144, 245). Interestingly, certain mutations in motifs 1 and 2 of TERT can promote nucleolytic cleavage of the primer rather than elongation (32, 225). Analysis of the cleavage products indicates that cleavage occurs most often at the 5' end of the template. Therefore, the purpose of this activity may be to ensure that either there is no read-through reverse transcription or that non-telomeric sequences are not extended (58, 113, 144, 221). Thus, the nuclease activity may enhance the fidelity of telomerase (58, 144, 221, 245). Alternatively, it may ensure that a substrate can be generated when the complexes have been stalled (144, 245). The establishment of an assay that detects nuclease activity separately from elongation will be necessary to understand this role of telomerase.

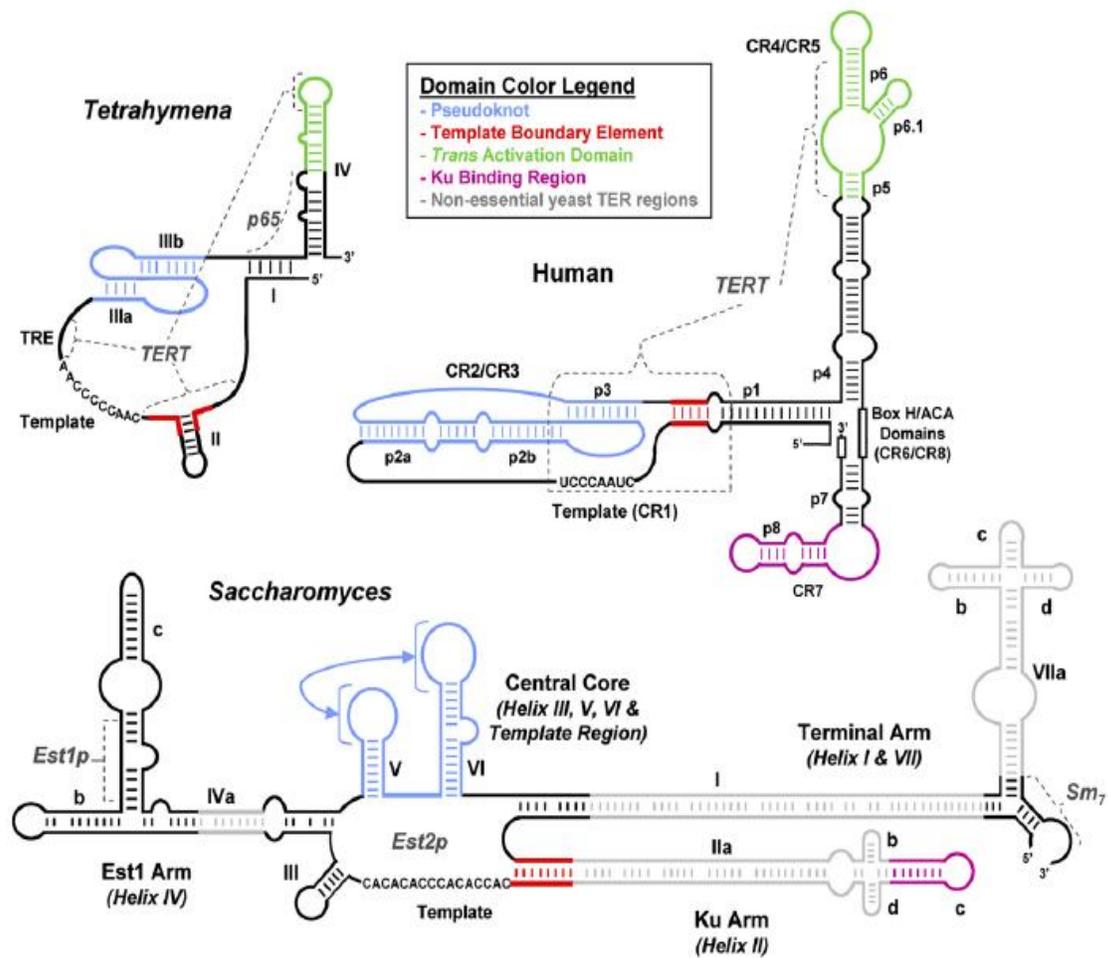


FIG. 7. Secondary structures of TER subunits from *Tetrahymena thermophila*, *Saccharomyces cerevisiae* and humans. Conserved features of all TERs such as the template, template boundary element and the pseudoknot are indicated. Binding sites of TERT and species-specific proteins are also indicated by dotted lines. Figure was taken from (177).

### *Regulation of TERT*

Telomerase is subjected to various types of regulation, at the levels of transcription, splicing, post-translational modifications, transport and subcellular localization. hTERT is transcriptionally repressed in somatic cells, but it is upregulated in immortal cells (61). Transcription factors Sp1 and c-myc have been implicated in the activation of hTERT transcription (reviewed in (61)). C-myc has been shown to activate hTERT transcription and activity in primary fibroblasts (325). The hTERT promoter is also subject to negative regulation by transcription factors such as p53, Mad1 and pRB (reviewed in (61)). hTERT mRNA is subject to alternative splicing that is expected to lead to production of inactive TERT isoforms. However, these proteins have not been detected. Nonetheless, the spliced transcripts can have a dominant negative effect on telomere length maintenance (159).

TERT is also subjected to post-translational modifications such as phosphorylation and ubiquitination and these modifications might provide additional control of telomerase activity. Multiple kinases such as Akt have been reported to act on hTERT (reviewed in (61)). Phosphorylation of hTERT is proposed to positively regulate telomerase activity (152). An ubiquitin ligase, MKRN1 was shown to interact with hTERT through yeast-two hybrid assays (160). Overexpression of MKRN1 causes degradation of TERT and results in decreased telomere lengths and activity (160). Therefore, MKRN1 functions in telomere maintenance by modulating TERT protein stability.

Telomerase can also be regulated by controlling its localization. Amino acids in the N-terminus of hTERT have been identified that function in its nucleolar localization. Analysis of hTERT localization during the cell cycle suggest that hTERT is present at subnuclear foci that do not correspond to either nucleoli or cajal bodies for most of the cell cycle (308). At S phase, hTERT is redistributed to cajal bodies, where hTR is

present, thus implicating cajal bodies in the biogenesis/assembly of the telomerase RNP (308). Therefore, sequestration of telomerase components is another mechanism for regulation of activity.

### **Telomerase RNA**

Telomerase RNA, termed TER in *S. pombe* and ciliates, TR in vertebrates and TLC1 in *S. cerevisiae*, provides the template for repeat addition. TERs from various species have been identified and similarities and differences can be noted. While both hTR and TLC1 are the products of RNA polymerase II transcription (45, 80), ciliate TERs are transcribed by RNA polymerase III and have a run of uridine residues at the 3' end (118). TLC1 is polyadenylated *in vivo*; however, it is subject to processing events that result in the removal of poly A tails in the mature TLC1 (45). TERs have diverged quite rapidly and there is no primary sequence conservation among TERs. However, secondary structures of TERs are found to be highly conserved (Fig. 7).

Phylogenetic analysis was used to determine the secondary structure of TERs in ciliates, yeast and vertebrates (50, 68, 272, 352). All TERs contain a single-stranded template sequence that corresponds to 1.5 copies of the telomere repeat. Besides the template, all TERs contain a 5' template boundary element (TBE) and a pseudoknot region downstream of the template (Fig. 7). The TBE functions to prevent telomerase from copying nucleotides outside the templating domain in TER. The pseudoknot domain is a high affinity TERT binding site (47, 201). It is essential for telomerase activity *in vivo* as it is involved in dimerization of the RNA and formation of a stable RNP (103, 202). In addition, other structural elements in the RNA are required for telomerase processivity, accumulation and regulation by binding proteins.

### *Secondary structure of ciliate TERs*

Ciliate TERs represent the smallest TERs identified and range from 150 nt (in *Tetrahymena thermophila*) to 200 nts (in *Paramecium* species). The *Tetrahymena* TER was the first to be identified through biochemical purification of telomerase. This set the stage for identification of TERs from other ciliates such as *Euplotes crassus* (286) and *Oxytricha nova* (190), which have different telomere repeats and therefore were predicted to have different template regions. Paramecia have a single TER gene like other ciliates, but synthesize variable telomere repeats due to the misincorporation of a thymine nucleotide by telomerase (216).

Phylogenetic analysis led to a consensus secondary structure for ciliate TERs. A pseudoknot structure that comprises helix IIIa and IIIb is predicted (Fig. 7). The ciliate pseudoknot is important for RNP assembly *in vivo* (103), however it has not been confirmed by structural studies. Two other conserved helices (helix I and IV) are also present. Helix IV is the site of interaction of TERT and is important for telomerase processivity (171). TERT also binds the ciliate TER at a conserved motif close to helix II (172). This sequence present 5' of the template functions as a template boundary element (TBE) in ciliates (11, 170). It is possible that the TERT protein itself acts sterically to inhibit reverse transcription into non-template sequences. Finally, ciliate TERs also possess a region next to the template known as the template recognition element (TRE) which is implicated repeat addition processivity (240).

### *Secondary structure of yeast TERs*

*S. cerevisiae* TLC1 is the largest known TER at approximately 1.3 kb. TLC1 was identified through a novel genetic screen for suppressors of telomeric silencing in yeast (287). Only recently the *S. pombe* TER1 was identified. The RNA was found

simultaneously by two groups using biochemical purification (181, 329). SpTER1 is similar to TLC1 in size (~1.2 kb). Secondary structure analysis for yeast TERs has been difficult because of their large size, sequence diversity and limited availability of sequences from closely related species. However, a working secondary structure model for TLC1 has been proposed (68, 352). TER sequences diverge rapidly and therefore TLC1 homologs from other yeasts in the “sensu stricto” group were cloned based on synteny of surrounding protein coding genes (68). The four TLC1 homologs cloned share only 43% identity; nevertheless these sequences were used for phylogenetic analysis. The predicted secondary structure (Fig. 7) contains a core region from which three helical arms extend out (68, 352). As expected, the central core contains a single-stranded template region. Template boundary definition is achieved by an RNA structure (helix I) which is present immediately 5' of the template (311). A potential pseudoknot was also identified in the secondary structure model. However, experimental studies have not detected the presence of pseudoknots in yeast TLC1. Rather two hairpins are proposed in this region (68). It is possible that TLC1 switches between these two conformations (see below). The putative pseudoknot region is the site of interaction with *S. cerevisiae* Est2 (TERT) (46, 188). Separate stem loops have also been identified that act as binding sites for Est1 (a holoenzyme component) and Ku (250, 281).

The large size of TLC1 has made it difficult to reconstitute activity *in vitro* due to misfolding of the RNA. However, a 500 nt truncated version of TLC1 called mini-T RNA reconstitutes activity with Est2p (353). Mini-T RNA contains the essential protein interaction sites for Est1, Ku and Sm proteins along with the central core that binds to Est2. The smallest RNA that works in the reconstitution assay is 384 nts long. Thus, most of the yeast TLC1 RNA is not required for telomerase activity (353).

The identification of TER1 from *S. pombe* has made it possible to draw comparisons between TER1 and TLC1. Like TLC1, TER1 also harbors binding sites for the TERT and Est1 (181, 329). Interestingly, interactions between TER1 and Ku were not identified (329). A preliminary secondary structure model for TER1 (Fig. 8) predicts a central core containing the template, template boundary element (TBE), a potential pseudoknot region and two long arms emanating from the core region (329). This model suggests that the heterogeneity in *S. pombe* telomere sequences could arise as a result of breathing in the TBE, which allows TERT to read beyond the template. Overall, the TER1 secondary structure is very similar to that of TLC1.

#### *Secondary structure of vertebrate TERs*

Subtractive screening of cDNA libraries from telomerase positive and negative cells led to the identification of hTR (80). Subsequently, vertebrate RNAs from various organisms representing different phyla were cloned (50). Vertebrate TERs are the most diverse in terms of size ranging from 319 nts in teleost fish to 559 nts in sharks; while human TR is approximately 451 nts in length (50, 343). These sequences were used to construct a secondary structure model for vertebrate TR (Fig. 7). The structure closely resembles that of the ciliate TER. Similar to ciliate TERs, TRs contain a single-stranded template region, a 5' template boundary element and a pseudoknot region downstream of the template (Fig. 7). In humans, the P1b helix upstream of the template acts as the TBE (49). Interestingly, mouse telomerase RNA lacks the P1b helix and the template boundary is established two nucleotides downstream of the 5' end (49). The pseudoknot region is a high-affinity binding site for TERT (231). In hTR, the pseudoknot is considered to be a dynamic structure in equilibrium with an alternative structure

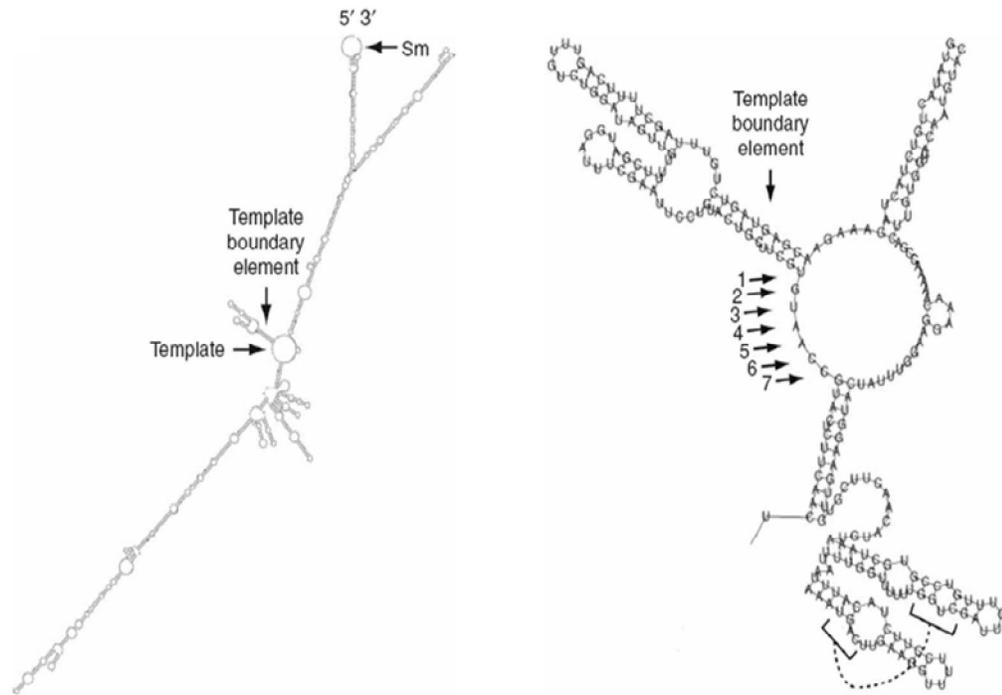


FIG. 8. Proposed secondary structure of *S. pombe* TER1. On the right, the central region of TER1 is shown in more detail. The secondary structure shows a single-stranded template (indicated by arrows 1-7), a template boundary element, a potential pseudoknot and a Sm binding motif. Taken from (329).

containing two hairpins. The switching of the pseudoknot between the two alternate structures is important as mutations in hTR that abolish this switching result in the telomerase deficiency disease dyskeratosis congenita (60). This might also be the reason why some studies have failed to identify the pseudoknot domain *in vivo* (3).

In addition to the pseudoknot region, conserved regions CR4/CR5 of hTR, which resembles stem IV of ciliate TER, bind TERT (172, 227, 231). The P6.1 stem of the CR4/CR5 region of hTR (Fig. 7) is absolutely essential for telomerase activity (51). It has been suggested that this stem might be involved in a long-range interaction with the template at the 5' end of hTR (312). Another conserved stem in the CR4/CR5 region, p6a, is however not required for telomerase activity (231). A minimal functional telomerase can be reconstituted with hTERT and two independent hTR domains (the pseudoknot and CR4/CR5 domains) (227). Both these regions are present in the 5' end of hTR. The 3' region of hTR contains two important regions: the H/ACA box as well as the CR7 domain that includes a cajal body motif (CAB) (50). These regions are required for RNA stability and accumulation (48). The H/ACA box forms a 'hairpin-hinge-hairpin-tail' structure similar to that found in small nucleolar RNAs (3). The role of the 3' region of hTR is in the biogenesis of the telomerase RNP (see below).

### **Telomerase RNP Composition and Biogenesis**

Telomerase activity can be reconstituted *in vitro* when TERT and TER are added to a crude extract such as rabbit reticulocyte lysate (331). Telomerase function requires core components and additional factors that may be involved in stability, folding or function of the RNP complex (Table 1). Some of the factors required for holoenzyme assembly *in vivo* have been identified in various species and dissociation of these factors most likely induces RNP turnover. Interestingly, TERT is not required for the

**Table 1 Telomerase associated proteins and their functions**

<b>Proteins involved in Telomerase RNP biogenesis, stability and localization</b>	<b>Reference</b>
<i>Ciliates</i>	
p65	(336)
p43	(1, 2)
<i>Yeast</i>	
Sm proteins	(282)
<i>Humans</i>	
Dyskerin	(229)
Gar1	(75)
Nhp2	(252)
Nop10	(252)
Pontin	(315)
Reptin	(315)
SmB	(93)
SmD3	(93)
p23	(141)
Hsp90	(141)
14-3-3	(280)
<b>Proteins involved in Telomerase recruitment</b>	
<i>Ciliates</i>	
p45	(336)
p75	(337)
<i>Yeast</i>	
Est1	(189, 300)
Est3	(146, 189)
Ku70/80	(82, 250)
<i>Humans</i>	
hEst1A	(262, 290)
hnRNP A1	(86)
hnRNP C1	(85)
<b>Proteins involved in Telomerase regulation</b>	
<i>Yeast</i>	
PinX1	(187)
Pif1	(279, 355)
<i>Humans</i>	
Akt	(61)
MKRN1	(160)
PinX1	(15, 356)
Pif1	(214)

stability of TERs in either yeast or vertebrates (55) as evidenced by hTR accumulation in cells without TERT. However, one report suggests that the half-life of hTR can be increased in the presence of TERT (350). In this section, the biogenesis or the process of transcription of the telomerase RNA until its incorporation into a functional RNP will be discussed.

#### *Ciliate telomerase biogenesis*

The ciliate TERs are products of RNA polymerase III transcription (118). In both *Tetrahymena* and *Euplotes*, affinity purification of TERT recovered proteins containing a La motif, called p65 and p43 respectively (2, 336). La proteins function in maturation of polymerase III transcripts. *Tetrahymena* p65 co-purifies with telomerase activity and recent studies indicate that it binds to stem IV of the *Tetrahymena* TER. This association results in an altered conformation that allows TERT to bind TER efficiently (295). Genetic depletion of p65 results in reduced TER and TERT accumulation and therefore p65 is an essential part of the telomerase holoenzyme (336). Thus, the biogenesis of ciliate TERs shares similarities with other polymerase III transcripts.

The *Tetrahymena* holoenzyme also consists of the proteins p20, p45 and p75 that were identified by affinity purification of telomerase (336). Epitope-tagged p45 co-purifies telomerase and genetic depletion of the p45 protein results in telomere shortening without affecting RNP accumulation (336). A similar phenotype is observed for the protein p75 (337). It is possible that these proteins might be involved in telomerase recruitment to the chromosome end. *Tetrahymena* p20 is also known as Skp1 and is part of the ubiquitin ligase machinery (337). Depletion of Skp1 promotes telomere elongation and therefore it is thought to have a negative role in telomere

maintenance (337). However, the mechanism of Skp1 function in the telomerase RNP is currently unknown.

In another purification of telomerase from *Tetrahymena*, two other proteins p80 and p95 were identified (59). Both these proteins bind TER and telomeric DNA. Furthermore, disruption of p80 and p95 leads to telomere lengthening (98, 224). However, it seems that these proteins are not specific to the telomerase RNP but rather bind a variety of RNPs in the cell (212).

#### *Yeast telomerase biogenesis*

*S.cerevisiae* TLC1 is transcribed by RNA polymerase II. The transcribed RNA has a poly A tail (approximately 150 nts), but it is immediately removed and this “tail minus” form of TLC1 is more abundant in the cell (45). TLC1 biogenesis involves nucleo-cytoplasmic transit that is dependent on other holoenzyme components (95). TLC1 RNA has a 5'-2, 2, 7-trimethylguanosine or TMG cap similar to small nuclear RNAs (snRNA) like U1 (282). Another feature common to both snRNAs and TLC1 is the presence of a uridine-rich motif that is the binding site of the Sm proteins. Sm proteins are heptameric complexes that promote the stability of small nuclear RNAs (158). Deletion of this motif as well as depletion of one of the members of the Sm complex leads to reduced levels of TLC1 (282). Immunoprecipitation with Sm proteins revealed that most telomerase activity is associated with the Sm complex. Therefore, the biogenesis pathway of TLC1 parallels that of snRNAs.

Inspection of *S. pombe* TER1 sequence also revealed the presence of a Sm motif (181). Deletion of this motif led to rapid shortening of telomeres and accumulation of an unprocessed TER1 transcript, suggesting that this motif is essential for TER1 function in fission yeast.

### *Vertebrate telomerase biogenesis*

Human TR is a product of RNA polymerase II transcription and is expressed in most tissues (80). Like TLC1, hTR also contains a 5'-TMG cap (80, 93). hTR has two important regions in its 3' end that are necessary for proper processing and stability: the H/ACA box and the CR7 domain. The H/ACA motif is required for accumulation and *in vivo* function of telomerase (92). This motif is common to small nucleolar RNAs (snoRNAs) which are bound by the proteins dyskerin, Nhp2, Nop10 and Gar1. The snoRNP complex functions in pseudouridylation of other RNAs such as ribosomal RNA or snRNAs (219). hTR is co-transcriptionally bound by the dyskerin complex of proteins (46,18,41), but so far no pseudouridylation target has been identified for this complex. Instead, the role of dyskerin complex is to stabilize the RNP and perhaps to constrain hTR so that hTERT can bind to it. Recent evidence suggests that the core telomerase consists of two molecules of hTERT, hTR and dyskerin (53). Dyskerin is an essential gene (129) and mutations in dyskerin lead to the disease dyskeratosis congenita (see below). A recent purification of hTERT identified two ATPases, pontin and reptin that bind to both TERT as well as dyskerin (315). Depletion of these proteins resulted in loss of hTR as well as dyskerin, and co-immunoprecipitation of either protein brings down TERT that has reduced catalytic activity (315). The data suggest that pontin and reptin may function in the assembly of the telomerase complex.

In addition to the H/ACA motif, assembly of a functional telomerase RNP also requires a loop in the CR7 domain. This loop contains a consensus cajal body (CAB) localization signal (151). Cajal bodies are nuclear compartments that act as sites of modification and assembly of RNPs. The presence of both H/ACA motif and a CAB box make hTR similar to a group of RNAs called small cajal-body RNAs (scaRNA) (151). It may be that the cajal bodies function in post-transcriptional maturation or assembly of

the telomerase holoenzyme. hTR has been observed to accumulate in cajal bodies throughout most of the cell cycle (151, 359). At S phase, hTR co-localizes with hTERT at foci near telomeres enabling telomerase to act at telomeres (308). Substitutions of the CAB box do not affect telomerase activity, but recent data indicates that these mutants are not able to recruit telomerase to the telomeres and hence cells undergo shortening of telomeres (64). The necessity of this motif indicates that cajal bodies are involved in maturation of hTR enabling it to bind to telomeres. Alternatively, cajal bodies may be involved in transporting telomerase to the telomeres.

Unlike TLC1, hTR does not contain a Sm binding motif. However, immunoprecipitation experiments with antibodies to SmB and SmD3 proteins pull down both hTR and telomerase activity (93). The association to Sm proteins seems to be mediated by the CAB motif. This is in contrast to yeast telomerase RNP where the Sm motif mediates binding of Sm proteins. Therefore, the biogenesis pathway of hTR is similar to that of scaRNAs, while TLC1 biogenesis is similar to snRNAs.

### **Other Telomerase-associated Proteins**

#### *Telomerase-associated proteins involved in enzyme recruitment to telomeres*

In order for telomerase to function at telomeres *in vivo*, it needs to access the 3' G-overhang. In budding yeast, this access is mediated largely by the holoenzyme components: Est1 and Est3. Epitope tagged Est1 and Est3 co-purify telomerase activity, but strains lacking Est1 or Est3 do not show reduced telomerase activity (146, 189). Furthermore, these components are not required for telomerase activity *in vitro*. Est1 binds a bulged stem of TLC1 RNA independently of Est2, whereas binding of Est3 to TLC1 requires Est2 (189, 281, 352). Recent data also shows that TLC1 accumulation in the nucleus is affected in mutants of Est1, Est2 and Est3 (95). When Est1 was fused to

the DNA-binding domain (DBD) of the G-overhang binding protein Cdc13, it promoted telomere lengthening (78). Furthermore, Est1 is not required for telomere maintenance when an Est2-Cdc13 DBD fusion protein is present in the cell (78). These data argue that Est1 functions in telomerase recruitment. One model for the recruitment of telomerase in yeast is the binding of Est1 to telomerase through TLC1 and the subsequent recruitment to the telomere by the interaction of Est1 with the end-binding protein Cdc13 in S-phase (300). Another model, proposed by the Zakian lab based on chromatin immunoprecipitation data, suggests that telomerase is always present on the telomere, however it is activated in S-phase by the binding of Est1 (300).

Telomerase recruitment in budding yeast also involves the DNA binding Ku heterodimer. Ku also localizes telomeres to the nuclear periphery (133, 173). In addition, Ku directly contacts telomerase through an interaction with a 48 nt stem loop present in TLC1 (250). Disruption of the TLC1-Ku interaction results in critically short telomeres with very long G-overhangs (112, 293). Loss of Ku also affects TLC1 nuclear localization (95). Therefore, Ku is thought to direct telomerase to telomeres through its interaction with TLC1 and this interaction promotes Est2-telomere association in G1 phase (82). However, other factors are required to convert telomerase to an active form in S phase. The prevalent mode of recruitment of telomerase is still under investigation.

EST protein homologs have also been identified in other organisms. *S. pombe* Est1 is also a holoenzyme component; it interacts with TER1 independently of Trt1 (TERT) and may also function in telomerase recruitment (181, 329). Similarly, human Est1a is associated with telomerase *in vivo* and when overexpressed causes telomere length elongation and fusions (262, 290). Human Est1b is also associated with telomerase and binds TERT directly. However, defining a role for hEst1a and hEst1b in telomerase recruitment has been confounded by their involvement in the nonsense-

mediated decay pathway (262, 290). *Arabidopsis* EST proteins have no known role in telomere biology. Rather, AtEst1b is important for exit from meiosis (264).

Other factors linked to telomerase recruitment in humans are the heterogeneous nuclear RNP proteins (hnRNPs). These proteins are very abundant and are associated with most RNA polymerase II transcripts. Several observations suggest that hnRNPs could function in telomerase recruitment. Mice deficient in hnRNP A1 display telomere shortening (169). hnRNP A1, hnRNP C1/C2 can also bind simultaneously to both hTR and a telomeric DNA substrate (81, 85, 169). Immunoprecipitation of hnRNP A1 pulls down telomerase activity. However, it is not known whether these abundant proteins are always associated with telomerase, as loss of some members of the complex do not affect telomere maintenance (reviewed in (86)).

Overall, the mechanisms controlling telomerase recruitment to the telomere are poorly understood and involve interactions between telomerase components and telomere binding proteins.

#### *Telomerase-associated proteins involved in RNP biogenesis, stability and localization*

The molecular chaperones Hsp90 and p23 directly associate with hTERT and are necessary for telomerase activity (141). These proteins are stably associated with telomerase and are involved in the proper assembly of the telomerase RNP. It is hypothesized that chaperones could facilitate the conformational change required for the translocation step of telomere synthesis (141). Genetic screens have identified Staufen, L22, TEP1 and SMN (survival motor neuron) as binding partners of telomerase (14, 125, 174). The requirement of these proteins for telomerase function *in vivo* is unknown.

14-3-3 was identified by yeast-two hybrid assay as a binding partner for hTERT (280). 14-3-3 is proposed to increase the nuclear localization of hTERT by inhibiting

hTERT binding to an export factor (280). Two other hTERT interacting proteins are the nucleotide triphosphatases (NTPase) NAT10 and GNL3L (94). The functions of these proteins are unknown but their overexpression results in telomere shortening (94). It is proposed that the NTPases function in telomerase assembly or localization.

#### *Telomerase-associated proteins involved in telomerase regulation*

A protein that is involved in negatively regulating telomerase function *in vivo* is PinX1 (356). PINX1 was first identified as a TRF1 interacting protein. Overexpression of PinX1 inhibits telomerase activity and causes telomere shortening (356). *In vitro*, PinX1 binds directly to hTERT, and represses telomerase activity by binding to the assembled telomerase complex (15). Yeast PinX1 binds to the RNA binding domain of Est2p forming inactive telomerase complex that prevents TLC1 from binding (187). PinX1 localizes to both the nucleolus as well as telomeres because of its association with TRF1. Thus, it can function as a potent regulator of telomerase.

Pif1, a 5'→3' DNA helicase, was first identified in *S. cerevisiae* as a protein important for maintenance of mitochondrial DNA (89). ScPif1 is also implicated in inhibition of *de novo* telomere addition (279). Accordingly, *pif1Δ* mutants in yeast display long telomeres and double-strand breaks in *pif1Δ* mutants are repaired by telomere addition (279, 355). ScPif1 can remove telomerase from telomeres *in vivo* and *in vitro* and is therefore thought to negatively regulate telomerase action by unwinding telomeric DNA: RNA hybrids (33). Human Pif1 binds to hTERT and this interaction is not dependent on RNA (214). However, the role of human Pif1 in telomere length maintenance is controversial (214, 354).

## Dimerization of Telomerase

Dimerization has been reported for telomerase from several species (human, yeast and *E. crassus*) (20, 254, 326). Dimerization of hTERT has been observed (4, 231). Specifically, the N-terminal domain of TERT can interact with the RT and C-terminal domain in trans and reconstitute activity (20). In humans, the catalytic core of telomerase is composed of two molecules each of TERT, hTR and dyskerin (53, 333). However, purification of telomerase from *Tetrahymena* indicates that the complexes contain a single TERT and TER indicating that this enzyme is active only as a monomer (38, 336). Dimerization has also been observed *in vitro* for the both human and yeast TERs (105, 202). Non-denaturing polyacrylamide gel electrophoresis was used to show that hTR dimerization occurs through the formation of a trans-pseudoknot (202). However, it is not known whether this trans-pseudoknot is formed *in vivo*.

Why does telomerase need to dimerize? Two models have been suggested (254, 333)(Fig. 9). The first one called the parallel extension model postulates that by dimerization, telomerase can extend two substrates simultaneously, for instance it can work on two sister chromatids. The other model called the template switching model envisions that the substrate is passed between two active sites so that processive elongation can take place. Support for the latter model comes from work in *S. cerevisiae* where introduction of an inactive template mutant RNA into yeast resulted in telomeres with both mutant and wild-type repeats (254, 255). Work from the Blackburn lab suggests that a telomerase dimer can processively use a single template for extension (271). Therefore, it is still not clear which model is the preferred mode of telomerase action.

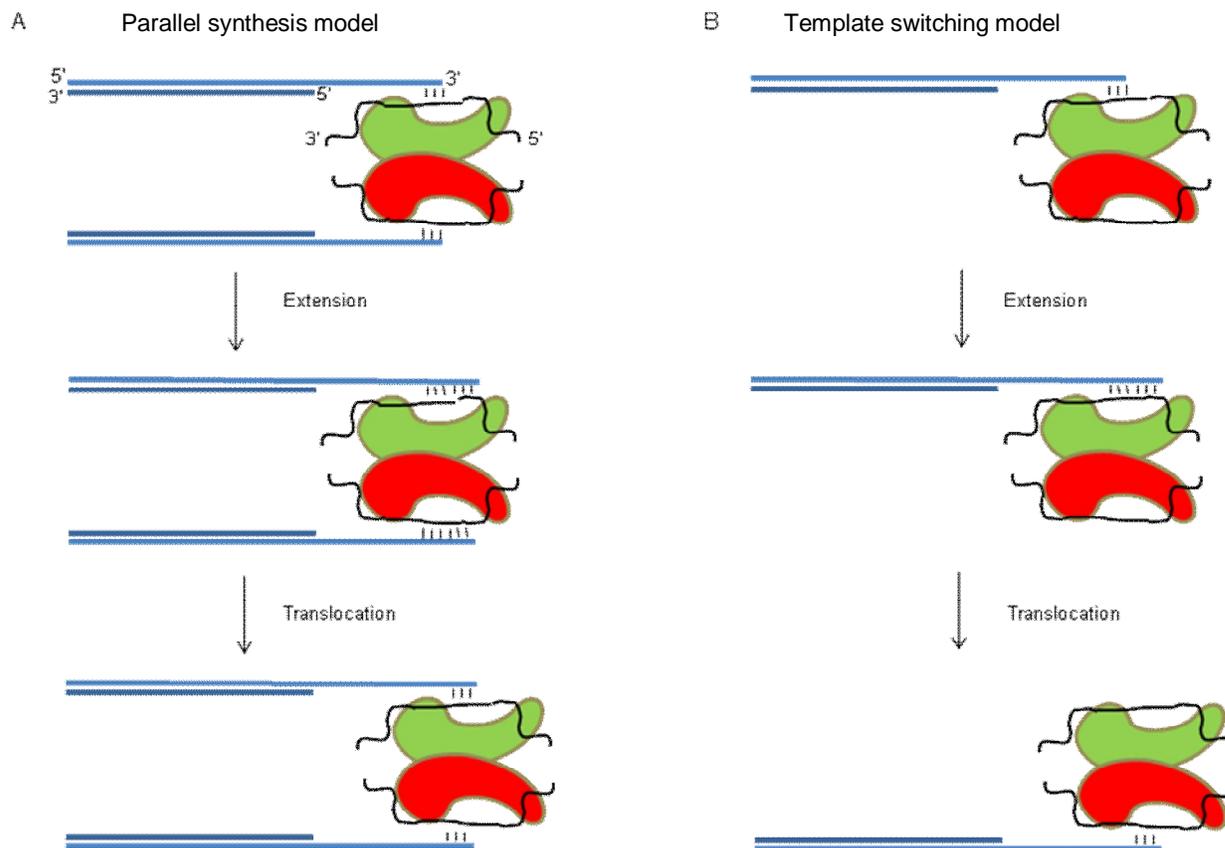


FIG. 9. Models for telomere elongation by a telomerase dimer. (A) In the parallel synthesis model, the telomerase dimer acts on two substrates (sister chromatids) simultaneously. (B) The template switching model envisions processive telomere elongation by switching of the DNA primer between two RNPs (254, 333).

## Consequences of Telomerase Dysfunction

Telomere synthesis is restricted to the S phase of the cell cycle (332, 340). In highly proliferative tissues even when telomerase is present, it does not act at every chromosome end in every cell cycle. Studies have established that telomerase is preferentially recruited to only short telomeres (135, 283, 304) and yet a **species-specific telomere length is established and is essential for long term cell survival**. In this section, the consequences of telomerase loss and haploinsufficiency are detailed.

### *Loss of telomerase function*

Telomerase is abundant in unicellular eukaryotes, whereas telomerase is limiting in multicellular eukaryotes. Moreover, in mammals, telomerase is not active in most somatic cells and therefore telomere shortening results which in turn limits the proliferation potential of cells. Telomerase is upregulated in most cancers and therefore it seems that limiting telomerase is necessary to be able to form a barrier against cancer.

A complete loss of TERT or TER by gene deletion results in a heritable loss of telomere length. This is well demonstrated in the case of mice lacking the telomerase RNA component (mTR).  $mTR^{-/-}$  are viable and early generations of  $mTR^{-/-}$  mutants do not display any abnormalities (30). This is due to the inherently long telomeres (20-150 kb) possessed by laboratory mice. However, late generation  $mTR^{-/-}$  mutants display severe growth and reproductive defects and a shortened life span as a result of critically shortened telomeres and accumulation of chromosomal fusions (175).

Loss of *Arabidopsis* TERT also results in progressive telomere shortening that ultimately results proliferation defects, genome instability and sterility (84, 268). Even though telomeres in *Arabidopsis* are only 2-5 kb,  $tert^{-/-}$  plants survive up to nine generations, losing 200 bp per generation (84, 268). When telomeres become critically

short at approximately 1 kb, they are subject to nucleolytic attack and end-joining activities that lead to fusion events (130). The increasing number of fusion events in late generation *tert*<sup>-/-</sup> plants leads to worsening phenotypes in these plants.

#### *Haploinsufficiency of telomerase*

Mating of *mTR*<sup>+/-</sup> mice having long telomeres with wild-type mice having short telomeres resulted in progeny with populations of long and short telomeres in the same cell. Surprisingly, *mTR*<sup>+/-</sup> from this cross are unable to lengthen the shortest telomeres (128). These mice display haploinsufficiency for telomere length maintenance, but are able to maintain the short telomeres from the cross. Similarly, heterozygosity of mTERT also leads to defects in the maintenance of longer telomeres, although it can rescue the short telomeres (76, 194). Haploinsufficiency of telomerase is also the underlying cause of the disease dyskeratosis congenita (see below).

In yeast, haploinsufficiency of the telomerase RNA subunit, TLC1, leads to telomere length defects (235). None of the other components of yeast telomerase display haploinsufficiency. However, additive haploinsufficiency has been observed in the case of *tlc1 est1* double mutants (180). Surprisingly, none of the known *Arabidopsis* telomerase components (AtTERT and AtPOT1a) are haploinsufficient for telomere length maintenance (84, 296). This might mean that *Arabidopsis* requires less telomerase to maintain its twenty telomeres than humans (92 telomeres) or yeast (64 telomeres).

#### *Mutations in telomerase components lead to mammalian diseases of cell proliferation*

Purification of telomerase from cancer cells indicated that the core telomerase RNP is a dimer of TERT, TER and dyskerin (53). The importance of this core complex is

further supported by the finding that mutations in any of these components can lead to telomerase deficiency (Fig. 10) (101). Such mutations are a hallmark of several disease phenotypes.

While telomerase deficiency is the underlying cause of several diseases including aplastic anemia, pulmonary fibrosis and Hoyeraal-Hreidarsson syndrome (56), the most severe disease associated with telomerase deficiency is X-linked dyskeratosis congenita (DC). Approximately 1 in 1,000,000 individuals are affected by this premature ageing disease (101). Males who inherit a mutant dyskerin allele suffer from a variety of symptoms such as nail dystrophy, abnormal skin pigmentation and progressive bone marrow failure (210). In general, tissues requiring constant renewal like skin and bone marrow are the most affected by the disease.

Various mutant alleles of dyskerin have been identified in patients with dyskeratosis congenita and most of these are single amino-acid missense changes that cluster around the N-terminal domain and the archaeosine-specific transglycosylase (PUA) domain of dyskerin (261). Both these domains play a role in binding hTR. Patients with X-linked DC have lower levels of hTR accumulation, lower telomerase activity and also shorter telomeres than age-matched controls (229). However, the levels of other H/ACA snoRNAs or rRNA processing are not affected in these patients (229, 338), suggesting that defects in telomere maintenance are the cause of the disease phenotype.

The autosomal dominant form of DC is caused by mutations that affect the human telomerase RNA (320). The mutations in hTR that culminate in autosomal dominant DC affect either the 3' end of the RNA or the pseudoknot and CR4/CR5 region of hTR (48). Mutations in the H/ACA domain or the CAB box in the 3' end of hTR affect

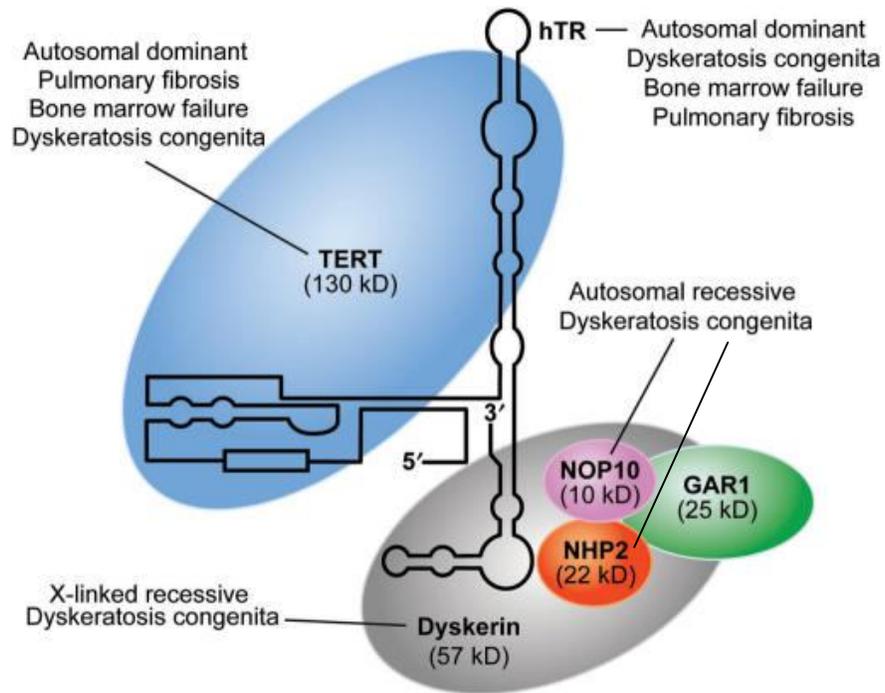


FIG. 10. Mutations in telomerase components lead to Dyskeratosis congenita and other diseases. The human telomerase RNP and the diseases associated with mutations in each telomerase component are shown. Adapted from (101).

RNP biogenesis as well as RNA accumulation, resulting in lower levels of hTR which in turn lead to telomerase haploinsufficiency (320). The mutant form of hTR is not detectable in these patients.

Mutations in the pseudoknot region and the CR4/CR5 region of hTR result in a stable but inactive form of the RNA. This mutant hTR is incorporated into an RNP, but it is not competent for telomere maintenance resulting in telomerase haploinsufficiency (92). Furthermore, missense mutations in the TERT subunit have also been found to lead to the autosomal recessive form of DC (5). Strikingly, genetic anticipation is also observed in DC families with mutations in either TERT or TER. Successive generations who inherit the mutant gene and the accompanying short telomeres show an earlier onset of the disease phenotype and increased disease severity relative to their parents (101, 321).

Recently, mutations in both Nop10 and Nhp2 proteins, which are associated with the telomerase RNP as part of the dyskerin complex, have been linked to autosomal recessive DC (319, 323). Thus, the common thread in all DC patients, regardless of the inheritance pattern, is the presence of short telomeres. Telomerase deficiency in these patients leads to loss of telomere maintenance which is detrimental to the renewal of regenerative tissues. DC particularly affects the stem cells of the hematopoietic system which leads to bone marrow failure in these patients (165).

### **Additional Roles of Telomerase Components**

The presence of TERT at telomeres in phases of the cell cycle when it does not act on the G-overhang suggests that TERT may perform roles besides telomere extension (288, 300). In both yeast and mammals, there are separation of function mutations in telomerase that affect either cell viability or telomere length maintenance

(reviewed in (25)). Mutants that are unable to function in telomere elongation are nevertheless capable of preventing chromosomal fusions. A hypomorphic TERT allele that cannot prevent telomere shortening can still prolong cellular lifespan (357), while overexpression of catalytically inactive TERT alleles can cause cell death without significant changes in bulk telomere length (161). These results suggest that even low levels of telomerase or defective telomerase can protect telomeres from fusion events by physically capping the chromosome termini.

Finally, hTR also has an additional role apart from telomerase. Overexpression of hTR inhibits activity of the protein kinase ATR and this effect is independent of hTERT (156). In response to UV damage, ATR levels are increased and the downstream targets like p53 are phosphorylated, leading to cell cycle arrest. In this situation, hTR levels are slowly increased, leading to inhibition of ATR activity and reinitiation of the cell cycle (156). However, the mechanism of hTR upregulation is still unknown.

### ***Arabidopsis* as a Model for Telomere Biology**

*Arabidopsis thaliana* belongs to the Brassicaceae family of angiosperms. It is the model plant system to work with because of its sequenced genome (125 Mb) and short generation time. Several species related to *Arabidopsis thaliana* such as *Arabidopsis lyrata* and *Capsella rubella* are also being sequenced. The information obtained from these sequencing projects will be useful for comparative genomics projects to identify evolutionarily conserved proteins and non-coding sequences (277, 278).

A variety of *Arabidopsis* mutant collections including T-DNA mutant lines, EMS-mutagenized lines and activation-tagged lines are available to researchers. With T-DNA lines, on average 1.5 insertions events occur. In cases where mutant lines are unavailable, overexpression, antisense or RNA interference constructs can be efficiently

transformed into plants. Furthermore, transformation efficiencies are higher in *Arabidopsis* than in other plant species. Crosses between mutants can be easily performed to generate double and triple mutants. Recently, synchronized *Arabidopsis* cell cultures have also become available (222). This has proven to be a great resource for telomere biology as telomerase is extremely abundant in cell culture.

*Arabidopsis* telomeres are much shorter than in mouse, the other major genetic model for telomere biology in higher eukaryotes. Telomeres range in size from 2 to 5 kb in the Columbia ecotype (283) and therefore even slight differences in telomere length between various backgrounds can be easily determined. For analysis of the length of individual chromosome arms, researchers can exploit the unique subtelomeric regions present on most of the chromosomes (130). Homologs to nearly all known telomere proteins in humans have also been identified in *Arabidopsis*, making it possible to draw conclusions about similarity/divergence of protein function across species.

*Arabidopsis* TERT is similar to other known TERT proteins. Its pattern of expression closely mirrors hTERT in that it is expressed only in reproductive organs and other highly proliferative cells (83, 159, 238). *Arabidopsis tert* mutants survive for up to nine generations indicating that both telomere dysfunction and genome instability can be tolerated by plants (268). Another component of the *Arabidopsis* telomerase RNP is AtPOT1a, which is structurally similar to other POT1 proteins, but is unique in its association with telomerase (296). This suggests that components of the telomerase RNP are evolving rapidly and this may be due to the divergence of the TER subunit (55). However, TER has not yet been identified in any plant species. In summary, the composition of the *Arabidopsis* telomerase RNP is relatively unknown and identification and characterization of other members of this complex is the focus of this dissertation.

## Overview

In this dissertation, I describe the identification and characterization of essential components of the *Arabidopsis* telomerase RNP complex. In Chapter II, the role of dyskerin (AtNAP57) in the *Arabidopsis* telomerase RNP is described. I demonstrate that, like its human homolog, AtNAP57 is associated with active telomerase and this association is most likely mediated through the RNA subunit. Although, null alleles of AtNAP57 are embryonic lethal, missense mutants of AtNAP57 could be studied. These plants showed defects in telomere length maintenance. Through *in vivo* and *in vitro* analysis, I demonstrated that dyskerin is required for proper telomere maintenance in *Arabidopsis* and thus is a conserved component of the telomerase RNP in higher eukaryotes.

In Chapter III, the identification and characterization of two putative *Arabidopsis* telomerase RNA subunits are described. Unexpectedly, this work uncovered two novel telomerase RNAs TER<sub>1G7</sub> and TER<sub>5G2</sub>. The two RNAs display different levels of gene expression in plants and are abundant in tissues with telomerase activity. When co-expressed with AtTERT, both TERs can reconstitute telomerase activity *in vitro*. Moreover, *in vivo* knockdown and mutagenesis studies suggest that both RNAs function in the telomerase RNP *in vivo*. Interestingly, a distinct set of interactions of telomeric proteins with the two TERs were observed *in vivo* suggesting similarities and differences in the function of the two TERs.

In Chapter IV, the search for homologs of TER candidates in other plant species is presented. Through synteny searches and degenerate PCR approaches, candidate TERs from several other plant species were identified. A preliminary working secondary structure model of the *Arabidopsis* TER<sub>1G7</sub> is also described. This model predicts

secondary structure features including a single-stranded template, pseudoknot domain and template boundary elements that are conserved in other known TERs.

Finally, in the appendix, I describe bioinformatics approaches that were adopted initially to identify telomerase RNA candidates. I characterized two candidates and none showed the properties expected for *Arabidopsis* TER *in vivo*. Although these approaches were unsuccessful, they laid the foundation for characterization of the bona fide TER species in *Arabidopsis*.

In total, the data presented in this dissertation support the conclusion that *Arabidopsis* is an important and unique model organism for the study of telomere biology.

**CHAPTER II**  
**DYSKERIN IS A COMPONENT OF THE *ARABIDOPSIS* TELOMERASE RNP  
REQUIRED FOR TELOMERE MAINTENANCE \***

**Summary**

Dyskerin binds the H/ACA box of human telomerase RNA and is a core telomerase subunit required for RNP biogenesis and enzyme function *in vivo*. Missense mutations in dyskerin result in dyskeratosis congenita, a complex syndrome characterized by bone marrow failure, telomerase enzyme deficiency, and progressive telomere shortening. Here we demonstrate that dyskerin also contributes to telomere maintenance in *Arabidopsis thaliana*. We report that both AtNAP57, the *Arabidopsis* dyskerin homolog, and AtTERT, the telomerase catalytic subunit, accumulate in the plant nucleolus, and AtNAP57 associates with active telomerase RNP particles in an RNA-dependent manner. Furthermore, AtNAP57 interacts *in vitro* with AtPOT1a, a novel component of *Arabidopsis* telomerase. Although a null mutation in *AtNAP57* is lethal, AtNAP57, like AtTERT, is not haploinsufficient for telomere maintenance in *Arabidopsis*. However, introduction of an *AtNAP57* allele containing a T66A mutation decreased telomerase activity *in vitro*, disrupted telomere length regulation on individual chromosome ends *in vivo*, and established a new, shorter telomere length set point. These results imply that T66A NAP57 behaves as a dominant-negative inhibitor of telomerase. We conclude that dyskerin is a conserved component of the telomerase RNP complex in higher eukaryotes that is required for maximal enzyme activity *in vivo*.

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## Introduction

An essential step in the maturation of ribosomal RNA is the conversion of uridine to pseudouridine by H/ACA ribonucleoproteins (RNPs) (219). Components of H/ACA RNPs include small nucleolar RNAs (snoRNAs), Gar1, Nhp2, Nop10, and the pseudouridine synthase, dyskerin. Dyskerin is an essential gene and its loss results in embryonic lethality in mice (128). In addition to its role in rRNA maturation, dyskerin also binds the H/ACA box of human telomerase RNA (hTR) and is involved in hTR processing and stabilization (48, 229). Mass spectrometry studies indicate that the core telomerase complex is composed of a dimer of a catalytic telomerase reverse transcriptase (TERT), TR (which acts as a template for TERT), and dyskerin (53). Notably, the dyskerin homolog in yeast, Cbf5p, is not stably associated with the telomerase RNA (73), and a different constellation of proteins is required for telomerase RNP biogenesis and enzyme function in this organism (55).

Mutations in human dyskerin are the cause of X-linked dyskeratosis congenita (DC), a rare disease that affects regenerative tissues and is characterized by abnormal skin pigmentation and bone marrow failure (134). Patients suffering from X-linked DC have shorter telomeres relative to age-matched controls (229). Most mutations in patients with X-linked DC cluster around the PUA (pseudouridine synthase and archeosine transglycosylase) domain of dyskerin, which is responsible for RNA binding (261). One of the most commonly identified dyskerin mutations, A353V, perturbs rRNA pseudouridylation and also results in reduced levels of TR, decreased telomerase activity, and shorter telomeres in mouse embryonic stem cells (230). Similarly, hypomorphic mice that express low levels of dyskerin display the clinical symptoms of DC and exhibit shorter telomeres, but only in later generations (273). While rRNA

processing is affected in some dyskerin mutants, the T66A mutation in humans appears to exclusively affect the telomerase-associated functions of dyskerin (229).

Recent data indicate that bone marrow disease can also arise through reduction of other telomerase RNP constituents. Heterozygous mutations in hTR, which reduce its accumulation and perturb its structure, lead to an autosomal dominant form of DC through haploinsufficiency of the RNA subunit (40, 106, 320). Similarly, haploinsufficiency of TERT has been implicated in DC and in aplastic anemia (5, 322, 345). Limiting abundance of telomerase subunits may help to facilitate the fine balance of telomerase repression and activation associated with differentiated cells and their stem cell progenitors (124).

The flowering plant *Arabidopsis* is a useful model for telomere biology (218). In contrast to mouse, *Arabidopsis* telomere tracts are relatively short (2-5 kb) and are abutted by unique sequences on most chromosome arms (130), making it possible to study the dynamics of individual telomeres. Moreover, *Arabidopsis* is exceptionally tolerant to telomere dysfunction and genome instability. Disruption of *AtTERT* results in a slow, but progressive loss of telomeric DNA (84). Beginning in the sixth generation (G6), *tert* mutants exhibit a low level of end-to-end chromosome fusions and the onset of growth and developmental defects (268). Remarkably, plants survive for up to five more generations with worsening phenotypes until they ultimately arrest growth in a miniature, de-differentiated state unable to produce a germline (37).

Aside from the presence of AtTERT, little is known about telomerase RNP composition and biogenesis in plants. The TR subunit has not yet been identified in any plant species, owing to the rapid evolution of the TR nucleotide sequence. However, recent studies indicate that AtPOT1a, an OB-fold containing protein whose counterparts in yeast and mammals associate with telomeres (17), functions as a telomerase RNP

accessory factor in *Arabidopsis* (296). This observation implies that the composition and/or role of telomerase subunits may vary among higher eukaryotes.

*Arabidopsis* encodes a dyskerin homolog, *AtNAP57* (182, 203) and here we examine its contribution to telomerase biochemistry and telomere maintenance. We demonstrate that *AtNAP57* localizes to the nucleolus along with *AtTERT* and associates with enzymatically active telomerase RNP particles in an RNA-dependent fashion. Although a null mutation is lethal, *AtNAP57* is not haploinsufficient for telomere maintenance. However, transgenic plants carrying an *AtNAP57* allele with a T66A mutation exhibit decreased telomerase activity *in vitro* and *in vivo*, deregulated telomere tracts on individual chromosome ends, and shorter, but stable telomeres. We conclude that dyskerin is a conserved component of the telomerase RNP in multicellular organisms that is required for telomere maintenance.

## Materials and Methods

*Plant materials, genotyping, c-DNA synthesis, site-directed mutagenesis and transformation*

*Arabidopsis* seeds with T-DNA insertions in the *AtNAP57* (SALK\_031065) and *AtKU70* (SALK\_123114) genes were purchased from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, Ohio), cold-treated overnight at 4°C, and then placed in an environmental growth chamber and grown under a 16 h light/8 h dark photoperiod at 23°C. *Arabidopsis* suspension culture cells were maintained as described (222). Siliques from wild-type and *AtNAP57* heterozygotes were dissected 10 days after fertilization and photographed using a Zeiss Axiocam digital camera coupled to a Zeiss microscope.

For genotyping, DNA was extracted from flowers and PCR was performed with the following sets of primers for *AtNAP57*: D5: 5' GTCGACATCTCACACTCGAA 3' and D 8: 5' GTCTCACTTTGTTCCAGAGT 3' and for *AtKU70*: Ku 1: 5' TTACTTTGTTGTTTCGGGTGC 3' and Ku 2: 5' CTCTTGGCAAGTACACGCTTC 3'.

Total RNA was extracted from 0.5 g of plant tissue using Tri Reagent solution (Sigma). cDNAs were synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen). Oligo dT primers were incubated with 2 µg of total RNA in the supplied buffer at 65°C for 5 min. Reverse transcription was carried with 100 U of Superscript III at 55°C for 60 min. RNA was degraded with RNase H (USB). For amplifying *AtNAP57*, we used primers D5 (above) and D2: 5' GCCATCACAGATGTTGTCATC 3'.

The genomic copy of *AtNAP57* (*AtNAP57* cDNA + 1kb promoter) was amplified by PCR and ligated into a binary vector pCBK05 (269) lacking the 35S CaMV promoter. To generate the T66A mutation, site-directed mutagenesis was performed with *Pfu* turbo polymerase (Stratagene) on the genomic version of *AtNAP57* in pCBK05 using the primers M1: 5' CCTCAACGTCCGTGCCGGTAC 3' and M2: 5' GTGACCGGCACGGACGTTGAGG 3' according to the manufacturer's guidelines. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101. Transformation of *AtNAP57* heterozygous plants was performed by the *in planta* method as described in (269). Transformants were selected on 0.5 Murashige and Skoog basal medium supplemented with 20 mg/L of phosphinothricine (Crescent Chemical) and Kanamycin (50 µg/mL) and then genotyped. To generate epitope-tagged protein, *AtNAP57* cDNA was amplified and ligated it into pCBK05 with an N-terminal 3X FLAG tag. This construct was transformed into *Agrobacterium* and then transformed into wild-type plants as described above.

### *TRF analysis, PETRA, TRAP and quantitative TRAP assays*

DNA from individual whole plants was extracted and TRF analysis was performed with *Tru1* (Fermentas) restriction enzyme and [<sup>32</sup>P] 5' end-labeled (T<sub>3</sub>AG<sub>3</sub>)<sub>4</sub> oligonucleotide as a probe (84). The peak value for bulk telomere length (FIG. 15D) was determined by ImageQuant software. PETRA analysis was conducted on DNA from whole plants as described (328). TRAP protein extraction and assays were performed on flowers as previously described (83).

Real-time quantitative TRAP was performed as described (137), but with the following modifications. 10.5 µl of a 4.8 ng/µl protein extract dilution, 1 µl of 10 µM forward primer (5' CACTATCGACTACGCGATCAG 3'), and 12.5 µl SYBR Green PCR Master Mix (NEB) were incubated at 37°C for 45 min. 1 µl of reverse primer (5' CCCTAAACCCTAAACCCTAAA 3') was added and products were amplified for 35 PCR cycles with 30 sec at 95°C and 90 sec at 60°C. Threshold cycle values (C<sub>t</sub>) were calculated using an iCycler iQ thermal cycler (BIO-RAD) and the supplied Optical System Software. Samples were analyzed in triplicate, with inactivated samples and lysis buffer serving as negative controls.

### *Western blotting, immunoprecipitation and immunofluorescence*

Plant extracts were made by grinding 0.3 g of flowers in buffer A (50 mM Tris-Cl pH7.5, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT and plant protease inhibitors (SIGMA)). Western blotting was performed with a 1:1000 dilution of anti-FLAG antibody (SIGMA) and a 1:10,000 dilution of HRP-conjugated anti-Mouse IgG (SIGMA). For immunoprecipitation, 50 µl of α-FLAG beads (SIGMA) were washed four times with buffer A and incubated with 500 µl of extract for 2 h at 4°C. Beads were then washed three times with buffer A and eluted using the 3X FLAG peptide for 30 min.

A peptide antibody against AtTERT was raised in rabbits and affinity purified (Covance). The peptide used was N-CIKHKRTL SVHENKRRKRD DNVQP corresponding to residues 180-202 of AtTERT. Peptide antibodies against mouse dyskerin (230) were a gift from Dr. Monica Bessler.

*Arabidopsis* suspension culture extracts were made as above, diluted in buffer W-100 (20 mM TrisOAc pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.2 M NaCl, 1% NP-40, 0.5 mM sodium deoxycholate and 100 mM potassium glutamate) and pre-cleared with Protein-A agarose (PIERCE). Extracts were incubated with antibody and pre-blocked beads. Beads were washed three times with W-300 (W-100 containing 300 mM potassium glutamate) and once with TMG (10 mM TrisOAc pH 7.5, 1 mM MgCl<sub>2</sub> and 10% glycerol). The beads were then used for either TRAP or western blotting. Proteins were expressed in rabbit reticulocyte lysate (RRL) (Promega) according to the manufacturer's protocol and used in immunoprecipitation experiments as above. The fraction of enzymatically active telomerase particles that was associated with AtNAP57 was determined by calculating the efficiency with which the TERT antibody immunoprecipitated telomerase activity in a TRAP assay (relative to input) using ImageQuant software. This value was compared to the amount of AtNAP57 signal obtained by western blot analysis following immunoprecipitation of these same samples using QuantityOne software.

For immunofluorescence, *Arabidopsis* suspension culture cells and floral buds were fixed with 3.5% formaldehyde in 1X PBS for 30 min and then washed with 1X PBS. Cells were soaked in 1X citric buffer (10 mM sodium citrate and 10 mM EDTA) for 10 min. Citric buffer was removed and enzyme mix (1% pectinase, 4% cellulose and 1% macerozyme) was added and incubated at 37°C for 40 min. Cells were rinsed with 1X PBS and spun down onto poly-lysine coated slides in a swinging bucket rotor centrifuge

for 3 min at 300 xg. Slides were removed from the centrifuge and immediately soaked in 1X PBS with 0.2% Triton X-100 for 30 min to permeabilize the cells. Slides were washed with 1X PBS and treated with Image-IT solution (Molecular probes). Primary antibodies (1:200 dilution for AtTERT and 1:400 dilution for dyskerin) were added to the slides, covered with a plastic coverslip, and incubated overnight at room temperature. After washes in 1X PBS and PI, secondary antibody (Goat anti-rabbit IgG conjugated to Texas Red 1:200 dilution) was added and incubated for 4 h. Slides were washed, Vectashield containing DAPI was applied, and images were captured using a CCD camera coupled to a Zeiss epifluorescent microscope.

#### *Co-immunoprecipitation and yeast two-hybrid assays*

Co-immunoprecipitation was performed as previously described (154) using full-length AtTERT, AtNAP57, AtKU70, AtKU80 and AtPOT1a proteins expressed in RRL. All components of the yeast two-hybrid system were obtained from Clontech Laboratories. AtNAP57 was subcloned from FLAG-AtNAP57-pCBKO5 into pAS2-1. KU70-pAS2-1, KU80-pAS2-1 and NAP57-pAS2-1 were transformed into the yeast strain AH109. AtKU80 and AtPOT1a were cloned into the prey vector pACT2 and then transformed into Y187 strain. Yeast mating assays were performed as detailed in the Clontech yeast protocols handbook (# PT3024-1). Double selection (SD/-leucine/-tryptophan) was used to obtain diploids and triple selection (SD/- leucine/-tryptophan/-histidine) was used to screen for interaction. To confirm interactions,  $\beta$ -galactosidase assays (colony-lift filter assay) were performed on the colonies that grew on triple selection plates and development of the blue color was followed for several hours.

## Results

### *Characterization of Arabidopsis AtNAP57*

*AtNAP57* is encoded by a single gene (At3g57150) on the third *Arabidopsis* chromosome (203) and has only a single exon and no introns. RT-PCR experiments revealed that expression of the 1.6 kb *AtNAP57* transcript is ubiquitous (FIG. 11A), as for mammalian dyskerin (126). *AtNAP57* mRNA translates to a highly basic protein with a molecular mass of 63 kDa. A heterologous antibody directed at mouse dyskerin (230) immunoprecipitated recombinant *AtNAP57* expressed in rabbit reticulocyte lysate (RRL) (FIG. 11B) as well as the endogenous plant protein (see FIG. 12D). This antibody was used to examine the subcellular localization of *AtNAP57* in *Arabidopsis* suspension culture cells. Consistent with a role for the plant *AtNAP57* in rRNA processing, a bright signal for *AtNAP57* was detected exclusively in the nucleolus (FIG. 11C). A similar finding was observed by Lermontova et al. in a recent study of *Arabidopsis* dyskerin (182). We next asked whether *AtTERT* also localized to this compartment using a peptide antibody raised against *AtTERT*. The anti-TERT antibody recognized recombinant *AtTERT* expressed in RRL (FIG. 11B) as well as the endogenous protein from suspension culture extracts (data not shown). Immunolocalization experiments reveal that *AtTERT*, like *AtNAP57*, localized to the nucleolus (FIG. 11C). Nucleolar localization of *AtTERT* was not detected in *tert*<sup>-/-</sup> flowers indicating that staining is specific. These findings imply that telomerase biogenesis may occur in the plant nucleolus.

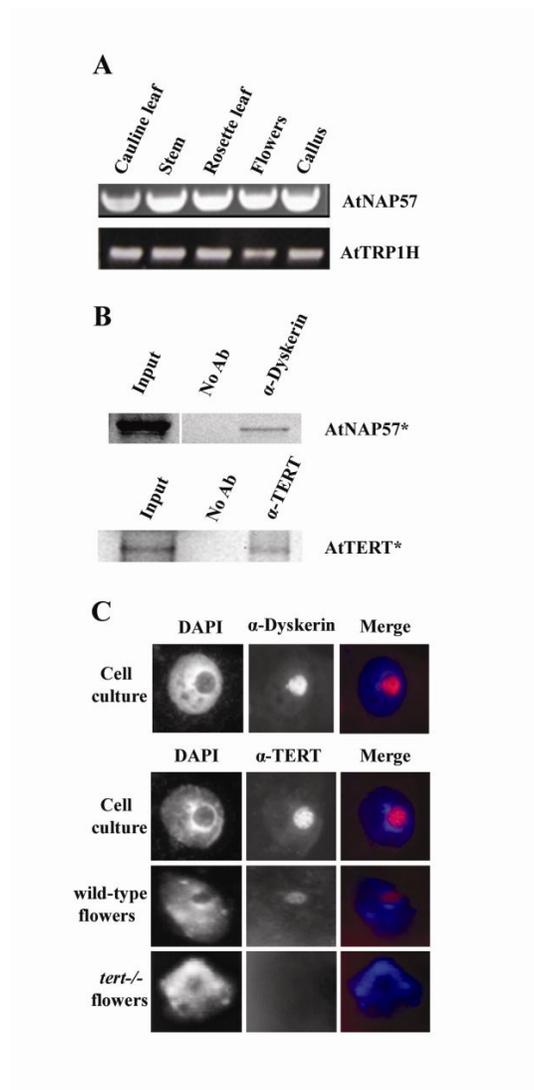


FIG. 11. Expression and localization of AtNAP57. (A) RT-PCR analysis of the AtNAP57 transcript in different plant tissues. AtTRP1H encodes a putative double-strand telomere binding protein (23) and was used as a loading control. (B) Recombinant AtNAP57 and AtTERT proteins were expressed in RRL and labeled with [<sup>35</sup>S] methionine (\*). Proteins were immunoprecipitated with an antibody (Ab) raised against mouse dyskerin ( $\alpha$ -dyskerin) or an antibody raised against an N-terminal peptide in AtTERT ( $\alpha$ -TERT). Relevant lanes are shown. (C) Immunolocalization of AtNAP57 and AtTERT in *Arabidopsis* suspension culture cells and in floral buds. Nuclei were stained with DAPI or the antibodies discussed above.

*AtNAP57 is a component of the Arabidopsis telomerase RNP*

We asked if AtNAP57 physically associates with the *Arabidopsis* telomerase RNP. A fusion construct was generated containing three copies of the FLAG epitope inserted at the N-terminus of AtNAP57 coding region under the control of the robust cauliflower mosaic virus 35S promoter. This construct was transformed into wild-type *Arabidopsis* and transformants were analyzed by western blotting using a FLAG antibody. Approximately 1 in 10 of the herbicide resistant transformants generated detectable levels of the FLAG-AtNAP57 protein (FIG. 12A; data not shown). These plants were used for further study. Complexes containing FLAG-AtNAP57 were immunoprecipitated from transgenic plants and eluted using 3X FLAG peptide. As expected, AtNAP57 was immunoprecipitated from transgenic plants, but not from their wild-type counterparts (FIG. 12B).

To monitor AtNAP57 association with telomerase, the telomere repeat amplification protocol (TRAP) was performed on FLAG-AtNAP57 and wild-type immunoprecipitates. Telomerase activity was immunoprecipitated from FLAG-AtNAP57 plants, but not from wild-type plants lacking FLAG-AtNAP57 (FIG. 12C). To verify that the AtNAP57 interaction with telomerase was specific, we performed a reciprocal immunoprecipitation experiment using the TERT peptide antibody to pull-down AtNAP57 from suspension culture cell extract. Telomerase activity was immunoprecipitated, and as expected, pre-treatment of the extract with RNase A abolished telomerase activity (FIG. 12D). Notably, western blot analysis revealed a strong enrichment of AtNAP57 in the  $\alpha$ -TERT immunoprecipitate, but not when the extract was pre-treated with RNase A prior to immunoprecipitation (FIG. 12D, bottom panel). To determine the relative amount

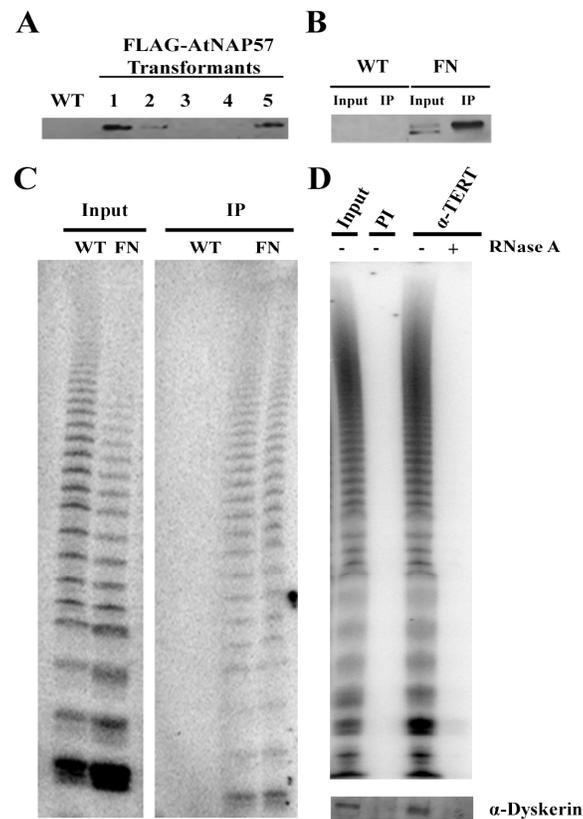


FIG. 12. AtNAP57 associates with *Arabidopsis* telomerase RNP. (A) Western blot analysis with FLAG antibody on plant extracts from the wild type (WT) or transformants bearing FLAG-tagged AtNAP57 (FN). (B) Western blot analysis of input or immunoprecipitates (IP) obtained with FLAG antibody on extracts from the WT and FLAG tagged AtNAP57 transformants. Four percent of input and 30% of IP was loaded on the gel. (C) TRAP assay results for WT and FN extracts before (input) or after immunoprecipitation (IP) with FLAG antibody. Immunoprecipitates were assayed in duplicate. (D) Top panel, TRAP assay results for cell culture extracts immunoprecipitated with preimmune serum (PI) and anti-TERT ( $\alpha$ -TERT) peptide antibody. The sample shown in the far-right lane was pretreated with 100  $\mu$ g/ml of RNase A prior to immunoprecipitation. Bottom panel,  $\alpha$ -TERT immunoprecipitates were subjected to Western blot analysis using the dyskerin antibody ( $\alpha$ -dyskerin). Fifteen percent of input and 60% of IP was loaded on the gel.

of telomerase that was associated with AtNAP57, we compared the efficiency of the AtTERT IP, which was determined to be approximately 10% from the experiment in FIG. 11B, to the amount of AtNAP57 recovered. From these data, we estimate that more than 90% of the active telomerase precipitated by the TERT antibody is associated with AtNAP57. These findings indicate that AtNAP57 is associated with catalytically active telomerase RNP particles and that this interaction requires RNA.

In mammals, dyskerin primarily associates with telomerase through TR. Because TR has not yet been identified in *Arabidopsis*, we asked whether AtNAP57 interacts with the known telomerase-associated proteins *in vitro* using co-immunoprecipitation. As expected in our control reactions (269) we detected the formation of AtKU70-AtKU80 heterodimers, while no interaction was observed for AtKU70 alone (FIG. 13A). We failed to observe binding of T7-tagged AtNAP57 to radiolabeled full-length TERT protein (FIG. 13A), and similarly in reciprocal co-immunoprecipitation experiments with T7-tagged AtTERT and labeled AtNAP57, no interaction was detected (data not shown). Furthermore, we did not detect binding of AtNAP57 to segments corresponding to the N-terminus, middle and C-terminus of TERT. Although there was a high background in the AtPOT1a control reaction with beads alone, AtPOT1a abundance was reproducibly higher in the immunoprecipitate of T7-tagged AtNAP57 (FIG. 13A; data not shown). The AtNAP57-POT1a interaction appears to be specific as a closely related protein, AtPOT1b (284) was not precipitated with T7-tagged AtNAP57 (data not shown). We confirmed the AtNAP57-AtPOT1a interaction using a yeast two-hybrid mating assay.

Yeast strains containing different plasmids were mated and diploids were selected on triple selection (FIG. 13B). To monitor reporter gene activity,  $\beta$ -galactosidase assays were performed. Blue staining, indicative of interaction, was observed for AtKU70- AtKU80 within one hour, and after four hours staining was detected for

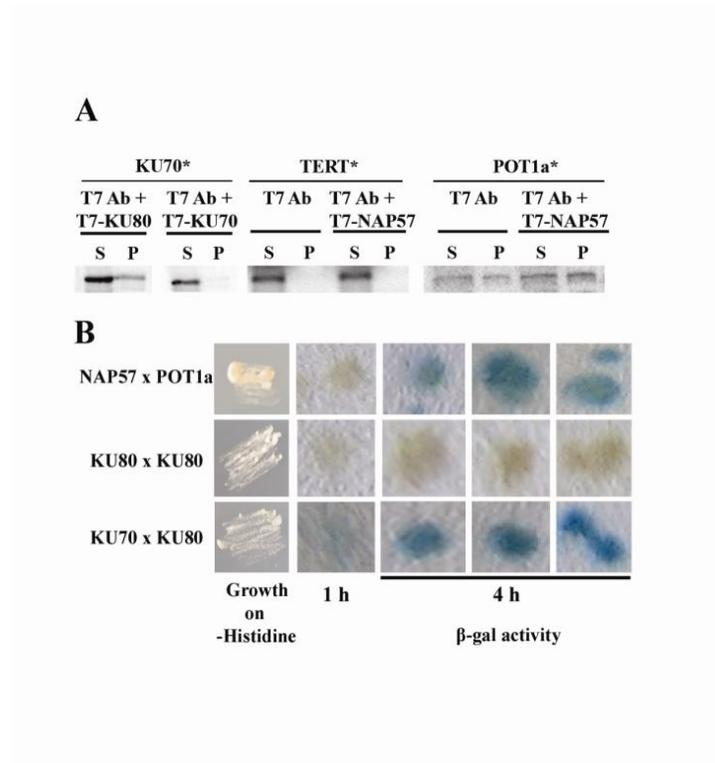


FIG. 13. AtNAP57 weakly associates with AtPOT1a. (A) Coimmunoprecipitation experiments were performed with the full-length recombinant AtKU70, AtTERT, and AtPOT1a proteins, labeled using [<sup>35</sup>S]methionine (\*), and T7-tagged AtKU80, AtKU70, and AtNAP57. Proteins were incubated with either T7 antibody (Ab) beads (control) or T7 beads and the indicated T7-tagged unlabeled proteins. The supernatant (S) and pellet (P) fractions were loaded in equal amounts. (B) Results of yeast two-hybrid analysis are shown. The indicated yeast crosses were performed and plated on medium lacking leucine, tryptophan, and histidine. Results of a colony lift β-galactosidase (β-gal) assay are shown. The blue color is indicative of protein interaction.

AtNAP57-AtPOT1a (FIG. 13B). Thus, the interaction between AtNAP57 and AtPOT1a is weak, but specific. Taken together, these data argue that AtNAP57 is physically associated with the *Arabidopsis* telomerase RNP.

*AtNAP57 is not haploinsufficient for telomere maintenance in Arabidopsis*

To investigate the role of AtNAP57 in telomere maintenance, we obtained a mutant line (SALK\_031065) carrying a T-DNA insertion in the extreme 5' end of the gene corresponding to the 18<sup>th</sup> amino acid of the AtNAP57 ORF (FIG. 14A). Although we genotyped a population of more than 50 progeny from this line, we did not recover any homozygous mutants (data not shown). Dissection of siliques (seed pods) from the heterozygous mutants revealed a reduced seed set in which approximately 25% of the seeds failed to form viable embryos (FIG. 14B) This outcome implies that AtNAP57 is an essential gene, a conclusion consistent with a recent report for plants with homozygous mutation in AtNAP57 (182).

Heterozygous AtNAP57 mutants were indistinguishable from wild-type in their growth and development over successive plant generations. Furthermore, terminal restriction fragment (TRF) analysis conducted on ten first generation (G1) *nap57<sup>+/-</sup>* mutants revealed some variability in bulk telomere length, but in all cases, telomeres were in the wild-type 2-5 kb size range (FIG. 14C, left panel; data not shown). A similar result was obtained when telomeres from G2 *nap57<sup>+/-</sup>* were examined (FIG. 14C, right panel; data not shown). These data imply that AtNAP57 is not haploinsufficient for telomere length maintenance in *Arabidopsis*.

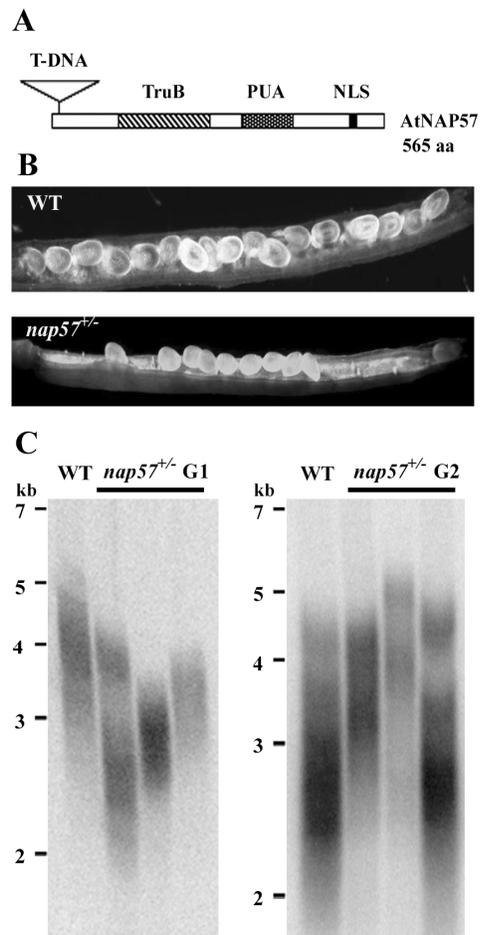


FIG. 14. *AtNAP57* is an essential gene in *Arabidopsis*. (A) Schematic diagram of the *AtNAP57* coding region showing the position of the T-DNA insertion, pseudouridine synthase domain (TruB), pseudouridine synthase, archeosine transglycosylase domain (PUA), and nuclear localization signal (NLS). (B) Siliques (seed pods) from wild-type (WT) or *nap57<sup>-/-</sup>* plants were visualized by microscopy. A reduced seed set was observed for *nap57<sup>+/-</sup>* plants, implying that the homozygous mutation is lethal. (C) TRF analysis of WT, first-generation (G1), or second-generation (G2) *nap57<sup>+/-</sup>* plants. Molecular size markers are indicated.

*The T66A mutation in AtNAP57 results in a new shorter telomere length set point*

The T66A missense mutation in dyskerin culminates in DC in humans and is associated with a defect in telomere maintenance (229). Since threonine 66 is conserved in AtNAP57 (FIG. 15A), we asked whether an alanine substitution at this site would lead to a telomere-related phenotype in plants. Plants heterozygous for the T-DNA insertion in *AtNAP57* were transformed with an *AtNAP57* gene carrying the T66A mutation under the control of its native promoter (FIG. 15B). We expected to obtain plants heterozygous or homozygous with respect to the T-DNA insertion, and which also carried the exogenous T66A NAP57. Surprisingly, we failed to recover plants that were homozygous for the T-DNA insertion in *AtNAP57* and also contained the T66A transgene. Thus, the *AtNAP57* gene bearing the T66A mutation is unable to rescue plants homozygous for the T-DNA insertion.

To test the effect of T66A NAP57 on bulk telomere length, TRF analysis was performed on first generation of transformants expressing T66A NAP57 (T1). Based on previous transformation experiments (FIG. 12; refs. (284, 328)), we expected that any detrimental consequences of the T66A mutation would be evident in a population of 10-20 transformants (T1 generation). Accordingly, we examined twenty independent transgenic lines. While for most plants bulk telomeres were in the wild-type range (FIG. 15C, lanes 2 and 3; data not shown), a subset of telomeres in several plants were significantly shorter than wild-type and their shortest telomere tracts trailed down to below 1.6 kb in length (FIG. 15C, lanes 4 and 5). To determine whether telomeres would continue to shorten in subsequent generations, we monitored the progeny of one T1 plant (FIG. 15C, lane 4; FIG. 15D). For all of the T2 progeny, the shortest telomere tracts migrated below 2 kb (FIG. 15C, lanes 7-10), and for one plant, the range of telomere

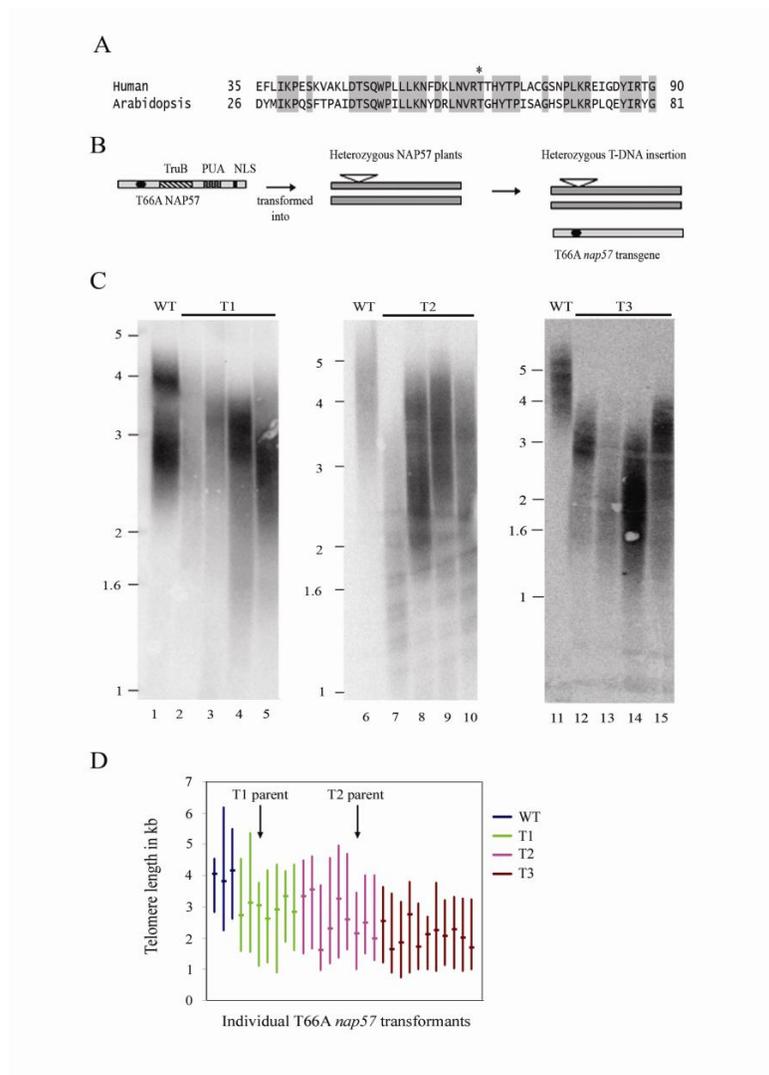


FIG. 15. The T66A mutation in AtNAP57 results in the establishment of a shorter telomere length set point. (A) Sequence alignment of human dyskerin and AtNAP57 proteins. Conserved residues are highlighted in gray boxes, and the threonine residue targeted for mutagenesis is denoted by an asterisk. (B) Overview of the process for introduction of the T66A mutation in AtNAP57 into *nap57*<sup>-/-</sup> plants. (C) TRF analysis of first, second, and third (T1, T2, and T3) generations of T66A transformants. The T1 plant whose telomeres were analyzed in the left panel, lane 4, was used as the parent for T2 progeny plants analyzed in the middle panel. The T2 plant represented in the middle panel, lane 7, was the parent for the T3 progeny analyzed in the right panel. DNA samples were not run as far into the gel shown the right panel as in the other two gels. (D) Graphic representation of bulk telomere length size range and peak telomere length (indicated by -) for WT and T66A transformants is shown. Arrows indicate telomere length measurements for plants used as T1 and T2 parents.

lengths was nearly identical to its T1 parent, spanning ~1.2 to 3.5 kb (FIG. 15C, compare lanes 4 and 7). When this T2 plant was propagated to T3, the telomere tracts of all four progeny were of similar lengths to their T2 parent (FIG. 15C, lanes 12-15; FIG. 15D). The loss of longer telomeres was more pronounced in T3 plants (FIG. 15D). The average bulk telomere length in wild-type plants (~ 4 kb) was reduced by 2 kb in T3 T66A mutants to ~2.1 kb (FIG. 15D). We detected no additional shortening in two T4 plants monitored (data not shown). Furthermore, none of the shortest telomeres fell below 1 kb in the four generations the transgenic plants were propagated (FIG. 15D). Thus, the T66A mutation perturbs telomere length regulation, but does not result in progressive telomere shortening. Instead, this mutation appears to promote the establishment of a new shorter length set point.

*The T66A mutation in AtNAP57 deregulates telomere length on individual chromosome ends*

To further examine telomere length dynamics in T66A *nap57* mutants, we monitored telomeres on three individual chromosome arms: the right arm of chromosome 2 (2R), the left arm of chromosome 3 (3L), and the right arm of chromosome 4 (4R) using Primer Extension Telomere Repeat Amplification (PETRA). As expected, PETRA produced a single diffuse band for each telomere in wild-type samples (FIG. 16A, lanes 1-3), consistent with tight regulation of telomere tracts on homologous chromosomes (283). A similar profile was observed in *nap57<sup>+/-</sup>* mutants (FIG. 16A, lanes 4-6). A different result was obtained with T66A *nap57* mutants. In the T1, T2 and T3 mutants that displayed shorter bulk telomere lengths, individual telomere tracts appeared as a cluster of several sharp bands (FIG. 16A, lanes 10-18; FIG. 16B).

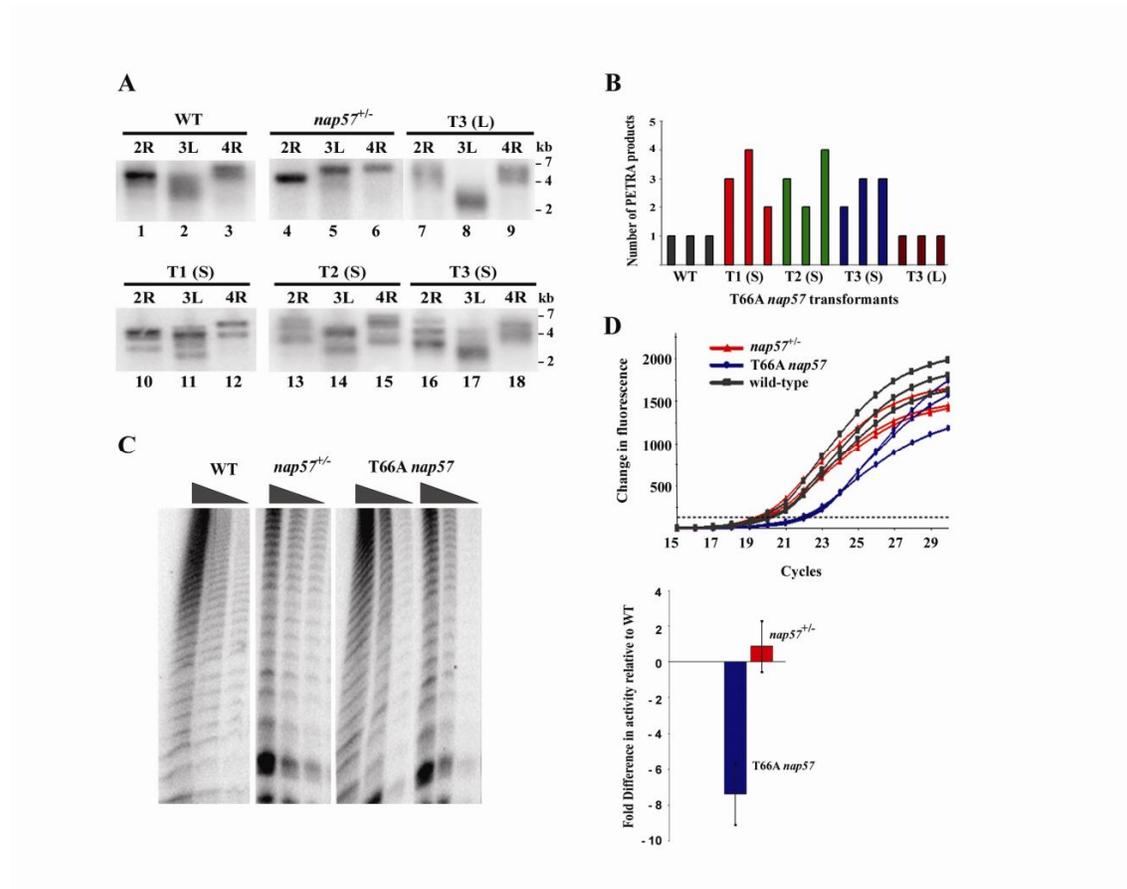


FIG. 16. The T66A mutation in AtNAP57 affects telomere length regulation on individual chromosome ends and decreases telomerase activity *in vitro*. (A) PETRA results are shown for the WT and individual *nap57<sup>+/-</sup>*, T1, T2, and T3 T66A *nap57* transformants with short telomeres (S) and a T3 T66A *nap57* transformant with wild-type-length telomeres (L). The telomeres monitored are indicated. 2R, right arm of chromosome 2; 3L, left arm of chromosome 3; 4R, right arm of chromosome 4. (B) Graphic representation of PETRA products obtained in each reaction as determined by visual inspection. (C) TRAP assay results for the WT and *nap57<sup>+/-</sup>* and T66A *nap57* transformants. Reactions were conducted using 1:50, 1:500, and 1:5,000 dilutions of protein extracts. (D) Results of real-time TRAP. The top panel shows raw data for three (each) of the WT and *nap57<sup>+/-</sup>* and T66A *nap57* transformants. The dashed line represents the threshold cycle for TRAP product detection. The bottom panel shows a histogram of the telomerase activity levels for *nap57<sup>+/-</sup>* and T66A transformants relative to those for the WT. Extracts from 10 individual plants from each genotype were monitored.

In contrast, the siblings of these plants whose bulk telomeres fell within the wild-type range showed a PETRA profile that more closely resembled to wild-type plants (FIG. 16A, lanes 7-9; FIG. 16B). This finding implies that the telomere length regulation on homologous chromosomes is perturbed in T66A *nap57* mutants. As discussed below, this phenotype is consistent with decreased telomerase activity.

*The T66A mutation in AtNAP57 decreases telomerase activity in vitro and in vivo*

To determine whether the T66A mutation in AtNAP57 decreased telomerase enzyme activity *in vitro*, TRAP assay was performed. Extract titration experiments revealed no detectable difference in the level of telomerase activity in wild-type versus *nap57<sup>+/-</sup>* mutants (FIG. 16C), supporting the conclusion that AtNAP57 is not haploinsufficient in *Arabidopsis*. In contrast, TRAP conducted at the highest dilution (1:5000) of protein extract reproducibly revealed decreased *in vitro* telomerase activity in T66A transformants relative to *nap57<sup>+/-</sup>* siblings (FIG. 16C). To more precisely gauge the level of telomerase activity in these mutants, we performed quantitative real-time TRAP following a method developed to monitor telomerase activity levels in human cells (137). As expected, we found no significant difference in telomerase activity in extracts prepared from *nap57<sup>+/-</sup>* versus wild-type plants. In contrast, T66A *nap57* mutants showed a seven-fold decrease in enzyme activity (FIG. 16D).

To examine the effect of the T66A *nap57* mutation on telomerase activity *in vivo*, we studied the consequences of this mutation in *ku70* mutants. KU70 is best known for its role in the non-homologous end joining (NHEJ) DNA repair pathway (266), but in *Arabidopsis* it also acts as a potent negative regulator of telomerase (96, 269). Telomeres in *ku70* mutants expand to two to three-fold the size of wild-type in a single generation and this elongation is dependent on telomerase (269, 328). Thus, we

predicted that incorporation of T66A *nap57* into the telomerase RNP would diminish the enzyme's ability to elongate telomeres in a *ku70* background. To test this hypothesis, we crossed the T66A *nap57* transformants with *ku70*<sup>-/-</sup> plants (FIG. 17A). In the first (F1) generation, we generated plants heterozygous for *AtKU70* and *AtNAP57* and selected for the T66A mutant transgene. In the second generation (F2), we recovered plants that were *ku70*<sup>-/-</sup> *nap57*<sup>+/-</sup> and carried the mutant T66A transgene. TRF analysis of the F2 population showed that *ku70*<sup>-/-</sup> *nap57*<sup>+/-</sup> plants elongated their telomeres to 5-8 kb (FIG. 17B, lane 8), while telomeres remained short in the presence of the T66A transgene (FIG. 17B, lanes 1-7). We conclude that expression of the T66A *nap57* allele prevents telomerase from hyper-elongating telomeres when its negative regulator *KU70* is inactivated. Altogether, our data argue that *AtNAP57* is essential for maximal activity of the *Arabidopsis* telomerase RNP *in vivo*.

## Discussion

### *A conserved pathway for telomerase biogenesis in higher eukaryotes*

The telomerase RNP is evolving at a rapid pace. The TR and TERT subunits have diverged dramatically, and a distinct set of proteins has emerged in higher and lower eukaryotes to promote RNP biogenesis and enzyme action at the chromosome terminus (48, 55). The yeast TR (TLC1) bears a Sm-protein binding motif and has adopted an RNP biogenesis scheme similar to snRNPs (282), while vertebrate TRs have acquired a 3' H/ACA box domain found in snoRNAs and are bound by the dyskerin complex. Thus, although dyskerin's function in catalyzing pseudouridylation of ribosomal RNAs is conserved, in mammals it has evolved an additional, more specialized role as an integral component of the telomerase RNP (53, 229).

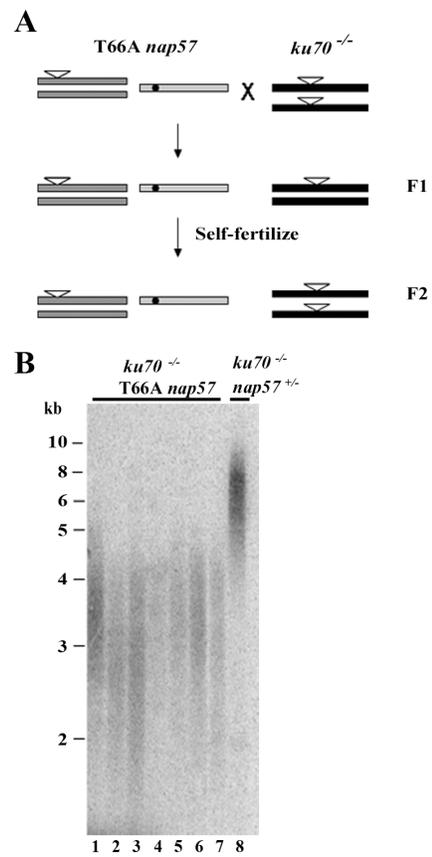


FIG. 17. The T66A mutation in AtNAP57 reduces telomerase activity *in vivo*. (A) Schematic diagram of genetic crossing scheme to generate  $ku70^{-/-}$  mutants carrying the T66A *nap57* allele. (B) TRF analysis of seven  $ku70^{-/-}$  T66A *nap57* plants and one  $ku70^{-/-}$  *nap57^{+/+}* control plant is shown.

In this study we provide several lines of evidence that telomerase enzymes from higher eukaryotes share a requirement for dyskerin. First, we found that AtNAP57 and AtTERT proteins co-localize to the nucleolus in *Arabidopsis*. Nucleolar localization of telomerase could be especially advantageous for *Arabidopsis*, since telomeres cluster at the nucleolar periphery (9). Second, we showed that AtNAP57 physically associates with enzymatically active telomerase particles. The major interaction partner for AtNAP57 in the plant telomerase RNP is likely to be TR, since AtNAP57 association with telomerase is abolished following RNase A treatment. Intriguingly, we also discovered a novel, but weak, interaction for AtNAP57 with AtPOT1a. AtPOT1a is an OB-fold bearing protein that physically interacts with catalytically active telomerase in *Arabidopsis* and promotes enzyme function *in vitro* and *in vivo* (296). Recent studies indicate that human telomerase associates with TPP1, an hPOT1 binding partner that also harbors a OB-fold motif (324, 344). Whether the human dyskerin contacts TPP1 is unknown. Third, and most importantly, we demonstrated that AtNAP57 is crucial for the function of *Arabidopsis* telomerase. Transgenic plants bearing a mutant AtNAP57 allele display reduced levels of telomerase activity *in vitro* and perturbed telomere length regulation *in vivo* (see below).

#### *Arabidopsis is not haploinsufficient for its known telomerase components*

Essential components of the telomerase RNP are limiting in mammals and yeast. In both *terc*<sup>+/-</sup> (TR) and *tert*<sup>+/-</sup> ES mouse cells, telomere maintenance is compromised (76, 128, 194). Indeed, haploinsufficiency of hTR is directly linked to autosomal dominant DC and the reduced hTR levels along with shorter telomeres in these patients results in disease anticipation (321). Similarly, recent studies indicate that TLC1, the TR component in *S. cerevisiae*, is haploinsufficient for telomere maintenance (235).

Moreover, yeast heterozygous for both TLC1 and EST1, a telomerase-associated protein, exhibit a phenomenon referred to as additive haploinsufficiency, where telomere tracts are even shorter than in either single heterozygote (180, 189).

In contrast, the known telomerase-associated proteins in *Arabidopsis*, AtTERT (84), AtPOT1a (296), and AtNAP57 (this study) are not haploinsufficient for telomere maintenance. Plants heterozygous for these components display wild-type levels of telomerase activity *in vitro*, and maintain telomeres in the wild-type size range through multiple generations. While it is possible that TR will prove to be present in limiting quantities, *Arabidopsis* may simply require a very low level of telomerase to maintain genome stability. We note that the *Arabidopsis* genome is comprised of only 10 chromosomes (twenty telomeres), significantly fewer than in diploid budding yeast (64 telomeres) or in human (92 telomeres) cells.

*The T66A mutation in AtNAP57 acts as a dominant negative allele to decrease telomerase activity in vitro and in vivo*

The T66A mutation in human dyskerin leads to DC through reduction in the steady state level of hTR, decreased telomerase activity and progressive telomere shortening (229). To determine if *Arabidopsis* would exhibit similar defects in telomerase, we generated transgenic plants harboring the corresponding mutant allele. As for humans (229), the T66A mutation in AtNAP57 did not grossly affect rRNA processing in *Arabidopsis* (K. Kannan and D. Shippen, unpublished data). Nonetheless, this mutation is highly deleterious; expression of this allele could not rescue the lethality associated with plants lacking both copies of the wild-type AtNAP57. Furthermore, although plants harboring one wild-type copy of AtNAP57 and the T66A *nap57* transgene were viable, they displayed decreased telomerase activity both *in vitro* and *in*

*vivo*. We suspect that this outcome is a consequence of reduced stability of telomerase RNA, but testing this hypothesis awaits identification of this molecule. Nonetheless, in marked contrast to the fate of human telomeres in T66A DC cells (229), telomeres in T66A *nap57* transgenic plants did not undergo progressive shortening. Instead, telomeres were stably maintained at a length approximately 2 kb shorter than in wild-type.

Data from PETRA on individual chromosome ends argue that this new length set point for telomeres is a consequence of limiting telomerase activity. In the PETRA assay, wild-type telomeres on homologous chromosomes appear as a single heterogeneous band (FIG. 16A, ref.(283)). Because DNA is analyzed from an entire plant, these results mean that individual telomere tracts are subjected to extremely tight regulation during plant growth and development (283). Strikingly, in T66A *nap57* mutants PETRA generates a complex profile of multiple sharp bands, indicating that telomere length is deregulated on individual chromosome ends. Previous studies in yeast (304), mammals (135), and in *Arabidopsis* (283) show that telomerase acts preferentially on the shortest telomeres in the population. We hypothesize that substrate preference is exacerbated in plants with reduced levels of telomerase (e.g. T66A *nap57* mutants), resulting in elongation of only a subset of telomeres in a fraction of cells. Bulk telomeres can then establish a new length set point when equilibrium between the compromised telomerase and forces that shorten telomeres (eg. the end-replication problem, nuclease action and recombination) is attained. Because the demand for telomerase activity is significantly greater in humans, we suspect such cells bearing the T66A *nap57* mutation fail to achieve a new telomere length set point and suffer progressive telomere erosion.

How does the T66A mutation in AtNAP57 inhibit telomerase activity in *Arabidopsis*? Since AtNAP57 is not haploinsufficient for telomerase function in plants,

but telomerase is inhibited when the T66A *nap57* allele is introduced into this background, the data argue that T66A NAP57 acts as a dominant negative inhibitor. Catalytically active human telomerase is a 670 kDa dimer comprised of two TERT, two TR, and two dyskerin molecules (53). *Arabidopsis* telomerase is approximately the same molecular mass and like hTERT (20), AtTERT is capable of dimerization *in vitro* (C. Cifuentes-Rojas, K. Kannan and D. Shippen, unpublished data). Thus, the plant telomerase may also harbor two copies of AtNAP57. Accordingly, incorporation of a mutant form of this protein into the telomerase RNP may compromise enzyme function.

**CHAPTER III**  
**IDENTIFICATION AND CHARACTERIZATION OF *ARABIDOPSIS* TELOMERASE**  
**RNA SUBUNITS**

**Summary**

The ends of eukaryotic chromosomes are capped with repetitive G-rich telomeric repeats that are necessary for genome stability. Telomeres are primarily maintained by the specialized reverse transcriptase, telomerase. Telomerase has a catalytic component, TERT, and an intrinsic RNA subunit, TER, that serves as the template for TERT. Here, we describe the identification and characterization of the TER subunit in the model plant, *Arabidopsis thaliana*. Biochemical purification of *Arabidopsis* telomerase led to the identification of two highly related RNAs termed TER<sub>1G7</sub> and TER<sub>5G2</sub>. The two TERs display differences in expression levels and interaction partners. Reconstitution experiments reveal that both TERs can function as templates for telomerase *in vitro*. Expression of TER<sub>1G7</sub> containing a mutant template in plants led to generation of mutant telomerase activity indicating that TER<sub>1G7</sub> acts as a template for telomerase *in vivo*. Genetic analysis of TERs indicates that both RNAs function redundantly in telomere length maintenance. Antisense experiments suggest that telomerase activity and telomere length maintenance are affected in plants with reduced TER levels. Thus, the finding of two TERs in *Arabidopsis* reveals unanticipated complexity in telomerase RNP composition and regulation.

**Introduction**

The end replication problem refers to the inability of the conventional DNA replication machinery to completely replicate the ends of eukaryotic chromosomes (289).

Without a solution to this problem, terminal chromosome sequences will be lost after every round of replication, ultimately leading to cell arrest or death. Eukaryotic organisms can avert this disastrous outcome by the addition of short G-rich repeats called telomeres to the ends of their chromosomes. Telomeres are primarily maintained by the enzyme telomerase. The action of telomerase, together with telomere-binding proteins, enables organisms to protect chromosome ends from debilitating activities such as chromosomal fusions and nucleolytic degradation, and to maintain genome integrity.

Telomerase is a ribonucleoprotein that was first identified in the ciliate *Tetrahymena thermophila* (117). It principally consists of two subunits: the catalytic telomerase reverse transcriptase (TERT) and the telomerase RNA (TER). These two subunits are sufficient to reconstitute telomerase activity *in vitro* when expressed in a cell free eukaryotic system such as rabbit reticulocyte lysate (331). *In vivo*, several additional proteins are necessary for maximal telomerase activity. For example, Est1 and Est3 subunits of the *S. cerevisiae* telomerase function to regulate access of telomerase to the telomere *in vivo*, but are not required *in vitro* (54, 146).

The addition of G-rich telomeric repeats is specified by a short template region in the telomerase RNA that is complementary to 1.5 telomere repeats (reviewed in (24)). TERs have no significant primary sequence conservation (55). However, phylogenetic analysis of ciliate, vertebrate and yeast TERs has led to the identification of several conserved elements of secondary structure (50, 68, 272, 352). These conserved elements are required for telomerase enzyme activity and TERT binding. TER subunits diverge in domains that are required for species-specific biogenesis and regulation. For example, hTR is similar to ciliate TER in secondary structure except that its 3' region contains motifs necessary for the binding to the protein dyskerin (229). Dyskerin is a

component of the core telomerase complex in humans (53). Mutations in any of the core telomerase components (TER, TERT and dyskerin) can lead to the devastating disease dyskeratosis congenita (DC) (210). DC results in loss of telomere maintenance and this in turn affects the regenerative capacity of cell types such as bone marrow cells (5, 229, 320). Bone marrow failure is the cause of death in DC patients (210).

Telomere structure is conserved throughout eukaryotes. The telomere repeat in most plants, TTTAGGG (263), differs from the human repeat, TTAGGG, by only a single nucleotide. Interestingly, some members of the plant kingdom display different telomere repeats. For example, some species in the monocot order of Asparagales have the human type telomere repeat, while members of the Alliaceae family including *Allium cepa* (onion) lack any of the known telomere repeat sequences (298). These changes in the telomere repeat are most likely due to mutations in the template of TERs, but so far TER homologs have not been identified from any plant species.

Several proteins that play a role in telomere biology have been identified in *Arabidopsis*. Recent work has shown that the *Arabidopsis* telomerase RNP consists of the proteins AtTERT, AtPOT1a and AtNAP57(dyskerin) (84, 153, 296). Loss of AtTERT results in progressive telomere shortening that leads to genome instability and ultimately complete sterility and developmental arrest in the tenth generation (268). *tert* mutants lose an average of 200-500 bp per generation (84). The same rate of loss of telomere length is seen in *pot1a* mutants (296). AtPOT1a was identified based on similarity to a family of single-strand telomere binding proteins characterized by an oligosaccharide /oligonucleotide binding fold (OB-fold). AtPOT1a is a positive regulator of telomere length and a telomerase RNP component that acts in the same genetic pathway as AtTERT (296). AtNAP57 is a homolog of human dyskerin recently found to be a conserved component of the plant telomerase RNP (153). As in mammals, mutations in

AtNAP57 affect telomere maintenance in plants. AtTERT, AtPOT1a and AtNAP57 display only weak interactions with each other *in vitro* (153, 296). Therefore, it is possible that as in the case of *S. cerevisiae* TLC1 (TER) (352), *Arabidopsis* TER is a bridging factor that brings the protein components of the RNP together for telomerase to function.

To identify the *Arabidopsis* TER subunit, biochemical purification of telomerase from suspension cell culture was performed (C. Cifuentes-Rojas and D. Shippen, unpublished data). We show that *Arabidopsis* is a unique model system for telomere biology as it harbors not one, but two distinct TERs. Biochemical purification of telomerase indicated that both TERs co-purify with enzyme activity. Interestingly, the template sequence in the two RNAs is identical but is situated at different positions in the two RNAs. *In vitro*, both TERs interact with AtTERT to reconstitute telomerase activity. The two TERs are expressed at dramatically different levels in plants, but display the same overall pattern with the highest levels of RNA found in telomerase positive organs. T-DNA mutations in either TER<sub>1G7</sub> or TER<sub>5G2</sub> genes have no obvious effect on telomere length. However, antisense experiments indicate that reducing the levels of both TERs decreases telomerase activity, implying that the two RNAs may play overlapping roles in promoting telomerase activity. Interestingly, the two TERs interact with a different set of proteins *in vitro* and *in vivo*, suggesting that this may be a means of regulating telomerase.

## **Materials and Methods**

### *Plant materials and genotyping*

*Arabidopsis* seeds with T-DNA insertions in TER<sub>1G7</sub> (SAIL\_25\_H08 and SALK\_089362) and TER<sub>5G2</sub> (SAIL\_556\_A04) genes were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH). The seeds were cold

treated overnight at 4°C, and then placed in an environmental growth chamber and grown under a 16-h light/8-h dark photoperiod at 23°C. For genotyping, DNA from flowers was extracted and PCR was performed with the following sets of primers: for TER<sub>1G7</sub>, LP (5' GAAAGACCTCAGCATCAGTGC 3') and RP (5' GGACTTTTTGAAAACAATTACAAATC 3'); for At*KU70*, Ku 1 (5' TTACTTTGTTGTTTCGGGTGC 3') and Ku 2 (5' CTCTTGGCAAGTACACGCTTC 3'). For TER<sub>5G2</sub>, 5G2 38 (5' GACGACA ACTAAACCCTACGCTTACA 3') and 5G2 45 (5' CGATGTTGTTTTCTGCTTAGGACACA 3') primers were used for amplifying the product from the wild-type allele. For the allele with the T-DNA insertion, the T-DNA primer was used along with 5G2 8526-01 fwd (5' GAGACGCAGCGAGCGATAGCCGATAG 3') primer.

Homozygous TER<sub>1G7-1</sub> and TER<sub>5G2-1</sub> mutants were crossed to obtain double heterozygous plants. The progeny of these plants were genotyped in order to find homozygous mutations in both TER<sub>1G7</sub> and TER<sub>5G2</sub>.

#### *Antisense RNA constructs, site-directed mutagenesis and plant transformation*

For antisense constructs, full-length TER genes were cloned into the gateway destination vector pB7WG2 in the antisense orientation. The constructs were then introduced into *Agrobacterium tumefaciens* strain GV3101. At*Ku70* heterozygous plants were transformed by the in planta method (269). Transformants were selected on 0.5 Murashige and Skoog basal medium supplemented with 20 mg/liter of phosphinothricin (Crescent Chemical) and kanamycin (50 µg/ml) and then genotyped.

To generate template mutations in TER<sub>1G7</sub>, site-directed mutagenesis was performed with *Pfu* turbo polymerase (Stratagene) on TER<sub>1G7</sub>-pDONR221 using the primers M1: 5' GCCTATCAGACGACA ACTAAAGGCTACACGCTTACA 3' and M2: 5'

TGTAAGCGTGTA GCCTTTAGTTGTCGTCTGATAGGC 3' according to the manufacturer's guidelines.

#### *Purification of telomerase*

*Arabidopsis* suspension culture cells were maintained as described previously (222). Telomerase was purified from nuclei extracted from *Arabidopsis* cell culture. Briefly, cells were collected from 4.5 L of cell culture and approximately 320 g of dry tissue were ground in liquid nitrogen. The resulting powder was resuspended in nuclei isolation buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA, 10 mM KCl, 250 mM sucrose, 1.5 mM MgCl<sub>2</sub>, 0.3% Triton X-100, 1 mM DTT, 1 mM spermine, 1 mM spermidine, 10 mM ribonucleoside vanadyl complex, 1X complete protease inhibitors (Roche), 0.4 mM Pefablock SC Plus (Roche)) and spun at 4000 xg for 20 minutes. The pellet was resuspended in nuclei isolation buffer containing 1% Triton X-100 and spun at 2000 xg for 1 min and then at 4000 xg for 1 min. The pellet was resuspended in nuclei extraction buffer (20 mM Hepes pH 8.0, 1.5mM MgCl<sub>2</sub>, 0.2 mM EDTA, 300 mM NaCl, 10% glycerol, 1% triton X-100, 0.1% NP40, 5mM DTT, 10 mM ribonucleoside vanadyl complex, 1X complete protease inhibitors (Roche), 0.4 mM Pefablock SC Plus (Roche)), incubated with rotation at 4°C for 30 min and spun for 15 min at 14000 xg at 4°C. The supernatant was collected, loaded into a 10 ml Q-sepharose column (Amersham Pharmacia) and eluted using a 100 mM-1 M NaCl gradient at 2 ml/min. The fractions were collected and tested for telomerase activity by TRAP assay. The peak of activity fractions were dialyzed to 100 mM, loaded in a 5 ml Heparin Agarose column (Amersham Pharmacia) eluted using a 100mM-1M NaCl gradient at 2 ml/min. The fractions were collected and tested for telomerase activity by TRAP assay.

### *RNA isolation*

RNA was extracted from the heparin column peak of activity fractions using a buffer containing 50 mM Tris HCl pH 9.0, 100 mM NaCl, 2% SDS, 10 mM EDTA and 20 mM  $\beta$ -Mercaptoethanol and standard acid phenol:chloroform extraction was performed. Samples were precipitated at  $-80^{\circ}\text{C}$  in ethanol, sodium acetate and glycogen and spun at 14000 xg for 30 min at  $4^{\circ}\text{C}$ . The pellet was washed with cold 70% ethanol, air dried at room temperature and resuspended in Tris-EDTA buffer. RNA was 3' end labeled with Cytidine-3',5' - bis (phosphate), [5'- $^{32}\text{P}$ ] (Molecular Probes) in an standard overnight T4 RNA ligation reaction and resolved in a 6% acrylamide gel. All the labeled RNAs were individually isolated and a RT-PCR performed, using penta-decamers for first strand synthesis using SuperScript III (Invitrogen). cDNA was sequenced using seven different permutations of the predicted *Arabidopsis* RNA template (CUAAACCCUA) and the resulting sequences were used in a BLAST search against the *Arabidopsis* transcripts and nucleotide collection databases.

### *TRF analysis, TRAP and real time TRAP assay*

TRF and TRAP assays were performed as previously described (83, 84). TRAP products from mutant telomerase were amplified using the primers mutant forward (5' CACTATCGACTA CGCGATTAG 3') and mutant reverse (5' GGCTAAAGGCTAAAGGCTAAAG 3').

Real time TRAP was performed as described previously (137), but with the following modifications. 10.5  $\mu\text{l}$  of a 4.8 ng/ $\mu\text{l}$  protein extract dilution, 1  $\mu\text{l}$  of 10  $\mu\text{M}$  forward primer (5' CACTATCGACTACGCGATCAG 3'), and 12.5  $\mu\text{l}$  SYBR Green PCR Master Mix (NEB) were incubated at  $37^{\circ}\text{C}$  for 45 min. 1  $\mu\text{l}$  of reverse primer (5' CCCTAAACCCTAAACCCTAAA 3') was added and products were amplified for 35 PCR

cycles with 30 sec at 95°C and 90 sec at 60°C. Threshold cycle values ( $C_t$ ) were calculated using an iCycler iQ thermal cycler (BIO-RAD) and the supplied Optical System Software. Samples were analyzed in triplicate.

*RT-PCR, real-time RT-PCR and RNA 5' and 3' mapping*

Total RNA was extracted from 0.5 g of flowers or other tissue using Tri Reagent (Sigma). cDNAs were synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen). Random pentadecamers were incubated with 2 µg of total RNA in the supplied buffer at 65°C for 5 min. Reverse transcription (RT) was carried out with 100 U of Superscript III at the following temperatures 37°C for 20 min, 42°C for 20 min and 55°C for 20 min. Enzyme was inactivated by incubation at 70°C for 10 min and subsequently RNA was degraded with RNase H (NEB). 1.5 µl of cDNA was used in PCR.

For real-time RT-PCR, 2 µl of the above cDNA was used at a 1:10 dilution in a 20 µl reaction containing 10 µl of SyBr green Master mix (NEB), 2 µl of each primer (2 µM) and 4 µl of water. PCR was performed for 40 cycles with 30s at 95°C and 60s at 60°C.

Primers used for real-time PCR are as follows: 1G7 Q4F: 5'

CCCATTTTCGTGCCTATCAGACGAC 3' and 1G7 Q4R: 5'

TCTCCGACGACCATTCTCTCGATAC 3'; 5G2 38: 5'

GACGACAATAAACCCCTACGCTTACA 3' and 5G2 40: 5'

CAGGATCAATCGGAGAGTTCAATCTC 3'; Actin 2F 5'

TCCCTCAGCACATTCCAGCAGAT 3' and Actin 2R 5'

AACGATTCTGGACCTGCCTCATC 3';  $\beta$ -6-tubulin F 5'

ACCACTCCTAGCTTTGGTGATCTG 3' and  $\beta$ -6-tubulin R 5'

AGTTCACTGCGAGCTTCCTCA 3'

For identification of the 5' and 3' ends of RNAs, total RNA from the heparin column peak of activity fraction were ligated at the 3' end to a primer (miRNA linker-1 (IDT)). cDNA was synthesized with a primer specific to the linker and circularized using CircLigase (Epicentre) according to the manufacturer's protocol. PCR was performed using primers 301fwd (5' ACAGAGAACGATGTTCCAAC 3') and template rev (5' CTCCTTGAGAATCTCAGCGAGT 3') for TER<sub>1G7</sub>. Products were then sequenced.

#### *Antibodies, western blotting and immunoprecipitation*

Antibodies to AtKu70 were a gift from Dr. Karel Riha (Gregor Mendel Institute, Vienna). A peptide antibody against *Arabidopsis* ATR was raised in rabbits and affinity purified (Covance). The peptide used was [C]-GKKRHIEDESTYKRKRQKV corresponding to residues 419-437 of ATR.

*Arabidopsis* suspension culture cells were maintained as described (222). Immunoprecipitation experiments using cell culture were performed following the protocol in (153). Western blotting was performed with a 1:2000 dilution of anti-Ku70 antibody and a 1:10,000 dilution of peroxidase-conjugated light chain-specific mouse anti-rabbit secondary antibodies (Jackson Immunoresearch).

#### *Telomerase reconstitution*

For telomerase reconstitution experiments, TERT-pET28a plasmid with an N-terminal T7 tag was used. Reactions were assembled with 8 µl of a mix containing Rabbit reticulocyte lysate (Promega), amino acids, RNase inhibitors, T7 RNA polymerase, 100 ng of TERT-pET28a plasmid and 0.5 pmol of gel purified DNA template encoding TER driven by a T7 promoter. The reactions were incubated for 90 min at 30°C. In parallel, T7 agarose beads (Novagen) were blocked with buffer W-100 (20 mM

TrisOAc [pH 7.5], 10% glycerol, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.2 M NaCl, 1% NP-40, 0.5 mM sodium deoxycholate, and 100 mM potassium glutamate) containing 0.5 mg/ml BSA, 0.5 mg/ml lysozyme, 0.05 mg/ml glycogen, 1 mM DTT and 1 µg/ml yeast tRNA. Beads were blocked four times for 30 min and after the last blocking; beads were spun down and resuspended in W-100 to get a final volume of 200 µl per reaction. After 90 min of incubation, the RRL mix was added to the beads and incubated for 2h at 4°C with rotation. Beads were then washed with 800 µl of W-300 buffer (W-100 containing 300 mM potassium glutamate) for five times and with 800 µl of TMG buffer (10 mM TrisOAc [pH 7.5], 1 mM MgCl<sub>2</sub>, and 10% glycerol) for three times. After the final wash, beads are left with about 30 µl of TMG. 2 µl of beads were used for TRAP assays as in reference (83).

## Results

### *Identification of TER<sub>1G7</sub> and TER<sub>5G2</sub>*

Several bioinformatics approaches to identify candidate TERs were tried but were unsuccessful (Appendix A). As an alternative strategy, we undertook a biochemical approach (details in Materials and methods) to identify the *Arabidopsis* TER from *Arabidopsis* suspension cell culture (83) (C. Cifuentes-Rojas and D. Shippen, unpublished data). The expected template sequence for the *Arabidopsis* TER is 5' CUAACCCUA 3' corresponding to 1.5 telomere repeats. This sequence was found in one RNA that co-purified with telomerase activity after column purification. BLAST search revealed a hit for this RNA in the 5' region of At1g71310 on chromosome 1. Interestingly, BLAST also identified a second related gene (At5g24670) on chromosome 5 that also contained the predicted template. RT-PCR revealed that RNAs corresponding to the sequence identified by BLAST were enriched in highly purified

telomerase fractions (C. Cifuentes-Rojas and D. Shippen, unpublished data). The two RNAs were named TER<sub>1G7</sub> and TER<sub>5G2</sub> based on their chromosomal positions.

The 5' and 3' ends of the RNAs were mapped and the sizes of TER<sub>1G7</sub> and TER<sub>5G2</sub> were found to be 748 and 784 nts respectively (C. Cifuentes-Rojas and D. Shippen, unpublished data). In TER<sub>5G2</sub>, the putative template is only 8 nts from the 5' end of the RNA (FIG. 18A). This is similar to the template in mouse TER (50). In TER<sub>1G7</sub>, the putative template is located 241 nts away from the 5' end of the RNA, more like the human TER. Interestingly, TER<sub>1G7</sub> and TER<sub>5G2</sub> share 90% identity in a 220 nt region that includes the putative template; however in TER<sub>5G2</sub> this region is separated by an insertion that is not shared (FIG. 18A). The first 144 nts of TER<sub>5G2</sub>, including the template, are 85% identical to TER<sub>1G7</sub> and the region encompassing the nts 694-748 in TER<sub>5G2</sub> shares 96% identity with TER<sub>1G7</sub> (FIG. 18B).

The two TERs display dramatic differences in expression levels. Real time RT-PCR revealed that both TER<sub>1G7</sub> and TER<sub>5G2</sub> are expressed in all tissues and follow a similar pattern of expression with the peak of expression being in flowers and cell culture (FIG. 19A). Interestingly, TER<sub>5G2</sub> is expressed at ~ 13 fold lower levels than TER<sub>1G7</sub> in flowers, cauline and rosette leaves. In stems, the difference in expression levels between TER<sub>1G7</sub> and TER<sub>5G2</sub> is about 30 fold; while in cell culture, the difference is approximately 60 fold. The increased levels of TERs in flowers and cell culture coincides with the abundance of telomerase activity in these cells (FIG. 19B) (83). In contrast, in telomerase negative tissues (leaves and stem), the expression levels of TERs are correspondingly lower. The difference in the expression levels of TERs may be important for their function *in vivo*.

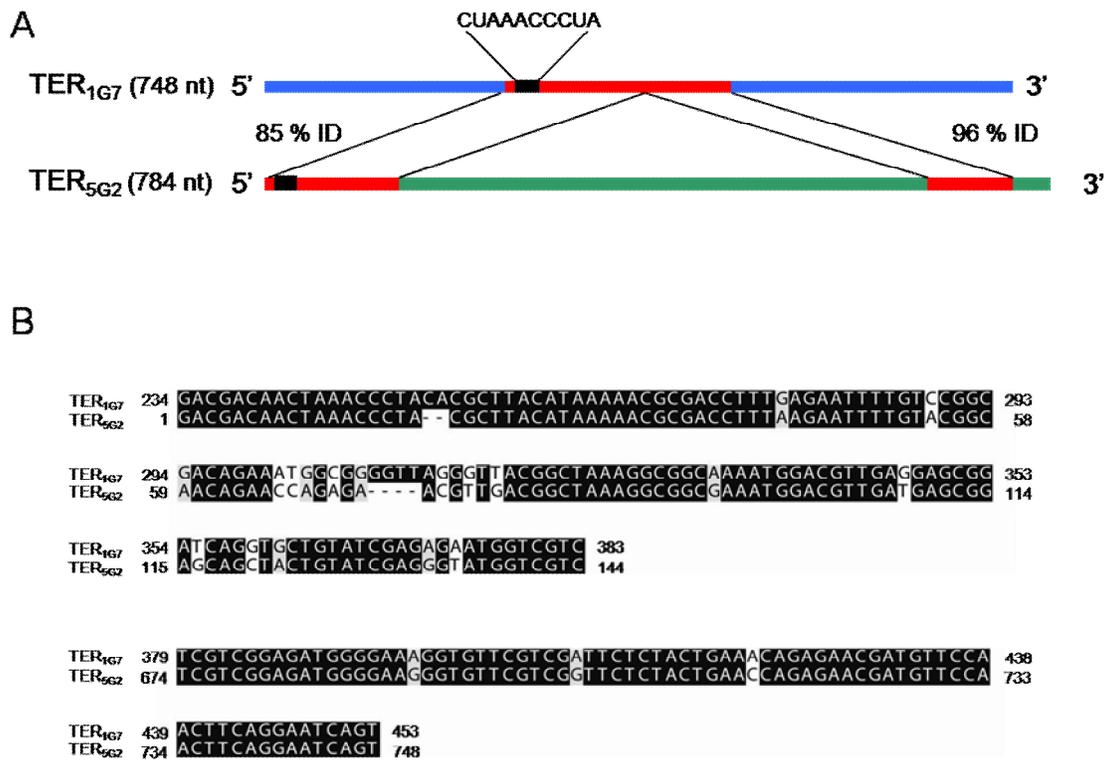


FIG. 18. *Arabidopsis* contains two TER genes. A. Schematic representation of TER<sub>1G7</sub> and TER<sub>5G2</sub> genes. Red region which includes the template (black box) represents the region shared by the two genes. In TER<sub>5G2</sub>, this region is interrupted by an insertion. B. Alignment of regions shared by TER<sub>1G7</sub> and TER<sub>5G2</sub>. The two TERs share 85% identity in the first region and 96% identity in the second region.

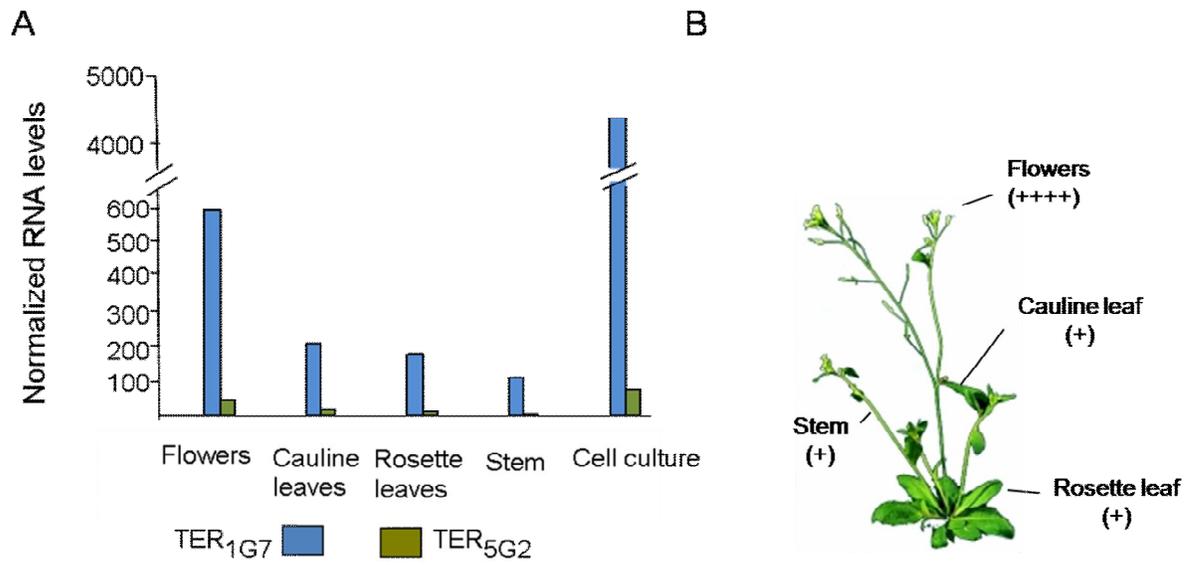


FIG. 19. TERs are expressed at different levels in plant cells. A. Graphical representation of TER<sub>1G7</sub> and TER<sub>5G2</sub> levels in plant tissues and cell culture as determined by real-time RT-PCR. TER levels were normalized to actin mRNA in all cases. B. Telomerase activity levels in different plant tissues are indicated by '+' signs. Telomerase activity is abundant in flowers and cell culture.

*TER<sub>1G7</sub> and TER<sub>5G2</sub> reconstitute telomerase activity in the presence of AtTERT*

To determine whether the TER candidates could serve as a template for TERT *in vitro*, they were used in an *in vitro* telomerase reconstitution assay. Human and ciliate telomerase are reconstituted with TERT and TER expressed in rabbit reticulocyte lysate (RRL) (10, 331). Therefore, each *Arabidopsis* TER was expressed from a PCR product containing a T7 promoter along with T7-tagged AtTERT in RRL. Immunoprecipitation was performed with a T7 antibody followed by TRAP. Co-expression of either TER with AtTERT gave rise to a ladder of TRAP products (FIG. 20, lanes 3 and 6), whereas incubation of RRL with TER (FIG. 20, lane 2) or AtTERT alone (FIG. 20, lane 1) did not result in telomerase activity. Furthermore, addition of RNase A to the reaction mixture abolished TRAP activity as expected (FIG. 20, lanes 4 and 7). Treatment with both RNase A and RNase inhibitors simultaneously prevented degradation of TER and restored activity (FIG. 20, lanes 5 and 8). The above experiments suggest that reconstitution of telomerase activity is dependent on both intact TER and TERT.

When reconstitution experiments were performed with AtTERT and an antisense version of TER<sub>1G7</sub> (FIG. 20, lane 9) or another non-coding RNA that contained a telomere template sequence (FIG. 20, lane 10), telomerase activity was not observed. These data indicate that telomerase reconstitution is specific for either TER<sub>1G7</sub> and TER<sub>5G2</sub> and not any RNA with a template region. Other controls confirmed that telomerase activity was dependent on AtTERT and could not be substituted by another telomeric protein (FIG. 20, lane 11). Thus, both TER<sub>1G7</sub> and TER<sub>5G2</sub> are capable of providing a template for TERT during telomere synthesis *in vitro*.

Striking results were obtained when reconstitution experiments were performed with varying amounts of PCR template (C. Cifuentes-Rojas and D. Shippen,



unpublished data). For reconstitution with TER<sub>1G7</sub>, the lowest amount of the PCR product that supported detectable telomerase activity in our assay was 0.1 pmol. When TER<sub>1G7</sub> PCR product concentration was increased, a corresponding increase in activity was observed. Reconstitution with TER<sub>5G2</sub> followed a different pattern. Preliminary titration experiments showed no telomerase reconstitution with 0.1 pmol of the TER<sub>5G2</sub> PCR product. However, when the concentration of TER<sub>5G2</sub> PCR product was decreased to 1 fmol, robust activity is obtained (C. Cifuentes-Rojas and D. Shippen, unpublished data). Thus, the biochemical properties of the two TERs appear to be different. Moreover, the concentration dependence of these reactions suggest that TER<sub>5G2</sub> may be active as a monomer and TER<sub>1G7</sub> as a dimer.

#### *Template mutations in TER<sub>1G7</sub>*

To ask if the template sequence within TER<sub>1G7</sub> serves as a template for telomerase *in vivo*, we made a mutation in this region of TER<sub>1G7</sub> that should promote incorporation of mutant telomeric repeats. The template of TER<sub>1G7</sub> was mutated from CTAAACCCTA to CTAAAGGCTA by site-directed mutagenesis. The expected mutant telomeric repeat is TTTAGCC. The mutated TER<sub>1G7</sub> will be referred to as TER<sub>1G7-CC</sub>.

To verify that TER<sub>1G7-CC</sub> could reconstitute telomerase activity with AtTERT *in vitro*, reconstitution was performed as described above, but with a reverse primer corresponding to the mutant repeat. TRAP products were not amplified in this reaction (data not shown). The 3' terminus of the original TRAP forward primer (5' CACTATCGACTACGCGATCAG 3') forms two base-pairs with the template of wild-type TER<sub>1G7</sub> and TER<sub>1G7-CC</sub> (FIG. 21A, panel 1 and 2). We reasoned that the primer contacts with the template of TER<sub>1G7-CC</sub> might not suffice for proper alignment and extension. Therefore, a point mutation was introduced into the TRAP forward primer (5'

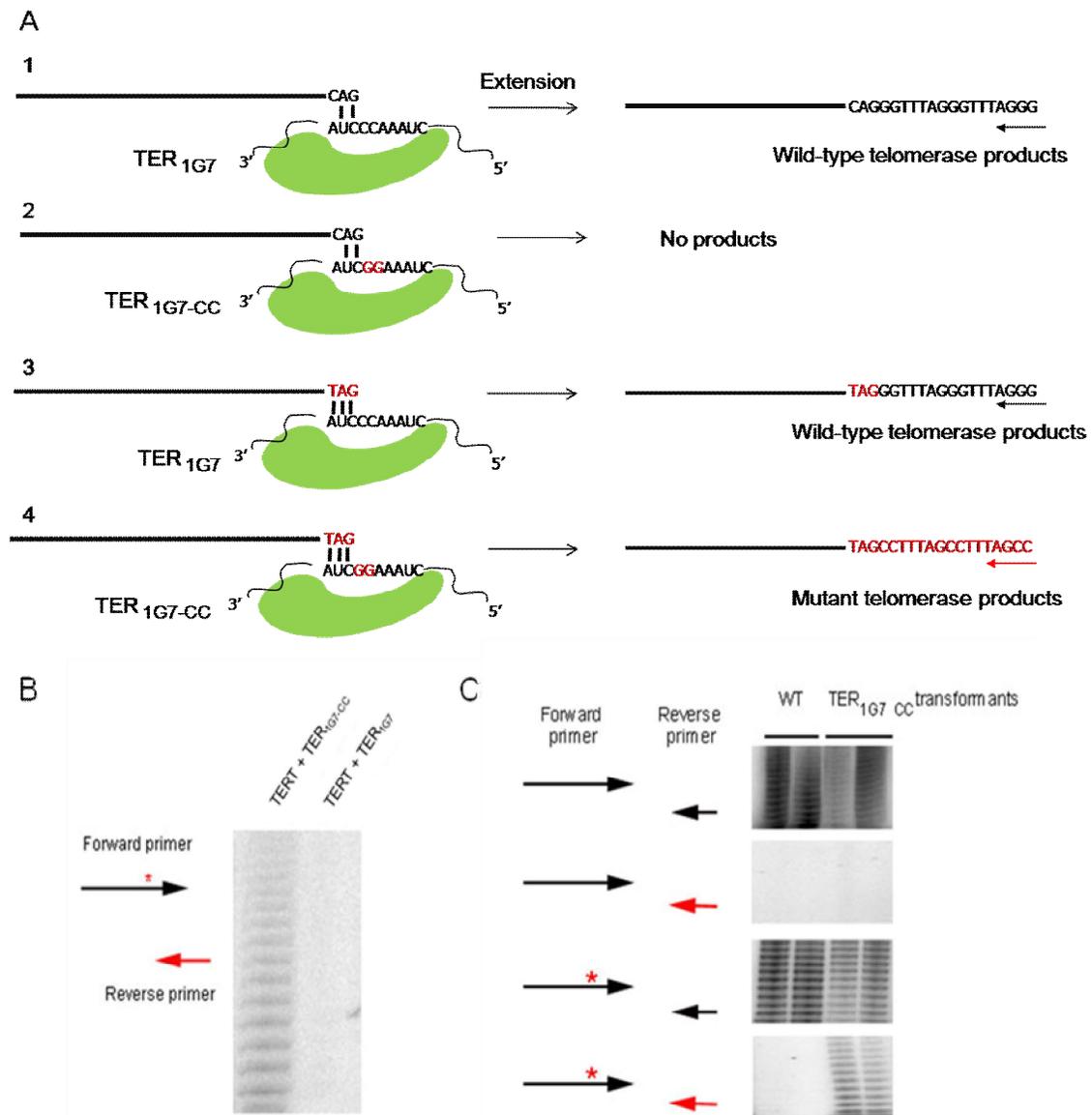


FIG. 21. Template mutation in TER<sub>1G7</sub>. A. Schematic showing alignment of original forward primer (black nucleotides) and mutant forward primer (ending in red nucleotides) and with either wild-type template (black) or mutant template (red). After extension by telomerase, either mutant reverse primer (red-specific to mutant repeats) or wild-type reverse primer (black) were used to generate TRAP products. To be able to observe incorporation of mutant repeats, mutant forward and reverse primers were used. B. Reconstitution experiments were performed as before with AtTERT and either TER<sub>1G7-CC</sub> or with TER<sub>1G7</sub>. TRAP assay was performed with a mutant forward primer (indicated by black arrow with red asterisk) and a reverse primer specific to the mutant repeats (indicated by a red arrow). C. TRAP assay on wild-type and TER<sub>1G7-CC</sub> transformants. TRAP assay was performed with combinations of the mutant forward primer, regular forward primer (black arrow), regular reverse primer (small black arrow) and mutant reverse primer.

CACTATCGACTA CGCGATTAG 3') to enable it to make an additional base-pair with the mutant template (CTAAAGGCTA) (FIG. 21A, panel 4). The mutant forward primer should also work for wild-type TER<sub>1G7</sub> reconstitution (FIG. 21A, panel 3). When TRAP was performed on the reconstitution reaction of TER<sub>1G7-CC</sub> and AtTERT using the mutant forward primer and the mutant reverse primer, amplification products were obtained (FIG. 21B). However, no TRAP products were obtained when these primers were used in reactions with wild-type TER<sub>1G7</sub> (FIG. 21B). These findings indicate that TER<sub>1G7-CC</sub> is capable of reconstituting telomerase activity *in vitro*.

To determine whether the mutant template could be used by telomerase *in vivo*, wild-type plants were transformed with TER<sub>1G7-CC</sub> under the control of the powerful 35S CaMV promoter. As expected, TRAP assays performed with extracts from transformants using the wild-type reverse primer and either of the two forward primers generated TRAP products (FIG. 21C). This activity represents the wild-type telomerase present in the plants. No activity was observed when the wild-type forward primer and the mutant reverse primer were used (FIG. 21C, panel 2). However, TER<sub>1G7-CC</sub> transformants showed TRAP activity with the mutant forward primer and the mutant reverse primer (FIG. 21C, panel 4). We conclude that mutant TER<sub>1G7</sub> can be incorporated into the telomerase RNP *in vivo* and can function to direct the synthesis of telomere repeats by telomerase. Similar experiments to examine TER<sub>5G2</sub> templating function *in vivo* are underway.

#### *Analysis of TER<sub>1G7</sub> and TER<sub>5G2</sub> T-DNA mutants*

We obtained T-DNA insertion lines for both the TER candidates. For TER<sub>1G7</sub>, we have a line with a T-DNA insertion (SAIL\_25\_H08) 40 nts upstream of the template termed TER<sub>1G7-1</sub> (FIG. 22A). To determine if TER<sub>1G7</sub> transcripts were generated in

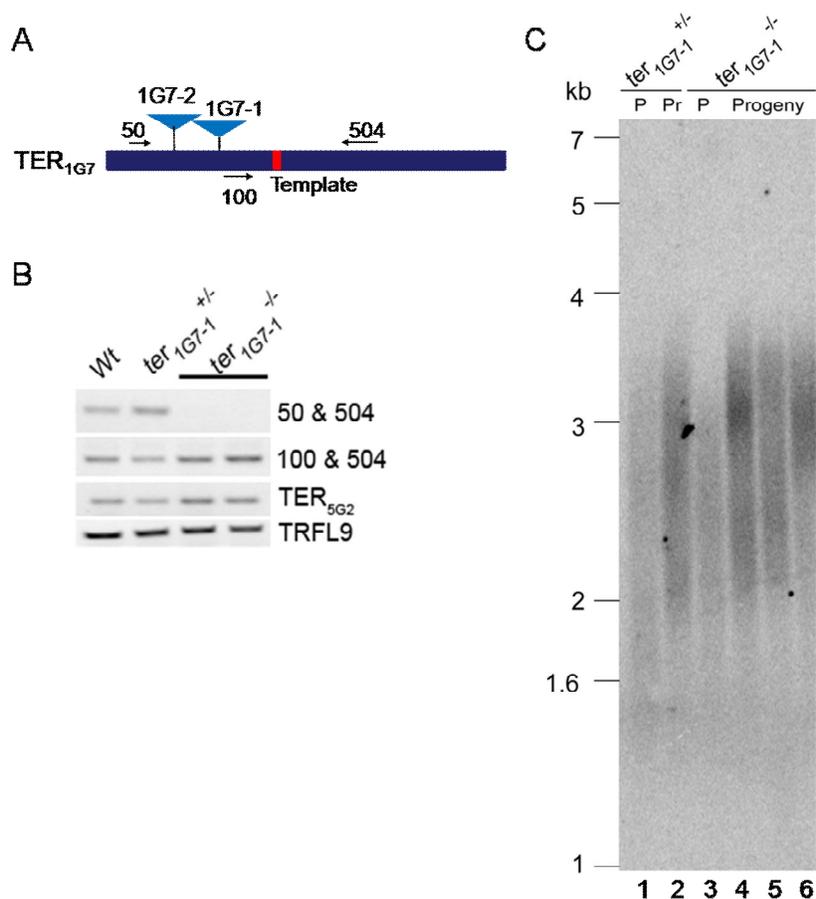


FIG. 22. T-DNA insertion in TER<sub>1G7</sub>. A. Schematic of TER<sub>1G7</sub> gene with template (red box) and T-DNA insertions TER<sub>1G7-1</sub> and TER<sub>1G7-2</sub> (blue triangles) upstream of template. Primers used for RT-PCR (below) are also indicated. B. RT-PCR analysis of TER<sub>1G7</sub> transcript in wild-type, *ter*<sub>1G7-1</sub><sup>+/+</sup> and *ter*<sub>1G7-1</sub><sup>-/-</sup> plants using the primer-pairs 50 & 504 or 100 & 504 (shown in A). Transcripts of TER<sub>5G2</sub> and a control double-stranded telomeric protein (TRFL9) were also monitored. C. TRF analysis of DNA from *ter*<sub>1G7-1</sub><sup>+/+</sup> and *ter*<sub>1G7-1</sub><sup>-/-</sup> (P represents G1 parent and Pr represents G2 progeny). The parent plants (in lanes 1 and 3) are siblings. In lane 2, DNA from the heterozygous progeny of the plant in lane 1 was analyzed. In lanes 4, 5 and 6, the homozygous progeny of the plant in lane 3 were analyzed.

homozygous  $TER_{1G7-1}$  mutants, RT-PCR was performed. Unexpectedly, a  $TER_{1G7-1}$  transcript was produced in the homozygous mutants (FIG. 22B). Cloning and sequence analysis revealed that this  $TER_{1G7}$  transcript lacked ~200 nts from the 5' terminus, but still retained an intact template.  $TER_{1G7-1}$  was able to reconstitute telomerase activity *in vitro* when co-expressed with AtTERT, albeit to a much reduced level (C. Cifuentes-Rojas and D. Shippen, unpublished data). Thus,  $TER_{1G7-1}$  is not a null allele.

Homozygous  $TER_{1G7-1}$  mutants were further analyzed by TRAP and TRF analysis. By real time TRAP, we observed no significant changes in telomerase activity levels compared to wild-type plants (data not shown). Consistent with this finding, telomere length was in the wild-type range in both the homozygous mutant and its heterozygous sibling in the first generation (FIG. 22C, compare lanes 1 and 3). When these plants were propagated to the next generation, no change in telomere length was observed between heterozygous and homozygous  $TER_{1G7-1}$  mutants (FIG. 22C, compare lane 2 to lanes 4-6). Homozygous mutants of another  $TER_{1G7}$  T-DNA line ( $TER_{1G7-2}$ ) which is located further upstream in the  $TER_{1G7}$  gene (FIG. 22A) also showed no telomere length defects (data not shown). Thus, the two  $TER_{1G7}$  T-DNA lines available are not null.

For  $TER_{5G2}$ , a line with a T-DNA insertion (SAIL\_556\_A04) directly in the template region termed  $TER_{5G2-1}$  was available. No  $TER_{5G2}$  transcript was produced in the homozygous  $TER_{5G2-1}$  mutants indicating that this is a true null allele (C. Cifuentes-Rojas and D. Shippen, unpublished data). Homozygous  $TER_{5G2-1}$  mutants did not show any obvious defects in telomere maintenance in the first generation. However, real time TRAP revealed a 2.8 fold increase in telomerase activity in homozygous  $TER_{5G2-1}$  mutants when compared to wild-type (C. Cifuentes-Rojas and D. Shippen, unpublished

data). Although this increase in telomerase activity is modest, the data suggest that  $TER_{5G2}$  may function as a negative regulator of telomerase *in vivo*.

Since neither single mutant showed obvious telomere length defects nor loss of telomerase activity, we reasoned that the two RNAs may act redundantly. Therefore, we crossed  $TER_{1G7-1}$  and  $TER_{5G2-1}$  in order to generate a double mutant. However, since the  $TER_{1G7-1}$  mutation results in only a knockdown of  $TER_{1G7}$ , we still may not observe defects in telomerase activity, but this possibility will be tested by real-time TRAP and TRF.

#### *Antisense knockdown of $TER_{1G7}$ and $TER_{5G2}$*

In parallel with analysis of the T-DNA insertion lines, antisense constructs targeting both RNAs were transformed into plants in order to reduce TER levels. To optimize chances of detecting a telomere maintenance defect, we transformed plants heterozygous for  $AtKu70$  with these constructs.  $AtKu70$  is a strong negative regulator of telomerase (269). In  $ku70^{-/-}$  plants, telomeres are hyperelongated from the wild-type length of 2-5 kb to about 6-8 kb in one generation (FIG. 23A). This elongation is dependent on telomerase. We reasoned that when levels of TERs are lowered, telomerase would be unable to extend telomeres in a  $ku70^{-/-}$  mutant (FIG. 23A). In  $ku70^{+/-}$  with reduced telomerase, we predicted that telomeres would stay in the wild-type range or shorten slightly (FIG. 23A). Thus, a difference in telomerase function would be apparent when the telomere lengths of these transformants are analyzed by TRF.

RT-PCR analysis of the first generation of  $TER_{AS}$  transformants (T1) revealed plants in which both TERs were knocked down relative to wild-type. In the eight transformants analyzed, real-time RT-PCR revealed that  $TER_{1G7}$  levels were reduced twelve-fold on average, while  $TER_{5G2}$  levels were reduced four-fold on average

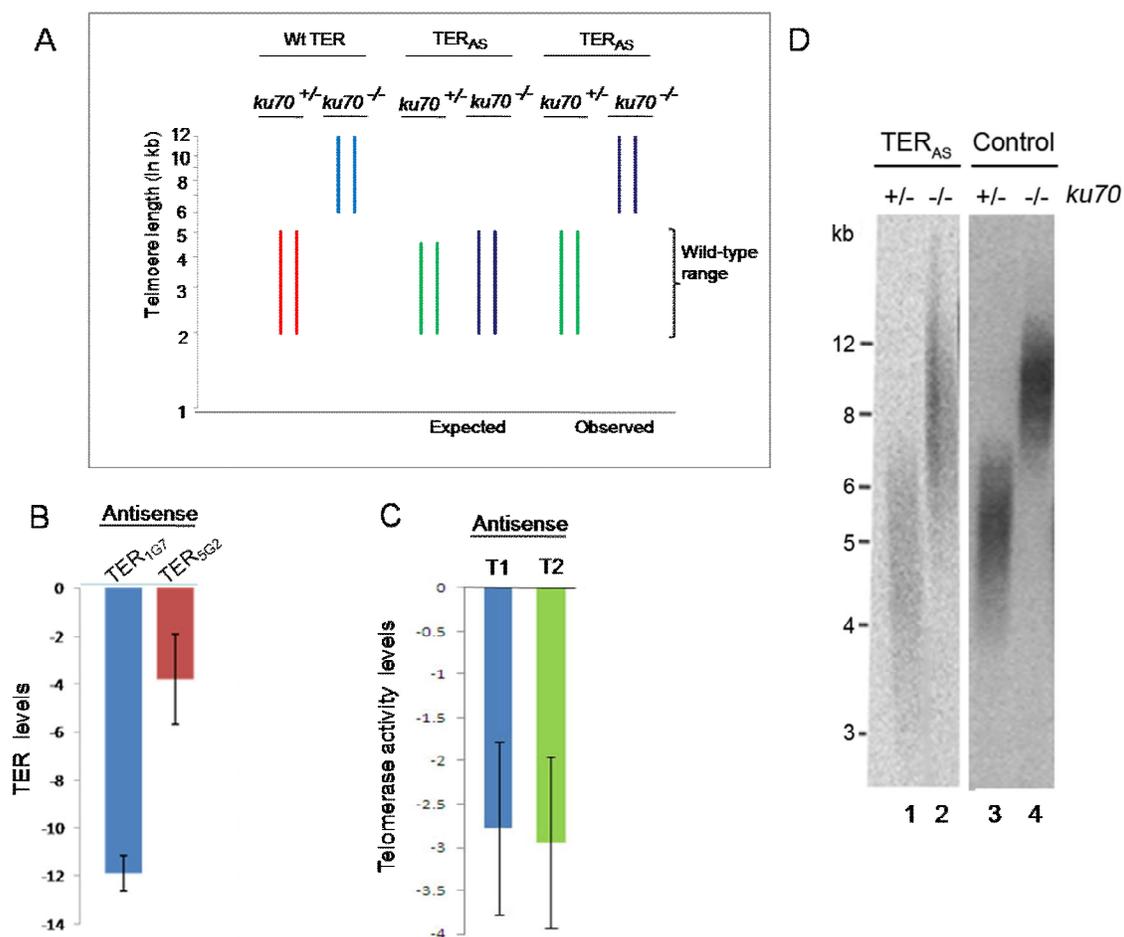


FIG. 23. Antisense constructs directed at TER subunits result in lower telomerase activity. A. Schematic of experiment. Telomere lengths of *ku70*<sup>+/-</sup> and *ku70*<sup>-/-</sup> plants are indicated. If TER levels are decreased in plants, the telomere length in *ku70*<sup>-/-</sup> is expected to be short and in the wild-type range of 2-5 kb. In fact, the observed telomere lengths of TER<sub>AS</sub> *ku70*<sup>-/-</sup> plants are the same as *ku70*<sup>-/-</sup> mutants. B. Real time RT-PCR analysis of TER<sub>1G7</sub> (blue bars) and TER<sub>5G2</sub> (red bars) transcripts in antisense transformants. C. Results of quantitative telomerase activity assay on T1 and T2 antisense transformants. D. TRF analysis of one TER<sub>AS</sub> *ku70*<sup>+/-</sup> (lane 1) and one TER<sub>AS</sub> *ku70*<sup>-/-</sup> (lane 2) is shown. In the right panel, TRF analysis of control untransformed *ku70*<sup>+/-</sup> (lane 3) and *ku70*<sup>-/-</sup> (lane 4) are shown.

(FIG. 23B). We next asked whether telomerase activity was affected in the TER<sub>AS</sub> transformants that showed a knockdown of TER levels. By real-time TRAP assay, we observed a decrease of approximately 2.7 fold in telomerase activity in first generation of transformants (T1) (FIG. 23C). We asked if the decrease in telomerase activity leads to telomere length defects. TRF analysis revealed no obvious change in the telomere length in T1 TER<sub>AS</sub> transformants relative to *ku70*<sup>+/-</sup> mutants (FIG. 23D, lanes 1 and 3). *ku70*<sup>-/-</sup> mutants with lower levels of TERs were still able to elongate telomeres to the same extent as in first-generation *ku70*<sup>-/-</sup> mutants (FIG. 23D, lanes 2 and 4). This result suggested that TER levels may have to be reduced to a greater extent in order to observe a telomere length defect.

An unexpected result was obtained when TER<sub>AS</sub> transformants were propagated to the next generation (T2). Real-time TRAP indicated ~2.9 fold reduction in telomerase activity indicating that the antisense construct was active in the second generation of transformants (FIG. 23C). Since, telomerase activity was reduced in T2 plants, we expected telomere lengths in TER<sub>AS</sub> *ku70*<sup>+/-</sup> plants to remain in wild-type range or become slightly shorter (FIG. 24A). However, TRF analysis of T2 TER<sub>AS</sub> transformants indicated that many telomeres in these plants were grossly elongated, spanning 3- 12 kb with an average size of 5.2 kb (FIG. 24B, lanes 3-12). Genotyping of the ten T2 transformants revealed that all were heterozygous for AtKu70 (FIG. 24C). Since telomeres are in the wild-type range in *ku70*<sup>+/-</sup> mutants (FIG. 23D, lane 3; ref (269)), it appears that the combined loss of one allele of AtKu70 and depletion of TER levels in the T2 generation led to loss of negative regulation of telomere length and as a consequence gross telomere elongation.

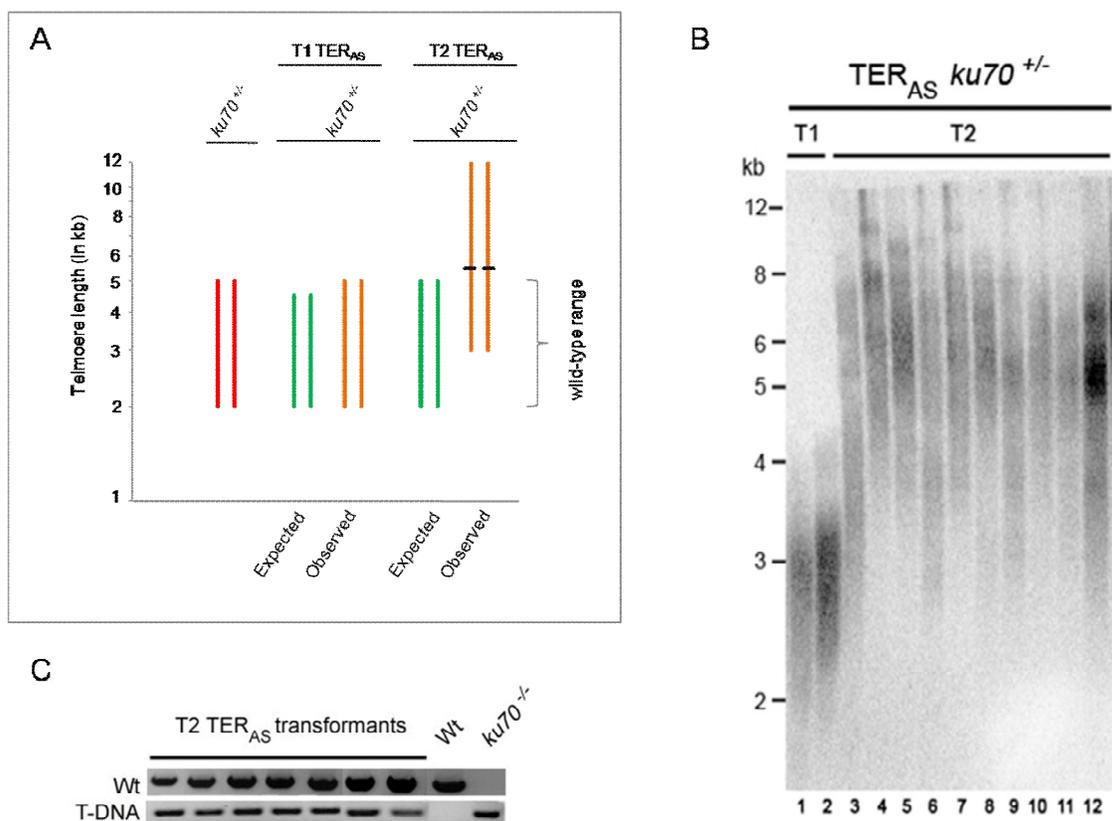


FIG. 24. Reduced levels of AtKu70 and TERs lead to telomere elongation. A. Schematic of telomere lengths in *ku70*<sup>+/-</sup> mutants. Expected telomere lengths in T1 and T2 TER<sub>AS</sub> *ku70*<sup>+/-</sup> and observed telomere length in T1 and T2 TER<sub>AS</sub> *ku70*<sup>+/-</sup> mutants are shown. The average telomere length of 5.2 kb, observed in T2 TER<sub>AS</sub> *ku70*<sup>+/-</sup> mutants is indicated by a dash. B. TRF analysis of first (T1) and second (T2) generation of TER<sub>AS</sub> *ku70*<sup>+/-</sup> plants is indicated by a dash. C. Genotyping of TER<sub>AS</sub> transformants with primers to detect wild-type and T-DNA mutant alleles of AtKu70. The presence of PCR products for both wild-type and T-DNA mutant alleles indicate that TER<sub>AS</sub> transformants are heterozygous for AtKu70. Wild-type and *ku70*<sup>-/-</sup> mutants were used as controls.

*TER<sub>1G7</sub> and TER<sub>5G2</sub> bind a distinct set of proteins*

TER subunits must interact with TERT in order to reconstitute telomerase activity. To verify this interaction, AtTERT was immunoprecipitated from *Arabidopsis* suspension cell culture using a peptide antibody against AtTERT (153). As expected, immunoprecipitation (IP) of AtTERT pulled down telomerase activity (FIG. 25A, lane 6) but not when antibody was omitted during IP (FIG. 25A, lane 5). Furthermore, IP with AtTERT antibody pulled down both TER<sub>1G7</sub> and TER<sub>5G2</sub> (FIG. 25C, lanes 7 and 8).

TER subunits bind a variety of proteins that function in conferring stability to the RNP, biogenesis or the recruitment of telomerase to the telomere (reviewed in (55)). In *Arabidopsis*, several telomere-related proteins have been identified, but their mechanism of action is unknown. For example, although AtPOT1a is a positive regulator of telomerase, stably associated with the telomerase RNP (296), the interaction of AtPOT1a with AtTERT and AtNAP57 (dyskerin) is very weak (153, 296). We reasoned that AtPOT1a could be associated with telomerase through TER<sub>1G7</sub> or TER<sub>5G2</sub>. *In vitro* co-IP experiments suggest that AtPOT1a binds to TER<sub>1G7</sub> and not to TER<sub>5G2</sub> (C. Cifuentes-Rojas and D. Shippen, unpublished data).

To test whether the specific interaction between AtPOT1a and TER<sub>1G7</sub> is seen *in vivo*, IP was performed with *Arabidopsis* suspension cell culture using a peptide antibody against AtPOT1a (296). Antibodies to AtPOT1a pulled down telomerase activity as expected (FIG. 25A, lane 3). Telomerase activity was not precipitated when antibody was omitted (FIG. 25A, lane 2). RT-PCR performed on the IP confirmed that TER<sub>1G7</sub> was precipitated with the AtPOT1a antibody and not when antibody is omitted (FIG. 25C, lanes 5 and 6, lower panel). IP with AtPOT1a does not pull down TER<sub>5G2</sub> (FIG. 25C, lane 6, upper panel) arguing that AtPOT1a is anchored to the telomerase RNP via its

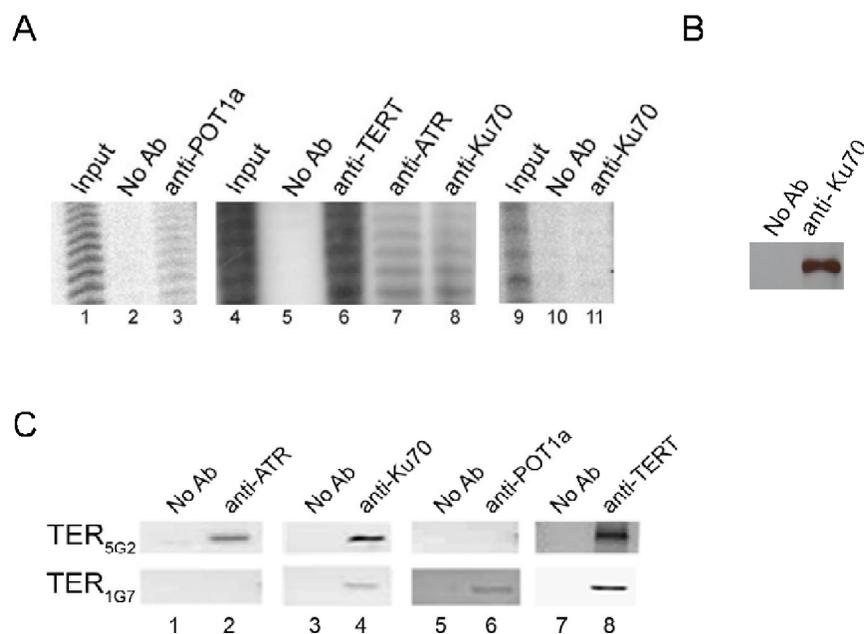


FIG. 25. TER subunits associate with different telomere-related proteins. A. POT1a, TERT and ATR immunoprecipitate telomerase activity. Proteins were immunoprecipitated from cell culture extracts with either no antibody (No Ab) and indicated antibodies and followed by TRAP assay. Immunoprecipitation with Ku70 antibody gave different results- telomerase activity was obtained after IP sometimes and not at other times (compare lanes 8 and 11). B. Immunoprecipitation of AtKu70 from cell culture. Cell culture extracts were immunoprecipitated with either no antibody or with a Ku70 antibody and immunoprecipitated material was subjected to western analysis using Ku70 antibody. C. Differential association of TERs with telomere-related proteins. RNA was extracted from immunoprecipitated material and subjected to RT-PCR analysis using primers specific to TER<sub>1G7</sub> and TER<sub>5G2</sub>.

interaction with TER<sub>1G7</sub>. Furthermore, the data suggest that AtPOT1a is in a distinct RNP complex that does not include TER<sub>5G2</sub>.

As discussed above, AtKu70 is a known negative regulator of *Arabidopsis* telomerase (97, 269). However, the basis of this regulation is unknown as no interactions have been detected *in vitro* between Ku and AtTERT (M. Heacock and D. Shippen, unpublished data). In yeast and mammals, Ku70/80 binds directly to TER (250, 307). Therefore, an *in vitro* co-IP experiment was performed to check whether AtKu70 bound either of *Arabidopsis* TERs. The results indicated that AtKu70 bound TER<sub>5G2</sub> specifically and not TER<sub>1G7</sub> (C. Cifuentes-Rojas and D. Shippen, unpublished data). A similar IP as described above was performed using a Ku70 antibody to look for Ku association with TER<sub>5G2</sub> *in vivo*. AtKu70 was pulled down only when IP was carried out using the AtKu70 antibody and not when antibody was omitted (FIG. 25B). However, we obtained contrasting results when TRAP assays were performed after IP. In some experiments, telomerase activity could be precipitated with Ku70 antibody (FIG. 25A, lane 8), but not in others (FIG. 25A, lane 11). This finding suggests that the association of AtKu70 with telomerase may be regulated. RT-PCR after IP indicated that unlike AtPOT1a, AtKu70 binds to both TER<sub>1G7</sub> and TER<sub>5G2</sub> (FIG. 25C, lane 4), although more TER<sub>5G2</sub> was pulled down than TER<sub>1G7</sub>. This finding suggests that the two TERs could be in the same telomerase sub-complex *in vivo*.

Another telomere-related protein is the checkpoint kinase ATR, which has been implicated in telomerase recruitment in yeast (303). In *Arabidopsis*, *atr tert* double mutants show a faster rate of shortening than *tert* mutants alone, implicating ATR in telomere maintenance (317). We obtained a peptide antibody specific for *Arabidopsis* ATR. Western blots conducted with this antibody recognized a 200 kDa protein that corresponds to ATR in wild-type extracts but not in *atr*<sup>-/-</sup> extracts (L. Vespa and D.

Shippen, unpublished data). IP of cell culture extracts with ATR antibody pulled down telomerase activity, suggesting that ATR physically interacts with the telomerase complex (FIG. 25A, lane 7). Surprisingly, IP of ATR pulled down TER<sub>5G2</sub> and not TER<sub>1G7</sub> (FIG. 25C, lane 2). Therefore, ATR may be associated with a sub-complex of active telomerase that contains TER<sub>5G2</sub> alone. Altogether, these data suggest that TERs are found in different RNP complexes, and that these complexes may have distinct functions in telomere maintenance.

## Discussion

In 1989, the first telomerase RNA was identified by several steps of biochemical purification of *Tetrahymena* extracts (118). A similar approach was adopted to identify the *Arabidopsis* TERs. Unexpectedly, we uncovered two related TERs. TER duplication is unique to *Arabidopsis*. The genome of *Arabidopsis thaliana* has undergone several rounds of duplication (29, 294). Previous work in our lab indicates that both double and single-strand telomere binding proteins are present in multigene families (154, 284). However, not all genes encoding telomere-related proteins are duplicated. For example, TERT and dyskerin are single copy genes (84, 153). Thus, the duplication of TER may be biologically significant. In support of this conclusion, TER<sub>1G7</sub> and TER<sub>5G2</sub> have distinct expression patterns, biochemical properties and protein interaction partners. Thus, the two TERs appear to represent an example of neofunctionalization, when one or both in a pair of duplicated genes acquire a novel function distinct from its single copy ancestor (185).

Telomerase is known to act as a dimer in many species including humans, and dimerization has been reported for both TERT and TER subunits (20, 105, 202, 231, 254, 271, 326, 333). However, in *Tetrahymena*, telomerase acts as a monomer (38).

Thus, telomerase can function both as dimer and monomer. *Arabidopsis* TERT is capable of dimerization *in vitro* (data not shown) and the size of purified *Arabidopsis* telomerase RNP is consistent with a dimer (C. Cifuentes-Rojas and D. Shippen, unpublished data). Our data suggest that core *Arabidopsis* telomerase RNP may exist in different forms: monomers, homodimers and heterodimers (FIG. 26). *In vitro* telomerase reconstitution with low levels of TER<sub>5G2</sub> result in robust telomerase activity, suggesting that TER<sub>5G2</sub> may be more active as a monomer. On the other hand, robust telomerase activity was obtained with TER<sub>1G7</sub> only when levels of TER<sub>1G7</sub> PCR product were ~100 fold higher than for TER<sub>5G2</sub>. This observation suggests that TER<sub>1G7</sub> favors dimer formation for maximal telomerase activity. Other sub-complexes of telomerase containing either TER subunit may also be formed *in vivo*.

#### *TER<sub>1G7</sub> and TER<sub>5G2</sub> act as templates for telomerase*

The classical experiment for confirming TER template function is mutation of the template and the detection of mutant telomeric repeats on chromosome ends (217, 351). To study TER<sub>1G7</sub> template function, this region was mutated so that telomeres with TTTAGCC repeats would be synthesized. Telomerase reconstitution experiments indicate that TER<sub>1G7-CC</sub> can function as a template for telomerase *in vitro* (FIG. 21B). Furthermore, TRAP assays revealed that TER<sub>1G7-CC</sub> transformants synthesize both wild-type and mutant telomere repeats (FIG. 21C). Thus, TER<sub>1G7</sub> can function as a template for telomerase *in vitro* and *in vivo*.

To determine if TER<sub>1G7</sub> can be used to incorporate telomere repeats onto chromosome ends, we performed primer extension telomere repeat amplification (PETRA) assays with TER<sub>1G7-CC</sub> transformants to amplify and clone the most 3' telomeric repeats (130). With this method, it is possible to target individual chromosome arms by

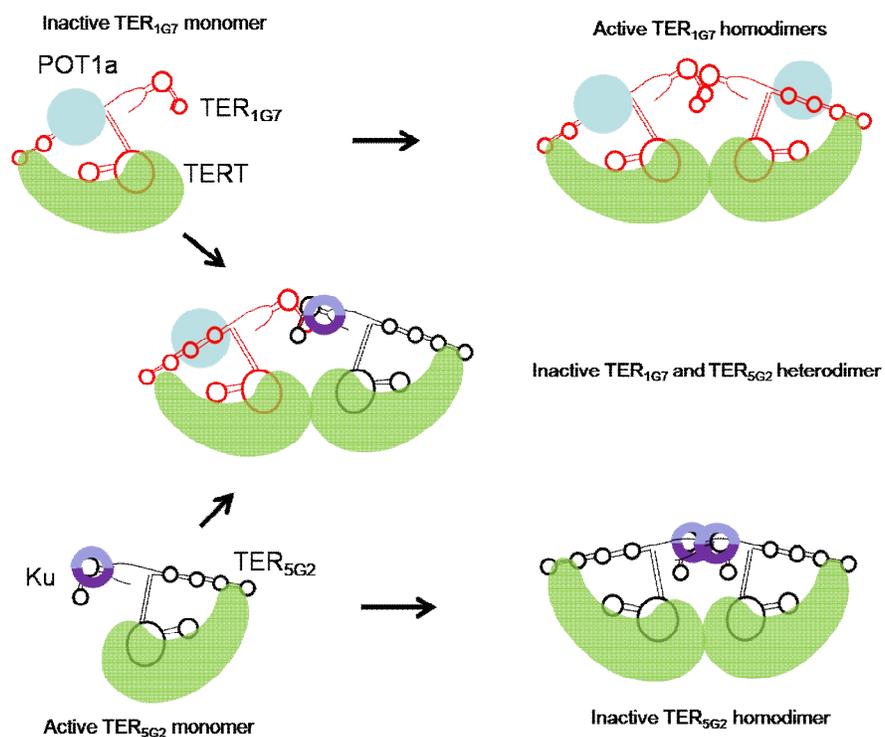


FIG. 26. Model for *Arabidopsis* telomerase complexes. TER<sub>1G7</sub> and TER<sub>5G2</sub> can both form monomers and dimers with TERT. TER<sub>1G7</sub> is more active as a dimer, and is bound by AtPOT1a. TER<sub>5G2</sub> is active as a monomer and it is bound by Ku70. The two TERs can also heterodimerize forming an enzymatically inactive telomerase complex.

using specific subtelomeric primers and thus the sequences of the repeats at a specific chromosome end can be determined. Sequencing of one PETRA product which was 700 nts in length, revealed the presence of one mutant repeat after sixty-five wild-type repeats (data not shown). Therefore, mutant repeats are added onto chromosome ends by TER<sub>1G7-CC</sub>.

To examine whether TER<sub>5G2</sub> can also function as a template for telomerase *in vivo*, a TER<sub>5G2</sub> construct with a template mutation was transformed into TER<sub>5G2-1</sub> homozygous mutants. This template mutant called TER<sub>5G2-Rsa</sub> can reconstitute telomerase activity *in vitro* (C. Cifuentes-Rojas and D. Shippen, unpublished data). The *in vivo* analysis of TER<sub>5G2-Rsa</sub> transformants is underway.

#### *TER<sub>5G2</sub> may act as a negative regulator of telomerase*

Several lines of evidence suggest a novel role for TER<sub>5G2</sub> as a negative regulator of telomerase. First, although TER<sub>5G2</sub> is expressed at a very low level in telomerase positive tissues, complete loss of TER<sub>5G2</sub> transcript leads to an increase in telomerase activity in homozygous TER<sub>5G2-1</sub> mutants. Second, when reconstitution experiments are performed with both TER<sub>1G7</sub> and TER<sub>5G2</sub> PCR products, increasing the levels of TER<sub>5G2</sub> PCR products result in lower telomerase activity (C. Cifuentes-Rojas and D. Shippen, unpublished data). We hypothesize that increased levels of TER<sub>5G2</sub> relative to TER<sub>1G7</sub> may promote the formation of heterodimers that are enzymatically inactive (FIG. 26). Third, TER<sub>5G2</sub> interacts *in vivo* and *in vitro* with AtKu70, a known negative regulator of telomerase. Thus, negative regulation of telomerase might involve the interaction between AtKu70 and TER<sub>5G2</sub>. This prediction is supported by our antisense experiments which suggest that simultaneous depletion of both AtKu70 and TER<sub>5G2</sub>, lead to a dramatic increase in telomere length in T2 TER<sub>AS</sub> *ku70*<sup>+/-</sup> plants.

The only other example of a telomerase component which acts as a negative regulator is the *Tetrahymena* Skp1p (337). The genetic depletion of this protein causes telomere lengthening in *Tetrahymena*, but it is not clear if this is a direct consequence of affecting the telomerase holoenzyme (337). Thus, the presence of a negative regulator in the *Arabidopsis* core telomerase RNP is a completely novel finding.

*Depletion of TER<sub>1G7</sub> and TER<sub>5G2</sub> in Arabidopsis leads to a decrease in telomerase activity*

Loss of TER subunits in various organisms lead to progressive telomere shortening (80, 181, 287, 329). However, the presence of two TER candidates in *Arabidopsis* made our genetic analyses complicated. A double mutant abolishing production of both TERs is required to see if a telomere shortening phenotype typical of other TER mutants could be observed. Unfortunately, we do not have a null mutant for TER<sub>1G7</sub>. Therefore a double null mutant cannot be generated at this time.

As an alternative approach, we transformed antisense constructs aimed at both TERs into *ku70*<sup>+/-</sup> plants. Ku is a potent negative regulator of telomere length (269). We reasoned that if TER levels are reduced in *ku70*<sup>-/-</sup> mutants, telomeres will not be elongated. TER<sub>AS</sub> transformants displayed diminished levels of both TER<sub>1G7</sub> and TER<sub>5G2</sub> and reduced telomerase activity. However, there was no effect of reduced TER levels on telomere length in the first generation of antisense transformants. This could be due to the slow turnover of telomerase complexes *in vivo*. In support of this hypothesis, in second generation TER<sub>AS</sub> *ku70*<sup>+/-</sup> transformants, telomeres were grossly extended ranging from 3-12 kb. We hypothesize that the extended telomeres in T2 antisense transformants result from the combined depletion of the two negative regulators AtKu70 and TER<sub>5G2</sub>. We propose that the regulation of TER<sub>5G2</sub> is necessary to control

telomerase action *in vivo*. In this model, the action of the negative regulators AtKu70 and TER<sub>5G2</sub> controls the enzymatic activity of TER<sub>1G7</sub> homodimers by formation of heterodimers and thus helps maintain a telomere length set point (FIG. 27). In TER<sub>AS</sub> transformants, however, levels of both TERs are reduced and telomerase activity is also reduced. However, the residual TER<sub>1G7</sub> in these plants is able to function at telomeres and in the absence of negative regulators, telomeres are extended (FIG. 27).

#### *Different binding partners for Arabidopsis TERs*

IP experiments indicate that both TERs are involved in a distinct set of protein interactions. These interactions may be required for biogenesis of the complex, recruitment of telomerase to the telomeres or regulation of enzyme activity *in vivo*. Our experiments suggest that AtPOT1a may perform a role similar to Est1 in yeast (79). Est1 binds to yeast TER (TLC1) and is thought to recruit telomerase to the telomeres via its interaction with the G-overhang binding protein Cdc13 (78, 281). Alternatively, Est1 may bind to telomeres at S-phase and interact with Cdc13 to activate telomere-bound telomerase (300). Analogous to Est1, AtPOT1a binding to telomerase through TER<sub>1G7</sub> may help in recruitment of telomerase to the telomere. AtPOT1a does not bind telomeric DNA on its own, but by chromatin IP assays it was shown to associate with telomeres in S-phase when telomerase is known to act (296). How AtPOT1a binds to the telomere is unknown, but our data suggest that AtPOT1a can function as a positive regulator of telomerase through interaction with TER<sub>1G7</sub>.

*Arabidopsis* Ku is a negative regulator of telomerase (96, 269). Our data suggests that *Arabidopsis* Ku is similar to its homologs in yeast and humans in its ability to bind to telomerase RNA (250, 293, 307). Interestingly, however, Ku is a positive regulator of telomere length in yeast (34, 112, 253) and a negative regulator of telomere

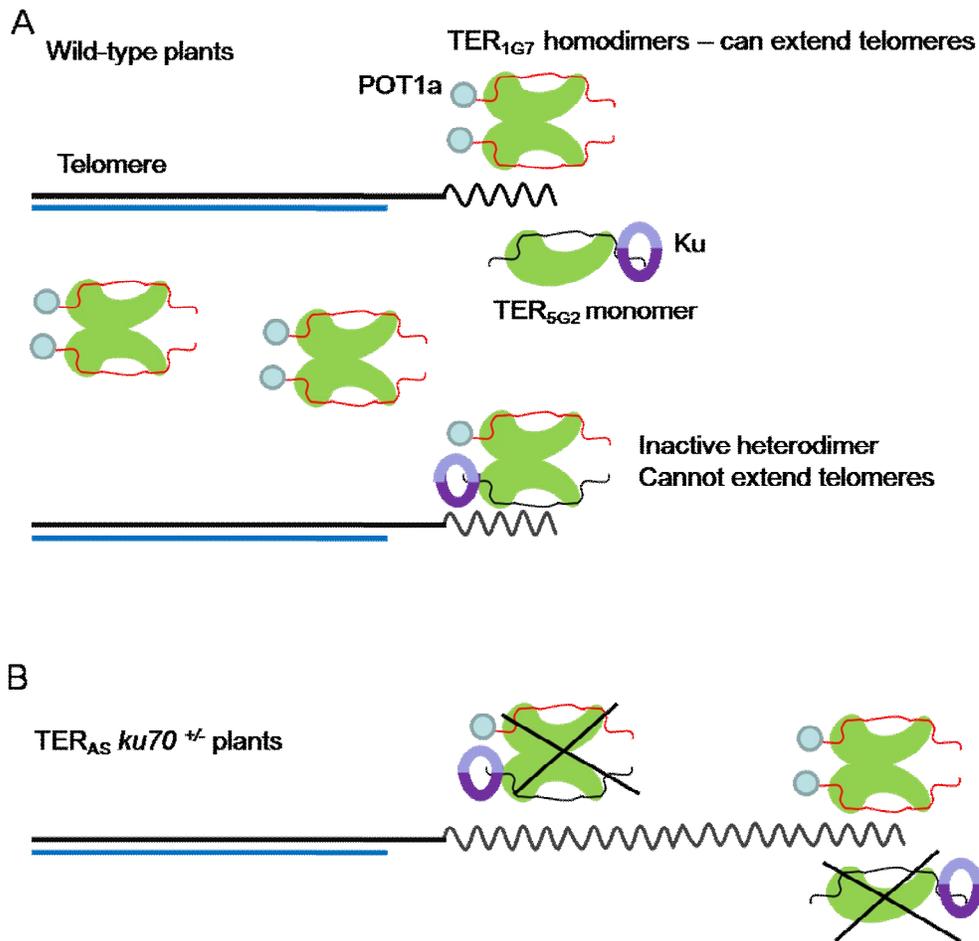


FIG. 27. Speculative model for negative regulation of *Arabidopsis* telomerase. Loss of negative regulators AtKu70 and TER<sub>5G2</sub> leads to telomere elongation. A. In wild-type plants, the action of TER<sub>1G7</sub> homodimers on telomeres is regulated by TER<sub>5G2</sub> monomers that are bound by Ku70. TER<sub>5G2</sub> can also form enzymatically inactive heterodimers with TER<sub>1G7</sub>. B. In TER<sub>AS</sub> *ku70*<sup>+/-</sup> plants, the level of TER<sub>5G2</sub> and TER<sub>1G7</sub> are much lower. The negative regulation exerted by TER<sub>5G2</sub>-Ku70 complex is reduced and therefore, the residual TER<sub>1G7</sub> in these plants can elongate telomeres.

length in *Arabidopsis*. Perhaps, the switch in regulation occurred with the duplication of TER subunits and an increased affinity of AtKu70 for the inhibitory TER subunit. Our TRAP results indicate that AtKu70 may be associated with both active and inactive telomerase complexes. Since these experiments were not performed with synchronized cell cultures, these results may reflect the association of AtKu70 with telomerase at different stages of the cell cycle. However, we hypothesize that AtKu70 primarily interacts with telomerase through TER<sub>5G2</sub> and this interaction may limit telomerase action (FIG. 26).

#### *TER<sub>5G2</sub> is linked to the DNA damage response protein ATR*

ATR plays an important role in the replication of telomeres during S phase (302). Studies in yeast have established a role for ATR in telomerase recruitment to short telomeres. In late S phase, Mec1 (ATR) is recruited to telomeres by the MRX complex (303). Mec1 binding leads to binding of Cdc13 and Est1 to telomeres and subsequently telomerase activation (303). How these events are orchestrated in higher eukaryotes is unknown. Here we show that *Arabidopsis* ATR is physically associated with the telomerase RNP and specifically with TER<sub>5G2</sub>.

One ATR interacting factor identified in our lab is the OB-fold containing protein ATPOT1b (L. Vespa and D. Shippen, unpublished data). AtPOT1b was shown to interact directly with TER<sub>5G2</sub> by *in vitro* co-immunoprecipitation experiments (C. Cifuentes-Rojas and D. Shippen, unpublished data). Therefore, we hypothesize that *Arabidopsis* ATR is involved in recruiting the TER<sub>5G2</sub>-TERT monomer to telomeres by modification of factors such as AtPOT1b. Since TER<sub>5G2</sub>-TERT monomer is extremely proficient at telomere synthesis, targeting of this complex to short telomeres may promote efficient lengthening

of telomeres. The identification of other components of this pathway will throw more light on telomerase recruitment in *Arabidopsis*.

In conclusion, the identification of two distinct *Arabidopsis* TER subunits has revealed unanticipated complexity in telomerase RNP composition, and the potential for novel modes of telomerase regulation.

## CHAPTER IV

### IDENTIFICATION OF PUTATIVE TER GENES FROM OTHER PLANT SPECIES

#### Summary

The enzyme telomerase is a ribonucleoprotein complex that maintains telomere length by addition of telomeric repeats to the ends of eukaryotic chromosomes. Telomerase RNA (TER) provides the template for addition of telomeric repeats by the catalytic component, TERT. TER species have been identified in various ciliates, yeast and vertebrates. TERs are highly divergent in both size and primary sequence, but display a conserved secondary structure. However, TER species have not been identified in any plant species and therefore a gap remains in our understanding of evolution of TER architecture. In the previous chapter, we discussed the identification of two *Arabidopsis* TER subunits that can function as templates for telomerase. Here, we investigate the presence of these TER genes in other plant species using both gene synteny and PCR approaches. We find evidence for the presence of TER genes in other members of the Brassicaceae family as well as in *Oryza sativa* and *Medicago truncatula*. Furthermore, we demonstrate that a single nucleotide deletion is present in the *Asparagus* TER gene and may be responsible for the human type telomere repeats found in this organism.

#### Introduction

Telomerase is a ribonucleoprotein complex that adds short G-rich telomeric repeats to the ends of eukaryotic chromosomes. Telomerase consists of a reverse transcriptase subunit (TERT) and an integral RNA subunit, TER. TER provides the template for the addition of repeats onto chromosome ends. The TERT subunit is

characterized by several conserved RT motifs as well as telomerase-specific domains (reviewed in (12)). These conserved motifs have made it possible to identify TERT subunits from a variety of species (191).

The TER subunit, on the other hand, has diverged very rapidly (48). Although *Tetrahymena* TER was identified years before the first TERT subunit (118, 191), the absence of any significant primary sequence conservation made it difficult to recover TERs from other species by homology searches. Instead, biochemical and genetic approaches were adopted to identify the array of TER sequences available today (80, 117, 287).

Primary sequences of TER species can be used to construct secondary structure models. These models can be tested by mutational analyses which provide an insight into the roles played by different regions of the RNA. Thus, a structure-function relationship can be established. For secondary structure determination, both experimental and phylogenetic approaches can be used. Phylogenetic covariation has been used to establish the secondary structure of TERs from ciliates, vertebrates and yeast (50, 68, 272, 352). To establish the vertebrate telomerase RNA secondary structure, homologous sequences from five different classes of the vertebrate phylum were cloned (50). Work from the Wellinger lab has shown that a reasonable secondary structure model can be deduced from as few as four sequences from closely related species. For optimal results, it is required that the sequences to be aligned have at least 60 – 80% identity (68). These sequences can then be used to build a secondary structure model by phylogenetic covariation analysis (246).

The basic tenet of this method is that homologous sequences from different organisms will exhibit a similar structure. Therefore, if a helix is important for the function of the RNA, then the pairing potential will be retained in homologous sequences too

(246). *In vitro* mapping experiments can then be performed to test these secondary structure models. Surprisingly, secondary structure models of TERs from ciliates, yeast and vertebrates revealed a high degree of conservation (50, 68, 272, 352). Therefore, it seems that TERs are similar in their functional domain composition rather than in primary sequence.

The striking features of the secondary structure models for TER include a single-stranded template region that corresponds to 1.5 telomeric repeats. The template is the region of TER that is transcribed by TERT reiteratively to make the telomere. All TERs also have a pseudoknot region downstream of the template. The pseudoknot domain is a high affinity binding site for TERT (46, 188, 231). It is considered to be essential for telomerase activity *in vivo* as it is involved in the formation of a stable RNP as well as dimerization of the RNA (103, 202). TERs also contain a template boundary element that limits reverse transcription to the template region of TER.

Other sites in TERs are important for binding to TERT and other factors that aid in telomerase recruitment and RNP biogenesis (48, 55). For example, TLC1 is bound by proteins involved in telomerase recruitment such as Est1 and Ku (250, 281). The 3' region of human telomerase RNA contains two essential motifs that contribute to its accumulation and localization in the cell. The H/ACA motif in hTR is bound by the protein dyskerin, that is necessary for the stability of the RNA *in vivo* (229). The CAB box is necessary for directing hTR to cajal bodies where presumably RNP assembly is coordinated (151, 359). Mutations in the pseudoknot region and the H/ACA domain (48) of hTR affect RNA accumulation or telomerase activity resulting in haploinsufficiency of telomerase and defective telomere maintenance (306). Loss of telomere maintenance in bone marrow cells is the underlying cause of autosomal dominant dyskeratosis congenita (229).

TER subunits have not been identified in the plant kingdom. Plants represent a eukaryotic branch that diverged from yeast approximately 1.6 billion years ago (131). Currently, there is a huge gap in our understanding of TER subunit divergence which could be bridged by the determination of a secondary structure for plant TERs. In *Arabidopsis*, we have identified two novel and related TER subunits: TER<sub>1G7</sub> and TER<sub>5G2</sub>. These two RNAs share a 220 nt region (includes the template) with 90% identity. From *in vitro* analysis, both TERs can function as templates for repeat addition (CHAPTER III). Thus, *Arabidopsis* is the only species that has two TER subunits.

In order to understand more about TER subunits in plants, we sought to identify these subunits in other plant species closely related to *Arabidopsis thaliana*. Here, we report the identification of several TER<sub>1G7</sub> subunits from species in the Brassicaceae family (to which *Arabidopsis* belongs) as well as in Rice, *Medicago* and *Asparagus* suggesting the conservation of this gene in other plant species. The identification of these TERs is also necessary for the establishment of a secondary structure for plant TERs. We present a preliminary secondary structure model proposed by our collaborator Dr. Yehuda Tzfati that provides evidence for conserved secondary structure elements in *Arabidopsis* TER<sub>1G7</sub>. Finally, we investigated the occurrence of TER gene duplication in the Brassicaceae family and find support that this duplication event dates to about 15 million years.

## **Materials and Methods**

### *Alignments and BLAST searches*

Sequence alignments were generated using the program CLUSTAL W and the Boxshade program was used to show identical nucleotides. BLAST searches were

conducted from NCBI BLAST web page using full length At1g71310 and At5g24670 genes against the Rice and *Medicago truncatula* databases.

#### *PCR & Southern Blotting*

Different combinations of the following primers from *Arabidopsis thaliana* TER<sub>1G7</sub> and TER<sub>5G2</sub> were used to amplify TER genes from *Arabidopsis lyrata*, *Olimarabidopsis pumila*, *Capsella rubella* and *Brassica oleraceae*. Primers used were 1G7-1 (5' TTAAGCTTTTCTTCTTCTCTGT 3'), 1G7-2 (5' AGGGTTTAGCAAACCTGGAGCA 3'), 1G7-3 (5' GACGACAATAAACCTACAC 3'), 1G7-4 (5' AATAATACACCAAATGTCA 3'), 1G7-5 (5' CGAACCCAGAGAACAAGAGA 3'), 1G7-6 (5' GAAGTGAAGATTAGTGACCCT 3') and 1G7-7 (5' TTCTATTTGGGTTTAGTGT 3'). For TER<sub>5G2</sub>, the following sets of primers were used: 5G2-1 (5' GACGACAATAAACCTACGCTTACA 3'), 5G2-2 (5' TCACGTCTCTCTTACGTCGTC 3'), 5G2-3 (5' CGATGTTGTTTTCTGCTTAGGACACA 3'), 5G2-4 (5' CACCCATAATTTTATTTTATCTATTCT 3'), 5G2-5 (5' TGTAAGCGTAGGGTTTAGTTGTCGTC 3'), 5G2-6 (5' AATTCTGTGTAGCTATGATCTTGTGGCA 3') and 5G2-7 (5' AGGAGATTTCTTCTACTCACCATACCC 3').

For cloning the Asparagus TER, primers were designed in the region surrounding the putative Rice template in chromosome 1. The template is located in the region corresponding to nucleotides 47737-47746 of the Rice PAC AP003261. The following primers used for PCR with Asparagus DNA as template: 47680 fwd (5' AGAGCTAACGAGTGTAGGGAG 3'), 47700 fwd (5' GCAGTGACATAAGAATAGGCCA 3'), 47720 fwd (5' CCAAAGGCATTCAGGCCT 3'),

47780 rev (5' TTCTGAGTGTGATTGATTACA 3'), 47800 rev (5' GATAAATAGCTGGTGACAATTCT 3') and 47850 rev (5' ATCCGTA ACTCAATGTGAACA 3'). PCR products were cloned into pDrive (QIAGEN) and sequenced.

For southern blotting analysis, DNA from the above species was digested with *EcoRI* and *XhoI* and then resolved on an agarose gel overnight. The DNA was transferred to a membrane and hybridized with a PCR product labeled using [ $\alpha$ - $^{32}$ P] dCTP and HighPrime (Roche). The probe spanned nucleotides 234-383 of TER<sub>1G7</sub>. Following overnight hybridization at 65° C, washes were performed twice with 2X SSC and 0.1% SDS and twice with 0.2 X SSC and 0.1% SDS for 15 min each. The blot was exposed to a phosphorscreen overnight.

## Results

### *Identification of TER<sub>1G7</sub> orthologs in other plant species*

*Arabidopsis* is the only known organism that has two TER genes. We were curious to know whether these TER genes were also present in other plant species. For this purpose, we sought to identify these genes from plants closely related to *Arabidopsis*.

*Arabidopsis thaliana* belongs to the Brassicaceae family. Other closely related members of Brassicaceae family include *Arabidopsis lyrata*, *Olimarabidopsis pumila*, *Capsella rubella* and *Brassica oleracea*. The evolutionary distance between *A. thaliana* and the other members of the Brassicaceae ranges from 5 million years to 20 million years. *A. thaliana* is thought to have diverged from *A. lyrata* approximately 5 million years ago (mya); whereas the evolutionary distance between *A. thaliana* and *O. pumila* is about 15 mya (FIG. 28A). The genomes of these plant species are still unknown

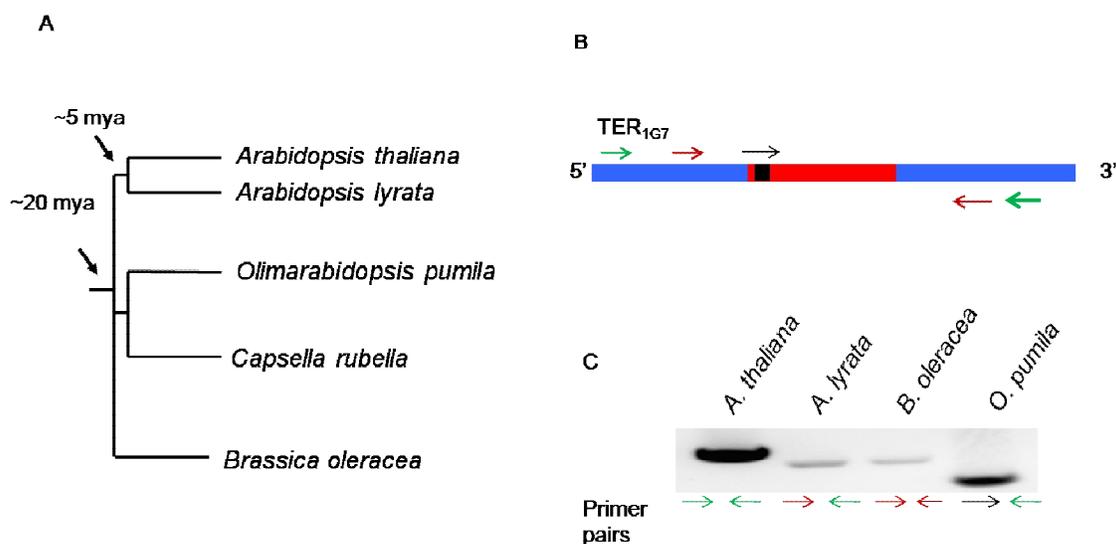


FIG. 28. PCR approach to identify TER genes in plant species closely related to *A. thaliana*. A. Phylogenetic tree of some of the Brassicaceae species. The estimated dates of divergence at different nodes are indicated in millions of years (mya). Adapted from (121). B. Schematic of TER<sub>1G7</sub> gene with red box indicating region shared with TER<sub>5G2</sub> and black box representing the template region. Positions of different primers used in (C) are also indicated. C. Results of PCR amplification using indicated primer pairs and DNA from *A. thaliana*, *A. lyrata*, *B. oleracea* and *O. pumila*.

although sequencing projects are underway for two close *A. thaliana* relatives (*Arabidopsis lyrata* and *Capsella rubella*) (277). Therefore, we decided to use degenerate PCR to amplify TER<sub>1G7</sub> genes from these species. Using this approach, we were able to obtain PCR products which were then cloned and sequenced (FIG. 28C). Sequence alignment of the PCR fragments indicated that TER<sub>1G7</sub> ortholog from *A. lyrata* exhibited 93% identity to the *A. thaliana* TER<sub>1G7</sub> gene (FIG. 29). The *O. pumila* ortholog exhibited 87% identity to the *A. thaliana* TER<sub>1G7</sub> (FIG. 29). In contrast, TER<sub>1G7</sub> from *B. oleracea* (Cauliflower) showed only 30% identity to TER<sub>1G7</sub> from *A. thaliana* (data not shown). Therefore, cauliflower RNA might not be useful in secondary structure determination. For TER<sub>5G2</sub>, we have been unable to identify any orthologs in other plant species by PCR approaches even though many primer combinations were tried. The failure to identify orthologs for TER<sub>5G2</sub> suggests that this RNA could be diverging rapidly.

Several sequenced plant genomes were scrutinized to identify putative TER<sub>1G7</sub> orthologs by in silico analysis. Since TER is a rapidly diverging non-coding RNA, its sequence is not likely to be as well conserved as protein-coding genes flanking it. Therefore, we took advantage of gene synteny or chromosomal positioning of the gene in related plant species. This approach was used to identify TLC1 homologs in yeast belonging to the “sensu stricto” group (68). TER<sub>1G7</sub> from *Arabidopsis thaliana* is present in the 5' UTR of a gene called SnRK1 interacting protein 1 (At1g71310). Both genes are expressed in the same direction and the template in TER<sub>1G7</sub> lies just 59 bp upstream of the start of At1g71310 gene (FIG. 30). Therefore, the sequence of this gene was used as query to BLAST against sequenced genomes such as Rice (a monocot) and *Medicago truncatula* (a dicot). The evolutionary distance between Rice and *A. thaliana* is about 100 mya and the distance between *M. truncatula* and *A. thaliana* is about 140 mya. In spite of the large evolutionary distance between these species, a significant hit

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A. thaliana 1 CTAAACCCTACAC --- GCTTACATAAAAACGCGACCTTTGAGAATTTTGTCCGGCGACAG
A. lyrata 1 CTAAACCCTACAC --- GCCACATAAAAACGCGACCTTTAAGAATTTTGTCCGGCGATTG
O. pumila 1 CTAAACCCTACACAGACCTA AAAAAAACGCGACCTTTAAGAATTTTGTCCGGCGACAG

A. thaliana 58 AAATGGCGGGGTTAGGGTTACGGCTAAAGGCGGC AAAATGGACGTTGAGGAGCGGATTCAG
A. lyrata 57 TAAATGGCGGGGTTAGGGTTACGGCTAAAGGCGGC AAAATGGATGTTGAGGAGCGGAGCAG
O. pumila 60 AAATGGCGGGGTTAATGGTTACGGTTGAAGTCGGCGAAAATGGCGTTGAGGAGCGGAGCAG

A. thaliana 118 GTGCTGTATCGAGAGAATGGTCGTCCGAGATGGGGAAAGGTGTTGTCGTCGATTCCTCTACTG
A. lyrata 117 CTACTGTATCGAGGGGATGGTCGTCCGAGATGGGGAAAGGTGTTGTCGTCGATTCCTCTACTG
O. pumila 120 CTACTGTATCTTAG ----- GGAAATGGGGAAAGGTGTTGTCGTCGATTCCTCTCTG

A. thaliana 178 AAACAGAGAACGATGTTCCAACCTCAGGAATCAGTAGACCCTCGCTGAGATTCTCAAGG
A. lyrata 177 AAACAGAGAACGATGTTCCAACCTCAGGAATCAGTAGACCCTCGCTGAGATTCTCAAGG
O. pumila 168 AAGTTGAAACGATGTTCCAACCTCAGGAATCAGTAGACCCTCGCTGAGATTCTCAAGG

A. thaliana 238 AGCTTAACAAAAAGGTTCCAGATTCAGTCATCCGAACTCGAGTTGAAGATGGTCTCCA
A. lyrata 237 AGCTTAACAAAAAGGTTCCAGATTCAGTCATCCGAACTCGAGTTGAAGATGGTCTCCA
O. pumila 228 AGCTTAACAAAAAGTTCCTGATTCAGTCATCCGAACAGAGTTGAAGATGGTCTCCA

A. thaliana 298 TCAAATACATCCCTTGGTAATCTCTTTCCTCA - GGGT - - - TTAGGGTCTTAAATGG
A. lyrata 297 TGAAATACATGTTCTA-TAATTGGGGTTTTTGA - GAGA - - - CTGAAATTTGTTTITTT - -
O. pumila 288 TGAAATACATCCCATGGTAATCTCTTTTITTTATTGGGAGTTTGAGAAACCTATTITC

A. thaliana 352 GGTITTTGGGTCAACTACTTACTCTTTCCTTGTTGATTCTCAGGCACATTG
A. lyrata 349 - - TTTTGGGGTCAACTACTTACT - - - TTCTTGTGTTGATTCTCAGGCACATTG
O. pumila 348 TGTITTT - - GTTTGGGGTCATACT - - - - - CTTGTTTGTGATTCTCAGGCACATTG

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FIG. 29. Alignment of partial sequences of TER<sub>1G7</sub> orthologs from *A. thaliana*, *A. lyrata* and *O. pumila*. The template region is indicated by a red line. Black nucleotides are identical in all species. *A. thaliana* and *A. lyrata* TER<sub>1G7</sub> orthologs share 93% identity and *A. thaliana* and *O. pumila* orthologs share 87% identity.

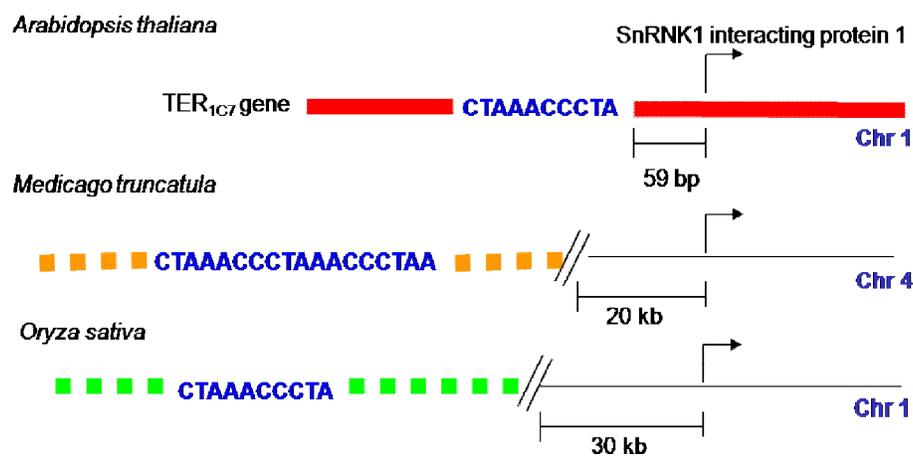


FIG. 30. Identification of putative TER genes in other plant species using a gene synteny approach. Schematic showing chromosomal position of *Arabidopsis* TER<sub>1G7</sub> relative to 5' end of At1g71310 gene which encodes SnRK1 interacting protein 1. Putative TERs from *M. truncatula* and *O. sativa* were identified based on conserved protein coding sequence of SnRK1 interacting protein 1 in those genomes. The positions of putative template sequences are also indicated relative to the conserved protein coding gene. The evolutionary distance between *A. thaliana* and *M. truncatula* is ~100 Mya and that between *A. thaliana* and *O. sativa* is ~140 Mya.

was registered with an E value of  $8e^{-06}$  for Rice and an E value of  $9e^{-24}$  for *M. truncatula*. More importantly, template sequences were identified approximately 30 kb upstream of the SnRK1 homologs in either case. The template in *Oryza sativa* (Rice) was 10 nts long and corresponded to 1.5 telomeric repeats as in *Arabidopsis* (FIG. 30). Interestingly, the template region in the dicot *Medicago truncatula* (a legume) was 18 nts long that corresponded to 2.5 telomeric repeats (FIG. 30). Similarly, gene synteny approaches for TER<sub>5G2</sub> were also employed, but this gene is flanked only by retrotransposon elements and no conserved protein-coding genes. As a result, we were unable to obtain significant hits from BLAST searches.

The plant kingdom is highly diverse and some species of the plants (like the *Asparagales* order) display a six base human-type telomere repeat (TTAGGG) instead of the *Arabidopsis* type repeat (TTTAGGG) (298). We hypothesize that the change in the telomere repeat of *Asparagus* to the human-type repeat is caused by a single nucleotide deletion in the template region of the TER gene. The *asparagus* genome sequence is currently unavailable, but it is thought to have diverged from Rice about 110 mya (335). Therefore, using primers from the region immediately flanking the template in the rice TER<sub>1G7</sub> candidate, we performed PCR on *Asparagus* DNA. A single PCR product of 300 nts was generated. Sequence analysis revealed that the region contained a template sequence CTAACCCTA that was identical to the template of human telomerase RNA. This mutation in the putative TER subunit of *Asparagus* could explain the emergence of human-type telomere repeats in this plant. Partial sequence alignment indicated that the Rice and the *Asparagus* TERs exhibited approximately 30% identity (FIG. 31). The identity in this partial sequence alignment is encouraging given the large evolutionary distance between these two species. In this context, we would expect the identity between TER<sub>1G7</sub> orthologs from *Arabidopsis* and cauliflower to be higher.

Rice	CCTAAACCTATTGAACC--AAGAATG-TGTAATC---AATCA-----CACTCA-----
Asparagus	CCTAA-CCCTATCGAGTCCTGAGAATAATTTAATCCAAAATCAGAGATATATTTATGTGG
Rice	-----GAATTGTCAC-CAGCTATTTATCT-----
Asparagus	AGAGAACTAATAAAGTTTTCACACATGAATTCCTTGTCGGTGACATAGAGAAGAACCAA
Rice	-----AAAATTAT--CACCCAAACATG-ATAGA-ATA--TG TTCACATTGAGTTAC
Asparagus	AAGCCAAGCAAAACTATTACACTCATGCATATATATATATGCCTG TTCACATTGAGTTAC
Rice	GGAT
Asparagus	GGAT

FIG. 31. Alignment of partial sequences of  $TER_{1G7}$  orthologs from Rice and Asparagus. The two RNAs share 30% identity. The putative template region in both RNAs is indicated by the red line.

### *A putative secondary structure model for TER<sub>1G7</sub>*

In the previous section, we provide evidence for TER<sub>1G7</sub> orthologs in other plant species. The sequences of TER<sub>1G7</sub> genes from other plant species can be used in phylogenetic analysis to generate a secondary structure model for plant TERs. As a starting point, a secondary structure model was generated for *A. thaliana* TER<sub>1G7</sub> by our collaborator, Dr. Yehuda Tzfati (Hebrew University) (FIG. 32). The secondary structure shows a single-stranded template region and a stem loop structure immediately 5' of the template. This stem is reminiscent of the template boundary element identified in the secondary structure of other TERs (49, 170, 311). A potential pseudoknot was also predicted. Using the identified elements as constraints, the rest of the sequence was then predicted. Interestingly, a stem resembling the p6.1 region of hTR was also identified (FIG. 32). This stem is part of the conserved region CR4/CR5 domain of hTR which is essential for telomerase activity (51). The 3' end of the RNA has a motif that is the consensus Sm protein binding motif. Both yeast and human TERs exhibit Sm protein binding although this motif is not present in hTR (93, 282). Therefore, TER<sub>1G7</sub> may be similar to hTR in its ability to bind both the dyskerin complex (228, 229) as well as the Sm protein complex (93). This initial secondary structure model will be improved upon by the addition of TER<sub>1G7</sub> sequences from closely related species.

### *Duplication of TER genes in plant species*

The unique feature of *Arabidopsis* TERs is the presence of two related genes. We wanted to know whether this duplication is prevalent among all plant species. As mentioned earlier, our attempts to clone TER<sub>5G2</sub> orthologs from plant species in the Brassicaceae were unsuccessful. Therefore, we adopted another strategy to ascertain

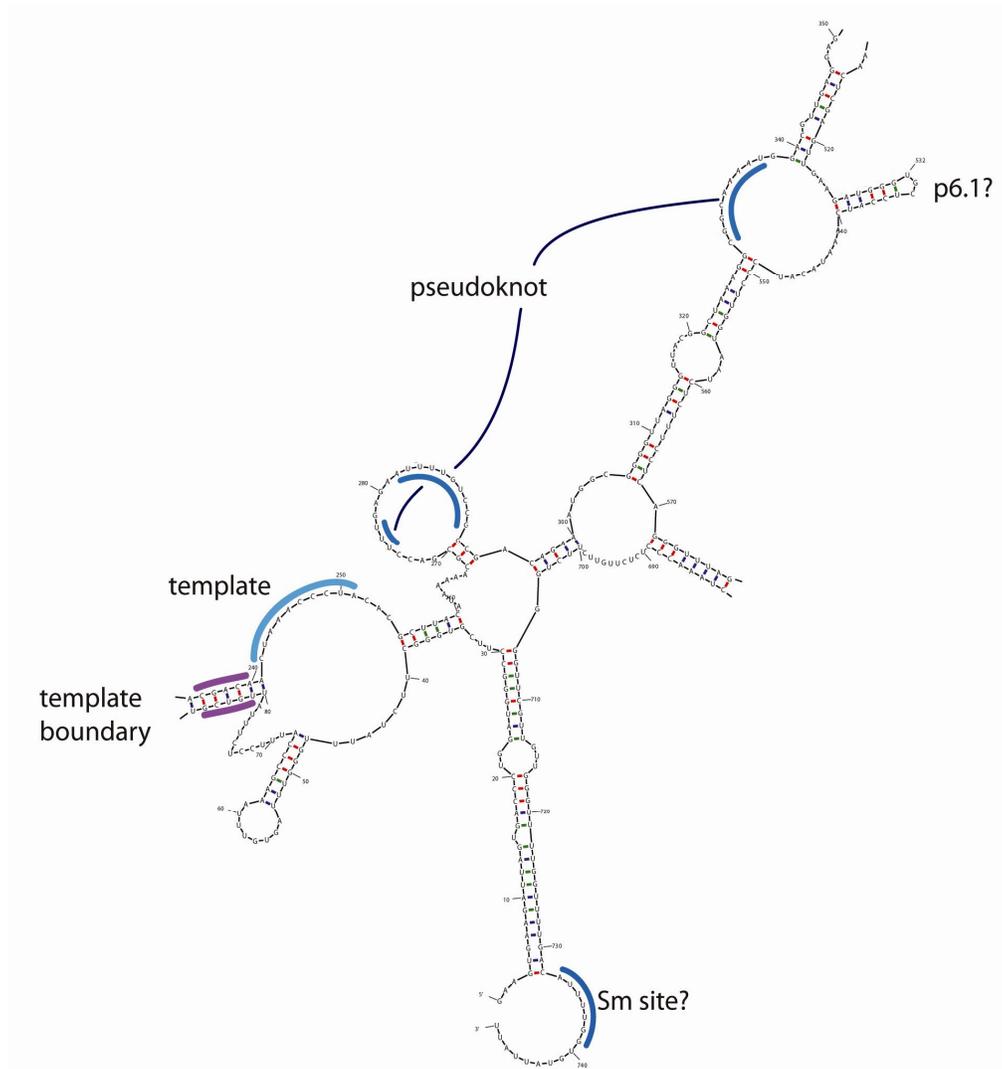


FIG. 32. Preliminary secondary structure model of *A. thaliana* TER<sub>1G7</sub>. The single-stranded template region (blue line) and the template boundary element (purple lines) are indicated. A sequence resembling a Sm site is also indicated as is a stem-loop structure resembling the p6.1 stem from human telomerase RNA. A potential pseudoknot domain is also indicated. The structure does not show the secondary structure for all regions of the RNA. (Model was generated by Dr. Yehuda Tzfati, Hebrew University).

the presence of TER gene duplication. A Southern blot was performed on genomic DNA from Brassicaceae species using a probe to the region of similarity shared by TER<sub>1G7</sub> and TER<sub>5G2</sub>. Interestingly, we were able to observe the presence of two bands by southern in both *A. lyrata* and *O. pumila* (FIG. 33). This suggests the presence of two TER genes in these species. We were able to detect only a single faint band in *C. rubella*. However, this does not rule out the possibility of a second gene in this species. Therefore, from this preliminary analysis of TER gene duplication, we conclude that TER gene duplication dates back to at least 15 mya.

## Discussion

### *Conservation of TER<sub>1G7</sub> in plant species*

All organisms studied so far have only one TER subunit. *Arabidopsis* is the only organism with two TER genes, both of which appear to be functional in telomere maintenance (CHAPTER III). The finding of these two TERs prompted many questions about plant TERs. The first question relates to the conservation of these TER species. Using gene synteny and PCR approaches; we obtained evidence for the conservation of TER<sub>1G7</sub> gene in several plant species. In the Brassicaceae family, we identified TER<sub>1G7</sub> orthologs that displayed a high identity to the *A. thaliana* TER<sub>1G7</sub> gene.

Draft genome sequence has already been released for *Carica papaya*; a distant relative of *A. thaliana* (226) and genome sequencing projects are underway for other species in the Brassicaceae family (277). Once complete sequences are published, gene synteny approaches can be used to identify other TER<sub>1G7</sub> homologs. Through gene synteny approach, we identified an extended template region in the putative TER from *Medicago truncatula*. It will be interesting to identify when the change from a 10 nt

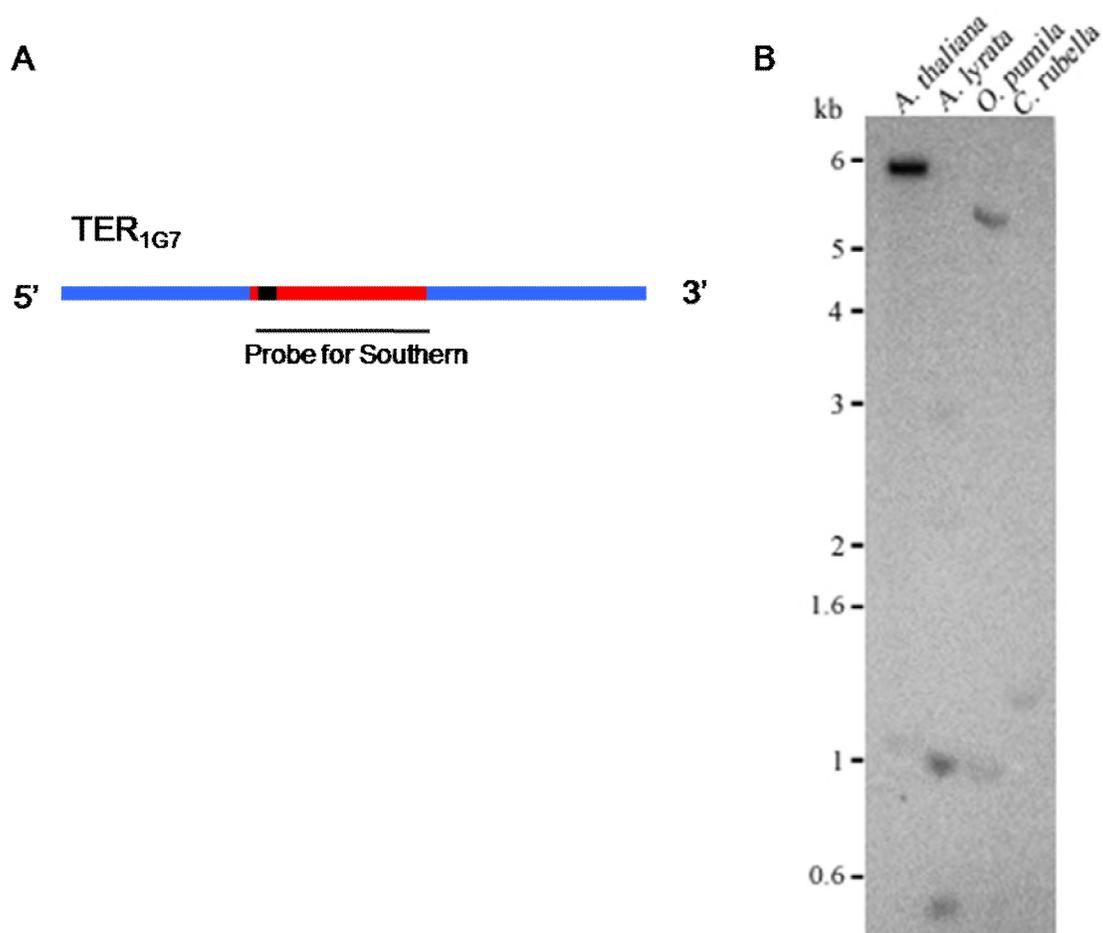


FIG. 33. Evidence for TER gene duplication in plant species closely related to *A. thaliana*. A. Schematic of TER<sub>1G7</sub> gene showing the region shared with TER<sub>5G2</sub> (red) and the template (black). The probe used for southern encompassed the region shared by the two TERs. B. Southern blot of digested DNA from *A. thaliana*, *A. lyrata*, *O. pumila* and *C. rubella* that were hybridized to the probe indicated in A.

template to a 18 nt template took place evolutionarily and also whether the extended template is prevalent in other plant species.

In this chapter, we provide evidence for a template mutation in the putative TER subunit of *Asparagus* that is likely to be responsible for the human-type repeats present in this organism. The sequence information from the putative *Asparagus* TER can be used to clone TER subunits from other closely related species such as *Allium cepa* (onion). Chromosome ends in *Allium cepa* do not terminate with any known telomeric repeats (298). Thus, cloning of the TER subunit from Onion will throw more light on the composition of telomeres in this organism.

#### *A secondary structure model for plant TERs*

The work presented in this chapter also provides a progress report on the generation of a secondary structure for plant TERs. A plant TER model is essential for understanding the evolution of the TER gene. The ciliate TERs are about 150 nts in length and are the prototype for TERs (50). The 5' half of human TER resembles ciliate TERs in its secondary structure. However, the 3' half of this molecule is not represented in the ciliate TER. This part of hTR may have evolved to provide additional regulation and to promote its biogenesis (reviewed in (48)).

The plant TERs (~750 nts) are closer in size to hTR (451 nts) than yeast TERs (~1.3 kb). Therefore, we predict that the secondary structure elements identified in hTR may also be conserved in plants. The working model proposed for TER<sub>1G7</sub> (FIG. 32) contains a stem region that resembles the p6.1 stem from hTR CR4/CR5 region that is essential for telomerase activity. Another element identified in the model of TER<sub>1G7</sub> (FIG. 32), is the template boundary element. It will be possible to disrupt these elements and then study the requirements of these various domains by *in vitro* reconstitution assays or

by *in vivo* complementation assays. Thus, we can begin to study the structure- function relationship of *Arabidopsis* TER<sub>1G7</sub>.

The secondary structure model for TERs is also expected to contain a pseudoknot domain, as this conserved domain is essential for telomerase function *in vivo* (103, 202). Mfold cannot be used to predict pseudoknot domains and hence the conserved regions identified by phylogenetic analysis will be necessary to predict this structure.

To obtain a good secondary structure model, it will be necessary to obtain sequences from additional plant species closely related to *Arabidopsis thaliana*. As few as four sequences have been used to create secondary structure models (68); however, the more the number of sequences, the better the model. A robust secondary structure model for vertebrate TERs was obtained from phylogenetic comparison of 32 TERs from five different classes (50). These sequences will also need to be divergent from the TERs in *A. thaliana* for reasonable phylogenetic comparative analysis. The sequences of TERs obtained from *A. lyrata* and *B. oleracea* may not be suitable for phylogenetic analysis as they are either too similar (as in the case of *A. lyrata*) or too distant (as in the case of cauliflower). Therefore, sequences from species closer to *O. pumila* and *B. oleracea* such as *Crucihimalaya himalaica* and *Caradamine amara* need to be identified, so that TER sequences suitable for phylogenetic analysis can be obtained.

Finally, we have also identified that AtTERT, AtKu70 and AtPOT1a bind to TER<sub>1G7</sub> *in vivo* (CHAPTER III). Once, a phylogenetic structure has been established for TERs, it will be possible to identify structural elements in TER<sub>1G7</sub> that support binding of these proteins. Ku70 has been shown to bind a stem-loop structure in both the yeast as well as the human TER subunit (250, 307). It will be interesting to find whether the

binding of the Ku proteins to *Arabidopsis* TERs is also mediated by a stem-loop structure.

### *Duplication of TER genes*

Studies of genomes have indicated that most of the Brassicaceae species have undergone duplication events and therefore multiple gene families are found in these species (294). In this chapter, we provide evidence for duplication of TER genes in at least some of the Brassicaceae species. With the available Southern data, this duplication event can be dated to have occurred about 15 mya. One of the reasons for retention of the duplicated genes in genomes may be to counter the dosage-sensitivity of some genes (278). Also, differential expression and neo-functionalization of genes may have led to retention of these duplicated genes (278). In this context, the two TERs are also bound by different sets of proteins (CHAPTER III) suggesting that they could be playing different roles *in vivo*. Additionally, the presence of two TER subunits could also be a means of regulating telomerase activity.

Work in our lab has indicated that POT1 genes, which are involved in telomere end protection and telomere length maintenance (284, 296), are also duplicated in the Brassicaceae species (E. Shakirov and D. Shippen, unpublished data). Furthermore, *Arabidopsis* POT1 proteins are unique in that they bind telomerase RNAs rather than DNA (C. Cifuentes-Rojas and D. Shippen, unpublished data). Therefore, it will be interesting to see if the duplication of TER genes extends to the plant species where duplication of POT1 genes has also been observed.

## CHAPTER V

### SUMMARY AND FUTURE DIRECTIONS

Telomerase has been the focus of many research groups as its enzyme activity has implications for both cellular aging and cancer. Telomerase activity is upregulated in many cancer lines and this is one of the key determinants for tumorigenesis (61). The telomerase RNP has been characterized in various organisms: from ciliates where it performs a housekeeping function to humans where its expression and action are highly regulated. Many differences are observed in telomerase RNP composition, assembly and regulation across species (55). In this dissertation, we extend the study of telomerase RNP to the plant kingdom and find commonalities with mammals (presence of dyskerin in the telomerase RNP) and we observe yet another layer of complexity: the presence of two TER subunits.

#### **Protein Composition of the *Arabidopsis* Telomerase RNP**

*Dyskerin is a component of Arabidopsis telomerase*

In chapter II, we investigated the role of AtNAP57 in the *Arabidopsis* telomerase complex. AtNAP57 is a homolog of the pseudouridine synthase, dyskerin, which is a core component of telomerase in humans (53). Dyskerin is essential for the biogenesis of human telomerase RNA and mutations in dyskerin compromise telomerase function and lead to the disease dyskeratosis congenita (165, 210, 229). This disease primarily affects tissues that require constant renewal such as bone marrow and skin (165, 210).

Our studies in *Arabidopsis* indicate that dyskerin is a conserved component of telomerase in plants as well. Both AtTERT and AtNAP57 localize in the nucleolus and immunoprecipitation of AtNAP57 from plants pulls down telomerase activity. However,

our experiments suggest that this interaction is mediated by the telomerase RNA subunit as treatment with RNase A abolished the pull down of dyskerin when AtTERT was immunoprecipitated. This is similar to the situation in mammals, where dyskerin binds to telomerase through the H/ACA motif of hTR (228, 229).

Dyskerin performs an essential role in the pseudouridylation of ribosomal RNAs (219), and therefore is an essential gene in mammals (129). Similarly, we found that AtNAP57 is also an essential gene in *Arabidopsis*. Interestingly, haploinsufficiency of telomerase components is observed in other systems and leads to dyskeratosis congenita in humans (124, 213, 235). But, none of the known *Arabidopsis* telomerase components AtTERT, AtPOT1a and AtNAP57 are haploinsufficient for telomere length maintenance (84, 153, 296). We hypothesize that plants require a lower amount of telomerase to maintain their telomeres. They have fewer telomeres when compared to humans or yeast.

Patients suffering from X-linked dyskeratosis congenita (due to mutations in dyskerin) have shorter telomeres and lower telomerase activity than normal people, suggesting that telomere maintenance is affected in these patients (229). Analysis of plants containing both a wild-type AtNAP57 allele and a T66A mutant AtNAP57 allele similarly indicated that telomere maintenance is compromised in these plants. Mutants have shorter telomeres than wild-type plants, but the shortening is not progressive. Rather telomeres are stabilized at a shorter set point. These mutants also show a seven-fold decrease in telomerase activity, suggesting that the mutant allele could disrupt the telomerase RNP in a dominant negative manner. Therefore, AtNAP57 is critical for telomerase function in *Arabidopsis*. The precise mode of AtNAP57 operation still needs to be addressed and this will throw more light on the biogenesis of the telomerase complex in plants. AtNAP57 shows a weak interaction with another telomerase

component, AtPOT1a (CHAPTER II), but not with AtTERT. However, our experiments suggest that the major binding partners for AtNAP57 are likely to be the TER subunits. Indeed, *in vitro* data suggests that both TER<sub>1G7</sub> and TER<sub>5G2</sub> are able to bind AtNAP57 (C. Cifuentes-Rojas & D. Shippen, unpublished data). Although putative H/ACA motifs have been identified in both TERs, it will be interesting to map the site of interaction of AtNAP57 to see if those motifs are necessary for binding.

#### *Colocalization of Arabidopsis telomerase components*

Studies in human cells have shown that both TERT and hTR accumulate at foci near cajal bodies only during mid S-phase (308). Interestingly, these foci also colocalize with a subset of telomeres (308). Therefore, control of telomerase component localization may be a way of restricting telomerase action to S-phase. The components of the *Arabidopsis* telomerase complex AtTERT and AtNAP57 both show nucleolar staining (153). Preliminary RNA in situ hybridization experiments suggest that TER<sub>1G7</sub> is also nucleolar (J. Lamb and D. Shippen, unpublished data). Since, *Arabidopsis* telomeres also localize to the nucleolar periphery (9), nucleolar localization of telomerase components may enable access to the telomere. To understand the biogenesis of the *Arabidopsis* telomerase RNP, it will be useful to follow the localization patterns of AtNAP57, AtTERT and the TER subunits through the cell cycle. These experiments will help in determining how localization of telomerase components is coordinated with telomerase action at telomeres.

#### *Identification of other mutants in AtNAP57 that compromise telomerase function*

To study the regions of AtNAP57 that are involved in telomerase function, it would be useful to have other mutant lines besides the one described above. EMS

mutagenized TILLING lines (136) would be a good starting point to identify mutations in AtNAP57 that are not lethal. The levels of TERT and TERs can be analyzed in these mutants as well as the telomere length phenotype.

*Identification of other putative telomerase associated proteins involved in telomerase biogenesis*

Dyskerin is part of a complex that consists of the proteins Gar1, Nop10 and Nhp2 (reviewed in (219)). All these proteins are conserved in *Arabidopsis*. However, some of these proteins are encoded by multigene families making it difficult to dissect their function. We predict that these proteins will also be associated with the *Arabidopsis* telomerase complex. Another protein Naf1, interacts with the dyskerin complex in humans and is required for association of dyskerin with small nucleolar RNAs (139). Interestingly, RNAi depletion of human Naf1 affected the accumulation of hTR levels in addition to that of snoRNAs (139), suggesting that hNaf1 may also play a role in the biogenesis of human telomerase RNP. Naf1 is a single-copy gene in *Arabidopsis* (At1g03530), and it interacts and co-localizes with AtNAP57 (182). We obtained a line with an insertion in AtNaf1 (SALK\_013589) and analyzed the telomere phenotype of null mutants. Telomeres in null *naf1* mutants were in the wild-type range, indicating that AtNaf1 is not required for telomere length maintenance. (K. Kannan and D. Shippen, unpublished data). However, the effect of AtNaf1 inactivation on telomerase activity as well as telomerase RNA accumulation remains to be tested.

Recently, two novel telomerase components were identified from human cells through affinity purification of TERT. These proteins called pontin and reptin are ATPases (315). Pontin and reptin interact with both TERT as well as dyskerin. Depletion of these proteins results in reduced hTR accumulation (315). Pontin and reptin may

transiently associate with telomerase during its biogenesis and then dissociate before telomere synthesis. Unfortunately, *Arabidopsis* contains multiple homologs to pontin and reptin and thus like the dyskerin complex, it will be difficult to dissect their role in plant telomerase biogenesis.

We are only beginning to understand the composition of the *Arabidopsis* telomerase RNP. Sequence homologs to telomerase components in other systems have been identified in *Arabidopsis*; however, the functions of these proteins are not always conserved. For example, there are two putative homologs to *S. cerevisiae* EST proteins in *Arabidopsis*, but neither of the plant proteins are components of the telomerase holoenzyme (264). Similarly, we have also looked at the interaction of AtTERT with Hsp90, p23 and 14-3-3 proteins, all of which interact with hTERT (141, 280). However, by co-immunoprecipitation experiments, no interactions were detected between AtTERT and the homologs of Hsp90, p23 and 14-3-3 (K. Kannan and D. Shippen, unpublished data). It may be that these proteins bind to the telomerase complex rather than to AtTERT alone. Another possibility is that these interactions are not conserved in *Arabidopsis*.

To identify additional telomerase components in *Arabidopsis*, yeast-two hybrid library screening could be performed using AtTERT as bait protein. Alternatively, mass spectrometry on purified telomerase complexes would enable the identification of novel components.

### **Identification and Characterization of *Arabidopsis* TERs**

In chapter III, the identification and characterization of *Arabidopsis* telomerase RNA subunit is described. Biochemical purification of telomerase from *Arabidopsis* cell culture led to the unexpected finding of two telomerase RNA subunits, TER<sub>1G7</sub> and

TER<sub>5G2</sub>. This is a novel finding as all other model organisms have only one TER subunit. The two TERs share a 220 nucleotide region of nearly 93% identity which includes the templating domain. Outside this region, there is little sequence similarity. TERs are expressed at dramatically different levels in plant tissues, but peak in highly proliferative cells where telomerase is active. This is similar to the situation in normal human cells, where hTR is abundant but its abundance further increases in cancer cells where telomerase activity is high (reviewed in (61)). It will be of interest to determine why *Arabidopsis* TERs are expressed at such different levels and how telomerase activity is impacted by altering the levels of RNAs *in vivo*.

#### *Strategies to reduce levels of TERs in plants*

In this dissertation, I have taken advantage of a variety of genetic strategies available to analyze gene function in *Arabidopsis*, including T-DNA insertion lines, antisense and RNAi. This has allowed analysis of the function of TERs *in vivo*.

Genetic analysis of two T-DNA insertions in TER<sub>1G7</sub> failed to uncover a null mutant. The truncated TER<sub>1G7</sub> transcript generated in TER<sub>1G7-1</sub> mutants can still function to reconstitute telomerase activity, although the level of activity is reduced (C. Cifuentes-Rojas & D. Shippen, unpublished data). Therefore, null mutants are unavailable for TER<sub>1G7</sub> as of now. A priority for future studies of TER subunits will be the identification of mutant lines which are null for TER<sub>1G7</sub>. In addition to T-DNA insertion lines available in the *Arabidopsis* biological resource center (ABRC), many collections of unmapped T-DNA lines are also available. About 33,000 such lines were screened by PCR to identify additional insertion lines for TER<sub>1G7</sub> (K. Kannan and D. Shippen, unpublished data). Unfortunately, we were able to identify insertions only in the 5' of TER<sub>1G7</sub> gene. However, more collections of unmapped T-DNA lines are available for screening. A null mutant is

available for TER<sub>5G2</sub>, but telomere maintenance is not compromised in the first generation of this mutant. This suggests that the two TERs may function redundantly in telomere maintenance.

Unexpectedly, TER<sub>5G2-1</sub> mutants display a 2.5 fold increase in telomerase activity (C. Cifuentes-Rojas & D. Shippen, unpublished data). This suggests that TER<sub>5G2</sub> acts an inhibitor of telomerase activity *in vivo*. This is a novel role for a TER subunit that has not been observed in other species and warrants more investigation. Parent-progeny analysis through at least two more generations will be necessary to understand the effects of the increased telomerase activity on telomere length in these mutants.

Another strategy to lower the levels of TERs in plants is to perform antisense and RNAi experiments. Given the regions of extensive similarity in the two RNAs, it is possible to target both TERs and to target each TER separately through their distinct regions. We transformed wild-type plants with an RNAi construct targeting 100 nts of the region shared by the two TERs, which included the template region. However, we were not able to identify any transformants in which TERs levels were knocked down significantly (K. Kannan and D. Shippen, unpublished data). We think that the RNAi machinery may not have been able to target TER since it is a nuclear RNA and this machinery mostly targets mRNAs present in the cytoplasm.

Next, we tried using an antisense approach to lower TER levels. To increase the sensitivity of detecting telomerase deficiency, we transformed antisense constructs into *ku70*<sup>+/-</sup> mutants. AtKu70 is a negative regulator of telomerase (97, 269). In *ku70*<sup>+/-</sup> mutants, telomeres are in the wild-type range of 2-5 kb, but in the first generation of *ku70*<sup>-/-</sup> mutants, telomeres get elongated to about 6-8 kb by telomerase (269). We expected that if TER levels are decreased, then telomere lengthening would not be observed in first generation *ku70*<sup>-/-</sup> mutants and this could be discerned by TRF analysis

(CHAPTER III, FIG. 23). In *ku70*<sup>+/-</sup> mutants, we expected telomeres to stay in the wild-type range or shorten slightly when TER levels are decreased in this background (CHAPTER III, FIG. 23).

Initial antisense experiments in *ku70*<sup>+/-</sup> mutants suggested that TER levels are decreased *in vivo*. But, a telomere phenotype was observed only in the second generation of transformants where telomeres are considerably elongated ranging from 3-12 kb. We suspect that this elongation is due to the combined loss of one allele of AtKu70 and TER<sub>5G2</sub> in these mutants. This data reveals an unanticipated role for TER<sub>5G2</sub> as a negative regulator of telomerase *in vivo* and suggests that proper telomere length maintenance in *Arabidopsis* requires action of TER<sub>5G2</sub>.

To further reduce TER levels, we transformed antisense constructs against TER<sub>1G7</sub> into a TER<sub>5G2</sub> null mutant. If this experiment lowers TER<sub>1G7</sub> levels to a great extent, and we expect to see a more significant loss of telomerase, and perhaps telomere shortening. These experiments are in progress.

#### *Contribution of TERs to telomere synthesis*

The duplication of TER genes in *Arabidopsis* raises the question of whether both TERs are involved in telomere repeat synthesis *in vivo*. Specifically, does TER<sub>5G2</sub> function as a regulator alone or is it also used for telomere synthesis? To address these questions, it would be useful to have a double null mutant for both TERs. These plants could then be transformed with template mutant constructs for both TERs under the control of their native promoters. Analysis of the telomere sequence in the transformed plants would reveal whether one or both of the TERs were involved in telomere repeat synthesis. For TER<sub>1G7</sub>, a template mutant TER<sub>1G7-CC</sub> was transformed into plants and

sequencing of telomeres in the transformants revealed a low level of incorporation of these repeats onto chromosome ends.

If a heterodimer of TERs is active then we might expect to observe alternating mutant repeats from each TER. We have already transformed TER<sub>5G2-1</sub> plants with a construct overexpressing TER<sub>5G2</sub> template mutation (TER<sub>5G2-Rsa</sub>). The levels of TER<sub>5G2</sub> in these transformants will be higher than that in wild-type plants and therefore if TER<sub>5G2</sub> is a negative regulator, these plants may not be useful in understanding whether TER<sub>5G2</sub> is involved in maintaining telomere tracts under physiological conditions. However, this experiment will allow us to gauge if TER<sub>5G2</sub> can be incorporated into telomerase RNP *in vivo* and if it can be used as a template by telomerase.

#### *Interactions of TER subunits with distinct proteins*

TERs bind a distinct set of proteins *in vivo* and *in vitro*. Both *Arabidopsis* TERs are bound by AtTERT (CHAPTER III). The telomerase component AtPOT1a binds only to TER<sub>1G7</sub> and not to TER<sub>5G2</sub>, whereas both ATR and AtKu70 interact only with TER<sub>5G2</sub> (CHAPTER III). Thus, different sub-complexes of telomerase are possible *in vivo*. It will be interesting to know when in the cell cycle these interactions occur and to know the roles of the different sub-complexes of telomerase *in vivo*. Studies in yeast have shown that Est2 (TERT) is situated at the telomeres at all times during the cell cycle, and that binding of Ku70/80 and Est1 (a component of the yeast telomerase holoenzyme) to telomerase is cell cycle regulated (82, 300). Loss of KU results in decreased accumulation of Est1 at telomeres and subsequently disrupts telomerase action at telomeres (82). Mec1 (ATR) binding to telomeres is also cell cycle regulated and is coordinated to enable telomerase action at telomeres during S-phase (302, 303). Thus,

telomere synthesis requires cell cycle regulated interactions of telomere-related proteins with telomerase.

In *Arabidopsis*, it is known that AtPOT1a is associated with telomerase and that AtPOT1a association with telomeres is increased in S-phase (296). It will be interesting to find out whether TERT is bound to telomeres at all times and when Ku70/80 binds to telomerase. How does the loss of any of these components affect the accumulation of other components at the telomere? We could also examine the localization of ATR with telomeres and telomerase through the cell cycle. These experiments will provide an insight into the dynamic nature of telomerase *in vivo*.

For all the interactions identified, it will be necessary to define the region of interaction on the TER subunit as well as the protein domains involved in binding. This study can be done by *in vitro* binding studies or by using the yeast-three hybrid system. *In vitro* data indicates that AtPOT1a binds to TER<sub>1G7</sub> through its OB-fold domains (C. Cifuentes-Rojas & D. Shippen, unpublished data). AtPOT1b is different from AtPOT1a in that it binds to TER<sub>5G2</sub> (C. Cifuentes-Rojas & D. Shippen, unpublished data). It will be interesting to see if the OB-folds of AtPOT1b are necessary for this interaction. If this is so, then it would be necessary to understand the differences between the OB-folds of AtPOT1a and AtPOT1b that allow recognition of different TERs. Studies in other systems have shown that protein-TER interactions are mediated by specific secondary structures such as pseudoknots or stem-loops in the RNA (reviewed in (305)). Similar secondary structure elements necessary for binding to TERT and Ku will also need to be identified in *Arabidopsis* TERs. These studies will be greatly aided by the generation of a secondary structure model for *Arabidopsis* TERs (see below).

The interaction of ATR with *Arabidopsis* telomerase is an exciting finding and implies that the DNA damage response proteins have a conserved role in telomerase

recruitment like in yeast and mammals (196) (CHAPTER III). In yeast, Mec1 (ATR) is recruited to telomeres during S-phase by the MRX complex and this recruitment is necessary for subsequent recruitment of telomerase by Cdc13 (G-overhang binding protein) and Est1 (302, 303). Therefore, we suspect that interaction of ATR with *Arabidopsis* telomerase is not a direct interaction, but may occur through telomere binding proteins that function in telomerase recruitment. The identification of these proteins will be crucial for understanding the role of ATR in the telomerase complex in a higher eukaryote. One candidate ATR interacting protein is AtPOT1b which binds to TER<sub>5G2</sub> like ATR. AtPOT1b appears to interact with ATR by co-IP and yeast-two hybrid analysis (L. Vespa, M. Jasti and D. Shippen, unpublished data). Both *atr* and *pot1b* single mutants do not show any telomere length phenotype but these mutants have been crossed to generate double mutants (E. Shakirov and D. Shippen, unpublished data) (317). Analysis of these mutants may provide further insight into the relationship between telomerase and the DNA damage response.

#### *Identification of novel protein interacting partners for Arabidopsis TERs*

Several proteins have been proposed to interact with TERs in other species (53, 55). The presence of two *Arabidopsis* TERs and the possibility of different sub-complexes of telomerase suggest that TERs may have other unique protein partners. The associations of these partners could be transient, but may be important in modification, regulation or transport of TERs.

Yeast- three hybrid library analyses can be performed with either TER as bait to find interaction partners. An alternate approach is to tag TERs with small tags such as aptamer tags (292) and introduce the tagged TER in plants null for the specific TER. Complexes can then be purified using the tags and mass spectrometry analyses can

then be performed. Novel protein components that are retrieved from these analyses can be characterized with respect to their role in telomere length regulation or telomerase activity.

Interestingly, yeast-two hybrid screens for AtPOT1a interacting proteins uncovered two proteins that have not been characterized previously (M. Jasti and D. Shippen, unpublished data). One of these proteins has a putative RNA binding motif. It remains to be seen if these proteins interact with *Arabidopsis* TERs and if they play any role in the telomerase RNP.

#### *Identification of the roles of TERT and TER domains by in vitro reconstitution studies*

Our experiments indicate that both TERs can reconstitute telomerase activity *in vitro* when co-expressed with AtTERT (C. Cifuentes-Rojas & D. Shippen, unpublished data). This result was important as it provided strong evidence that the TER subunits serve as a template for telomerase. The establishment of this assay has made it possible to address questions relating to function of the TERT domains and elements of the RNA involved in binding and processivity. For example, in humans, it was found that two conserved regions of hTR, the pseudoknot region and the CR4/CR5 domain are sufficient to reconstitute telomerase activity *in vitro* (227). Also, the contribution of different TERT and TER domains to telomerase processivity can be assessed. It would be interesting to know if the N-terminal domain of AtTERT plays an important role in telomerase processivity in plants as it does in humans and yeast (reviewed in (12)). Thus, we can begin to understand the functions of TER and TERT domains.

## Identification of TER Homologs

In chapter IV, we describe the approaches taken to identify TER homologs in species closely related to *Arabidopsis thaliana*. We wanted to determine when the TER gene duplication occurred and also to obtain sequences of TER homologs from related species to generate a secondary structure based on phylogenetic covariation analysis. Phylogenetic analysis has been used to determine the secondary structure of TERs in ciliates, yeast and vertebrates (50, 68, 272, 352) and has led to the identification of a conserved TER core. We would expect plant TERs to also contain these conserved secondary structure elements.

The identification of TER<sub>1G7</sub> homologs in species such as *Arabidopsis lyrata* and *Olimarabidopsis pumila* revealed these TER subunits exhibit 93% and 87% identity to *Arabidopsis thaliana* TER<sub>1G7</sub>. In contrast, the TER<sub>1G7</sub> homolog from *Brassica oleraceae* displays only 30% identity to *A. thaliana* TER<sub>1G7</sub>. Thus, it will be necessary to determine if the other Brassica TERs have diverged significantly from *A. thaliana* (e.g. TERs from *Brassica rapa*) or if *B. oleraceae* is an outlier. The genome of *B. rapa* is currently being sequenced and therefore it will be possible to identify TER<sub>1G7</sub> orthologs in this organism (278).

For secondary structure determination by phylogenetic analysis, it is preferable to have sequences exhibiting ~ 60-80% identity (68). The greater the number of sequences, the better the secondary structure model generated. Therefore, TER subunits need to be cloned from species closely related to *A. thaliana*. Some of the species that can be considered are *Crucihimalaya himalaica* and *Caradamine amara* that are separated from *A. thaliana* by 10-14 mya and 13-19 mya respectively (121, 168). Sequencing projects are underway for other species related to *A. thaliana* such as *Capsella rubella* and *Thellungiella halophila* (278). The availability of sequenced

genomes and degenerate PCR approaches should make the identification of TER subunits relatively straightforward and will allow us to generate a secondary structure model for TER<sub>1G7</sub>.

For TER<sub>5G2</sub>, we have been unable to find orthologs in species closely related to *A. thaliana* by degenerate PCR approaches (CHAPTER IV). Preliminary Southern blotting experiments performed using a probe to the region shared by both TER<sub>1G7</sub> and TER<sub>5G2</sub>; indicate the presence of two TER genes in both *A. lyrata* and *O. pumila* (CHAPTER IV). Perhaps, this RNA is diverging rapidly, and therefore PCR approaches need to be modified to focus on the region shared with TER<sub>1G7</sub>.

#### *Extent of TER duplication in plant species*

Preliminary Southern blotting experiments reveal that duplication of TER genes at least extends to a species that diverged from *A. thaliana* about 15 mya (CHAPTER IV). This analysis needs to be extended to other species closely related to *A. thaliana* to more precisely define the date for duplication. Southern blotting with a variety of probes specific to either TER or common to both TERs can be used to analyze the extent of duplication in Brassicaceae. This branch of angiosperms is interesting as species with different genome sizes and different duplication histories are present. For example, it is known that segments of *Arabidopsis* genomes are present in triplicate in the Brassica species (121). Several species within Brassicaceae have genomes that are smaller than *A. thaliana* including the plant *Cardamine amara*, whose genome size is one-third of *Arabidopsis* (121). Does TER duplication extend to this plant species? With more plant TER sequences, we can begin to address questions about the structure/function of RNAs and about the conservation of protein-binding sites in these RNAs.

### *Verification of the secondary structure model for TER<sub>1G7</sub>*

A secondary structure model was generated for *A. thaliana* TER<sub>1G7</sub> by our collaborator, Dr. Yehuda Tzfati (Hebrew university). This preliminary model is very encouraging as it shows elements conserved in other TER species. The presence of such elements (e.g. template boundary element, the pseudoknot and the p6.1 stem) can be verified through mutational analysis followed by reconstitution reactions *in vitro*. These mutants can also be transformed into plants null for TER<sub>1G7</sub> to study the effect of these changes on telomerase activity *in vivo*. Compensatory mutations that restore the secondary structure element can be introduced to check if telomerase activity is restored.

A consensus Sm motif was also identified in the preliminary secondary structure model for TER<sub>1G7</sub>. Heptameric Sm proteins function in biogenesis of snRNAs (260), and bind both yeast and human TERs (93, 282). It will be interesting to see if an Sm binding site is also seen in TER<sub>5G2</sub>. This analysis can be extended to see if the biogenesis pathway of *Arabidopsis* TERs is more similar to that of small nucleolar RNAs (involving dyskerin complex) or small nuclear RNAs (involving Sm proteins). *Arabidopsis* TERs may also be similar to human telomerase RNA which has a biogenesis pathway involving the dyskerin complex and is additionally also associated with members of the Sm protein complex (reviewed in (48)).

Human telomerase RNA contains an H/ACA motif at its 3' end which is bound by dyskerin (228, 229). This motif consists of an H box (ANANNAA) and an ACA box which together adopt a hairpin-hinge-hairpin-tail secondary structure (99). Our data suggest that *Arabidopsis* dyskerin (AtNAP57) is associated with telomerase through an RNA subunit (153). *In vitro*, AtNAP57 binds both TER<sub>1G7</sub> and TER<sub>5G2</sub> (C. Cifuentes-Rojas & D. Shippen, unpublished data). Both TERs contain many putative H/ACA motifs and

therefore a secondary structure model will help in resolving which of these motifs are functionally relevant.

Finally, secondary structure models for *Arabidopsis* TERs will be very useful in understanding the elements of TERs that are necessary for protein binding. Are *Arabidopsis* TERs also modular structures similar to yeast TLC1? Once protein binding elements have been mapped on TERs, experiments can be performed in which the positions of these elements are swapped in TERs and the impact of this swapping on TER function can be assessed.

In summary, a secondary structure model for TERs will help in understanding why certain features of TERs have been conserved and how plant TERs relate to TERs from other eukaryotes.

#### *Defining a minimal TER core*

The yeast TER, TLC1, is the largest TER identified at 1.3 kb (287). The secondary structure for TLC1 predicts a central core region that contains the template and pseudoknot domains and three long helical arms that emanate from this central core, which are necessary for binding to specific proteins (68, 352). Intriguingly, most of the RNA is not required for function: a 500 nt version of TLC1, called Mini-T RNA that contains only the central core and the protein-binding stems can maintain telomeres *in vivo* and reconstitute telomerase activity *in vitro* (353). Thus, Mini-T RNA represents the core of the telomerase RNA in yeast.

One observation that led us to think about a TER core for *Arabidopsis* TERs is that homozygous TER<sub>1G7-1</sub> mutants, that express a truncated TER<sub>1G7</sub>, display reduced activity *in vitro*. This truncated transcript is similar to TER<sub>5G2</sub> in that the template region is present at the 5' end. Thus, the 5' 200 nucleotides of TER<sub>1G7</sub> are apparently not required

for its function. Once, secondary structures have been defined for *Arabidopsis* TERs, it will be interesting to define the minimal TER<sub>1G7</sub> and TER<sub>5G2</sub> core that is necessary for telomerase function *in vivo* and study the differences in the two TER cores that contribute to their function. It may be that the shared region in the two TERs is sufficient to constitute the core TER. Once the core elements are defined, it may be possible to recreate a TER core that combines elements of both TER<sub>1G7</sub> and TER<sub>5G2</sub> (such as protein-binding structural elements). This RNA will be representative of the ancestral RNA before duplication of TER subunits occurred. It will be exciting to see if this ancestral RNA can function *in vivo*.

#### *Identification of TERs in plant species with non-canonical telomere repeats*

In chapter IV, we present the identification of a putative TER from *Asparagus*. *Asparagus* is one of several plant species in the Asparagales order of plants that display human-type telomere repeats (298). When the sequence of full-length *Asparagus* TER is available, the interaction of this TER with *Asparagus* POT1 can be checked. *Asparagus* POT1 was cloned in our lab and was shown to bind both *Arabidopsis* and human type telomeric DNA *in vitro* by gel-shift assays (E. Shakirov and D. Shippen, unpublished data). This is in contrast to AtPOT1a, which does not show any binding to telomeric DNA repeats *in vitro* and instead has evolved to bind TER<sub>1G7</sub> (296). Therefore, it will be interesting to know if *Asparagus* POT1 has retained both DNA and RNA binding activities.

The sequence information from the putative *Asparagus* TER can be used to clone TER subunits from other closely related species such as *Allium cepa* (onion). *Allium cepa* does not contain any of the known telomere repeat sequences (251, 298). Although strategies such as FISH and Southern blotting have been used to detect

telomere repeats in *Allium*, no G-rich repeat has been identified (251, 298). Cloning of the TER subunit and definition of the telomere template sequence from Onion will throw more light on the composition and evolution of telomeres in plants.

## **Conclusions**

In conclusion, we have characterized two of the core components of the *Arabidopsis* telomerase RNP. Our studies reveal similarities and intriguing differences between telomerase RNPs in *Arabidopsis* and other organisms. Future challenges will include elucidating the function of these components in the telomerase RNP and defining additional components. The proposed studies will provide insight into the evolution of the telomerase complex.

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**APPENDIX A**  
**ALTERNATIVE APPROACHES TO IDENTIFY TER CANDIDATES IN *ARABIDOPSIS***  
***THALIANA***

### **Summary**

Telomerase is the enzyme responsible for addition of telomeric repeats to the ends of chromosomes. The catalytic component of telomerase, TERT, is highly conserved among eukaryotes, while the RNA subunit of this enzyme, TER, is highly divergent in both sequence and size. Thus, it has been difficult to identify TER orthologs by homology searches. Here, we describe biochemical and in silico approaches that were adopted to identify the *Arabidopsis* TER subunit and the subsequent characterization of these TER candidates. We focused on identification of putative telomere template sequences in the *Arabidopsis* genome. Several potential candidates were identified. These candidates were subjected to Mfold and expression analysis, from which a suitable candidate, 4G3 RNA, was obtained. However, through subsequent biochemical and genetic analyses, we found that 4G3 RNA was not a true TER subunit. Other approaches taken to identify candidate TERs are also described. Although these attempts were unsuccessful, they laid the foundation for characterization of bona-fide TER subunits, TER<sub>1G7</sub> and TER<sub>5G2</sub>.

### **Introduction**

Telomerase is a ribonucleoprotein complex that serves to add G-rich telomeric repeats to chromosome ends. Telomerase functions to maintain telomeres, helping the cell avoid disastrous situations such as chromosomal fusions and nucleolytic degradation of chromosomal DNA. Telomeres and the proteins that bind telomeres

provide this protection by disguising the chromosomal ends from factors that can recognize the terminus as a double-stranded break.

Telomerase is minimally composed of two subunits: a reverse transcriptase subunit (TERT) and an RNA subunit (TER). TERT subunits are distinguished by several conserved RT motifs in the central region and a variable N and C-terminal region that aid in RNA binding and processivity (reviewed in (12)). In contrast, the TER subunits are highly divergent with sizes varying from 150 nts in ciliates to 1.3 kb in yeast (reviewed in (48)). There is no significant primary sequence conservation among the various TER subunits. Instead, the conservation is at the level of secondary structure. All TERs contain a single-stranded template region that is composed of sequence complementary to 1.5 telomeric repeats. Another conserved feature is a pseudoknot domain downstream of the template. This region is thought to be essential for telomerase activity (reviewed in (48)). The yeast TER, TLC1, is much larger, but recent data suggests that it has a smaller core that is sufficient for telomere maintenance (353). The truncated yeast TER called Mini-T contains the template and the pseudoknot region that forms the central core. In addition, Mini-T contains the binding arms for other proteins such as Est1, Ku and Sm proteins that enable its biogenesis and function (352, 353).

*In vitro*, telomerase activity can be reconstituted with TERT and TER in the presence of an extract such as rabbit reticulocyte lysate (331). For reconstitution of human telomerase activity, the 5' end of hTR is sufficient. This region contains the template and pseudoknot regions (227). The 3' region of hTR, which is required for its biogenesis and accumulation in cajal bodies (151, 228, 359), contains an H/ACA motif that serves as the binding site for the dyskerin complex of proteins (229). The dyskerin complex (which includes Gar1, Nhp2 and Nop10) binds to H/ACA small nucleolar RNAs (snoRNAs) and is involved in pseudouridylation of target residues in ribosomal RNAs.

Recent data suggests that dyskerin is also a member of the catalytic core of human telomerase (53). Purification of telomerase activity from cancer cells showed that the telomerase core enzyme consists of two molecules of TERT, hTR and dyskerin. The 3' region of hTR also contains a CAB box which is necessary for directing the RNA to cajal bodies (151). It is thought that these sub-nuclear structures may be important in the biogenesis and assembly for the telomerase complex as both hTERT and hTR localize to this compartment (48).

In *Arabidopsis*, the TERT subunit had been studied in some detail. Inactivation of AtTERT leads to loss of 200-500 bp of telomeric DNA per generation (84, 268). From the sixth generation (G6) onward, these plants begin to accumulate end to end chromosome fusions leading to genome instability (268). These mutants can survive up to nine generations, but ultimately arrest in a terminal vegetative state (268). Work in our lab has led to identification of two other protein constituents of the *Arabidopsis* telomerase complex- AtNAP57 (153) and AtPOT1a (296). AtNAP57 is homolog of human dyskerin, and AtNAP57 co-purifies with telomerase activity (153). AtPOT1a also co-purifies with telomerase activity and is required for maximal telomerase activity *in vivo* (296). The missing component of the *Arabidopsis* telomerase complex is the TER subunit.

In this section, we discuss the different biochemical and bioinformatics approaches taken to identify TER candidates in *Arabidopsis*. Although several candidate TERs were identified, knockdown and knock-outs of these RNA candidates did not lead to a telomere maintenance defect. Thus, using these approaches, we were unable to find real TER subunits.

## Materials and Methods

### *Bioinformatics strategy*

Identification of telomere template in the genome was performed by using the program called Patmatch (<http://Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl>) on the TAIR website. The sequence CTAAACCCTA, which corresponds to the putative TER template, was used as the query. Both DNA strands of the intergenic dataset of the *Arabidopsis* genome were searched. Hits that contained multiple templates in the same sequence were eliminated. Mfold analysis to predict the secondary structure of candidate TERs was performed using default parameters on the Mfold website (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>).

### *Plant materials and genotyping*

*Arabidopsis* seeds with T-DNA insertions in the 4G3 RNA (FLAG\_368C04) and At\_oRNA\_415 (SALK\_065403) genes were purchased from INRA (France) and the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, Ohio) respectively. Seeds were cold-treated overnight at 4°C, and then placed in an environmental growth chamber and grown under a 16 h light/8 h dark photoperiod at 23°C.

For genotyping, DNA was extracted from flowers and PCR was performed with the following sets of primers for 4G3 RNA: F1: 5' CGGATGCACTAAACCCTACA 3' and R1: 5' ACGTGAACGAAAGCTTCGT 3' and for 3G0 RNA: Fwd: 5' AATCACTTATTTCTCTAAAAATCT 3' and Rev: 5' ATATGGTGTTTGAACATATTTTGA 3'.

### *RNA extraction and c-DNA synthesis*

Total RNA was extracted from 0.5 g of flowers using Tri Reagent solution (Sigma). cDNAs were synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen). Oligo dT primers were incubated with 2 µg of total RNA in the supplied buffer at 65°C for 5 min. Reverse transcription was carried with 100 U of Superscript III at 55°C for 60 min. RNA was degraded with RNase H (USB). For amplifying cDNA, genotyping primers were used.

For recovering sequences of RNAs co-purifying with FLAG-TERT immunoprecipitation, the protocol described in references (147, 209) were followed. PCR products were cloned into pDrive (QIAGEN) and subsequently sequenced.

### *RNAi, antisense constructs and plant transformation*

For RNAi against 4G3 RNA, 100 nucleotides surrounding the template region were targeted. This region was cloned into the vector pKannibal (334) and then transformed into plants. For 3G0 RNA, the targeted regions were amplified by PCR and cloned into gateway compatible vectors pB7WG1GW2 for RNAi and pB7WG2 for antisense. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101. Transformation of wild-type plants was performed by the in planta method as described in (269). Transformants were selected on 0.5 Murashige and Skoog basal medium supplemented with 20 mg/L of phosphinothricine (Crescent Chemical) and Kanamycin (50 µg/mL) and then genotyped.

### *Immunoprecipitation and western blots*

Extracts were made by grinding 0.3 g of flowers in buffer A (50 mM Tris-Cl pH7.5, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT and plant protease inhibitors (SIGMA)). For immunoprecipitation, 50 µl of α-FLAG beads (SIGMA) were washed four times with buffer A and incubated with 500 µl of extract for 2 h at 4°C. Beads were then washed three times with buffer A and eluted using the 3X FLAG peptide for 30 min. Elution was performed three times and 2 µl of each eluate was used for TRAP assay and RNA was extracted from the rest using TRI reagent. For western blots, eluates were run on 10% SDS-PAGE gel and then transferred to nitrocellulose membranes. For western, POT1a peptide antibody (296) was used at 1:1000 dilution and peroxidase-conjugated light chain-specific mouse anti-rabbit secondary antibody was used as 1:10,000 dilution (Jackson Immunoresearch).

### *TRF analysis and TRAP assays*

DNA from individual whole plants was extracted and TRF analysis was performed with *Tru1I* (Fermentas) restriction enzyme and [<sup>32</sup>P] 5' end-labeled (T<sub>3</sub>AG<sub>3</sub>)<sub>4</sub> oligonucleotide as a probe (84). Extracts for TRAP assays were made from flowers as previously described (83). For the telomerase inhibition studies, the following oligonucleotides (at 1 µg/µl concentration) were boiled for 5 min and then 1 µl of oligonucleotide was incubated with 2 µl of wild-type protein extract for 30 min. TRAP assay was then performed as previously described (83). Oligonucleotide 7: 5' TGTAGGGTTTAGTGC 3'; Oligonucleotide 8: 5' ACTAGGGTTTAGACG 3'; Oligonucleotide 9: 5' CATAGGGTTTAGCAT 3' and Oligonucleotide 6: 5' TGTAGGGTTTAGTGC 3'.

## Results

### *Identification and characterization of 4G3 RNA*

The *Arabidopsis* telomere repeat is TTTAGGG and it differs from the human repeat TTAGGG by a single nucleotide. The template in hTR is 5' CUAACCCUA 3' (80). Therefore, we hypothesized that the permutation of the *Arabidopsis* telomere repeat (5' CUAAACCCUA 3') might be found in *Arabidopsis*, corresponding to 1.5 telomere repeats. We initiated an in silico analysis of the *Arabidopsis* genome to search for the presence of the template. A major impediment to our search was the presence of several interstitial telomere repeats in *Arabidopsis*. To avoid these elements in our search, we restricted the search to the intergenic regions of the genome. Our reasoning is based on a report that indicated that most H/ACA snoRNAs in *Arabidopsis* are located in intergenic regions (209). For our search, we also assumed that the *Arabidopsis* TER would be similar to the human TER, which contains an H/ACA box motif at the 3' terminus.

To start our search, we used the program Patmatch on the TAIR website. Patmatch is a pattern matching program that can be used to search for short (<20 residues) nucleotide sequences or patterns. Using CTAAACCCTA as query, we searched for patterns occurring in both strands of the genome in intergenic dataset. The program identified approximately 1700 hits in the intergenic region of the genome. To narrow the candidates, we eliminated hits that contained multiple templates in the same sequence. Further, hits that were in regions larger than 800 nts were also disregarded. This criterion was based on earlier work in our lab indicating that the size of the TER subunit was approximately 600 nts (M. Fitzgerald and D. Shippen, unpublished data). With these restrictions, the number of hits was reduced to 600.

In all TERs, the template region is single-stranded. In order to predict the single-stranded nature of the template region in the hits, we used the program Mfold (360). While Mfold can make accurate predictions of DNA/RNA secondary structures, it cannot predict pseudoknot structures. Mfold was also adopted by the Wellinger lab in their identification of TER subunits in the “sensu stricto” group of *Saccharomyces* (68). Mfold was used to identify candidate RNAs in which a single-stranded template was predicted among the lowest energy structures. By this method, we were able to reduce the number of candidates to 16 (Table 2).

The above candidates represented putative TERs; however, it was not known whether these candidate TERs were expressed *in vivo*. In humans, TER is expressed in all tissues, whereas telomerase activity is restricted to highly proliferative tissues (80). Similarly, in *Arabidopsis*, telomerase activity is restricted to flowers indicating that TER must be expressed in flowers. Therefore, we tested the expression of candidate TERs in flowers. Most candidates were expressed in wild-type flowers, while some candidates were eliminated as they were not expressed in flowers (FIG. 34A, lane 5; FIG. 34B, lane 5). Although, TER subunits are stable in the absence of TERT in humans and yeast (55), we checked to see if *Arabidopsis* TER might need to be stabilized by its association with TERT. We checked to see if the TER candidates were also expressed when TERT was absent. One TER candidate was well expressed in wild-type but not expressed in a *tert* mutant (FIG. 34A; compare lanes 7 and 8). The absence of the TER candidate was not a consequence of the genome instability associated with late-generation *tert*<sup>-/-</sup> mutants (268) as even in G3 *tert*<sup>-/-</sup> mutants, this TER candidate was not detected (FIG. 34B; compare lanes 9 and 10). We named this candidate 4G3 RNA and proceeded to characterize this RNA.

Table 2 List of TER candidates with a single-stranded template in Mfold analysis

Intergenic region	Template position	Total length of sequence
AT5G57280	797 - 706	818
AT5G64360	32 - 41	268
AT5G06200	582 - 591	695
AT3G14855	66 - 75	447
AT2G27980	33 - 42	478
AT1G03810	672 - 681	760
AT1G06720	154 - 145	324
AT1G14990	21 - 12	406
AT1G20530	67 - 58	752
AT1G47915	232 - 223	463
AT1G62120	61 - 52	614
AT2G44050	92 - 83	234
AT4G39300*	444 - 435	471
AT5G06950	185 - 176	253

\* This TER candidate was analyzed biochemically and genetically. See text for details.

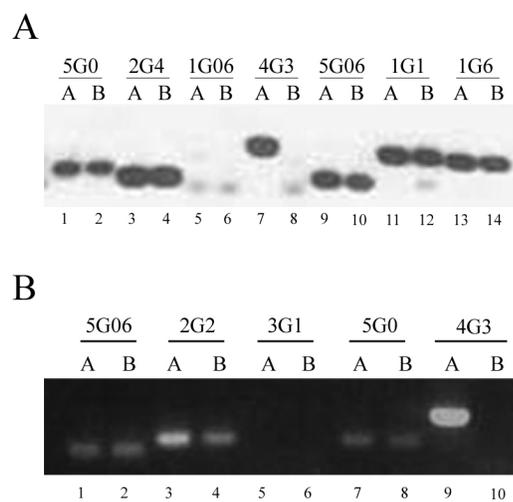


FIG. 34. Expression analysis of candidate TERs. (A) RT-PCR analysis of candidate TERs in wild-type flowers (lanes A) and G8 *tert*<sup>-/-</sup> flowers (lanes B). (B) RT-PCR of candidate TERs in wild-type (lanes A) and G3 *tert*<sup>-/-</sup> flowers (lanes B).

Telomerase activity can be inhibited by oligonucleotides complementary to the template region of TER (80). To see if oligonucleotides complementary to 4G3 RNA inhibit telomerase activity, several oligonucleotides of different lengths were designed and tested in a telomere repeat amplification (TRAP) assay (FIG. 35A). Oligonucleotides were incubated with extract from wild-type flowers and then subjected to TRAP assay. Results of the TRAP assay indicated that the shortest complementary oligonucleotide (Oligo 7) inhibited telomerase activity *in vitro* (FIG. 35B; lanes 3 and 4). Oligo 7 is a 15-mer predicted to anneal to the template and five adjacent nucleotides that surrounded the template (FIG. 35A). When the non-template bases of this oligonucleotide were modified, activity was not inhibited (FIG. 35B; lanes 5, 6, 7 and 8). Furthermore a longer oligonucleotide was also unable to inhibit telomerase activity (FIG. 35B; lanes 1 and 2). One reason why longer oligonucleotides might fail to inhibit telomerase activity is that they cannot stably bind to the template and adjacent region tightly. It is possible that the region surrounding the template is not available for binding to the oligonucleotide. The above data suggested that annealing of oligonucleotides to 4G3 RNA inhibits telomerase activity.

We then analyzed the consequences of a knock-out in the gene encoding 4G3 RNA. A mutant line with a T-DNA insertion in the 3' region of the RNA termed 4G3-1 was obtained from the INRA (France) collection (FIG. 36A). 4G3-1 mutant is in the Wassilewskija (WS) ecotype of *Arabidopsis*. Telomere length distribution varies among different *Arabidopsis* ecotypes (283). In the WS ecotype, telomeres are typically maintained in the 2-8 kb range (283) (FIG. 36B, lanes 1 and 2). Telomere lengths of both heterozygous and homozygous 4G3-1 mutants were analyzed and were found to be in the wild-type size range (FIG. 36B, lanes 3-7). Furthermore, telomerase activity

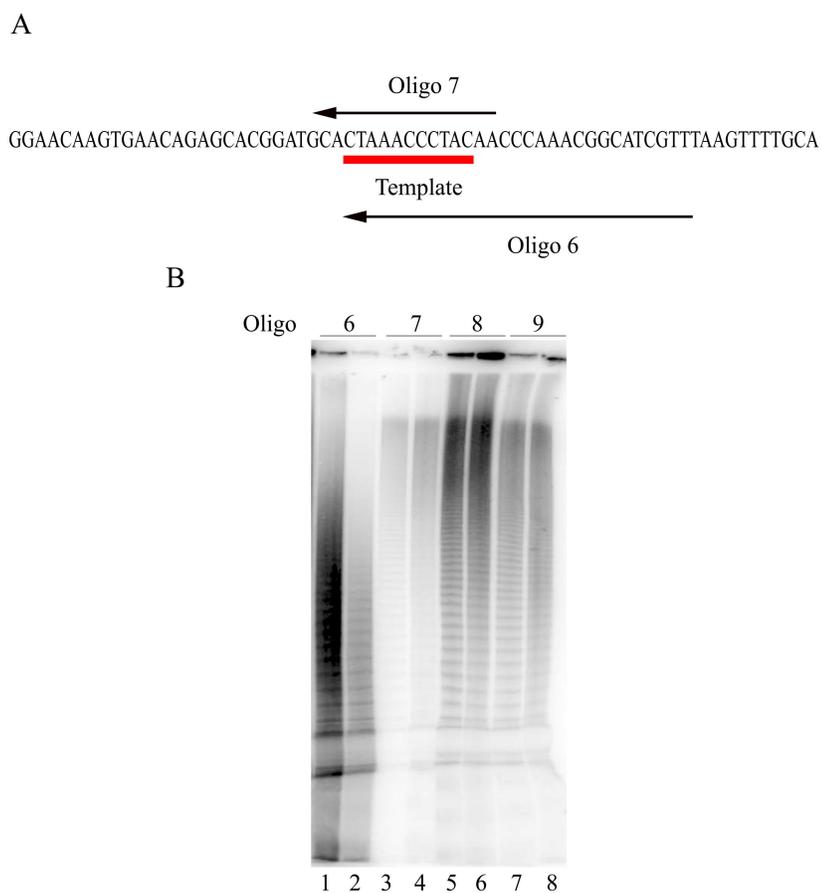


FIG. 35. Oligonucleotides complementary to the template region of 4G3 RNA inhibit telomerase activity. (A) Sequence of 5' region of 4G3 RNA. The template region is underlined in red. Oligonucleotide 7 is complementary to the template of 4G3 RNA and oligonucleotides 8 and 9 differ from oligonucleotide 7 only in the nucleotides that lie outside the template domain. Oligonucleotide 6 is a 22-mer specific to 4G3 RNA. (B) Protein extracts from flowers were incubated with the indicated oligonucleotides and tested for telomerase activity.

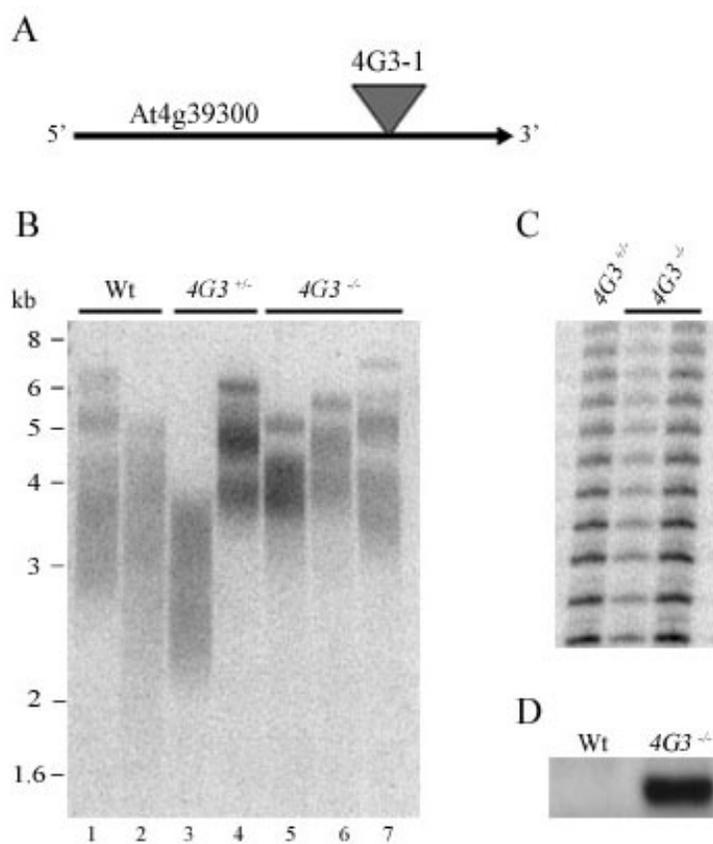


FIG. 36. Analysis of plants with T-DNA insertion in 4G3 RNA. (A) Schematic of T-DNA insertion in 4G3 RNA gene (4G3-1) (B) TRF analysis of wild-type,  $4G3^{+/-}$  and  $4G3^{-/-}$  plants. These mutants are in the WS ecotype of *Arabidopsis* where telomeres range from 2-8 kb (283) (C) TRAP assay was performed on extracts from  $4G3^{+/-}$  and  $4G3^{-/-}$  flowers. (D) RT-PCR using a 5' primer and T-DNA left border primer on RNA from wild-type and  $4G3^{-/-}$  flowers.

was not reduced in the homozygous mutants (FIG. 36C). However, RT-PCR analysis using primers directed at the template region of 4G3 and the left border of the T-DNA showed that a truncated 4G3 RNA was being produced in the homozygous mutants (FIG. 36D). This expression most likely results from a cryptic promoter in the T-DNA (223). Therefore, the 4G3-1 mutant is not null for 4G3 RNA.

To generate a more complete knockdown of 4G3 RNA, RNAi constructs directed at 4G3 RNA were transformed into wild-type plants. Transformants that showed a decrease in 4G3 RNA expression were obtained (FIG. 37A). However, these plants did not show a corresponding loss of telomerase activity (FIG. 37A) and telomeres remained in the wild-type range (FIG. 37B). These data suggested that 4G3 RNA is not required for telomerase activity. Therefore, other strategies were implemented to look for *Arabidopsis* TER candidates.

#### *Analysis of RNAs co-purifying with AtTERT*

Next, a biochemical approach was undertaken to identify TER candidates. Here, the goal was to sequence RNAs co-purifying with AtTERT. Therefore, to enrich for telomerase, we overexpressed AtTERT in plants. AtTERT cDNA was tagged with an N-terminal 3X FLAG tag and placed under the control of the 35S CaMV promoter. To ensure complete replacement of endogenous TERT for the tagged TERT, the construct was overexpressed in early generation *tert*<sup>-/-</sup> plants. This construct also contained the first intron of AtTERT present in frame with the rest of the coding sequence. We expected this transcript to be spliced and therefore not be targeted for degradation.

Transformants were obtained and whole cell protein extracts were made from flowers and subjected to western blots. Unfortunately, we could never detect a band of the right size corresponding to the FLAG-TERT (data not shown). However, since we

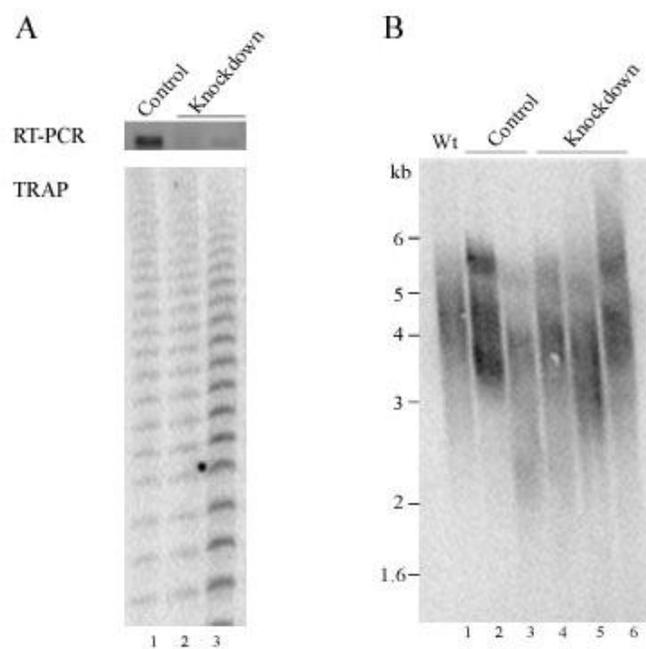


FIG. 37. Loss of 4G3 RNA does not diminish telomerase activity *in vitro* or telomere length *in vivo*. (A) RT-PCR analysis of 4G3 RNA levels in control non-transformed plants and plants transformed with RNAi construct directed at 4G3 RNA. TRAP analysis of extracts from control and knockdown plants. (B) TRF analysis of wild-type, control and plants showing knockdown of 4G3 RNA.

transformed this construct in a knock-out background, we checked to see if the protein extracts from flowers had telomerase activity. Indeed, activity was obtained in the FLAG-TERT transformants and this suggested that FLAG-TERT was being expressed (FIG. 38A).

Immunoprecipitation was then performed on the floral extracts using anti-FLAG beads. As expected, telomerase activity was recovered from FLAG-TERT immunoprecipitate, but not from wild-type immunoprecipitates (FIG. 38B). In addition, we were able to detect AtPOT1a by western in the FLAG-TERT immunoprecipitate and not in the wild-type immunoprecipitate (FIG. 38C). Thus, an intact telomerase RNP was purified. RNA was then extracted from the FLAG-TERT eluate. Using a protocol developed for cloning non-coding RNA (147), RNA from FLAG-TERT immunoprecipitate was ligated to 3' and 5' linkers sequentially and then reverse-transcribed to cDNA using primer specific to the 3' linker and then subjected to PCR amplification. PCR products were subsequently cloned and sequenced. Sequencing of the clones revealed ribosomal RNA to be a major contaminant and we were unable to retrieve any template containing RNAs by this strategy. Therefore, even though we were able to purify telomerase, we suspect that we were unable to clone TER candidates due to their low abundance in comparison to ribosomal RNA.

#### *Identification and characterization of 3G0 RNA*

In addition to performing our own *in silico* analysis, we also analyzed data from other labs that were involved in the search for non-coding RNAs in *Arabidopsis* (36, 138, 204, 209, 258). We were particularly interested in orphan RNA transcripts (138). Orphan RNAs are not annotated as the product of any particular gene and therefore represent

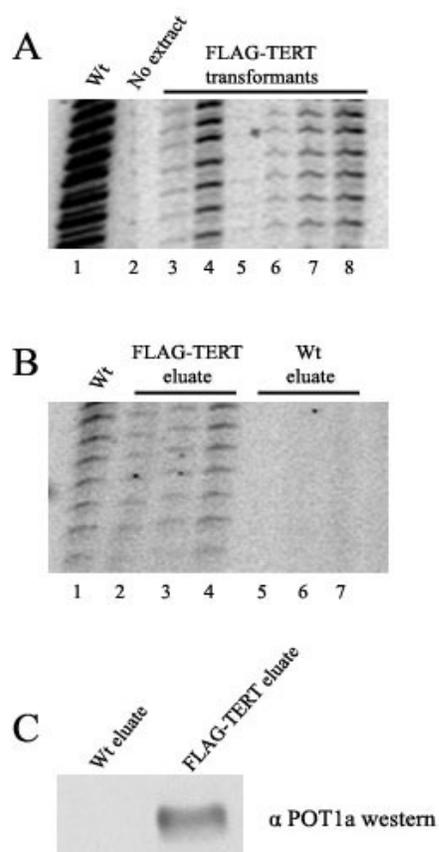


FIG. 38. Analysis of FLAG-TERT transformants. (A) TRAP assay of extracts from FLAG-TERT transformants. Extract from wild-type flowers was used as positive control for TRAP assay. (B) IP of wild-type and FLAG-TERT extracts using anti-FLAG beads. Eluates were tested for telomerase activity. (C) Wild-type and FLAG-TERT eluates were also subjected to western using POT1a anti-serum.

non-coding RNAs. TER is a non-coding RNA and therefore, we searched the orphan RNA database (<http://atornadb.bio.uni-potsdam.de/>) for TER candidates by looking for the template region in these RNAs. We identified five candidates that contained the predicted *Arabidopsis* template. Mfold analysis of the template containing orphan RNAs was also performed. Only one orphan RNA (At\_oRNA\_415), termed 3G0, was predicted to contain a single-stranded template region in the 5' region of the RNA. This RNA was expressed in all plant tissues (138). Interestingly, 3G0 RNA was not expressed in flowers from G3 *tert* mutants (FIG. 39A).

To further characterize the 3G0 RNA, we performed an immunoprecipitation experiment on *Arabidopsis* cell culture extracts using AtPOT1a antibody. AtPOT1a is a constituent of the telomerase RNP (296) and is associated with active telomerase. Therefore, we asked whether 3G0 RNA is associated with active telomerase in an AtPOT1a immunoprecipitation. *Arabidopsis* cell culture extracts were immunoprecipitated with either pre-immune serum or with AtPOT1a anti-serum. Immune complexes were analyzed by TRAP assay and RT-PCR. Telomerase activity was specifically pulled down using AtPOT1a anti-serum (FIG. 39B). Interestingly, 3G0 RNA was also recovered in same IP, but not in the IP with pre-immune serum (FIG. 39B). In contrast, the abundant U6 snRNA was present in both pre-immune and anti-POT1a immunoprecipitates. This suggested that 3G0 RNA is associated with active telomerase and that this interaction is specific.

The *Arabidopsis* database contained a line with a T-DNA insertion in the 3' end of the 3G0 RNA, termed 3G0-1 (FIG. 40A). Since the insertion was present in the 3' end, we checked to see if 3G0 transcripts were expressed in 3G0-1 mutants. RT-PCR analysis using a template primer and a T-DNA primer indicated that products

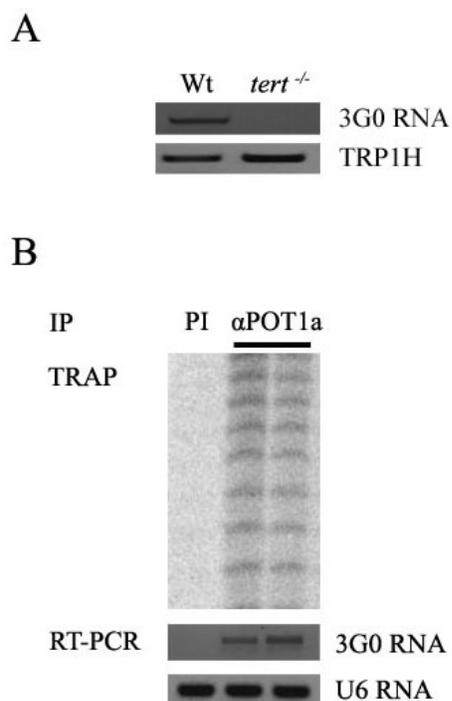


FIG. 39. 3G0 RNA is associated with telomerase. (A) RT-PCR analysis of 3G0 RNA in wild-type and *tert*<sup>-/-</sup> flowers. TRP1H is a double-stranded telomere binding protein and was used as a control for RT-PCR. (B) Immunoprecipitation (IP) of cell culture extracts was performed with pre-immune serum (PI) or anti-POT1a serum. Upper panel: TRAP assay of immunoprecipitates. Lower panel: RNA was also extracted from the immunoprecipitates and RT-PCR was performed for 3G0 and U6 RNA.

corresponding to this region were still expressed in the homozygous plants (FIG. 40A). Therefore, the T-DNA insertion does not seem to affect the abundance of 3G0 RNA as it is present only in the 3' end of the RNA. However, the loss of the 3' region may result in a non-functional RNA. Therefore, telomere lengths were analyzed in 3G0-1 mutants. TRF analysis indicated no change in telomere length among heterozygous and homozygous mutants in this line (FIG. 40B). Screening of DNA pools from T-DNA mutagenized plants, not included in the database, showed no other insertions in 3G0 RNA (data not shown).

We used two additional approaches to knock down 3G0 RNA in wild-type plants: antisense RNA and RNA interference (RNAi). An antisense RNA construct was generated by overexpressing full length 3G0 RNA in the antisense orientation. For RNAi, two constructs targeting 100 nts of 3G0 RNA template region (called template RNAi) or the nucleotides from 350-450 (called middle RNAi) were made. Antisense and RNAi constructs were transformed into wild-type plants. Transformants obtained from all three transformations were analyzed by RT-PCR. In all transformants analyzed, 3G0 RNA was undetectable even after 40 cycles of PCR whereas the mRNA for TRP1H, a double-stranded telomere binding protein control (154), was amplified in all transformants (FIG. 41A). TRAP assays revealed abundant telomerase activity in these transformants (FIG. 41B). Thus, the 3G0 RNA does not appear to be a viable TER candidate.

## **Discussion**

### *A bioinformatics approach to identify candidate TERs*

The bioinformatics approach was a good starting point for identification of *Arabidopsis* TERs since *Arabidopsis* has a sequenced and annotated genome.

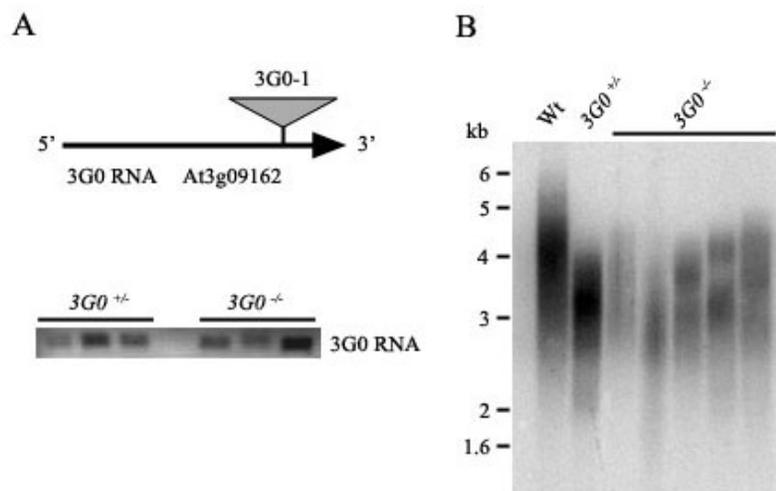


FIG. 40. Analysis of T-DNA insertion in 3G0 RNA. (A) Upper panel: Schematic of T-DNA insertion in 3' region of 3G0 RNA (At3g09162). Lower panel: RT-PCR of 3G0 RNA using template primer and T-DNA primer in 3G0 heterozygous and homozygous plants. 3G0 RNA is still expressed in these plants. (B) TRF analysis of wild-type, 3G0<sup>+/-</sup> and 3G0<sup>-/-</sup> plants.

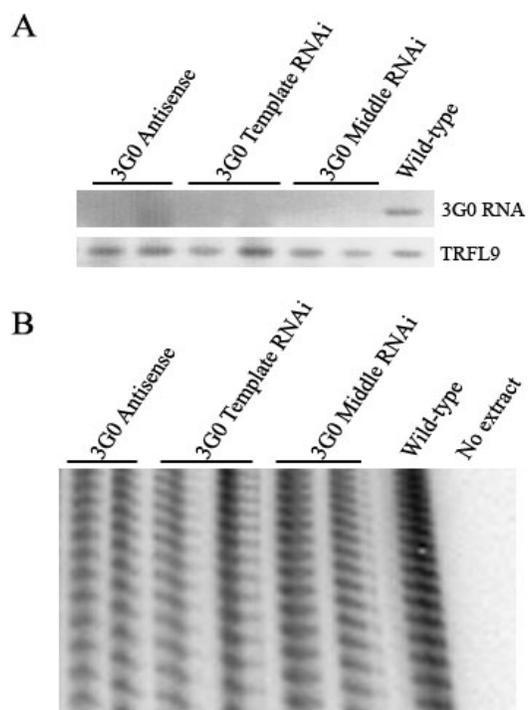


FIG. 41. Knockdown of 3G0 RNA. A. RT-PCR analysis of 3G0 RNA and TRFL9 (control) transcripts in antisense, RNAi and wild-type plants. B. TRAP assay was performed on extracts from 3G0 RNA antisense, RNAi and wild-type plants.

Furthermore, several tools are also available to analyze and filter candidates resulting from these approaches. Using the bioinformatics approach, we were able to identify many template-containing candidates. We restricted our search to the intergenic regions of the *Arabidopsis* genome based on work on the identification of other non-coding RNA genes in these regions of the genome (209). However, since our work, the annotation of the genome has changed, and many candidates that were considered to be located in intergenic regions are now part of coding regions. The *Arabidopsis* TERs, TER<sub>1G7</sub> and TER<sub>5G2</sub> (CHAPTER III), have their template sequence located in intergenic regions and were present in the initial list of candidates. Therefore, in spite of improper annotation, this was a good criterion to distinguish TER candidates. Another good criterion to filter TER candidates was the requirement for a single-stranded template region as determined by Mfold analysis (360). As mentioned earlier, this criterion was also used for TLC1 homologs in other yeast species (68). When determining the secondary structure of RNAs, it is important to know the sequence boundaries. This was not possible with the above TER candidates. Since, we did not know the 5' and 3' ends of these RNAs; we may have missed several other candidates. We also looked for the presence of H/ACA boxes in candidate TERs to account for the association of *Arabidopsis* dyskerin (AtNAP57) with the telomerase RNP. However, these motifs are degenerate: the sequence of the consensus H box is ANANNAA (where N is any nucleotide). This criterion did not help in ruling out candidates, as this sequence was present in nearly all candidates. In total, the lack of sequence information apart from the template region did not help us in setting appropriate criteria for candidate TERs.

While the bioinformatics approach we took was flawed, we still obtained a reasonable TER candidate. Several lines of evidence pointed to 4G3 RNA as a possible *Arabidopsis* TER subunit. First, this RNA is absent in *tert*<sup>-/-</sup> mutants. One interpretation

of the observation is that *Arabidopsis* TER is stabilized by association with AtTERT. Therefore, it may accumulate in flowers only when AtTERT is present. Second, by yeast-three hybrid assays, we were able to show an interaction between 4G3 RNA and both AtTERT and AtNAP57 (data not shown). Finally, the inhibition of telomerase activity by oligonucleotides aimed at 4G3 RNA template convinced us that this RNA was an excellent candidate. In retrospect, we realized that both AtNAP57 and AtTERT bind RNA non-specifically. Inhibition of telomerase by 4G3 RNA oligonucleotides is an accidental consequence of the sequence similarity in the template region between 4G3 RNA and *Arabidopsis* TERs. The 15-mer oligonucleotide that showed maximal inhibition of telomerase (oligo 7) shares 12 nts with TER<sub>1G7</sub> and 10 nts with TER<sub>5G2</sub>, and can thus bind to the template of *Arabidopsis* TERs and inhibit activity.

In the above context, the genetic analysis was very important. Homozygous mutants in 4G3 RNA showed no telomere length defects. Initially, we attributed the absence of telomere defects to the expression of a truncated 4G3 transcript that contained an intact telomere template. However, with RNAi against 4G3 RNA, we were able to conclusively prove that loss of 4G3 RNA did not affect telomerase activity or telomere length. We also considered the possibility that the absence of telomere length defects in the above mutants may be due to duplication of 4G3 RNA. However, a BLAST search of the genome revealed that 4G3 RNA is a single-copy gene. Thus, we were able to dismiss 4G3 as a viable TER candidate.

Many laboratories are involved in the cloning of non coding RNA species in *Arabidopsis* (36, 138, 204, 258). We were able to take advantage of the data generated from these laboratories and search for putative TER candidates. We screened 500 candidates and found 5 candidates that contained the template. Our search led to the identification of a single-strand template containing RNA that we named 3G0 RNA. This

RNA was expressed in all plant tissues (138). As for 4G3 RNA, genetic analysis showed that telomerase activity and telomere length maintenance is unperturbed in 3G0 mutants. Thus, 3G0 is not likely to be *Arabidopsis* TER.

One interesting feature of 4G3 and 3G0 RNAs is the inability to detect these RNAs in early generation *tert* mutants. Stability of both human and yeast TERs are not affected in the respective *tert*<sup>-/-</sup> backgrounds. Furthermore, accumulation of TER<sub>1G7</sub> and TER<sub>5G2</sub>, the bona fide *Arabidopsis* TERs, are stable in the absence of AtTERT (CHAPTER III). It is unclear why both 4G3 and 3G0 RNAs, which have a template region, are destabilized in a *tert*<sup>-/-</sup> background. One interesting possibility is that a surveillance mechanism is in place to regulate the levels of the non-coding RNAs and that this mechanism is triggered in response to telomerase inactivation. Microarray analysis of *tert* mutants would perhaps throw light on the factors contributing to the stability of other non-coding RNAs. More research is required to understand this mechanism and implications of this observation.

In summary, the bioinformatics approach is a good starting point for TER identification in sequenced, annotated genomes such as *C. elegans*. With the establishment of relevant criteria, this approach can be a useful tool for identification of TER orthologs in other sequenced plant genomes.

#### *PCR approach to clone TER candidates*

Purification of telomerase complexes is a good strategy to identify TER candidates and this approach was used was ultimately successful in identifying TER<sub>1G7</sub> and TER<sub>5G2</sub> in *Arabidopsis*. In this study, we took the first step in this direction by looking for RNAs that co-purified with tagged TERT. However, the presence of abundant contaminating ribosomal RNA fragments hindered our attempts to clone TER candidates

by the PCR approaches that have worked well for other non coding RNAs (147). Even with additional purification steps, it is difficult to avoid ribosomal RNA contamination (CHAPTER III). This problem was also faced by the Zakian group in their two-step purification of *S. pombe* telomerase (329).

Although the attempts described above to identify *Arabidopsis* TER were unsuccessful, we learned several valuable lessons. Multiple genetic approaches need to be taken to analyze putative TER function. Cryptic promoters in T-DNA insertions may lead to partially functional RNA transcripts. Therefore, antisense and RNA interference approaches can be useful to decrease the endogenous level of RNA. These lessons were very useful in analyzing the *Arabidopsis* TER genes described in chapter III.

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