TELOMERE DYNAMICS IN *ARABIDOPSIS THALIANA*

A Dissertation

by

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ABSTRACT

Telomeres are the physical ends of eukaryotic chromosomes. Because chromosome ends resemble double–strand breaks, telomere binding proteins mask telomeres from DNA damage response machinery. Consequently, telomere protection physically blocks telomere replication by the unique ribonucleoprotein (RNP) reverse transcriptase, telomerase. Telomerase access to telomeres is strictly regulated in the cell, and thus telomeres vacillate in status from telomerase accessible and telomerase inaccessible states.

Here, I report the mechanistic contributions of the telomerase accessory protein POT1a (Protection Of Telomeres 1) in *Arabidopsis* telomere dynamics. POT1a, one of three POT1 paralogs in *Arabidopsis,* is essential for telomere replication. My work revealed POT1a is an activator of telomerase and stimulates its enzymology. POT1a physically binds two telomere proteins, CTC1 and STN1, and all three proteins can associate with active telomerase *in vivo.* In contrast, POT1a competed with TEN1, a capping protein shown to negatively regulate telomerase activity, for an interface on STN1. Thus, POT1a contributes to telomere dynamics though its interactions with telomerase and telomere binding proteins.

Additionally, I examined the function of TER2, a non-canonical telomerase RNA that negatively regulates the TER1 (canonical) telomerase RNP. Null mutations in *ter2* result in mild telomere phenotypes. However, when *ter2* mutation was combined with the loss of POT1a, *pot1a ter2* double mutants exhibited severe rates of telomere

shortening and early onset defects in plant morphology and development. Thus, POT1a and TER2 represent two distinct regulators of telomere maintenance in *Arabidopsis thaliana*.

Lastly, I observed the consequences of POT1 gene duplication. Evolutionary analysis revealed POT1a post-duplication was under Darwinian selection pressure for non-synonymous changes in three amino acid sites. Reversion of these residues back to the ancestral (POT1b) state resulted in a reduced ability of these mutants to genetically complement the telomere maintenance defect of *pot1a* mutants. In addition, these mutants had a reduced affinity for CTC1 *in vitro*. Therefore, POT1a is under positive evolutionary selection for its role in telomere maintenance and its association with CTC1.

In summary, my work has elucidated the contributions of POT1a to *Arabidopsis* telomere dynamics, and how these functions contribute to its role in promoting telomere maintenance.

DEDICATION

This dissertation is dedicated to my wife Rachel, who somehow put up with me over the last several years. She is an amazing spouse, mother, and friend.

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First and foremost, special thanks go to my mentor and PI, Dorothy Shippen. Few people are as driven as her in their lives, and she has inspired me to obtain her level of passion in science and beyond. She has also taught me the importance of questioning the world around me, particularly as it relates scientific research. How can you ever know if anything is worth doing if you never ask the question why? I will always appreciate her contributions in my life and career.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The end game: A perspective on telomere biology

The architectural evolution of genomes poses a series of trade-offs. Eukaryotes typically have multiple linear chromosomes while prokaryotic organisms have one or few circular chromosomes. Circular chromosomes lack some of the complexities of and challenges faced by linear chromosomes, but are limited in biological processes such as meiosis and the consequent diversity that arise from sexual reproduction. The advent of linear chromosomes increases genetic diversity via recombination, but this invention requires more effort to maintain genomic stability (Ishikawa & Naito, 1999). The challenge is related to the physical similarity of chromosome ends to double-strand breaks as well as the inability of conventional DNA replication to fully replicate linear chromosomes (Fig. 1-1). These cellular inconveniences are known as the end-protection and the end-replication problems.

Often in evolution, necessity begets innovation (Carroll, 2001). Chromosome termini are composed of repeated telomeric DNA sequences and a host of associated protein complexes that circumvent cellular DNA damage responses by capping the chromosome ends with a variety of terminal protein complexes. The loss of end-protection results in severe genome instability and in many cases, cellular and organismal death. Moreover, eukaryotes have evolved a remarkable mechanism to accomplish complete DNA replication, which utilizes the unique polymerase,

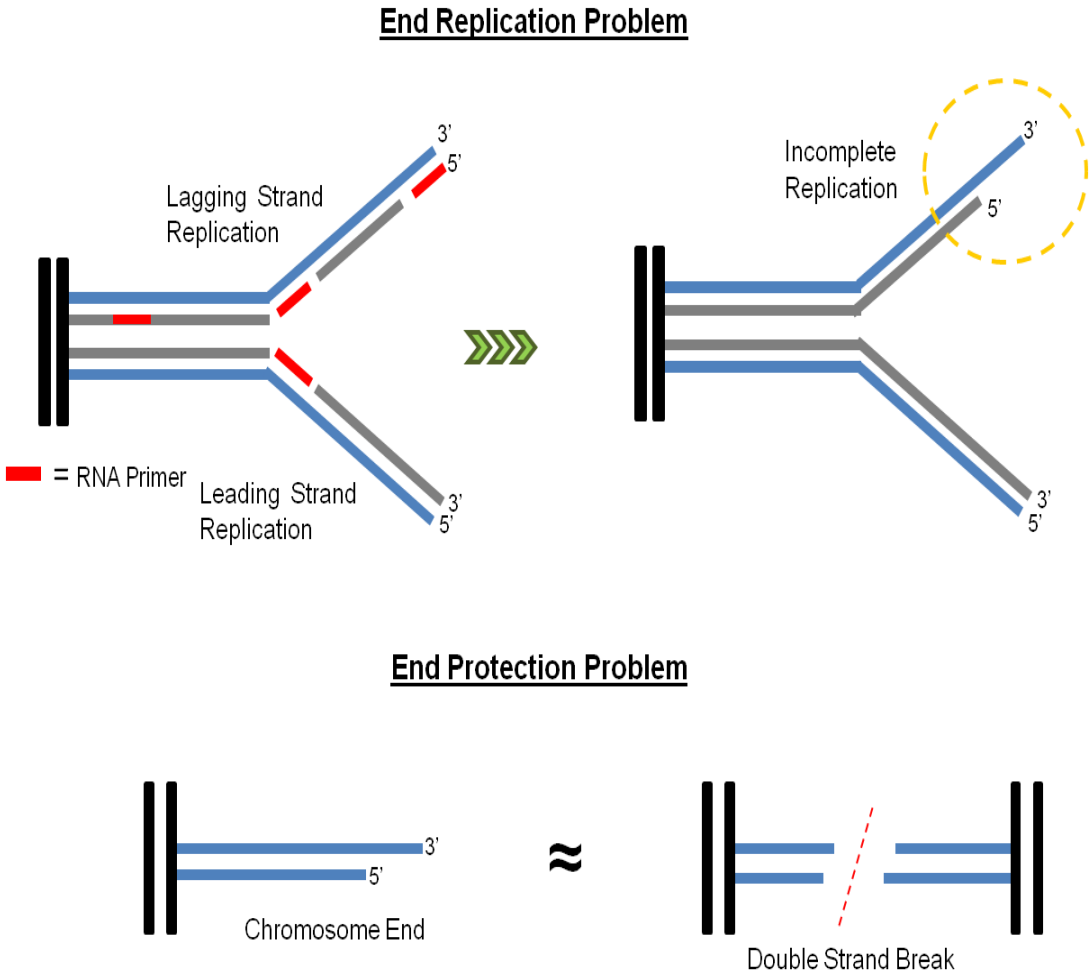


Figure 1-1. The end-replication and end-protection problems. (*Top*) During replication the leading strand is replicated fully by DNA polymerase. However, removal of the terminal RNA primer on the lagging strand results in an unreplicated gap. Without a 3' OH, DNA polymerase cannot fill in these gaps *de novo*. Consequently, this leads to progressive shortening at the terminus every time DNA is replicated. (*Bottom*) Chromosome ends naturally resemble DSBs. The cell must mask these structures to prevent elicitation of a DDR at natural chromosome termini.

telomerase. There is interesting variety across Eukarya in the ways the end-protection and end-replication problems are solved, but the goal remains the same; protect and replicate the ends to ensure complete genetic inheritance. The diversity of solutions to these problems underscore the importance of telomere biology. Multicellular organisms could not have evolved linear chromosomes without solving the end-replication and end-protection problems. This introduction is provides an overview of the interesting solutions that have evolved the field of telomere biology.

Telomere history

In the fourth decade of the 20th century, exciting work on chromosome biology by two independent researchers started to reveal the importance of chromosome ends in higher organisms. Barbara McClintock utilized maize to observe the consequences of chromosomal breakages. She discovered some broken chromosomes could fuse end-to-end, but normal chromosomes were protected from this by the natural properties of their termini (McClintock, 1938; McClintock, 1939). Chromosome end fusions could lead to dicentric chromosomes that do not segregate properly during mitosis resulting in chromosome bridges during anaphase. She hypothesized the cell naturally prevents these abnormalities in linear chromosomes despite being structurally similar to a broken chromosome at their distal regions (McClintock, 1941). Hermann Muller meanwhile was busy observing fragmented irradiated fruit fly chromosomes and found that the natural ends never resulted in fusion events suggesting there was something unique about the end portion of chromosomes (Muller, 1938). He coined the term telomere (telo-end,

mere-part) in reference to these terminal structures, realizing that their function was that of "sealing the end of the chromosome".

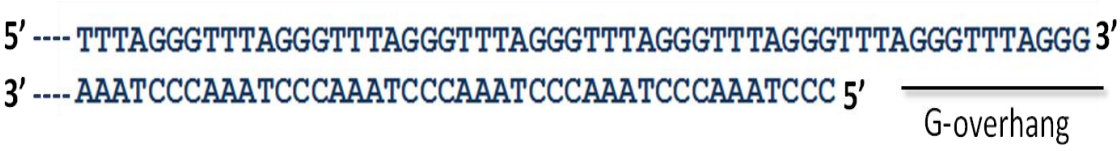
The discovery of duplexed DNA by Francis Crick and James Watson in the early 1950's radically changed our views on DNA replication (Watson & Crick, 1953). It wasn't until the 1970's that the Russian biologist Alexey Olovnikov with impeccable foresight predicted there was an end-replication problem with linear DNA molecules and that there was a mechanism (telomerase) to resolve it (Olovnikov, 1973). Moreover, he surmised how telomere shortening could be causal for replicative senescence. The end-replication problem was ultimately due to the inability of conventional DNA replication to fill in the gap left behind from RNA primer removal at the end of the 3' strand. Because replication is coordinated with cell division, this would lead to loss of that sequence every time a cell replicates its DNA without a mechanism to fill in these residual gaps.

In the late 1970's, the sequence of ciliate telomeres was reported for *Euplotes* and *Oxytricha* (Blackburn & Gall, 1978). The following decade achieved "telomere enlightenment" when a powerful model system met a determined biochemist. Elizabeth Blackburn took advantage of the abundance of linear rDNA chromosomes in *Tetrahymena thermophila* and revealed that the terminal sequences were not composed of unique genes assembled into common nucleosomes, but instead consisted of a stretch of tandem GGGGTT repeats (Blackburn & Chiou, 1981). In collaboration with Jack Szostack, Blackburn and colleagues inserted *Tetrahymena* telomeric onto the ends of of a linearized plasmid in budding yeast. Remarkably, the *Tetrahymena* sequences

remained stable and yeast telomere sequences were synthesized onto the ends of *Tetrahymena* repeats (Szostak & Blackburn, 1982). This result hinted at the possibility of wide scale conservation of telomeric functions across eukaryotes. Because yeast telomere sequences are different than *Tetrahymena* telomere sequences, this result also indicated DNA was not synthesized using the *Tetrahymena* DNA as a template, as occurs in typical DNA replication (Shampay et al, 1984). Finally, in collaboration with her graduate student, Carol Greider, Blackburn discovered a "telomere transferase" later named telomerase (Greider & Blackburn, 1985), and subsequently showed it was a ribonucleoprotein reverse transcriptase (Greider & Blackburn, 1987). This seminal work would ultimately earn them and Jack Szostack a Nobel Prize in medicine in 2009.

Telomeric DNA

Later work uncovered a variety of closely-related telomere sequences across lower and higher eukaryotes, adhering to the general principle of guanosine-rich tandem repeats (Fig. 1-2). Budding yeast telomeres are composed of irregular $TG_{1\text{-}3}$ repeats, while vertebrate and most plant telomeres consist of TTAGGG and TTTAGGG repeats, respectively (Moyzis et al, 1988; Richards & Ausubel, 1988; Shampay et al, 1984). Such similarity in telomere sequence suggested telomeres were an early evolutionary event that either was allowed or arose due to the linearization of DNA chromosomes. Although telomere sequences are mostly conserved, one of the most astounding examples of evolutionary diversity in telomere biology is the broad range in telomere length (Fig. 1-2). Budding yeast and *Tetrahymena* simply contain a few hundred repeats



Organism	Sequence	Telomere Length	G-Overhang	Ref.
A. thaliana	TTTAGGG	2-7kb	20-30nt	Richard and Ausubel, 1988
N. tabacum	TTTAGGG	40-160kb	n/a	Fajkus et al 1996
S. cerevisiae	$G_{2-3}(TG)_{1-6}$	~0.3kb	12-14nt	Shampay et al, 1984
T. thermophila	TTGGGG	~0.3kb	12-21nt	Jacob et al, 2004
Homo sapiens	TTAGGG	2-15kb	~250nt	Smogorzewska et al, 2000

Figure 1-2. **Telomere sequence and G-overhang conservation.** Telomeres consist of duplex DNA and a 3' ss protrusion termed the G-overhang. *Arabidopsis* possesses TTTAGGG repeat sequences, but this varies in other organisms. Telomere sequences are G- and C-rich, a feature that is conserved, but their lengths and G-overhangs are widely variable across eukarya.

while organisms such as tobacco and mice can possess telomeres in excess of 150kb (Fajkus et al, 1996; Jacob et al, 2004; Shampay et al, 1984). *Arabidopsis thaliana* and humans make up some of the middle ground with 2-7kb and 2-30kb, respectively (Smogorzewska et al, 2000; Zellinger & Riha, 2007). It is still unclear why telomere lengths are so variable among different organisms.

G-overhangs and t-loops

Perhaps the most conserved feature of telomeric structures is the presence of a 3' single-strand protrusion of the G-rich strand termed the G-overhang (Fig 1-2). G-overhangs were discovered in ciliate telomeres (Klobutcher et al, 1981), and it became apparent that telomerase selectively extended this strand when C-rich telomeric oligonucleotides were not extended by telomerase in *Tetrahymena* cell free extracts (Greider & Blackburn, 1985). G-overhang length, similar to overall telomere length, is diverse across kingdoms (Fig. 1-2). Ciliates (12-16nt), budding yeast (12-14nt), and *Arabidopsis* (20-30nt) have smaller overhangs while humans have considerably larger (~250nt) (Jacob et al, 2001; Klobutcher et al, 1981; Makarov et al, 1997; Riha et al, 2000; Wellinger & Zakian, 2012). Interestingly, despite the early discovery of this feature in telomere biology, the mechanism of G-overhang formation is still largely unanswered in the field. Removal of the most terminal RNA primer on the lagging strand would produce an overhang, but leading strand replication would presumably produce a blunt-ended telomere. Thus, generating a G-overhang on the end replicated by leading strand synthesis requires additional processing steps. Furthermore, RNA primers

are not precisely positioned on the end and also necessitate processing to achieve a standard G-overhang length at lagging strand telomeres. Indeed, G-overhangs are generated largely due to the action of nuclease cleavage post replication (Chai et al, 2006; Jacob et al, 2003). Surprisingly, recent work has shown half of *Arabidopsis* telomeres are blunt-ended and protected by the double-strand telomere binding protein Ku (Kazda et al, 2012). This finding suggests there is some interesting divergence in plant telomere biology.

Another conserved feature of telomeres is the t-loop (Fig. 1-3). Implicated in chromosome end protection, the t-loop is characterized by G-strand invasion into the duplexed region of telomere DNA. These structures were first identified through electron microscopy by Jack Griffith in 1999 (Griffith et al, 1999). To date they have been reported in mammals, plants, *Caenorhabditis elegans*, ciliates, and the yeast *Kluyveromyces lactis* (Cesare et al, 2008; Cesare et al, 2003; Murti & Prescott, 1999; Raices et al, 2008). Formation of t-loops in mice has been shown to be dependent on the telomere-protection protein TRF2 (Doksani et al, 2013).The dynamics of t-loops are still unclear because replication would require exposure of the G-overhang substrate in S phase. Additional work is needed to elucidate changes in these higher order structures throughout the cell cycle.

Telomerase

The discovery of telomerase and its contribution to cellular and organismal stability, as well as its implications in cancer biology, ignited a firestorm of interest in

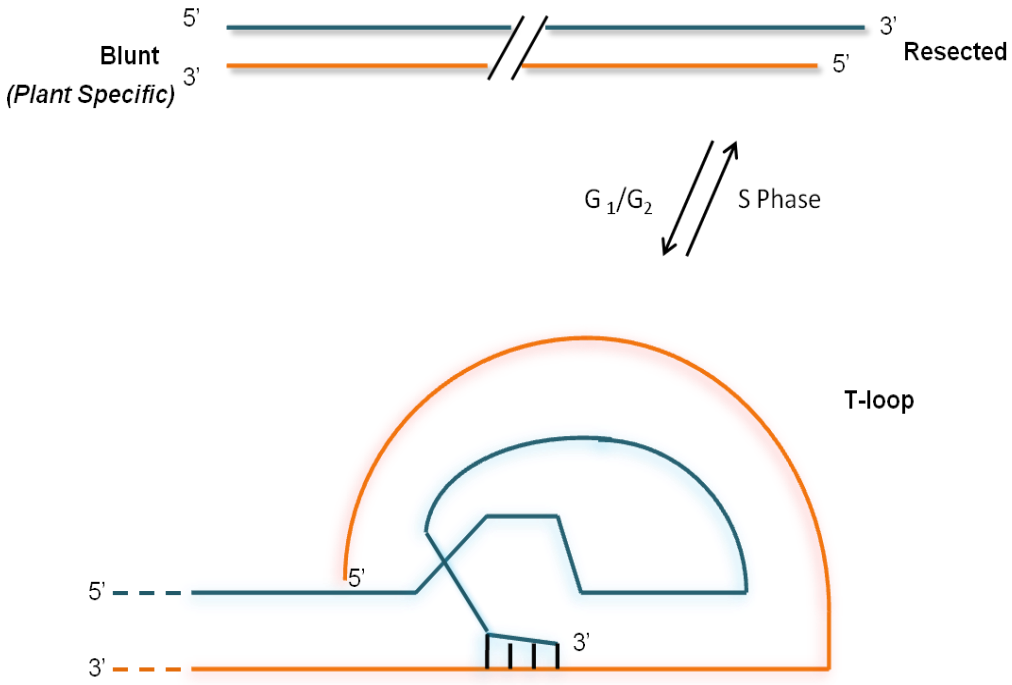


Figure 1-3. Telomere structure. Resected telomeres with G-overhangs shift between open and closed (t-loop) states. T-loops are formed by the invasion of the ss 3' overhang into the ds region of the telomeres. T-loops have been identified in plants and mammals, as well as other species (Griffith et al, 1999; Cesare et al, 2003). *Arabidopsis thaliana* telomeres have recently by discovered to be blunt ended on one side of each chromosome (Kazda et al, 2012).

telomere biology. As Alexey Olovnikov and James Watson would predict, the cell must have a means to overcome the end replication problem. Without telomerase, telomere shortening would eventually lead to replicative senescence due to the finite number of cellular divisions possible in non-stem cells. This concept was first proposed by Leonard Hayflick who challenged the paradigm that normal human cells were immortal (Hayflick & Moorhead, 1961). Hayflick showed empirically there was a finite number of population doublings that could occur in normal human cell lines in culture before they would stop dividing (Hayflick & Moorhead, 1961). The number of cell divisions is known as the Hayflick limit. Ultimately, cellular aging would be defined by a critical telomere length threshold. The grander idea that telomeres contribute to organismal aging was then proposed (Harley et al, 1990).

The Hayflick limit is overcome in cultures of human cancer lines or single cell eukaryotic organisms by constitutive expression of telomerase and its ability to solve the end-replication problem and thereby halting telomere shortening. The discovery that human somatic tissues lacked telomerase activity and their telomeres progressively shortened suggested that telomerase mediates the proliferative capacity of cells (Hastie et al, 1990). Indeed, transgenic expression of telomerase in telomerase negative cell lines extended their *in vitro* lifespan indefinitely (Bodnar et al, 1998). But why would cells undergo cellular senescence instead of constitutively expressing telomerase to achieve immortality? The simple answer is that replicative senescence is a safeguard against tumor formation (Smith & Pereira-Smith, 1996). Interestingly, telomerase expression is not universally regulated even in multi-cellular eukaryotes. Mice retain telomerase

activity in somatic tissues, which may explain their increased rates of malignancies (Greenberg et al, 1998; Rudolph et al, 1999). In contrast, plants restrict expression to actively dividing tissues such as young seedlings, flowers, and siliques which is more similar to telomerase regulation in humans (Fitzgerald et al, 1996). Interestingly, plants do not suffer from cancer as found in animal systems (Doonan & Sablowski, 2010), however they still regulate telomerase expression. Telomerase regulation may have evolved for an economic reason as undividing vegetative tissues do not need constitutive telomerase expression.

Telomerase reverse transcriptase, TERT

Soon after the discovery of telomerase, the enzyme was shown to be a ribonucleoprotein complex dependent on both its RNA and protein subunits (Greider & Blackburn, 1987). Telomerase could synthesize telomere repeats on oligonucleotide primers *in vitro* without the need for an exogenous template DNA strand (Blackburn et al, 1989). Instead, a region within the RNA is complementary to telomere repeats and serves as a templating domain for telomere replication (Greider & Blackburn, 1989; Shippenlentz & Blackburn, 1990). This led to the conclusion that the "telomere terminal transferase activity" was in fact a reverse transcriptase function of telomerase (Fig. 1-4). This finding explained the earlier observation that yeast telomere sequences were synthesized *de novo* onto *Tetrahymena* linear plasmids terminating in telomere repeats (Blackburn et al, 1989).

The identification of the TERT gene was discovered by forward and reverse genetics. Vicki Lundblad's group discovered the catalytic subunit and three other telomere-related genes in a genetic screen of yeast mutants defective in telomere maintenance (Lendvay et al, 1996; Lundblad & Szostak, 1989). These classic mutants would be called Est for their 'Ever Shorter Telomere' phenotype. Est2 would eventually be shown to be the reverse transcriptase component of telomerase. Meanwhile, Tom Cech and Joachim Lingner purified telomerase from *Euplotes aediculatus* and identified the reverse transcriptase as TERT through mass spectrometry analysis (Lingner et al, 1997b). BLAST searches revealed homology to the yeast Est2 gene. Both displayed conserved hallmarks of viral reverse transcriptases (Lingner et al, 1997b). Intriguingly, yeast telomerase activity *in vitro* was not dependent on the other Est genes, including the originally identified Est1 (Lingner et al, 1997a; Lundblad & Szostak, 1989), but Est2 and telomerase RNA (TLC1) were required (Cohn & Blackburn, 1995; Lingner et al, 1997a). The presence of additional Est genes hinted that the process of telomere replication was regulated *in vivo.*

Subsequent discovery of TERT in humans, *Tetrahymena,* fission yeast, and *Arabidopsis* all revealed similar core reverse transcriptase hallmarks (Bryan et al, 1998; Fitzgerald et al, 1999; Harrington et al, 1997; Nakamura et al, 1997). The regulation of TERT expression is conserved and typically relegated to actively dividing tissues in multicellular organisms or within S-phase for single cell species. The fact that murine somatic cells are telomerase-positive in contrast to humans highlights the amount of divergence in telomerase regulation even within mammals (Prowse & Greider, 1995).

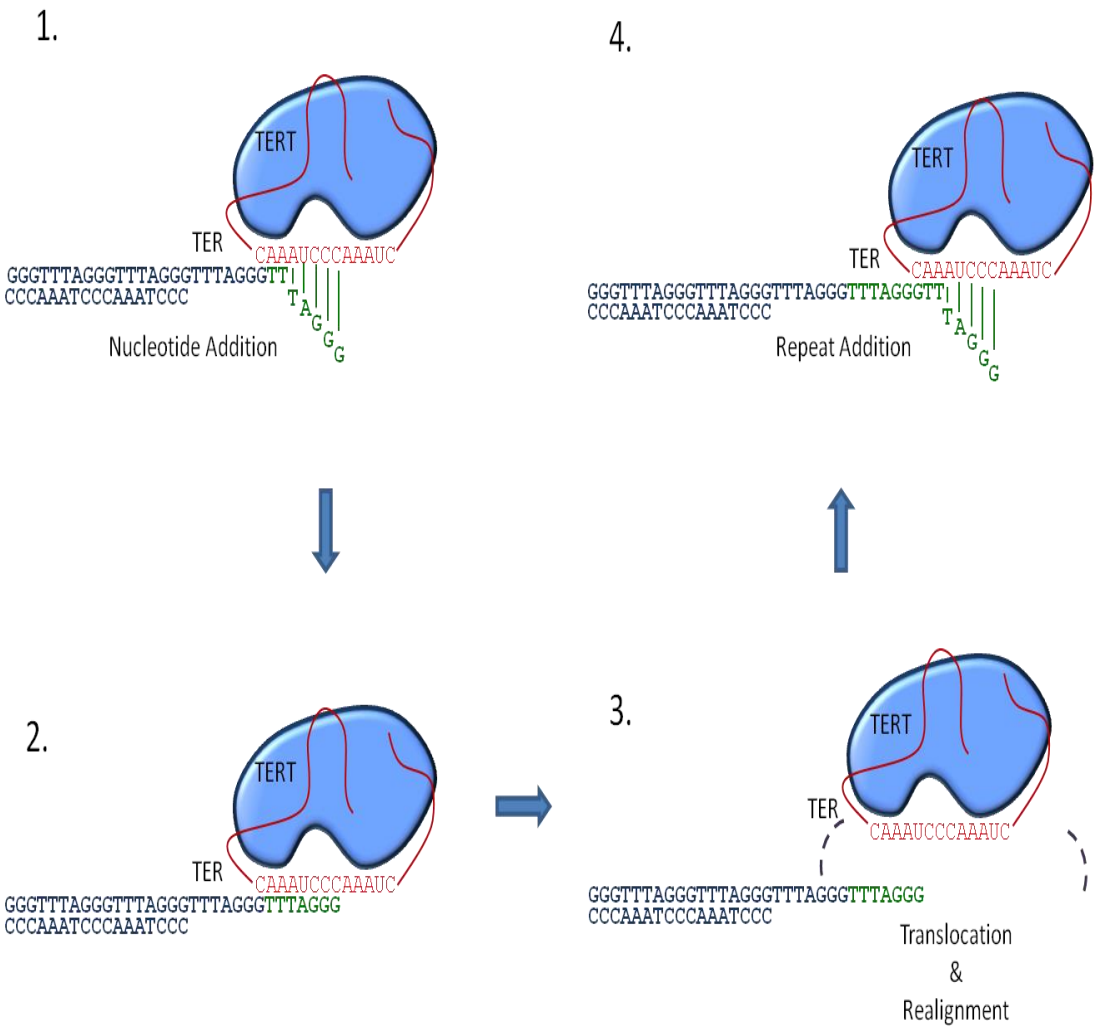


Figure 1-4. The mechanism of telomerase. Telomerase minimally consists of a catalytic reverse transcriptase (TERT) and an RNA subunit (TER). TER contains a limited complementary template domain (1.5x repeats) used to synthesize telomere repeats. Each nucleotide is added sequentially (nucleotide addition, 1 and 2). Telomerase must translocate and realign its template reiteratively (repeat addition, 3 and 4) to begin a new round nucleotide addition. The ability of telomerase to stay associated with its substrate while synthesizing repeats is known as repeat addition processivity (RAP). Telomerase RAP is facilitated by TER and other telomere proteins (Berman et al, 2011; Wang et al, 2007).

Telomerase RNA, TER

Soon after the discovery of telomerase, it was noted that RNase treatment could abolish telomerase activity (Greider & Blackburn, 1987). The telomerase RNA subunit, termed TER, assembles with TERT to form the core telomerase enzyme. Telomerase aligns its template sequence in a manner that allows sequential nucleotide addition to generate a full telomere repeat (Fig. 1-4). However, the template sequence is limited in length and consists of 1.5 telomere repeats. This implies either telomerase must realign this sequence reiteratively, or that telomerase reengages its substrate every repeat addition. The processive nature of ciliate telomerase and its corresponding six nucleotide periodicity was elegantly revealed by Carol Greider using direct primer extension telomerase activity assays (Greider, 1991). This unique characteristic of telomerase would be known as repeat addition processivity (RAP) (Fig. 1-4). It would take a number of years before the elements within TER that facilitate the processive nature of telomerase would be revealed (Berman et al, 2011; Mason et al, 2003). A recent model suggests flexibility within the single-stranded regions flanking the template element within TER act as a spring to reposition the enzyme as it iteratively synthesizes telomeric DNA (Berman et al, 2011).

In addition to providing the template for telomere replication, TER can act as a flexible scaffold for various protein accessory components of telomerase (Cifuentes-Rojas et al, 2011; Lustig, 2004; Zappulla & Cech, 2004). One of the best characterized telomerase RNAs is that of *S. cerevisiae* termed TLC1.The size of TLC1 is relatively large (1157nt) compared to vertebrates (~451nt), ciliates (148-209nt), and *Arabidopsis*

(748nt) (Chen et al, 2000; Cifuentes-Rojas et al, 2011; Collins, 1999). It has multiple essential binding sites for proteins such as Est1, Ku, and Sm7 (Seto et al, 2002; Seto et al, 1999; Stellwagen et al, 2003) that radiate out from a central core (Fig. 1-5). Interestingly, these domains can be structurally rearranged around the RNA and still maintain telomerase integrity suggesting some level of promiscuity in the three-dimensional architecture (Zappulla & Cech, 2004). Later work defined a minimal TLC1 RNA of 500nt composed of essential domains for catlalysis and found that it still conferred telomerase activity *in vitro*. However, cells expressing this minimal TLC1 had reduced fitness *in vivo,* signifying the flexible linker regions of the RNA are biologically important (Zappulla et al, 2005).

Recently it was discovered that *Arabidopsis* possesses two TER genes (TER1 and TER2) due to gene duplication (Cifuentes-Rojas et al, 2011). Interestingly, a processed TER2 isoform (TER2s) was discovered from total RNA extracts that was generated from splicing and 3' cleavage events (Cifuentes-Rojas et al, 2012a). Canonical telomere maintenance is attributed to TER1, while TER2 is a regulatory molecule. TER2 deficiency results in increased telomerase activity, although plants display wild type telomere lengths, suggesting telomerase is not deregulated in telomere replication. Furthermore, introduction of double-strand breaks (DSB) by the drug zeocin leads to a spike in TER2 expression coincident with a decrease in telomerase activity levels (Cifuentes-Rojas et al, 2012a). This observation suggests TER2 may be needed to down regulate the TER1 RNP at DSBs to prevent *de novo* telomere formation at these sites. Surprisingly, TER2 retains the highest affinity for TERT, but is normally lowly

expressed (Cifuentes-Rojas et al, 2012a). However, in the presence of DSBs, TER2 becomes the most abundant isoform. Thus, TER2 may sequester telomerase in a non-productive RNP complex. A biological role for TER2s has not been discerned at this time. Further insights into the function of TER2 are discussed below.

Telomerase accessory proteins

Soon after the discovery of telomerase it became apparent that proteins beyond TERT and TER could modulate telomere maintenance though regulation of telomerase itself (Lendvay et al, 1996). What was not readily obvious was that some of these proteins were actually accessory components of telomerase (Fig. 1-5). As discussed earlier, budding yeast Est1 was uncovered via a genetic screen for mutants with telomere maintenance defects and senescence phenotypes (Lundblad & Szostak, 1989). Immunoprecipitation of Est1 protein from yeast extracts co-precipitated TLC1 RNA and active telomerase enzyme (Lin & Zakian, 1995b; Steiner et al, 1996). Mutations in Est1 that disrupted the TLC1 RNA interaction resulted in senescence phenotypes (Zhou et al, 2000). Biochemical analysis identified a bulged stem region on TLC1 responsible for Est1 binding (Seto et al, 2002). The primary role for Est1 appears to be recruitment of telomerase to the chromosome end and perhaps telomerase activation (Chan et al, 2008; Taggart et al, 2002). Telomerase recruitment will be addressed in detail below.

Est3, is another budding yeast telomerase component that has remained functionally enigmatic. Like Est1, Est3 mutants display telomere maintenance defects (Lendvay et al, 1996). However, unlike Est1, Est3 binds Est2 (TERT) of telomerase, but

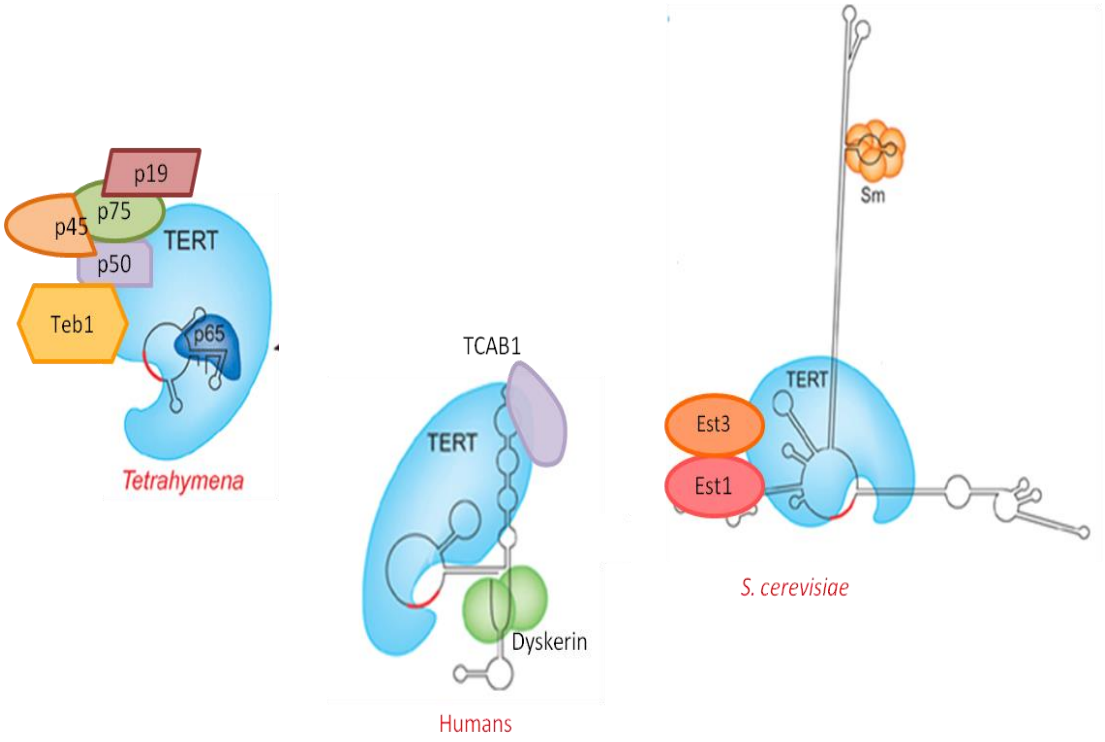


Figure 1-5. Telomerase RNP complexes from select organisms. *(Right)* Budding yeast telomerase possesses Est1, Est3, and the snRNP Sm7 accessory proteins. Est1 promotes telomerase recruitment and Est3 is required for telomere maintenance *in vivo* (Zapulla and Cech, 2004; Talley et al, 2011; Seto et al, 1999). *(Left) Tetrahymena* telomerase is the most well characterized holoenzyme consisting of a TASC complex (p45, p19, p75) that is recruited by TERT bound p50 (Min and Collins, 2009). The regulatory protein Teb1 stimulates Tetrahymena telomerase RAP and binds through contacts with p50 and TERT and TER bound p65 promotes RNP biogenesis (Min and Collins, 2009; Prathapam et al, 2005). (*Middle)* Less is known about human telomerase. It contains the RNP maturation factor dyskerin and the RNA chaperone TCAB1 at cajal bodies (Heiss et al, 1998; Stern et al, 2012). Figure is adapted from Teixeira and Gilson, 2007.

is not required for telomerase recruitment to telomeres *in vivo* (Talley et al, 2011; Tuzon et al, 2011). Rather, Est3 is proposed to activate telomerase potentially through its associate with Est1 protein (Tuzon et al, 2011). Recently, biophysical characterization of Est3 revealed similarities in the surface of Est3 with another telomerase interacting protein in humans, TPP1 (Rao et al, 2014). This and other recent studies are have established the importance of telomerase interacting proteins in promoting telomerase activity to facilitate telomere replication.

Telomerase has also been intensively studied in ciliated protozoa (Fig.1-5). *Euplotes crassus* telomerase shifts between lower and higher molecular weight complexes during its developmental life cycle. These higher molecular weight complexes have increased enzyme processivity *in vitro* suggesting that accessory proteins bestow or enhance telomerase RAP (Greene & Shippen, 1998). Telomerase purification from *E. aediculatus* identified a La-motif containing protein p43 responsible for stabilization and stimulation of telomerase complexes (Aigner & Cech, 2004; Lingner & Cech, 1996). *T. thermophila* also has a variety of telomerase-associated components including the p43 ortholog p65 (Prathapam et al, 2005). The telomere adaptor subcomplex (TASC) consisting of p75, p45, and p19 is recruited to the telomerase core by the p50 protein (Min & Collins, 2009). Once the single-strand telomere binding protein Teb1 interacts with telomerase, RAP is enhanced *in vitro* (Min & Collins, 2009). The usefulness of ciliate models for biochemical studies has significantly enhanced our understanding of the complexities of telomerase RNP maturation and regulation in the context of holoenzyme formation.

Identification of telomerase holoenzyme components in multicellular organisms has been limited thus far (Fig. 1-5). Telomerase RNP maturation requires dyskerin, a gene originally identified to be involved in the X-linked disease dyskeratosis congenita (DKC) in humans (Heiss et al, 1998; Mitchell et al, 1999). Dyskerin recognizes H/ACA motifs on small nucleolar RNAs (snoRNAs) including TER (Mitchell et al, 1999). Primary fibroblasts from DKC patients have reduced TER accumulation, and as a consequence reduced telomerase activity leading to shorter telomeres (Mitchell et al, 1999; Vulliamy et al, 2001). Later it was discovered that dyskerin is also a component of *A. thaliana* telomerase. As in humans, loss of function mutants in *Arabidopsis* dyskerin result in diminished telomere maintenance (Kannan et al, 2008a). A more detailed description of *Arabidopsis* telomerase accessory proteins will be presented later in this introduction.

Telomere associated proteins

Beyond the nucleic acid component of telomeres there exists numerous telomere associated proteins and complexes (Fig. 1-6). One critical function to solve the end-protection problem (de Lange, 2009). They also play important roles in telomere replication and telomerase regulation (Jain & Cooper, 2010). Therefore, understanding the functions of telomere proteins is central to understanding global telomere biology.

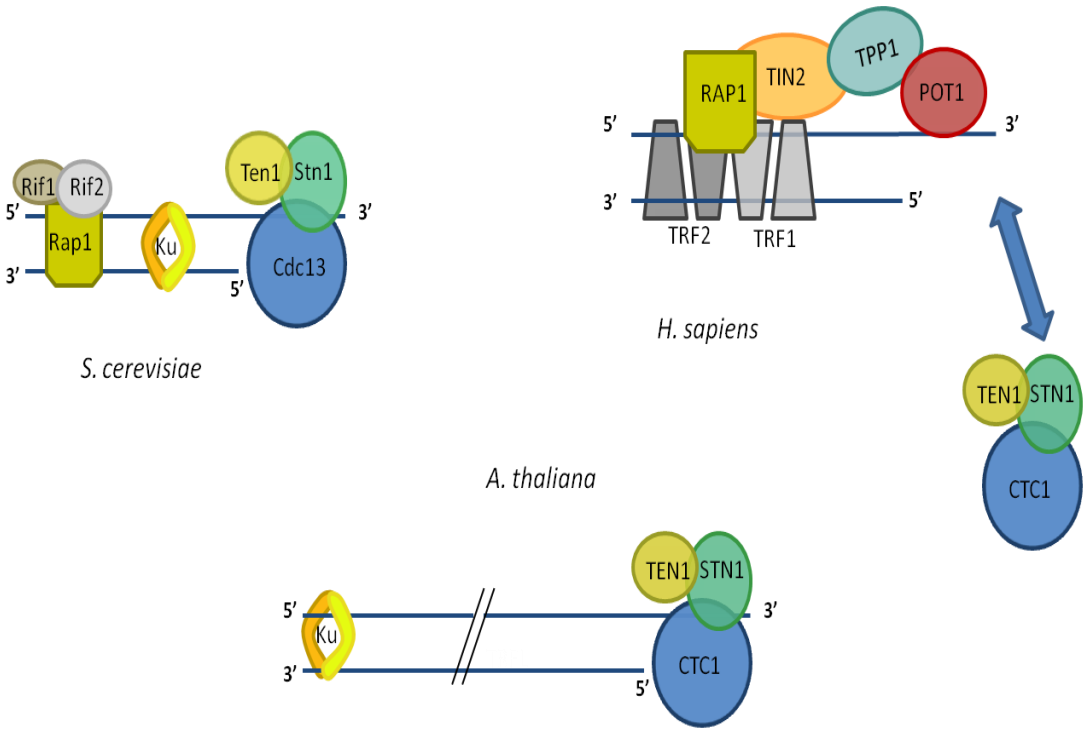


Figure 1-6. Telomere capping complexes in budding yeast, humans, and *Arabidopsis.* Budding yeast telomeres are composed of ds binding protein Rap1 and associated (RIF1 and 2) proteins that regulate telomere maintenance (Marcand et al, 1997). Ku binds ds telomeric DNA, but the exact context of Ku binding is not known. Cdc13/Stn1/Ten1 CST capping complex binds the G-overhang and promotes telomere integrity (Pennock et al, 2001). Human telomeres are protected by the shelterin complex (de Lange 2009). TRF1 and TRF2 are homodimers and bind ds telomeric DNA. POT1 binds the G-overhang and is bridged to the ds region via TPP1, TIN2, and RAP1. Human CST (CTC1/STN1/TEN1) terminates telomerase post replication and promotes C-strand fill-in (Chen et al. 2012; Gu et al, 2012). Lastly, *Arabidopsis* possesses a homologous CTC1/ST complex that primarily functions in chromosome end protection at G-overhangs (Price et al 2010). Ku protects and binds ds DNA at blunt ended telomeres (Kazda et al, 2012).

Double-strand binding proteins

Budding yeast Rap1, a repressor and activator of transcription, binds double-stranded (ds) telomeric DNA via a conserved Myb domain found in other ds telomere binding proteins (Berman et al, 1986; Longtine et al, 1989; Shore & Nasmyth, 1987). Rap1 has a direct role in regulating telomere length homeostasis (Conrad et al, 1990). Deletions of the C-terminal domain of Rap1 cause hyper-extended telomeres *in vivo*. Rap1 separation-of-function mutants were identified that disinguish telomere length regulation and transcriptional silencing through two Rap1 Interacting Factors (RIF1 and RIF2) that bind Rap1 at telomeres (Marcand et al, 1997b). The number of Rap1 molecules bound to telomeres provides a sensory mechanism so called "protein telomere counting" for determining the extent of telomere elongation at a particular chromosome end (Marcand et al, 1997b). Thus, telomerase elongation is not an arbitrary cellular process, but reflects a highly regulated process involving many cellular factors that achieve telomere length homeostasis.

The telomeres of budding yeast are also bound by the Ku heterodimer (Ku70/Ku80) (Gravel et al, 1998). Ku is widely conserved and functions in telomere biology and DNA repair. However, the fact that Ku associates with telomeres is paradoxical considering it functions in NHEJ (non-homologous end joining) (Fisher & Zakian, 2005), an activity that must be blocked at telomeres (Bertuch & Lundblad, 2003). At telomeres, Ku promotes silencing of telomere proximal genes (Boulton & Jackson, 1998), and blocks nucleolytic degradation of telomeric DNA (Bonetti et al, 2010). Therefore, Ku engages telomeres to promote telomere maintenance and

protection, but it is still not clear how Ku associates with chromosome ends. Because Ku binds dsDNA ends as a preformed ring (Walker et al, 2001), it is not known how Ku overcomes the presence of t-loops or other common pre-bound telomere proteins. Furthermore, Ku has other functions in telomerase recruitment that add to the complexity of understanding its dynamic functions (see below).

In addition to binding telomeric DNA, Ku also associates with telomere ends and telomerase RNA in humans and plants (Cifuentes-Rojas et al, 2012a; Hsu et al, 1999; Kazda et al, 2012; Ting et al, 2005). Inactivation of human Ku leads to loss of telomere integrity and chromosome end-to-end fusions (Myung et al, 2004). In marked contrast, loss of *Arabidopsis* Ku leads to hyper-elongated telomeres and no apparent chromosome fusions (Riha & Shippen, 2003). This may be due to the presence of blunt ended telomeres in *Arabidopsis* for which Ku seems to promote end-protection of (Kazda et al, 2012).

Shelterin complex

A bit perplexing is the evolution of telomere complexes in vertebrates. Shelterin is a six-membered complex that is anchored to the ds telomere region via TRF1/TRF2 and RAP1 and is molecularly bridged to the G-overhang binding protein POT1 by TIN2 and TPP1 (Jain & Cooper, 2010). Shelterin components are poorly conserved in budding yeast with RAP1 being the only known homolog. Oddly enough, shelterin orthologs are found in fission yeast (Jain & Cooper, 2010), but are mostly absent in *Arabidopsis* aside from three POT1 homologs and a number of TRF-like proteins (Nelson et al, 2014).

Thus, it seems despite the fairly well conserved nature of telomeric DNA sequence and structure, there is much divergence in the protein factors that mediate essential functions in telomere biology.

Mammalian shelterin promotes end protection by suppressing the DDR master kinases Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR) (de Lange, 2009). ATM and ATR monitor DSBs and accumulated ss DNA in the cell, respectively, and elicit checkpoint responses via phosphorylation pathways. Shelterin component TRF2 represses ATM signaling and distinguishes telomeres from DSBs (Denchi & de Lange, 2007). How TRF2 achieves this is currently unknown but TRF2 is believed to promote t-loop formation which may mask chromosome ends (Doksani et al, 2013). POT1, which binds ss G-overhangs, down regulates ATR signaling (Denchi & de Lange, 2007). Knockdown of POT1 results in increased telomeric γ-H2AX foci, as well as increased DNA damage checkpoint responses downstream of ATR kinase (Baumann & Cech, 2001a; Jain & Cooper, 2010; Veldman et al, 2004). Surprisingly, ATM and ATR promote aspects of telomere maintenance in mammals and in yeast (Shore & Bianchi, 2009; Verdun & Karlseder, 2006). Phosphorylation of downstream targets regulate facets of telomerase recruitment and potentially telomere dynamics.

In addition to TRF2 and POT1a, other shelterin components are similarly critical for telomere integrity. Human RAP1is less understood compared to its yeast counterpart. It lacks similar DNA binding capabilities and must be recruited to telomeres through an interaction with TRF2 (Li et al, 2009), but those studies have now become controversial (Arat & Griffith, 2012; Martinez & Blasco, 2011{Arat, 2012 #265). One potential

function of RAP1 is prohibiting NHEJ at telomeres through its interaction with TRF2 (Sarthy et al, 2009). TIN2 and TPP1 critically bridge the ds portion of shelterin with the ss binding protein POT1. TIN2 in conjunction with TRF1 can mediate telomere length regulation (Ye & de Lange, 2004). Lastly, TPP1 is required for POT1 recruitment to telomeres and is essential for telomerase recruitment and regulation(Liu et al, 2004b; Nandakumar et al, 2012; Wang et al, 2007). TPP1 will be discussed in more detail below.

Single-strand binding proteins

The first identified telomere binding components were found in the ciliate *Oxytricha nova* (Gottschling & Zakian, 1986). Telomere end binding protein alpha and beta (TEBPα/β) specifically bind ss telomeric DNA, and dimerize in a DNA dependent manner (Fang & Cech, 1993). Furthermore, their ability to shield telomeric DNA from Bal31 digestion indicated they protect telomere ends (Gottschling & Zakian, 1986). The concept of protein-mediated telomere capping introduced a new paradigm in telomere biology. The 3' terminus of G-overhangs need to be sequestered to mitigate nuclease resectioning and to prevent cellular DNA damage response mechanisms from treating the ends as breaks. In the same fashion, telomeres also need to be exposed to telomerase and central DNA replication machinery during S phase. The complexities involved in telomere dynamics will be discussed later in this Chapter.

One of these genes uncovered in the Est screen encoded the protein Cdc13, previously shown to have ss telomeric DNA binding and properties of a telomere

capping protein (Garvik et al, 1995b; Nugent et al, 1996a). The DNA binding domain of Cdc13 consists of an oligosaccharide-oligonucleotide motif (OB-fold) that binds ss G-rich telomeric DNA with high affinity (Mitton-Fry et al, 2004). Beyond its nucleic acid binding, Cdc13 has numerous interactions with other telomere proteins making it a hub for coordinating processes involved in telomere biology. Cdc13 interacts with the Est1 subunit of telomerase to mediate telomerase recruitment (Pennock et al, 2001; Tuzon et al, 2011). Cdc13 also binds Pol1 of the Pol α complex to facilitate C-strand fill in (Qi & Zakian, 2000), as well as two small OB-fold proteins, STN1 and TEN1 (see below)(Fig. 1-6).

Analysis of separation of function Cdc13 alleles revealed a binary role for this protein in telomere protection and telomere maintenance (Garvik et al, 1995b; Nugent et al, 1996a). Some Cdc13 mutant alleles resulted in increased DDR and elongated G-overhangs similar to Cdc13 deletions. Other alleles had an Est phenotype, but upheld telomere integrity at least initially. There are some conflicting reports as to the molecular consequences of these alleles (Chan et al, 2008; Chandra et al, 2001), but nonetheless these mutants reveal new insights into telomere dynamics (Reviewed below). Stn1, a binding partner of Cdc13 was identified as a suppressor of *cdc13* mutation (Grandin et al, 1997). Stn1 deficiency elicited a similar Rad9-activated DNA damage response as *cdc13* mutants as well as increased telomere length indicating it acts within the Cdc13 protection pathway (Grandin et al, 1997). Fusion constructs in which Cdc13 is physically tethered to Stn1 repressed telomere maintenance, but maintained chromosome end protection, suggesting Stn1 mediates the protective function of this complex at the

telomere (Grandin et al, 2000; Pennock et al, 2001). Ten1 is the last component in what would eventually be called the 'CST complex'. Ten1 forms a stable heterodimer with Stn1 (Grandin et al, 2001). Ten1 mutations cause similar defects in telomere protection like Stn1 and Cdc13. Thus, CST has been proposed to be a guardian of the chromosome end in budding yeast.

The role of CST is not resolved as some conflicting reports have proposed CST is not a capping complex, but a telomere specific RPA (replication protein A) like heterotrimer responsible for telomere replication (Chandra et al, 2001; Gao et al, 2007). Interestingly, the CST complex is conserved in humans, although Cdc13 is replaced by another large OB-fold bearing telomere binding protein, CTC1 (Conserved Telomere Maintenance Component 1) (Miyake et al, 2009; Surovtseva et al, 2009a). Similar to the proposed RPA like roles for yeast CST, the primary function for human CST is proposed to be a regulator of telomere replication rather than end protection (Gu et al, 2012; Stewart et al, 2012). Curiously, shelterin appears to fill the protection role in vertebrates (discussed above). Since budding yeast lacks most of these components, the RPA-like role for yeast CST would seem torequire some other unidentified mechanism of telomere protection.

Lastly, CST components have been identified in plants, but at least in *Arabidopsis thaliana,* they seem to function both in end-protection and replication (Fig. 1-6)(Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009a). Loss of any CST component results in severe telomere shortening, chromosome end-to-end fusions, and upregulated DDR {Boltz, 2012 #164}. The *Arabidopsis* CTC1 component interacts with

the POL α subunit ICU2 similar to CTC1 in humans (Price et al, 2010), and recent work from the Riha lab implicates STN1 in telomere replication (K. Riha, personal communication). Studies to clarify the roles of CST complex in *Arabidopsis* are discussed in Chapter II.

Telomere dynamics and telomerase recruitment

Because telomere protection must be coordinated with telomere replication during the cell cycle, maintaining telomere length homeostasis is a complicated affair. The number of different factors and interactions involved in this process reflect the dynamic nature of telomere and telomerase regulation. Soon after Cdc13 was discovered it became apparent there was duality in its telomere functions (Nugent et al, 1996a). One classic allele known as *cdc13-2*^{est} displayed an ever shorter telomere phenotype, which was identical to phenotypes associated with loss of Tlc1. When this allele was crossed to a *tlc1*Δ strain, the phenotype was the same as in either single mutant , suggesting it perturbed telomere maintenance (Nugent et al, 1996a). In contrast, the *cdc13-1*^{ts} allele did not display an 'est' phenotype and elicited cell cycle arrest, extensive ss telomere DNA, and conditional lethality at the restrictive temperature. Therefore, Cdc13 has two critical roles: telomere maintenance and telomere protection. Later work confirmed that Cdc13 binds telomerase as well as conventional replication enzymes and thus serves as a hub for telomeric processes (reviewed above). Importantly, Cdc13 can also inhibit telomerase action by sequestering the G-overhang substrate, thereby protecting the chromosome end from inappropriate resectioning by nucleases (Zappulla et al, 2009).

The role of Cdc13 during the cell cycle is defined by its binding partners (Fig. 1-7). When bound to Stn1 and Ten1, Cdc13 mediates end protection. However, Cdc13 in association with the Est1 subunit of telomerase mediates recruitment in S phase. These complexes are mutually exclusive and regulated by the cell cycle dependent kinase, Cdk1 (Li et al, 2009). Phosphorylation of Cdc13 shifts the binding preference from Stn1 and Ten1 to Est1 protein, thus altering telomere status to a replication inducive state. This mechanism is a cornerstone in the overall idea that telomeres are bimodal in nature and defined by a shift between telomerase accessible and inaccessible states.

Another interaction hub at the telomeres centers around budding yeast Ku which also binds TLC1 (Stellwagen et al, 2003), indicating Ku as a telomerase recruitment factor. Interestingly, RNA binding by Ku appears to be mutually exclusive of its DNA binding activity (Pfingsten et al, 2012) . Therefore, it is difficult to explain Ku's ability to positively promote telomere maintenance (Fisher & Zakian, 2005) via a role in telomerase recruitment by a model that necessitates mutual DNA and RNA binding events. An interesting model proposes Ku promotes nuclear accumulation of telomerase, and then once in the nucleus it releases Tlc1 in the presence of telomeric DNA due to its higher affinity for DNA (Gallardo et al, 2008; Pfingsten et al, 2012). Other recent studies have shown that telomerase extension of telomeres is dependent on the Ku-TLC1 interaction due to its ability to facilitate Est1 mediated recruitment of telomerase in S phase (Williams et al, 2014). More work is needed to clarify the exact contributions of

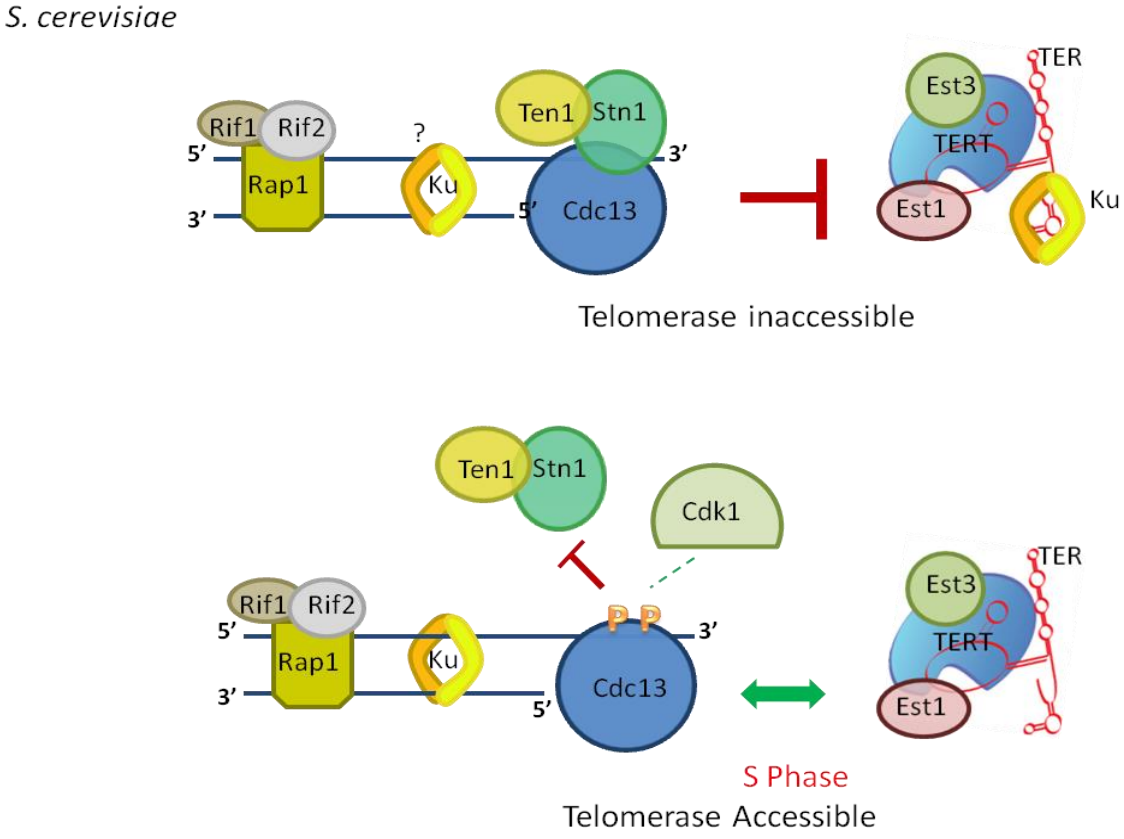


Figure 1-7. Budding yeast telomere dynamics. In G1, telomerase access to telomeres is mitigated by the CST complex (telomerase inaccessible). Upon S phase, Cdk1 phosphorylation of Cdc13 shifts its binding preference to Est1, thus removing Stn1-Ten1 (Li et al, 2009). Est1-Cdc13 association promotes telomerase recruitment and telomere maintenance (telomerase accessible; Wu et al, 2011). The role of Ku is still controversial due to its RNA and DNA binding properties. A proposed role is that it switches from TER to ds telomeric DNA during telomerase recruitment (Pfingsten et al, 2012).

Ku in yeast telomere biology and how that coalesces with other known aspects telomere maintenance and protection.

The co-existence of shelterin and CST in vertebrates complicates the intertwined regulatory networks that control telomere status (Fig. 1-8). For example, while shelterin exists as a complete six-subunit complex, there is evidence for subcomplexes that mediate different aspects of telomere metabolism (Diotti & Loayza, 2011; Liu et al, 2004a; Takai et al, 2010). Perhaps the most well studied example of this is the TPP1-POT1 heterodimer. POT1, as a G-overhang binding protein (Baumann & Cech, 2001b), can prevent elongation of telomerase substrates *in vivo* by sequestration of the 3' end of the G-overhang (Lei et al, 2005). However, when POT1 is bound to TPP1 as a heterodimer, the complex stimulates telomerase RAP (Wang et al, 2007). Although it is not clear in what biological context heterodimer formation occurs, the number of TPP1 and POT1 molecules is in excess of the G-overhang binding sites present in a cell, suggesting POT1-TPP1 has a capacity beyond its role in the shelterin complex (Takai et al, 2010).

A major recent discovery was that TPP1 directly contacts telomerase and thus is involved in telomerase recruitment (Nandakumar et al, 2012; Zhong et al, 2012). Thus, like yeast Cdc13, TPP1 appears to be a multifunctional protein essential for end protection in the context of shelterin and for telomerase-mediated replication. Telomerase recruitment can be inhibited by specific mutations in TPP1, eventually leading to cell death due to ineffectual telomere maintenance (Nakashima et al, 2013). Because these studies of TPP1 were performed in asynchronous cell populations, it was

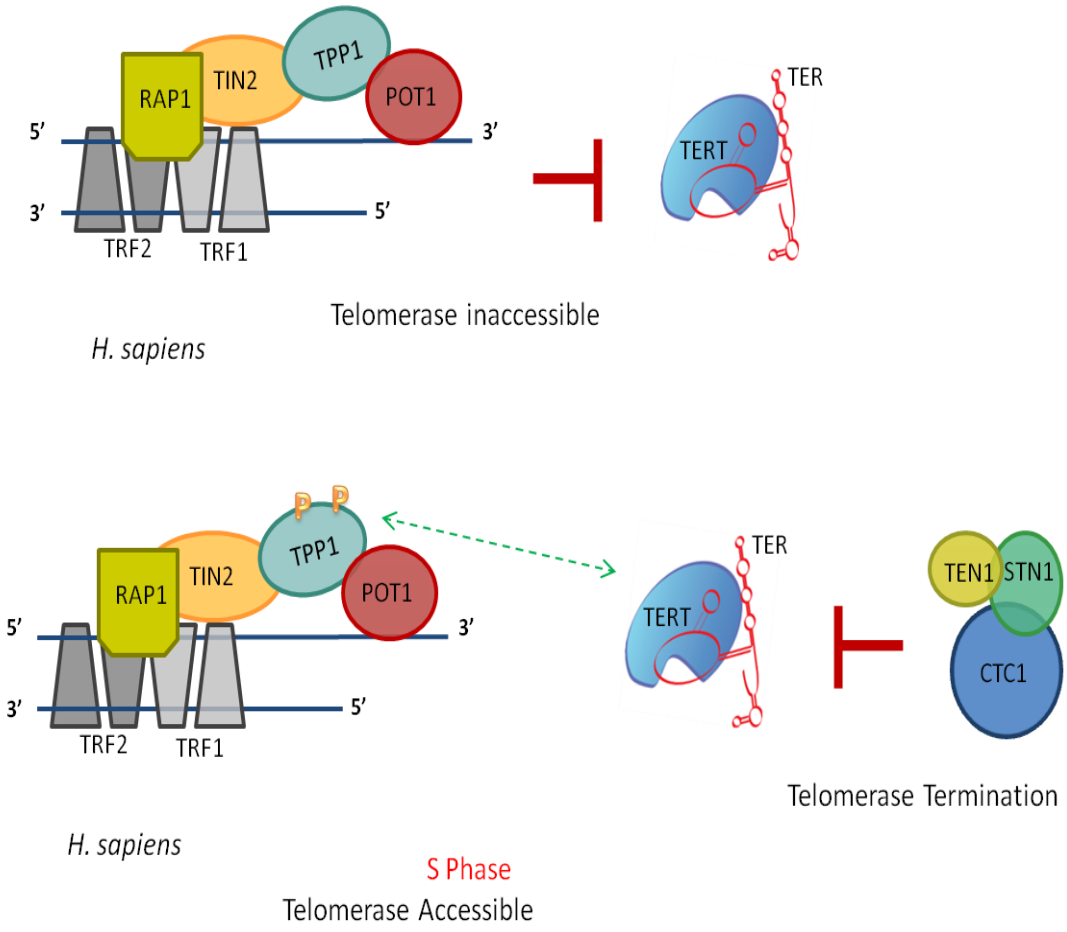


Figure 1-8. Human telomere dynamics. Shelterin complex promotes the telomerase inaccessible state in G1. Increasing phosphorylation of TPP1 leading up to S phase promotes its direct association with TERT thereby recruiting telomerase to the telomeres (telomerase accessible; Zhang et al, 2013; Nandakumar et al 2012). The CST complex terminates telomerase post replication and coordinates the transition into C-strand synthesis (Chen et al, 2012). After S phase, the telomeres are returned to an inaccessible state bound by shelterin complex.

unclear what events lead to telomerase recruitment in S phase. Analysis of putative phosphorylation sites on TPP1 in synchronized cells revealed multiple residues undergo increasing phosphorylation within S/G2 phase. Modification of these sites is needed to mediate telomerase recruitment and stimulate telomerase activity (Zhang et al, 2013a).

Little is known how the G-overhang is handed off from telomerase to DNA polymerase α. From a mechanistic view point, telomerase must be removed to allow initiation of C-strand fill-in. Work from the Lingner group revealed a novel role for human CST in terminating telomerase activity through contacts with POT1-TPP1 (Chen et al, 2012). Specifically, interactions between CTC1-POT1, CTC1-TPP1, and STN1-TPP1 were uncovered in yeast-two-hybrid assays. The mouse POT1 paralog, POT1b, was also reported to interact with CTC1 to facilitate fill-in synthesis and G-overhang processing (Wu et al, 2012). Therefore, the sequence of events that occur pre- and post-telomere replication is beginning to be mechanistically defined.

Telomere length homeostasis

One of the most perplexing aspects of telomere biology is the diversity in telomere length (Fig. 1-2). Telomeres stripped down to their nucleic acid skeleton are fairly well conserved in sequence, composition, and structure. What accounts for the diversity in the length of telomeric duplex and G-overhang? The answer to this question is unknown, but what is clear is that each organism has a set point for telomere length. The cellular consequences of insufficient telomere length are senescence and cell death, while overly elongated telomeres are signs of telomerase deregulation (Teixeira et al,

2004). In addition, extremely long G-overhangs elicit DNA damage responses due to the accumulation of ss DNA via the ATR pathway (Denchi & de Lange, 2007).

Telomeres can undergo shortening and lengthening activities. Telomere erosion and eventual replicative senescence is a tumor suppressor mechanism (Smith & Pereira-Smith, 1996). Therefore, restrictive expression of telomerase core components is a means for safeguarding cells. Telomerase access to the G-overhang can be physically blocked to prevent telomere lengthening by G-overhang binding proteins (Lei et al, 2005; Mitton-Fry et al, 2004; Zappulla et al, 2009). Nucleases are also a necessary part of telomere processing and help to keep telomere length at a specific set point. The Apollo nuclease is recruited by shelterin component TRF2 to process leading strand (blunt-ended) telomeres into G-overhangs (Wu et al, 2010). In yeast, a complex coordination of Ku, Rap1, Exo1 nuclease, and the Mre11-Rad50-Xrs2 (MRX) nuclease complex play a delicate balancing act to control resectioning of telomeres (Bonetti et al, 2010).

Telomere lengthening is primarily mediated by telomerase. Interestingly, telomerase is preferentially recruited to shorter telomeres and not longer ones. In yeast, Rap1 and its associated factors Rif1 and Rif2, provide a negative feedback mechanism by coating ds telomeric DNA and inhibiting telomerase in *cis* in correlation with telomere tract length (Marcand et al, 1997a). In addition, Tel1, the ATM homologue in yeast, mediates recruitment of telomerase to shorter telomeres as a preferential substrate (Bianchi & Shore, 2007; Chang et al, 2007). Similarly, shorter telomeres in mammals are also preferentially elongated (Hemann et al, 2001). Therefore, telomere length

homeostasis may be achieved by transient activation of DNA damage components at telomeres (Verdun & Karlseder, 2006).

One interesting feature of telomere length maintenance is the involvement of recombination based mechanisms that lead to lengthening and shortening of telomere tracts. Alternative Lengthening of Telomeres (ALT) was initially discovered in budding yeast when a fraction of cell populations deficient in Est1 failed to senesce and die (Lundblad & Blackburn, 1993). These survivors were dependent upon Rad50 or Rad51 recombination. In this situation, critically shortened telomeres can be maintained by rolling circle amplification using extra-chromosomal telomeric circles (ECTC) as a template for telomere extension (McEachern & Haber, 2006). ALT has been reported in humans, fission yeast, and plants (Akimcheva et al, 2008; Bryan et al, 1997; Nakamura et al, 1998). The unifying theme of ALT is its occurrence in cells with disrupted telomerase mediated maintenance.

Recombination can also lead to rapid truncations of telomere tracts. Telomere Rapid Deletion (TRD) involves excision of either intramolecular t-circles or t-loops formed from G-overhang strand invasion into the duplex region (Tomaska et al, 2009). The existence of ECTCs or t-circles and reports of TRD span across a diversity of organisms. Some studies have proposed TRD as a means to restore over-elongated telomeres and suggested TRD is a mechanism used by the cell to maintain telomere lengths within a prescribed range (Pickett et al, 2009; Watson et al, 2005). A mutant allele of TRF2 in humans generated stochastic deletions approximately the size of t-loops, and this was dependent on the protein XRCC3 implicated in resolution of

Holliday junctions (Wang et al, 2004). T-circle formation in *Arabidopsis* is repressed by the Ku heterodimer. In the absence of Ku, truncated chromosome termini are quickly extended by telomerase (Zellinger et al, 2007). The combination of ALT and TRD implies recombination is a critical tool selected by evolution to maintain telomere length. Moreover, roles for recombination and telomerase exemplify the amount of effort exerted by cells to sustain telomere length homeostasis.

Arabidopsis as a model system for telomere biology

Arabidopsis thaliana has proven to be impactful as a model for telomere biology. It is ironic that we return to plants where the origins of telomere biology began. *Arabidopsis* is the reference species for plants and serves as a comparative model for higher eukaryotes. *Arabidopsis* has a relatively short life span of ~6 weeks and a sequenced genome of ~130Mb. Perhaps the most practical advantage of *Arabidopsis* is its genetic tractability. The ability to transform *Arabidopsis* via *Agrobacterium* has led to large libraries of T-DNA insertion lines which contain mutations for many of the known telomere-related genes. Moreover, activation tagged and EMS-mutagenized lines provide an additional array of genetic tools for studying plant biology.

The small size of *Arabidopsis* telomeres (2-5kb) makes it possible to examine perturbations in telomere length using conventional Southern blotting procedures (Shakirov & Shippen, 2004). Furthermore, unique sequences upstream of telomere tracts provide distinct PCR primer binding sites for analyzing individual chromosome arm length and chromosome end-to-end fusion events (Heacock et al, 2007). Advancements

in molecular and biochemical methods in plants have made it possible to analyze mechanistic details of telomeric pathways. Numerous antibodies now exist from commercial vendors or have been generated from the Shippen lab to allow for informative molecular assays such protein function and interactions of *Arabidopsis* specific telomere complexes.

Arabidopsis is highly tolerant to telomere dysfunction unlike other model organisms. Mutations in telomere capping components or DDR mediators like ATM and ATR that lead to lethality in other systems are not lethal in *A. thaliana* (Watson & Riha, 2010). However, plants have many similarities to humans in the manner of telomere and telomerase regulation. Telomerase expression is restricted to dividing tissues, but absent in vegetative organs (Fajkus et al, 1996; Fitzgerald et al, 1996; McKnight & Shippen, 2004). As in yeast, disruptions in the TERT gene ultimately lead to senescence phenotypes, but plants lacking telomerase can persist for up to ten generations (Riha et al, 2001). Telomere sequence and structure is also quite similar to mammalian systems. Together these attributes make *Arabidopsis* a premier model system for comparative biology.

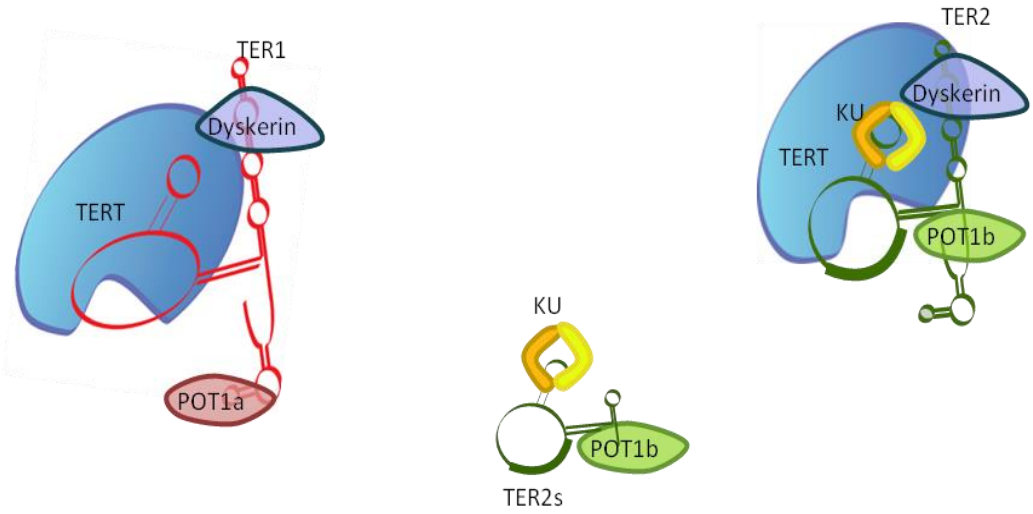
Because plants have undergone numerous genome and gene duplication events, the effects of selection on telomere-related genes are useful to investigate how and why gene function has changed over time. Moreover, the 1001 *Arabidopsis* genome project has compiled >1000 ecotype specific genome sequences. This data is invaluable for analyzing natural variation. As will be discussed in later Chapters, gene duplication events have had a profound impact on *A. thaliana* telomere biology.

Arabidopsis telomerase

Identification of the catalytic component of *Arabidopsis* telomerase, AtTERT, was facilitated by its significant homology to other TERT genes (Fitzgerald et al, 1999). *Arabidopsis* T-DNA insertion mutants within the TERT gene result in progressive telomere shortening phenotypes (Riha et al, 2001). While the first four generations of *tert* mutants are morphologically similar to wild type, later generation plants suffer leaf development abnormalities, enlarged shoot apical meristems, and eventual vegetative arrest with no discernible germline production. Moreover, loss of TERT leads to increasing sterility by way of seed loss and decreased pollen production (Riha et al, 2001). Because plants produce organs post-embryogenesis, TERT deficiencies lead to loss of proliferative capacity in shoot and root meristems, and ultimately the inability to differentiate into proper cell types (McKnight & Shippen, 2004).

Alternative telomerase RNPs

Brute force biochemistry was employed to identify the TER component of telomerase, but surprisingly this led to the discovery of two TER genes (Cifuentes-Rojas et al, 2011). TER1 and TER2 transcripts both assemble into telomerase RNP complexes *in vivo* and display telomerase activity *in vitro* (Fig. 1-9). However, TER1 is responsible for canonical telomere maintenance (Cifuentes-Rojas et al, 2011). The role for TER2 is



RNP	RNA Length	Attributes
TER1	748nt	Telomere maintenance, POT1a binding
TER2	784nt	Induced in response to DNA damage, TER1 RNP repression, POT1b/Ku binding
TER2s	219nt	Processed from TER2, accumulates in POT1b complexes *in vivo,* unknown function

Figure 1-9. *Arabidopsis* RNP complexes. Two distinct telomerase RNPs are formed due to TER1 and TER2 assembly with TERT protein. TER1 RNPs are bound by POT1a protein, known to positively regulate telomerase activity, and dyskerin (Surovtseva et al 2007; Cifuentes-Rojas et al, 2011). In contrast, the TER2 RNP is bound by dyskerin, the paralogous POT1b protein and Ku (Ciffuentes-Rojas et al, 2011). The interactions of POT1b and Ku with TER2 have not been characterized. Lastly, a processed isoform of TER2, TER2s, may or may not form an RNP with telomerase but does accumulate with POT1b and potentially Ku protein (Cifuentes-Rojas et al, 2011).

still somewhat mysterious, but *ter2* mutants display a constitutive DDR and are hypersensitive to zeocin induced DSBs (Cifuentes-Rojas et al, 2012a). Moreover, telomerase activity is elevated in these plants. A processed form of TER2, TER2s has also been reported. No readily identifiable function has been ascribed to TER2s, but it is well conserved amongst *A. thaliana* ecotypes. Analysis of TER2 will be a major focus of Chapter III.

TER1 and TER2 assemble into different RNP complexes with distinct protein composition (Cifuentes-Rojas et al, 2011). Dyskerin is associated with both TER1 and TER2. Interestingly, Ku has affinity for TER2 (Cifuentes-Rojas et al, 2011). Ku mutants have hyperelongated telomeres which suggests that Ku is primarily a negative regulator of telomerase (Riha et al, 2002), as has been proposed for TER2 (Cifuentes-Rojas et al, 2012a). POT1a, one member of the POT1 gene family in plants and homolog to human POT1, binds TER1 and functions as a positive regulator for telomerase (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007). POT1b, on the other hand, binds TER2, and may function in chromosome end protection (Shakirov et al, 2005). POT1c only exists in *A. thaliana* and its function is unknown. The functions and interactions of POT1 proteins from *Arabidopsis* are quite different than their counterparts in humans and fission yeast. AtPOT1a and AtPOT1b bind TER and do not seem to contribute to chromosome end protection.

Plant POT1 proteins

AtPOT1a was originally identified based on its homology to fission yeast POT1 (Baumann & Cech, 2001a). Despite its sequence conservation, AtPOT1a has functionally diverged away from chromosome end protection into a telomerase regulatory protein (Surovtseva et al, 2007). POT1a binds specifically to TER1 and associates with active telomerase complexes *in vivo* (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007). Moreover, POT1a lies within the telomerase pathway as double *pot1a tert* mutants have identical telomere shortening profiles as either single mutant. Extracts prepared from *pot1a* mutants have ~13 fold reduction in telomerase activity levels compared to wild type (Surovtseva et al, 2007), suggesting POT1a is necessary for maximal telomerase activity. Initial biochemical analysis of POT1a it was not able to bind telomeric DNA like it vertebrate and yeast counterparts (Shakirov et al, 2009a). However, recent work from the Shippen lab has demonstrated POT1a possesses specific telomeric ss DNA binding activity *in vitro* derived from its amino proximal OB fold domain (Dr. Amit Arora, personal communication). Thus, the ancestral function of DNA binding appears to be conserved in POT1a.

A duplication event has led to another full length POT1 gene in *Arabidopsis thaliana,* POT1b (Rossignol et al, 2007; Shakirov et al, 2005). Null *pot1b* mutations are wild type in their morphology and telomere length suggesting POT1b has diverged functionally from POT1a. Over-expression of the amino terminal half of POT1b leads to telomere shortening and chromosomal fusions indicating it may have retained a function in end protection (Shakirov et al, 2005). Furthermore, POT1b has a

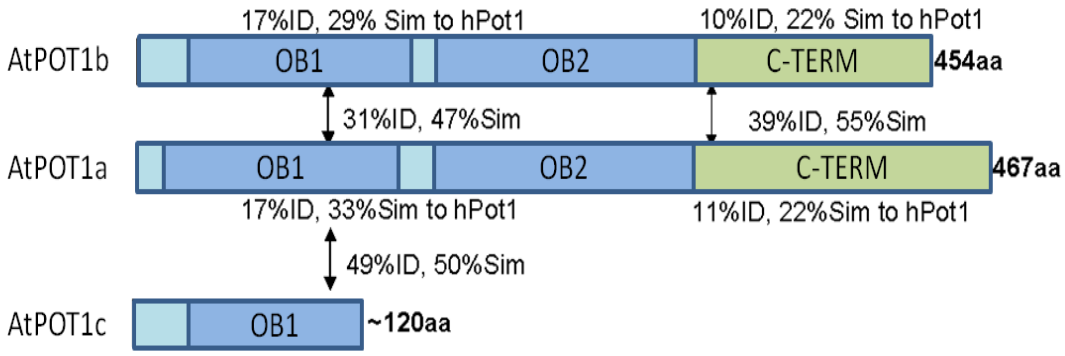


Figure 1-10. POT1 duplication in *Arabidopsis*. Gene duplication has led to three POT1 paralogs in *Arabidopsis.* POT1 proteins are highly sequence divergent relative to hPOT1 despite possessing two similar N-terminal OB-fold motifs, and are fairly divergent amongst the AtPOT1 gene family. POT1c duplication is specific to *A. thaliana* and has only retained a fraction of the POT1 locus with homology to POT1a OB1.

physical interaction, but the context of this interaction at telomeres is not clear at this time (Cifuentes-Rojas et al, 2011). POT1c represents a recent paralogous duplication of POT1a, and this gene is only found in *Arabidopsis* (Fig. 1-10). Truncation at the POT1c locus has led to retention of only a single OB fold domain with significant homology to the first OB fold of POT1a (Fig. 1-10). The lack of a T-DNA insertion line for POT1c has prevented assessment of its functional role at telomeres. Finally, it is interesting that the moss *Physcomitrella patens* encodes only a single POT1 locus. The role of PpPOT1 is similar to hPOT1 in that it promotes chromosome end protection (Shakirov et al, 2010). Thus, there are many outstanding questions concerning the evolution of POT1 in plants. Different aspects of AtPOT1 evolution is explored in Chapters IV and V.

Arabidopsis telomere components

Aside from POT1, the only other shelterin homologs that exist in *Arabidopsis* are TRF-like genes containing Myb domains that bind ds telomeric DNA *in vitro* (Karamysheva et al, 2004). However, null mutations in these loci do not lead to telomere-related phenotypes, suggesting they are divergent from their vertebrate TRF1 and TRF2. *Arabidopsis* telomeres are bound by the conserved CST complex (CTC1-STN1-TEN1) (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009a). As previously mentioned, the *Arabidopsis* CST complex is critical for abrogating cellular DDR and thus maintaining genome stability. However, recent work has revealed AtTEN1 is a molecular chaperone that stabilizes CTC1 in response to heat-shock induced cellular stress (Lee et al; 2014 submitted). Moreover, TEN1 represses

telomerase RAP and transiently associates with telomeres unlike its CST counterparts (Leehy et al, 2013), suggesting AtTEN1 has evolved an interesting role in telomerase regulation. The interplay of TEN1 and CST with POT1a will be further examined in Chapter II.

Duplication of plant telomere genes

One aspect of this dissertation is the examination of the effects of positive Darwinian selection in the AtPOT1a locus and how it evolved contrasting roles compared to its counterparts in other organisms. Positive selection has been increasingly documented in such cases as olfactory and fertilization genes in mammals (Gilad et al, 2000; Swanson et al, 2003). Positive selection itself is identified by examining the rates of synonymous vs. non-synonymous amino acid substitutions in genes (Gilad et al, 2000; Swanson et al, 2003)

When the rate of non-synonymous changes is greater than synonymous, this indicates these allelic substitutions are being retained by Darwinian selection and therefore increasing their frequency in the population. Conversely, when the rate of synonymous changes is greater than non-synonymous, this is evidence of purifying selection or loss of gene function (Delport et al, 2009). Ultimately positive selection is bestowed when changes result in advantageous traits.

Dissertation overview

The primary focus of this work was to elucidate the role of the telomerase regulatory protein POT1a in *Arabidopsis*. Specifically, the goal was to obtain mechanistic insight into how POT1a promotes telomere maintenance (Chapter II). In Chapter III, the fate of plants doubly deficient in POT1a and the telomerase negative regulator TER2 are examined. Chapter IV takes a broader look at the evolution of the POT1 locus in plants and ascribes a functional consequence to the POT1 gene duplication in *A. thaliana*. Lastly, the significance of these findings are discussed and a roadmap for future experiments is presented (Chapter V).

In Chapter I experimental results are presented that test the hypothesis that POT1a recruits telomerase to chromosome ends. These experiments showed that null *pot1a* mutants had no perturbation in telomerase recruitment. However, telomerase enzyme activity was altered as measured by a newly created telomerase processivity assay. The lack of POT1a resulted in reduced amounts of high molecular weight products, indicative of a defect in RAP. We examined whether POT1a was important for the activation of telomerase at telomeres. It was discovered that POT1a is required for telomerase to extend telomeres even though it is not needed to recruit telomere to the telomeres. POT1a interactions with the CST complex were also examined. POT1a was shown to directly bind CTC1 and STN1, but not TEN1. Intriguingly, these studies showed that STN1 bound POT1a and TEN1 in a mutually exclusive manner *in vitro,* suggesting competition for the STN1 interface may occur *in vivo*. It was also shown that CTC1 and STN1 associate with enzymatically active telomerase *in vivo,* similar to

POT1a. However, TEN1 did not recapitulate this result. Altogether, this work was used to establish a new model for telomere dynamics in which POT1a promotes telomerase activation and stimulates telomerase activity at telomeres through interactions with STN1 and CTC1, while TEN1 may oppose these functions.

In Chapter III, the role of TER2 in telomere maintenance was examined. Because *ter2* null mutants have very subtle phenotypes, TEr2 function was examined in plants doubly deficient in POT1a and TER2. Strikingly, double *pot1a ter2* mutants showed a highly unusual telomere profile. Telomeres eroded at significantly faster rates than single *pot1a* plants, and these mutants had early onset severe morphological aberrancies attributed to stem cell defects. Moreover, double mutants had high levels of seed loss and reduced viability through each generation. Surprisingly, these mutants had low levels of end-to-end fusions, suggesting that loss of POT1a and TER2 interfered with DDR activation at otherwise dysfunctional telomeres. Altogether, these data argue POT1a and TER2 are distinct regulators of telomerase and their simultaneous absence leads to synergistic telomere failure.

Chapter IV examines the consequences of Darwinian selection on the POT1a locus in *Arabidopsis*. Evolutionary analysis of the lineage leading up to POT1a revealed it is undergoing positive selection at three amino acid sites. Specifically, these residues have evolved over time to be non-conserved compared to more ancestral copies of POT1, including the POT1b paralog, but have been retained in the genome. Because POT1a interacts with CTC1 and STN1, we assessed whether reverting these residues in POT1a back to the ancestral amino acid state (POT1b sequence) would perturb this

interaction. Positive selection site mutants reduced the binding between POT1a and CTC1, but did not affect the POT1a-STN1 interaction. Moreover, POT1a bearing positive selection site mutations was unable to rescue the defects of POT1a null mutants in a genetic complementation assay, indicating that POT1a function is dependent on the status of these particular residues. Lastly, cross-species complementation of recent and distantly related plant POT1 genes revealed the POT1a locus is undergoing rapid diversification. This work supplements the previous analyses of POT1a (Chapter II) and provides greater insight into why *A. thaliana* POT1a is functionally divergent relative to POT1 copies in other organisms.

The appendix of this dissertation presents results for a collaboration with Dr. Katie Leehy in analyzing the function of TEN1 protein.

CHAPTER II

POT1a AND COMPONENTS OF CST ENGAGE TELOMERASE AND REGULATE ITS ACTIVITY IN *ARABIDOPSIS*

Summary

Protection of Telomeres 1 (POT1) is a conserved nucleic acid binding protein implicated in both telomere replication and chromosome end protection. We previously showed that *Arabidopsis thaliana* POT1a associates with the TER1 telomerase RNP complex, and is required for telomere length maintenance *in vivo.* Here we further dissect the function of POT1a and explore its interplay with the CST (CTC1/STN1/TEN1) telomere complex. Analysis of *pot1a* null mutants revealed that it is not required for telomerase recruitment to telomeres. However, telomere-bound telomerase required POT1a to maintain telomere tracts. We show that POT1a stimulates the synthesis of long telomere repeat arrays by telomerase, likely by enhancing repeat addition processvity. We demonstrate that POT1a binds STN1 and CTC1 *in vitro*, and further STN1 and CTC1, like POT1a, associate with enzymatically telomerase *in vivo.* We unexpectedly discovered that *in vitro* interaction of STN1 with TEN1 and POT1a was mutually exclusive, indicating that POT1a and TEN1 may compete for the same binding site on STN1 *in vivo*. Finally, unlike CTC1 and STN1, TEN1 was not associated with active telomerase *in vivo*, consistent with our previous data showing that TEN1 negatively regulates telomerase enzyme activity. Altogether, our data support a two-state model in which POT1a promotes an extendable telomere state via contacts with the telomerase RNP as well as STN1 and CTC1, while TEN1 opposes these functions.

Introduction

Eukaryotes face an end-protection and end-replication problem due to the linear nature of their chromosomes and the limitations of conventional DNA replication. Telomerase averts these crises using its RNA subunit (TER) as a template to reiteratively synthesize G-rich repeat sequences on the 3' single-strand extension (G-overhang) of the chromosome terminus. Both the single (ss) and double-strand (ds) portions of the telomere are host to protein complexes that modulate telomerase action and distinguish natural chromosome ends from double-strand breaks (de Lange, 2009; Jain & Cooper, 2010; Palm & de Lange, 2008; Price et al, 2010).

Telomeres vacillate between a telomerase extendable and a telomerase un-extendable state during the cell cycle (Blackburn, 2000; Teixeira et al, 2004). In G1, the G-overhang is sequestered, preventing the DNA terminus from eliciting a damage response, but also preventing telomerase access. In late S/G2 phase, telomerase is recruited to chromosome ends for DNA synthesis. Once telomerase extends the G-rich strand, the C-strand is replicated by DNA Polymerase □ □primase (Moser et al, 2009; Qi & Zakian, 2000), followed by terminal DNA processing to create the 3' G-overhang (Dai et al, 2010). The terminus is then sequestered once again. These reactions are highly coordinated, and driven by the exchange of large replication/processing complexes on the G-overhang.

One telomere complex under intensive scrutiny is CST (Cdc13/CTC1, Stn1, Ten1), an RPA-like heterotrimer (Gao et al, 2007; Sun et al, 2009) first identified in

budding yeast. Cdc13 anchors CST to ss telomeric DNA via its central oligosaccharide-oligonucleotide binding domain (OB-fold) (Mitton-Fry et al, 2004). Genetic analysis of separation-of-function alleles reveals that Cdc13 maintains genome integrity and regulates telomere maintenance (Garvik et al, 1995a; Nugent et al, 1996b). Stn1 and Ten1 are also essential for telomere integrity, and their association with Cdc13 renders telomeres into an un-extendable state (Grandin et al, 2001; Grandin et al, 1997; Pennock et al, 2001). However, the CST heterotrimer is not static, and recent data show that Stn1 and Ten1 make contributions distinct from Cdc13 (Holstein et al, 2014). In addition, phosphorylation of Cdc13 in late S phase shifts the binding preference from Stn1 and Ten1 to the telomerase accessory factor Est1 (Li et al, 2009; Liu et al, 2014). Est1 is a multifunctional protein that directly binds the TER subunit (Tlc1) as well as Cdc13. This interaction recruits telomerase to the chromosome end (Evans & Lundblad, 1999; Lin & Zakian, 1995a; Steiner et al, 1996; Wu & Zakian, 2011). Consistent with its critical role in telomere maintenance, Est1 deletion causes progressive telomere shortening (Lundblad & Szostak, 1989). In addition, Est1 stimulates the activity of telomerase on telomeric DNA (Evans & Lundblad, 1999; Taggart et al, 2002) likely through contacts with Cdc13 (DeZwaan & Freeman, 2009).

Mammalian telomeres are protected by an alternative complex termed shelterin. The six shelterin subunits include TRF1, TRF2, and RAP1, which are tethered to ds telomeric DNA and are bridged by TIN2 and TPP1 to the ss DNA binding protein POT1 (Baumann & Cech, 2001a; Palm & de Lange, 2008). All shelterin components are critical for genome stability, and like budding yeast CST, may shift between sub-

complexes during the cell cycle (Nandakurnar & Cech, 2013). Biophysical and biochemical data reveal that POT1 inhibits telomerase elongation *in vitro* by preventing substrate access (Lei et al, 2004; Lei et al, 2005). In contrast, the POT1-TPP1 heterodimer stimulates telomerase repeat addition processivity (RAP) by promoting substrate association and template translocation during telomerase extension (Latrick & Cech, 2010; Wang et al, 2007; Xin et al, 2007). In addition, TPP1 appears to directly contact TERT and thereby recruits telomerase to telomeres (Nandakumar et al, 2012; Zaug et al, 2010; Zhang et al, 2013b).

CST also exists in vertebrates and plants, although Cdc13 has been replaced by another large OB-fold containing protein, CTC1 (Leehy et al, 2013; Miyake et al, 2009; Song et al, 2008; Surovtseva et al, 2009b). In contrast to yeast where CST functions in both end protection and telomeric DNA replication (Price et al, 2010), vertebrate CST primarily serves to promote telomere replication by stimulating C-strand fill-in and genome-wide replication rescue (Gu et al, 2012; Kasbek et al, 2013; Stewart et al, 2012; Wang et al, 2012). In addition, CTC1 and STN1 directly contact the telomerase activator proteins TPP1/POT1 (Chen et al, 2012; Wang et al, 2007; Wu et al, 2012). Recent studies indicate that human CST negatively regulates telomerase by competing with TPP1/POT1 for telomeric DNA binding and by squelching the stimulation of telomerase RAP by TPP1/POT1 (Chen et al, 2012). Thus, the interaction of TPP1/POT1 with CST is proposed to terminate G-strand synthesis by telomerase. While the molecular basis for the dynamic exchange between shelterin, telomerase and CST is unknown, shifting interactions between shelterin constituents (Jun et al, 2013; Loayza & de Lange, 2003)

prompted through posttranslational modification (Garg et al, 2014; Liu et al, 2014; Miyagawa et al, 2014; Zhang et al, 2013b) likely control telomere transactions. *Arabidopsis* telomeres represent an intriguing blend of features from yeast and vertebrates. Only a subset of shelterin components can be discerned in plants, and while the *Arabidopsis* CST complex is structurally analogous to mammalian CST, it appears to play a role in chromosome end protection. Loss of any of the *Arabidopsis* CST subunits elicit dramatic telomere shortening, increased ss telomeric DNA, and chromosomal fusions (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009b), culminating in stem cell failure (Hashimura & Ueguchi, 2011). Notably, TEN1 is detected at a significantly smaller fraction of telomeres than CTC1 (Leehy et al, 2013; Surovtseva et al, 2009b). In addition, unlike plants lacking STN1 or CTC1, *ten1* mutants have higher levels of telomerase enzyme activity overall, and generate longer telomere repeat arrays *in vitro*, indicating that TEN1 negatively regulates telomerase activity (Leehy et al, 2013).

Arabidopsis harbors two TER genes encoding RNAs that assemble into different RNP complexes with opposing functions. TER1 is a canonical TER subunit required for telomere maintenance, while TER2 negatively regulates telomere synthesis by the TER1 RNP in response to DNA damage (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012b). *Arabidopsis* harbors several telomerase accessory factors, but notably the two Est1-like proteins play no obvious role in telomere maintenance and rather are implicated in the regulation of the meiotic cell cycle (Riehs et al, 2008). POT1a, one of three *A. thaliana* POT1 paralogs (Rossignol et al, 2007; Shakirov et al, 2005) exhibits

properties reminiscent of Est1. POT1a associates with TER1, and localizes to telomeres in S phase (Surovtseva et al, 2007). Moreover, plants lacking POT1a are defective in telomere maintenance, and undergo progressive telomere shortening. In addition, *pot1a* mutants have reduced telomerase activity *in vitro* (Surovtseva et al, 2007). These findings indicate that POT1a positively regulates telomerase enzyme activity and promotes telomere repeat synthesis on chromosome ends.

In this study, we further explore the role of POT1a. We report that POT1a is not required to recruit telomerase to telomeres, but is required for telomere-bound telomerase to maintain telomere tracts. Our biochemical data indicate that POT1a directly stimulates telomerase enzyme activity, likely by enhancing its RAP. We further show that POT1a directly contacts STN1 and CTC1 *in vitro*, and its association with STN1 is mutually exclusive of TEN1-STN1 binding. Finally, we demonstrate that CTC1 and STN1, but not TEN1, interact with enzymatically active telomerase *in vivo*. These findings suggest a model in which POT1a promotes telomere maintenance by activation of telomerase at chromosome ends. The data further suggest that the opposing functions of POT1a and TEN1 in telomerase regulation may contribute to the switch from telomerase extendable to the telomerase un-extendable state.

Materials and methods

Plant materials

Plants were housed in growth chambers with a 16 hr photoperiod at 22°C. *stn1-1*, *ctc1-1*, *tert*, *pot1a-1* and *ten1-3* mutants were used for crosses and genotyped as

described (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009b; Surovtseva et al, 2007). *pot1a ter2* crosses were generated from homozygous parents. F1 progeny was planted for selection by genotyping. F3 seedlings were used for ChIP assays and pTRAP.

Chromatin immunoprecipitation

Approximately 4-6 grams of *Arabidopsis* seven day-old seedlings were used for each genotype. The protocol was adapted from (Saleh et al, 2008) with minor changes. Sonication was performed on ice after crosslinking and nuclei extraction using (Fisher Scientific) with 4 cycles of 15 sec on and 1 min off per sample at 40% amplification. Immunoprecipitation (IP) was performed using rabbit anti-TERT antibody and Protein-A agarose/salmon sperm DNA beads (Millipore). Eluted DNA was subjected to Southern dot blotting using a telomeric ^{32}P 5' end-labeled oligonucleotide probe. Stripping and rDNA hybridization performed as previously described (Surovtseva et al, 2007). Quantification was performed using Quantity One software (Bio-Rad).

E. coli protein purification

Constructs for *E. coli* expression of TEN1 and POT1a OB1 were cloned in pET28a vector (Novagen). The POT1a OB1 domain was cloned from the POT1a start codon to residue 158. Four amino acids (SISS) were added to the C-terminus to increase protein solubility. Affinity column purification was achieved using Ni-NTA agarose resin (Qiagen) from BL21 DE3 lysates. Protein was eluted in imidazole buffer and

dialyzed overnight. POT1a OB1 was further purified using a Sephadex G-75 (GE Healthcare) size exclusion column. Protein fractions were analyzed for homogeneity on coomassie stained SDS-PAGE gels. Proteins were expressed in rabbit reticulocyte lysate (RRL) (Promega) as indicated according to the manufacturer's instructions with [35S] Met (Perkin-Elmer) to label the protein expressed from pCITE4a, and in some cases pET28a.

Protein interaction assays

POT1a, STN1, TEN1, and CTC1ΔN cDNA were cloned into pET28a (T7-tag fusion) and pCITE4a vectors (Novagen). Details for POT1a OB1, OB1+2, and C-terminus constructs are previously described (Cifuentes-Rojas et al, 2011). Co-IP with the RRL-expressed proteins was performed as described (Karamysheva et al, 2004). Competition assays were performed by incubating *E. coli* TEN1 protein with RRL-expressed STN1, and various amounts of *E. coli* POT1a OB1 or BSA. Equal loading for STN1 was achieved by evenly dividing a single master mix of RRL-expressed protein among the samples. Pull downs were performed by IP of TEN1 using purified TEN1 antibody (Leehy et al, 2013) and protein-A agarose beads (Pierce). Complexes were washed 10x with buffer W300 (Karamysheva et al, 2004) and eluted by boiling for 5 min in SDS loading dye. Samples were resolved on 12% SDS-PAGE gels followed by coomassie staining and then dried for analysis by autoradiography.

Protein immunoprecipitation

Extracts from ~5 grams of wild type and *pot1a* tissue were prepared as previously described (Fitzgerald et al, 1996) and pre-cleared using protein-A agarose beads (Pierce) with gentle rocking at 4°C for 1 h. IP was performed by adding 15 □g of affinity purified TERT, STN1, TEN1 or anti-GFP (Abcam) antibody (or pre-immune sera) overnight with gentle rocking at 4°C. Anti-rabbit STN1 antibody was raised from *E. coli* expressed and purified MBP-STN1 antigen. Protein-A agarose beads were added the following day for 2 hrs followed by 5x washes with buffer W300 (Karamysheva et al, 2004), and 2x washes with buffer TMG (Karamysheva et al, 2004). IP samples were left in a final 50:50 slurry in buffer TMG.

Telomere and telomerase assays

DNA from whole plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed using 50 µg of DNA digested with *Tru1*I (Fermentas) and hybridized with a 32P 5' end–labeled $(TTTAGGG)_4$ probe (Fitzgerald et al, 1996). Blots were developed using a Pharos FX Plus Molecular Imager (Bio-Rad) and data were analyzed with Quantity One software (Bio-Rad). Primer extension telomere repeat amplification (PETRA) was performed as described (Heacock et al, 2004). 2 µg of DNA was used per reaction for telomere extension, followed by PCR amplification. PETRA products were separated on an agarose gel and subjected to Southern blotting using the same telomeric probe mentioned above.

Protein for Telomere Repeat Amplification Protocol (TRAP) assays were extracted from 5 day-old seedlings and reactions were conducted as described (Fitzgerald et al, 1996). TRAP assays on STN1, TEN1, CTC1-CFP, or TERT IP samples were performed by using 1:l of the final IP slurry. Quantitative-TRAP (qTRAP) was performed as discussed (Kannan et al, 2008b). Telomerase processivity TRAP (TP-TRAP) protocol was adapted from (Szatmari & Aradi, 2001) and performed as previously described (Leehy et al, 2013).

Western blotting

Fifty micrograms of wild type, *stn1*, and *pot1a* extracts were used for input samples. IP samples were boiled for 5 min in SDS loading dye. Samples were run on a 12% SDS-PAGE gel followed by protein gel blotting. Proteins were transferred overnight at 4^{o}C onto a polyvinylidene difluoride (PVDF) membrane, followed by 2 hrs of blocking using 6% non-fat dried milk dissolved in 1x TBS-T (50mM Tris, 150mM NaCl, 0.1% Tween-20). Rabbit anti-STN1 antibody was diluted 1:5000 in TBS-T and incubated with the protein blot for 4 hrs followed by 3x washes with TBS-T. Secondary anti-rabbit horseradish peroxidase was diluted 1:7500 in TBS-T and incubated with the protein blot for 2 hrs, followed by 3x washes with TBS-T. Final detection was performed using an ECL prime protein blotting kit (GE Healthcare). Western blotting was performed as described for CTC1-CFP and POT1a (Surovtseva et al, 2007) and TEN1 (Leehy et al, 2013).

Quantitative RT-PCR

RNA was extracted from 5 day-old seedlings (Omega Bio-tek) followed by DNase I digestion (Zymogen) for 30 min at room temperature. RNA was phenol:chloroform extracted followed by EtOH precipitation. 1 µg of RNA was reverse transcribed (Quanta Supermix), then diluted 1:4 using thousand-fold diluted yeast tRNAs. 1 µl of cDNA was used for qRT-PCR using CFX Connect Real-Time System (Bio-Rad) in triplicate. Quantification is from three biological replicates.

Results

POT1a is not required for TERT association with chromosome ends

Chromatin immunoprecipitation (ChIP) was used to investigate whether POT1a is needed for telomerase recruitment to chromosome ends. As expected, the telomerase catalytic subunit TERT (Fitzgerald et al, 1999) could be detected at telomeres in rapidly dividing young wild type seedlings (Fig. 2-1A). However, there was no significant difference in the level of telomere-bound TERT in *pot1a* mutants versus wild type (Fig. 2-1A and C). One possible explanation is that the TERT signal includes telomere-bound TER2 RNP. Since POT1a does not interact with TER2 (Cifuentes-Rojas et al, 2012b), loss of this protein is not expected to perturb the alternative telomerase RNP. To address this possibility, we generated plants doubly deficient in POT1a and TER2. ChIP assays performed on *pot1a ter2* mutants revealed the same level of telomere-bound TERT as in wild type plants (Fig. 2-1A and C). We conclude POT1a is not required for TERT association with telomeres.

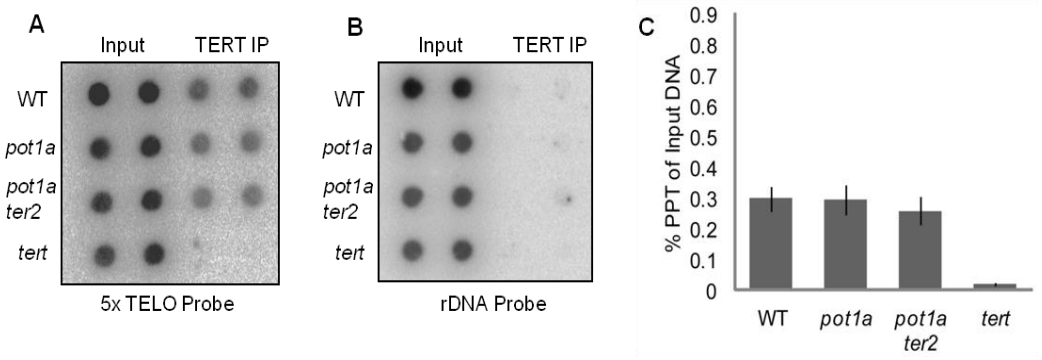


Figure 2-1. Telomerase associates with telomeres in the absence of POT1a. (A) Results of ChIP assays using TERT antibody in wild type, *pot1a*, *pot1a ter2*, and *tert* seedlings. Signal was assessed by dot blot using a telomeric probe. (B) Membrane was stripped and re-hybridized with a rDNA oligonucleotide probe. (C) Quantification of TERT ChIP. IP signal is represented as percent precipitation of input DNA. Error bars represent standard error of the mean.

POT1a stimulates activity of the TER1 telomerase RNP

If POT1a is not required to position telomerase at chromosome ends, how does it promote telomere maintenance? One possibility is that POT1a directly modulates telomerase enzyme activity. The conventional telomere repeat amplification protocol (TRAP) assay shows an ~13 fold decrease in telomerase activity in *pot1a* relative to wild type extracts (Surovtseva et al, 2007). This change in enzyme activity is not due to altered expression of TERT and TER1 transcripts or genes previously shown to inhibit telomerase activity such as TER2 and TEN1 (Fig. 2-2). Attempts to develop a direct primer extension assay in *Arabidopsis* have been unsuccessful thus far. To obtain a more accurate gauge of the distribution and quantity of the products of *Arabidopsis* telomerase, we used a modified version of the TRAP assay, telomerase processivity TRAP (TP-TRAP), designed to provide an indication of mammalian telomerase RAP (Leehy et al, 2013; Szatmari & Aradi, 2001). Pilot reactions with an oligonucleotide bearing five telomere repeats yielded a discrete band of the expected size (Fig. 2-3), indicating that the PCR amplification step of TP-TRAP gives a reliable assessment of the length of a telomere repeat array generated in the PCR reaction.

TP-TRAP performed with wild type *Arabidopsis* extract generated a broad distribution of elongation products, including high molecular weight species corresponding to the addition of at least 15 TTTAGGG repeats (Fig. 2-4A and C). As expected, extract from *ten1* mutants, but not *stn1* or *ctc1* mutants, generated slightly longer products than wild type (Fig. 2-4A and 2-5)(Leehy et al, 2013), supporting the conclusion TEN1 negatively regulates telomerase activity and further that this is a

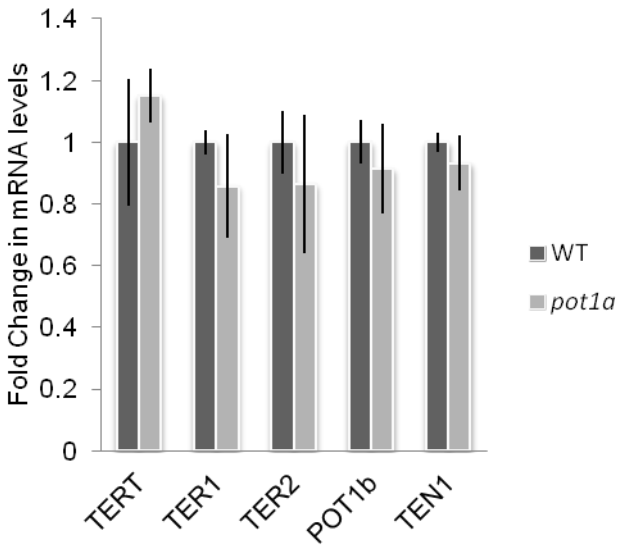


Figure 2-2. Quantitative Real Time PCR (qRT-PCR) of telomere gene transcripts in wild type and pot1a plants. Results are shown for three independent biological replicates. Error bars represent standard error of the mean.

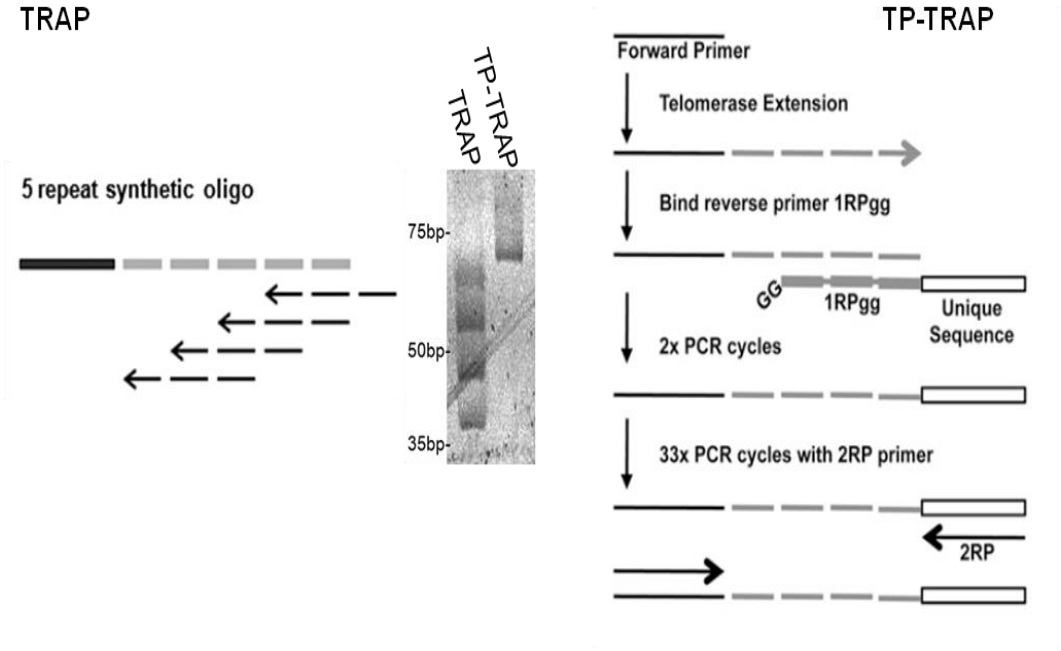


Figure 2-3. Telomerase processivity TRAP (TP-TRAP) assay. A five telomere repeat sequence attached to the typical TRAP substrate primer was used as a synthetic telomerase product control. Gel shows results from conventional TRAP reaction performed with a complementary telomere repeat reverse primer (left lane) and TP-TRAP reaction performed with the two unique reverse primers (right lane). Product size is slightly higher due to incorporation of the unique sequence tag.

unique property of this CST subunit. The TP-TRAP results for *pot1a* mutants were markedly different and showed a dramatic reduction in high molecular weight products relative to wild type (Fig. 2-4C). While standard TRAP assays show a general decrease in telomerase activity in *pot1a* mutants (Fig. 2-4B), the TP-TRAP indicated that the defect lies in the production of long arrays of telomere repeats (Fig. 2-4C). Because TP-TRAP, like conventional TRAP and the direct primer extension assays for telomerase, uses a large excess of substrate primer relative to enzyme, it is unlikely that telomerase re-binds and extends the same substrate. The data are consistent with the notion that POT1a stimulates RAP.

To determine if the decreased telomerase activity associated with *pot1a* mutants is specific to the TER1 RNP complex, we performed TP-TRAP on *ter2* seedling extracts. The product profiles were nearly identical to wild type (Fig. 2-6A), indicating the TER1 RNP efficiently synthesizes telomeric DNA in wild type plants. To confirm that POT1a modulates the TER1 RNP, we analyzed *pot1a ter2* mutants. Long products were reduced in the double mutants, but not to the same extent as *pot1a* (Fig. 2-6A). In agreement with previous results showing that TER2 negatively regulates TER1 RNP (Cifuentes-Rojas et al, 2012b), quantitative TRAP (qTRAP) revealed a higher level of telomerase activity in *ter2* mutants relative to wild type (Fig. 2-6B), which could explain why the TP-TRAP and qTRAP signal is higher in *pot1a ter2* than *pot1a* (Fig. 2-6A and B). Since the TER1 RNP is the only functional telomerase complex in *pot1a ter2* mutants, the data indicate POT1a distinctly modulates this complex.

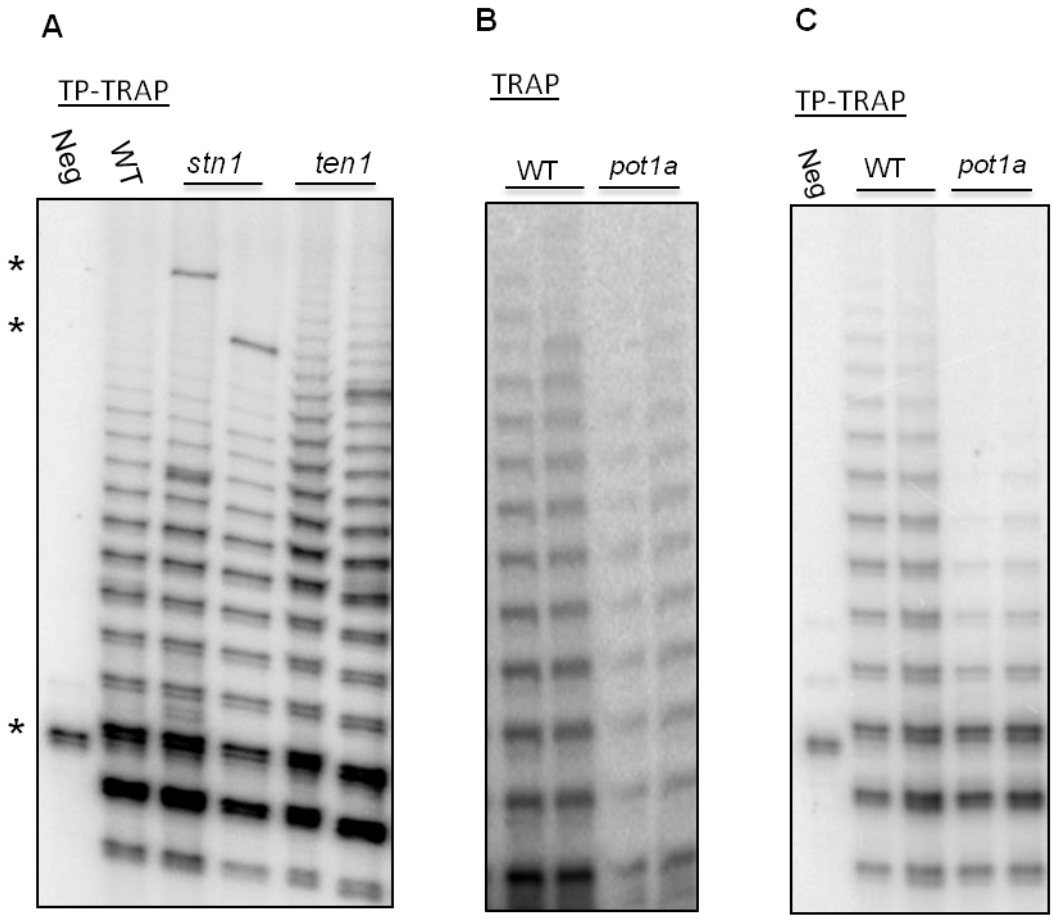


Figure 2-4. POT1a promotes synthesis of long telomere repeat arrays. (A) TP-TRAP assay results performed on flower extracts from wild type, and two independent *stn1*, and *ten1* mutants. Negative control is without extract to monitor PCR contamination. Asterisks indicate non-specific amplification products. (B) Conventional TRAP results from wild type and *pot1a* seedling extracts. Results from two independent seedling extractions are shown. (C) TP-TRAP results for *pot1a* and wild type seedling extracts. Results from two independent seedling extracts are shown. Negative control is without extract.

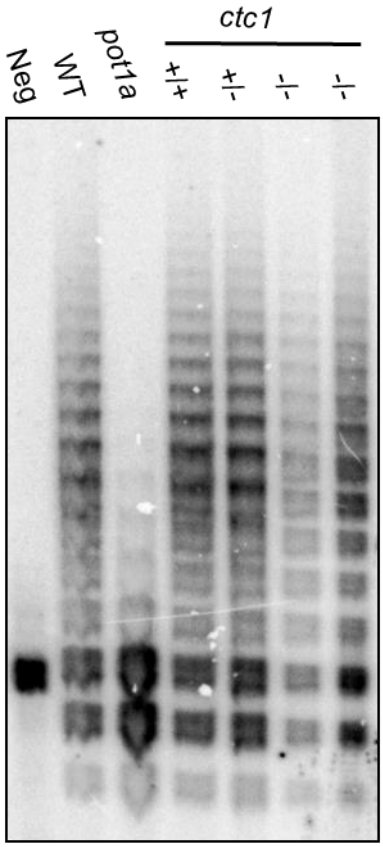


Figure 2-5. TP-TRAP analysis of ctc1 mutants. Results for flower extracts of wild type, *pot1a*, and *ctc1* sibling segregants of the genotypes indicated. Homozygous null plants display wild type product profiles.

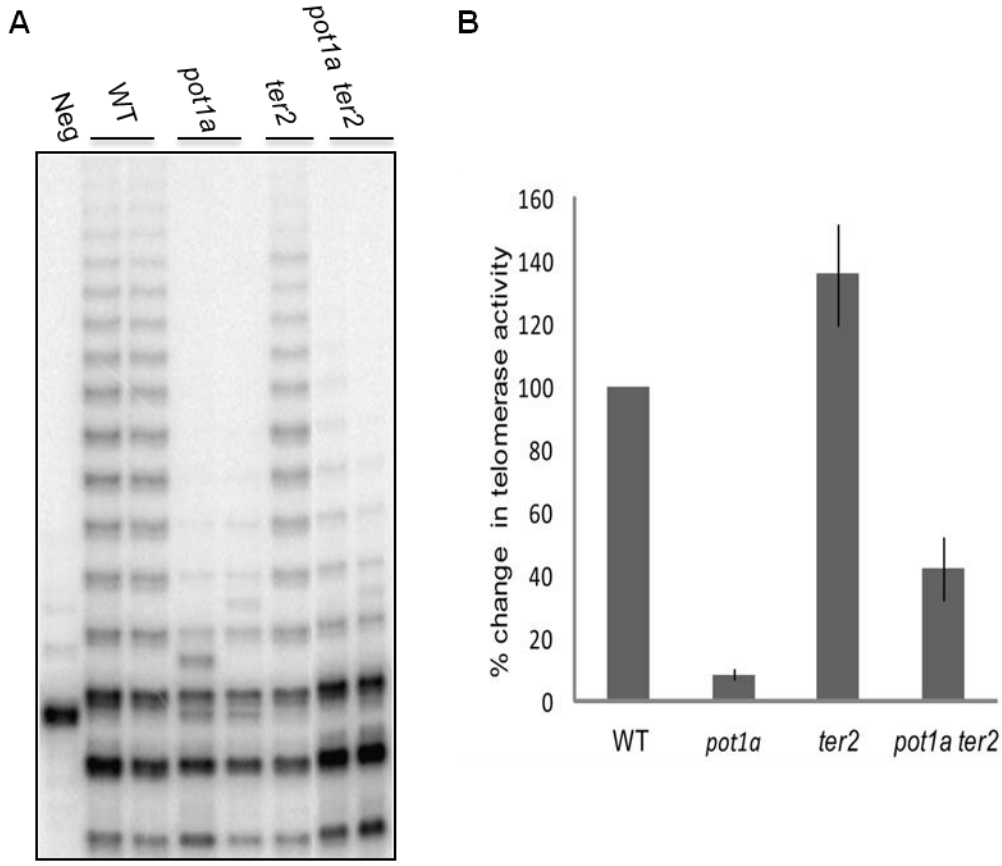


Figure 2-6. POT1a stimulates telomerase activity of the TER1 RNP. (A) TP-TRAP analysis from two independent biological replicates wild type, *pot1a*, *ter2*, and *pot1a ter2* mutants. (B) Results of quantitative TRAP. Error bars represent standard error of the mean from three biological replicates.

Telomerase partially rescues the telomere dysfunction of stn1 and ctc1 mutants

In both yeast and vertebrates, CST plays a key role in modulating access of the G-overhang to telomerase and DNA Pol-α (Chen et al, 2012; Price et al, 2010; Qi & Zakian, 2000). To test whether telomerase acts in concert with CST for telomere maintenance, we used a genetic approach. We previously showed that CTC1 and STN1 are critical for telomere stability in *A. thaliana* (Song et al, 2008; Surovtseva et al, 2009b). As expected, *ctc1* and *stn1* mutants exhibited severe morphological aberrancies including irregular phyllotaxy, fasciated stems, and reduced fertility (Fig. 2-7A and C, and 2-8A; (Song et al, 2008; Surovtseva et al, 2009b)). Notably, telomerase partially rescued the morphological defects of *stn1* and *ctc1* plants (Fig. 2-7A and 2-8A). The morphological phenotypes were more severe when telomerase was inactivated in *stn1* or *ctc1* plants. Progeny lacking CTC1/STN1 and TERT were rarely recovered, and when they were, double mutants arrested in a dwarf vegetative state without production of germline tissue (Fig. 2-7A and Fig. 2-8A). Telomere length was examined using Terminal Restriction Fragment (TRF) analysis or Primer Extension Telomere Repeat Amplification (PETRA) when sufficient material was unavailable for TRF. Consistent with previous studies, *stn1* or *ctc1* mutants displayed shorter, more heterogeneous telomere tracts than wild type plants (Song et al, 2008; Surovtseva et al, 2009b), while telomeres in *tert* mutants consisted of a discrete, homogeneous population of bands shorter than wild type (Fig. 2-7B and Fig. 2-8B) (Riha et al, 2001). In contrast, the telomeres of plants lacking either CTC1 or STN1 and telomerase were dramatically shorter with some telomeres dipping below the critical threshold of 1kb (Fig. 2-7B and

Fig. 2-8B), which triggers telomere fusions (Heacock et al, 2004). We conclude telomerase is present at telomeres devoid of CTC1 or STN1 and is capable of extending these termini. However, given the very severe telomere deprotection phenotype associated with the loss of CST, these epistasis experiments cannot rule out the possibility that STN1 or CTC1 engage telomerase and modulate its activity *in vivo*.

To determine if the partial rescue of STN1/CTC1 deficient plants by telomerase is dependent on POT1a, we evaluated *pot1a ctc1* and *pot1a stn1* double mutants. We were unable to recover viable *pot1a ctc1* mutants. However, *stn1 pot1a* mutants exhibited similar morphological defects as *stn1 tert* plants (Fig. 2-7C). In addition, molecular analysis revealed the same type of telomere aberrations (Fig. 2-7D). Thus, the absence of POT1a renders *stn1* mutants incapable of employing telomerase as a recovery mechanism, even though telomerase is present at chromosome ends (Fig. 2-7B). These findings support the conclusion that POT1a is required to activate telomere-bound telomerase.

POT1a associates with CTC1 and STN1, but not TEN1 in vitro

Recent studies show that human POT1 and mouse POT1b bind CTC1 and STN1 (Chen et al, 2012; Wu et al, 2012). Additional contacts between TPP1 and CTC1 and TPP1 and STN1 have been observed (Chen et al, 2012; Wan et al, 2009). Therefore, we asked if POT1a binds individual components of CST *in vitro* via co-immunoprecipitation assays using rabbit reticulocyte lysate (RRL) expressed proteins. We were unable to express full length CTC1 due to protein insolubility, and so we

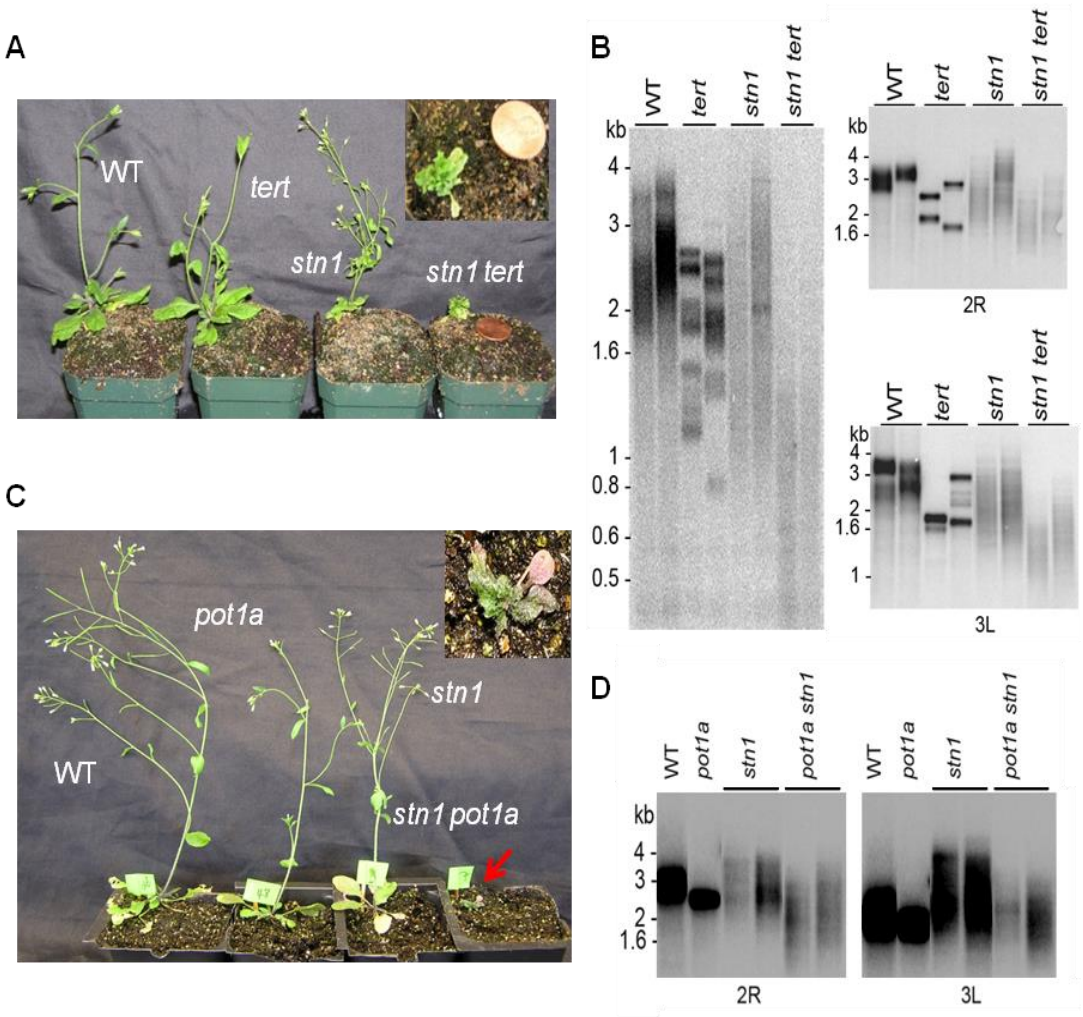


Figure 2-7. POT1a acts with telomerase to partially rescue the telomere dysfunction of stn1 mutants. (A) Morphology of wild type, *stn1*, *tert*, and *stn1 tert* double mutants. (B) Telomere length analysis assessed by TRF (left) and PETRA (right panels) for the genotypes indicated In each case, results for two independent plants are shown. For PETRA, telomeres on the right arm of chromosome 2 (2R) or the left arm of chromosome 3 (3L) were analyzed. (C) Morphology of wild type, *stn1*, *pot1a*, and *stn1 pot1a* double mutants. (D) Telomere length analysis by PETRA. Telomeres analyzed are indicated.

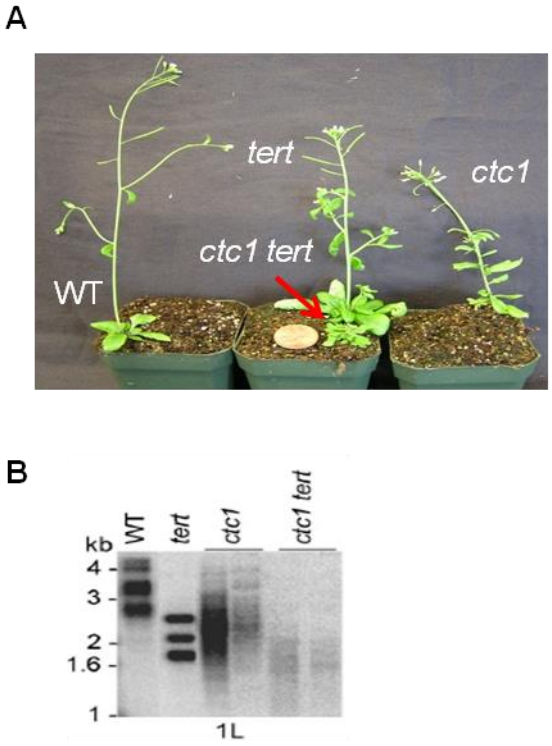


Figure 2-8. ***Morphological and telomere length analysis of ctc1 tert mutants.*** (A) Morphological analysis of wild type, *ctc1*, *tert,* and *ctc1 tert* segregants. (B) PETRA analysis for the indicated genotypes was performed using a primer corresponding to the left arm of chromosome 1 (1L).

employed an amino-terminal deletion construct (CTC1ΔN) that was sufficient to bind STN1 and the DNA Pol α subunit, ICU2 (Price et al, 2010; Surovtseva et al, 2009b) *in vitro*. POT1a was T7 tagged on its amino terminus and immunoprecipitation (IP) was performed using T7-antibody conjugated agarose beads. Binding was assessed by the ability of POT1a to co-precipitate ^{35}S-methionine labeled CTC1□N, STN1, or TEN1. We detected POT1a binding to CTC1ΔN and STN1, but no interaction between TEN1 and POT1a (Fig. 2-9A).

Since TEN1 and STN1 form a heterodimer, we considered the possibility that POT1a might compete with TEN1 for STN1 binding. To test if STN1 can simultaneously bind POT1a and TEN1, TEN1 was T7 tagged, and incubated with labeled STN1 (Fig. 2-9B, lane 4), POT1a (Fig. 5B, lane 6) or both proteins (Fig. 2-9B, lane 2) followed by IP. In the reaction containing STN1 and POT1a, only STN1 was detected in the TEN1 IP (Fig. 2-9B, lane 2). Because TEN1 does not bind POT1a (Fig. 2-9A and Fig. 2-9B, lane 6), this result argues that STN1 binding to TEN1 and POT1a is mutually exclusive.

Next, we asked whether POT1a could dislodge STN1 from TEN1. We expressed and purified *E. coli* TEN1 protein as well as the first OB-fold of POT1a (POT1a OB1), which is sufficient for POT1a-STN1 interaction *in vitro* (Fig. 2-10 and Fig. 2-9C, lane 5). A competition assay was performed by incubating TEN1 with RRL-expressed ^{35}S-methionine labeled STN1 in the presence of increasing amounts of POT1a OB1. Following TEN1 IP, *E. coli*-expressed proteins (TEN1 and POT1a OB1) were monitored

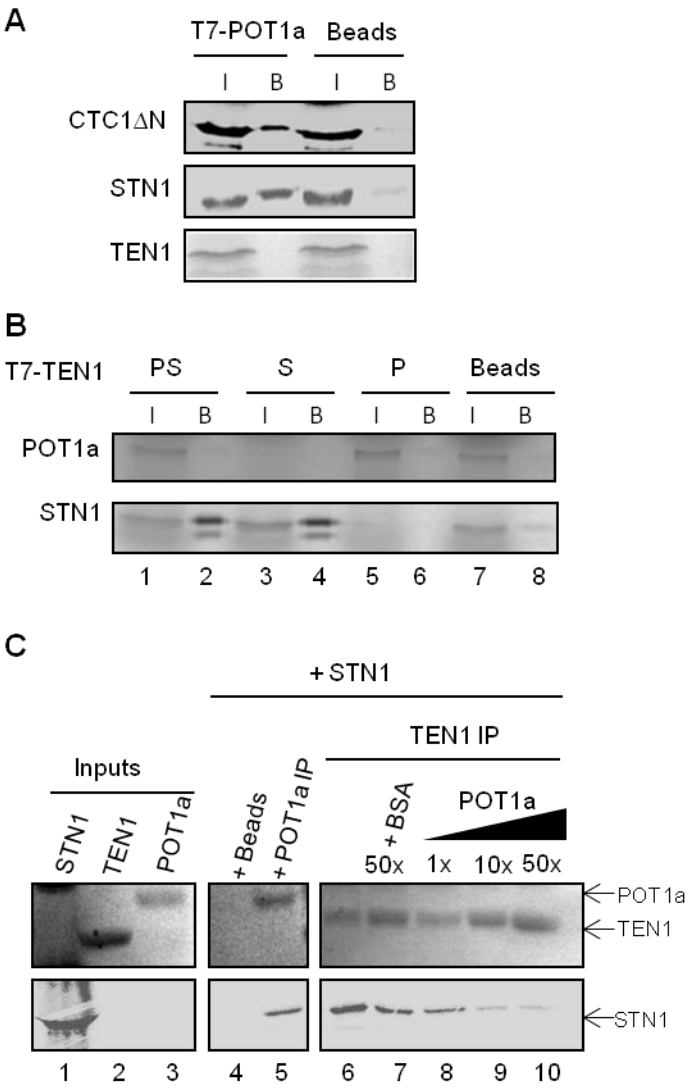


Figure 2-9. POT1a associates with CTC1 and STN1 in vitro. (A) *In vitro* co-immunoprecipitation (co-IP) results for RRL-expressed T7-tagged POT1a interactions with labeled CTC1ΔN, STN1, and TEN1. Negative control (beads conjugated with T7-tag antibody) was performed without tagged POT1a. (I) denotes protein input, (B) indicates bound protein. (B) Co-IP results for RRL-expressed T7 tagged TEN1 with labeled POT1a (P; lane 6), STN1 (S; lane 4) or both proteins ("PS", lane 2). The beads control contained no T7 tagged TEN1 (lane 8). (C) *In vitro* Co-IP competition assay using *E. coli*-expressed TEN1 and POT1a OB1 detected by coomassie stain, and RRL-expressed ^{35}S methionine labeled STN1 detected by autoradiography. Protein inputs are shown in lanes 1-3. TEN1 was incubated with STN1 and increasing concentrations of POT1a OB1 (lanes 8-10). 50x BSA was used as a control (lane 7) IP of POT1a was performed independently to verify its interaction with STN1 (lane 5). Beads alone was used to monitor background binding of STN1 protein (lane 4).

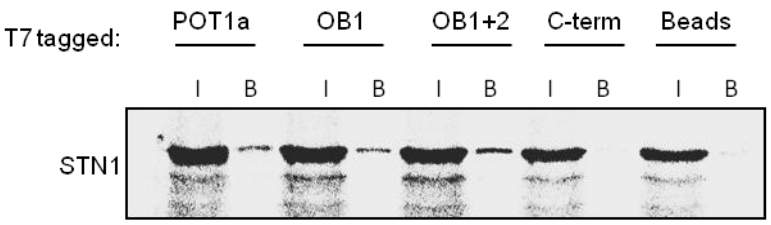


Figure 2-10. POT1a OB1 binds STN1 in vitro. RRL-expressed T7-tagged POT1a, OB1, OB1+OB2, and C-terminus was used to IP [^{35}S] labeled STN1. (I) Input and (B) bound are shown.

by coomassie stain (Fig. 2-9C *Top*)and STN1 by autoradiography (Fig. 2-9C *Bottom*). As expected, TEN1 pulled down STN1 (Fig 2-9C, lane 6). At an equal molar ratio of POT1a OB1 to TEN1, the TEN1-STN1 interaction persisted (Fig. 2-9C, lane 8). However, a ten-fold excess of POT1a OB1 significantly reduced STN1 in the TEN1 IP (Fig. 2-9C, lane 9). In contrast, 50-fold excess bovine serum albumin did not dislodge STN1 from TEN1 (Fig. 2-9C lane 7). Because *E. coli* POT1a OB1 directly binds STN1 (Fig. 2-9C, lane 5), these data support the conclusion that STN1 binding to POT1a and TEN1 is mutually exclusive. Moreover, because excess POT1a OB1 is required to disrupt the STN1-TEN1 interaction, the data indicate that STN1 has a higher affinity for TEN1 than POT1a OB1.

STN1 and CTC1, but not TEN1, associate with enzymatically active telomerase in vivo

The discovery of *in vitro* interactions between POT1a with STN1 and CTC1 raised the possibility that these CST components associate with enzymatically active telomerase *in vivo*. To test this idea, we generated a STN1 antibody that could be used for IP-TRAP. Western blot analysis confirmed that the antibody specifically recognizes STN1 (Fig. 2-11B). IP-TRAP using TERT antibody as a control revealed abundant telomerase activity (Fig. 2-11A). Strikingly, IP-TRAP with STN1 antibody gave a similar result. Western blot analysis verified that STN1 was precipitated in the reaction (Fig. 2-11B). Telomerase activity was not detected in an IP with pre-immune sera and was removed by RNaseA treatment, indicating that the STN1 interaction with

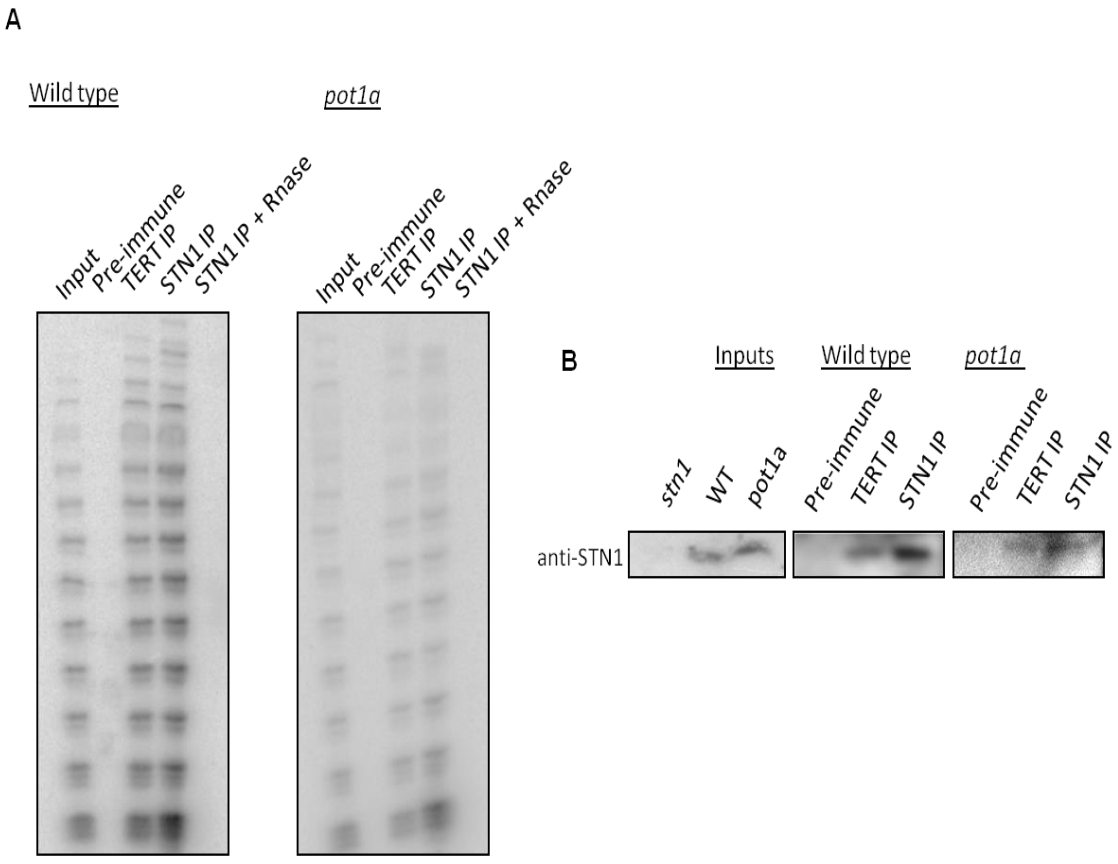


Figure 2-11. STN1 associates with enzymatically active telomerase in vivo. (A) Protein extract from wild type or *pot1a* seedlings was used for immunoprecipitation with STN1 or TERT antibody. IP samples and extract input were subjected to conventional TRAP (A) or western blot analysis with STN1 antibody.

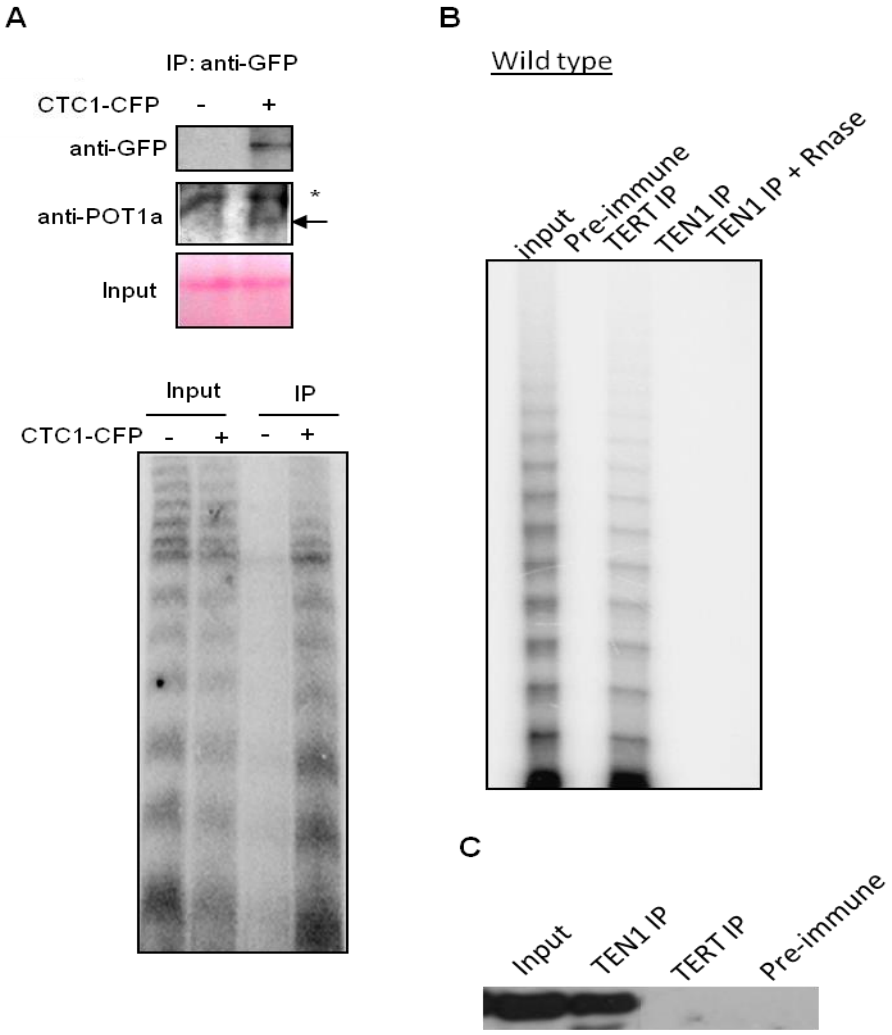


Figure 2-12. CTC1, but not TEN1 associates with active telomerase in vivo. (A) (*Top)* CTC1 and POT1a were detected by anti-GFP or anti-POT1a antibodies. Negative control is untransformed wild type tissue. (*Bottom) In vivo* pull down of transgenic CTC1-CFP followed by conventional TRAP assay to monitor telomerase activity. Negative control was performed in untransformed wild type tissue. (B) Protein extract from wild type seedlings was used for IP with TEN1 antibody. IP samples and extract input were subjected to conventional TRAP or (C) western blot to monitor for TEN1 protein after IP.

telomerase was specific. Importantly, STN1 protein was present in the TERT IP, confirming the association of these molecules *in vivo*. IP of a transgenic CTC1-CFP protein also pulled down active telomerase (Fig. 2-12A). These findings indicate that both STN1 and CTC1 are associated with enzymatically active telomerase *in vivo*

We asked if POT1a was essential for the STN1-telomerase interaction by repeating the STN1 IP-TRAP experiment in a *pot1a* mutant. Telomerase activity and TERT were detected in the STN1 IP of *pot1a* extracts (Fig. 2-11A and B). As expected, telomerase activity was visibly decreased in this background (Surovtseva et al, 2007); Fig. 2-11B). These data indicate that telomerase can associate with STN1 in the absence of POT1a. The data also support the conclusion that POT1a is not necessary for telomerase localization to telomeres. Rather, POT1a is necessary to promote the full activation of telomere-bound telomerase.

Finally, we performed IP-TRAP with our TEN1 antibody to test if TEN1 is associated with active telomerase. In marked contrast to STN1 and CTC1, telomerase activity was not observed in the TEN1 pull down (Fig 2-12B). Moreover, TEN1 protein could not be detected in the TERT IP (Fig. 2-12C). We conclude that TEN1 is not associated with enzymatically active telomerase *in vivo*, consistent with its role as a negative regulator of telomerase activity.

Discussion

Telomere accessibility to telomerase is tightly regulated during the cell cycle. Whereas aspects of telomerase recruitment are similar in yeast and vertebrates, many

questions remain unanswered, in part because the specific proteins that mediate these interactions are not well conserved (Nandakurnar & Cech, 2013). In this study, we investigated how the interplay between POT1a and CST in *Arabidopsis* promotes telomere maintenance. Like the budding yeast recruitment factor Est1 (DeZwaan & Freeman, 2009; Lin & Zakian, 1995a; Lundblad & Szostak, 1989; Steiner et al, 1996), POT1a directly contacts the canonical TER, TER1 (Cifuentes-Rojas et al, 2011), and is required for robust telomerase activity *in vitro* and telomere maintenance *in vivo* (Surovtseva et al, 2007). However, unlike Est1 (Chan et al, 2008), we found that POT1a is not necessary for the telomere localization of TERT. The TERT interaction with telomeres was also unperturbed in plants doubly deficient in POT1a and TER2, indicating TERT is not tethered to telomeres through the TER2 RNP. How telomerase is recruited to chromosome ends in the absence of POT1a is unclear. In yeast, Ku provides an alternative route for telomerase recruitment in G1 (Chan et al, 2008). However, Ku inhibits telomere synthesis in plants (Gallego et al, 2003; Riha et al, 2002), and thus this mechanism is not used to dock telomerase at *Arabidopsis* telomeres. One intriguing candidate for a telomere recruitment factor is HOT1, which stimulates telomerase recruitment in mammals through contacts with telomeric DNA and the telomerase RNP independent of shelterin (Kappei et al, 2013). Notably, *Arabidopsis* has a putative HOT1 ortholog, but lacks several of the core shelterin components, including TPP1, which is implicated in recruiting vertebrate telomerase (Nandakumar et al, 2012; Xin et al, 2007).

Although POT1a is not required to position telomerase at telomeres, it is required for the enzyme to extend telomere tracts *in vivo* (Surovtseva et al, 2007). Our data

indicate POT1a directly stimulates telomerase catalysis. Using a modified version of the TRAP assay (Fig. 2-3, (Szatmari & Aradi, 2001) to gauge the length of telomerase products, we discovered that POT1a is necessary for the synthesis of long telomere repeat arrays. An attractive model is that POT1a promotes telomerase RAP, as shown for other telomerase-associated OB-fold bearing proteins such as human TPP1 and *Tetrahymena* Teb1 (Min & Collins, 2009; Wang et al, 2007; Zaug et al, 2010). However, in the absence of a direct primer extension assay for *Arabidopsis* telomerase, we cannot exclude the possibility that POT1a affects some other parameter of telomerase enzymology (e.g. nucleotide addition processivity, nucleotide binding affinity or affinity for the DNA primer).

Once telomerase is positioned at the telomere, how is its activity controlled? CST has a central role to play in this regard, but precisely how this complex engages telomerase and whether this association stimulates or represses telomerase differs in yeast and vertebrates. Our analysis indicates that CST is not required to recruit *Arabidopsis* telomerase to chromosome ends. We found that telomerase can act on telomeres lacking CTC1 or STN1, partially rescuing the telomere dysfunction and the aberrant morphological defects associated with these mutations. Importantly, telomerase rescue of CTC1 and STN1 deficient plants is dependent upon POT1a, supporting the conclusion that POT1a is required to promote telomere maintenance.

In mammals, CST interaction with POT1 orthologs is linked to telomerase termination (Chen et al, 2012) and G-overhang maturation (Wu et al, 2012). However, in *Arabidopsis*, we find that STN1 and CTC1 like POT1a are associated with enzymatically

active telomerase, (Surovtseva et al, 2007);. 2-11A), implying that telomere extension by telomerase occurs in the presence of STN1 and CTC1. We postulate that POT1a works in concert with CTC1 and STN1 to facilitate telomerase-mediated telomere repeat addition onto G-overhangs (see below).

We found a direct interaction between POT1a with both STN1 and CTC1, but not TEN1 *in vitro*. Our data suggest that STN1 interaction with POT1a and TEN1 is mutually exclusive. In support of this conclusion, TEN1 unlike STN1 and POT1a is not associated with active telomerase *in vivo*. These observations are consistent with a role for TEN1 in negative regulation of telomerase enzyme activity (Leehy et al, 2013). Immunolocalization data suggest that TEN1 may transiently associate with *Arabidopsis* telomeres. CTC1 can be detected at ~50% of the *Arabidopsis* chromosome ends (Surovtseva et al, 2009b). Since only half of the *Arabidopsis* telomeres carry G-overhangs (Kazda et al, 2012), this finding suggests that essentially all of the G-overhangs are bound by CTC1. In contrast, TEN1 can only be detected at 11% of the telomeres (Leehy et al, 2013) and suggests that it may dynamically engage telomeres rather than functioning exclusively in the context of a trimeric CST complex.

Altogether, our data suggest a model in which POT1a facilitates telomere maintenance in two ways: by promoting the switch from the un-extendable to the extendable state and by stimulating telomerase enzyme activity (Fig. 2-13). In S phase, telomerase holoenzyme is recruited to the G-overhang through an unknown mechanism. The enzyme associates with CTC1 and STN1 through contacts with POT1a, and POT1a stimulates G-strand synthesis. One attractive hypothesis is that mobilization of POT1a to

the chromosome terminus helps to dislodge the telomerase negative regulator TEN1 from STN1 as part of the switch to the telomerase extendable state. Although STN1 has a higher affinity for TEN1 than POT1a, additional contacts between CTC1 and POT1a may stabilize its interaction with STN1. Furthermore, telomerase-CST interactions are likely to be governed by cell cycle specific posttranslational modifications such as those described for yeast Est1 and CST, as well as human TPP1 (Li et al, 2009; Liu et al, 2014; Zhang et al, 2013b). Once the G-strand is extended, telomerase action is terminated, perhaps with the assistance of TEN1. This clears the way for conventional replication machinery and processing enzymes to complete telomere replication and return the telomere to its fully protected un-extendable state. While additional studies are required to precisely delineate the telomere-telomerase interface and its control during telomere replication, our findings underscore the highly dynamic nature of telomerase-telomere transactions and suggest that modulation of telomerase enzyme activity at the chromosome terminus contributes to the bimodal switch in telomere states.

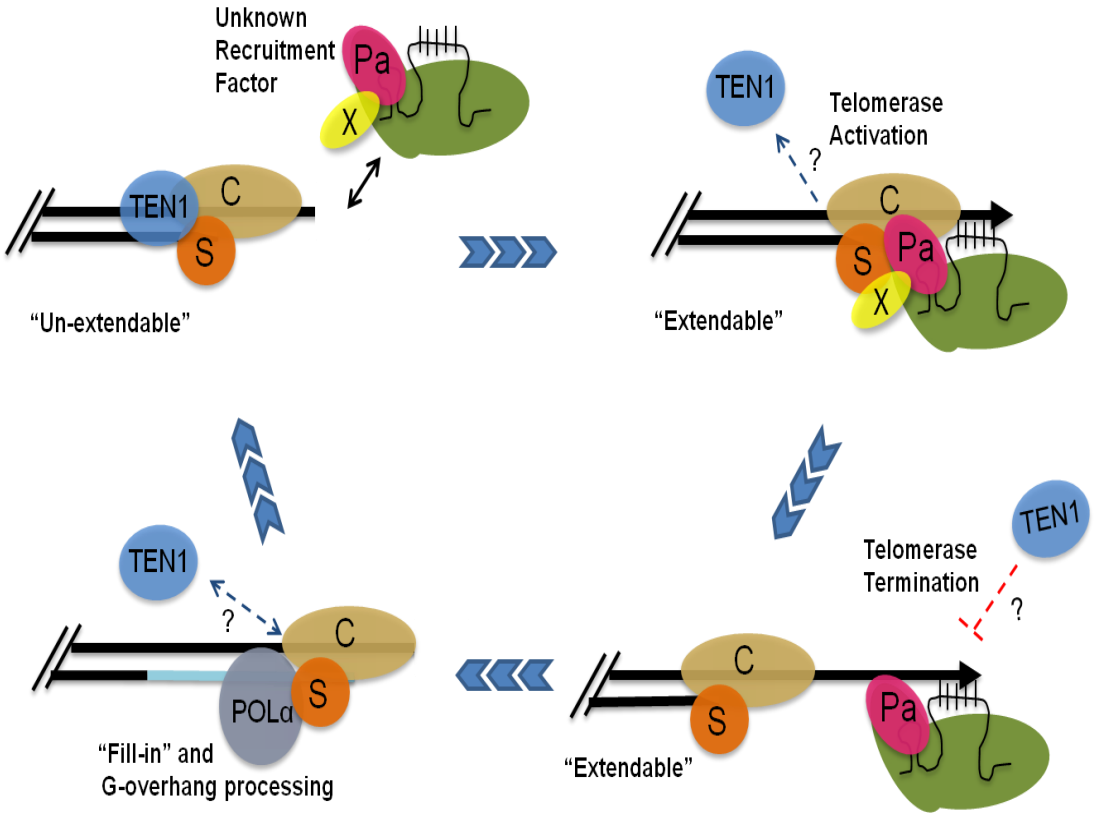


Figure 2-13. ***A model for telomere replication in Arabidopsis.*** In the un-extendable state, telomeres are bound by the heterotrimeric CST complex. The telomerase RNP is positioned at the chromosome terminus by an unknown recruitment factor (X) during S phase. POT1a (Pa) contacts STN1 (S) and CTC1 (C) to promote a telomere extendable state. POT1a also stimulates telomerase enzymatic properties. TEN1 represses telomerease activity and thus may help to terminate telomerase action. Telomerase is removed and replaced by POLα for C-strand fill-in and terminal DNA processing. The telomere is then converted into an un-extendable state.

CHAPTER III

POT1a AND TER2 ARE DISTINCT REGULATORS OF *ARABIDOPSIS* TELOMERE BIOLOGY

Summary

Arabidopsis thaliana harbors paralogs of two conserved telomere genes; Telomerase RNA (TER) and Protection of Telomeres 1 (POT1). TER1 is a canonical telomerase RNA that assembles with the catalytic reverse transcriptase TERT and uses its template domain to facilitate reiterative synthesis of telomere repeats onto the ends of chromosomes. Plants null for TER2 have wild type telomere length, which indicates TER2 does not maintain telomere length homeostasis. However, *ter2* mutants exhibit a constitutively increased DNA damage response (DDR) and higher telomerase activity levels *in vitro*. In addition, TER2 is upregulated in response to genotoxic stress coincident with repression of telomerase activity. These findings not only link TER2 to the DDR, they also indicate TER2 is a negative regulator of telomerase. Like TER, POT1 has been duplicated. The POT1a paralog binds TER1 directly, modulates telomerase repeat addition processivity *in vitro,* and is required for telomerase mediated telomere maintenance. POT1b, in contrast, assembles into an RNP with TER2. Because POT1a and TER2 are components of two distinct RNP complexes and represent two types of telomerase regulators, we examined the consequences of disrupting both genes to gain a broader perspective on telomerase regulatory pathways. Double *pot1a ter2* mutants exhibited accelerated telomere shortening and early onset stem cell defects including significant deficiencies in seed production. These indicate that POT1a and

TER2 act in two separate pathways, which synergistically promote telomere length maintenance and stem cell viability.

Introduction

Telomeres, the repetitive DNA sequences on the ends of linear eukaryotic chromosomes, vacillate in structure and composition throughout the cell cycle. They are mostly double-stranded, but they terminate in a short 3' overhang consisting of a guanosine rich DNA (G-overhang). Cells face an end-replication and end-protection problem (de Lange, 2009; Jain & Cooper, 2010). The former is caused by the inability of conventional DNA replication to fully copy chromosome end, and the latter is due to the similarity of chromosome termini to double-strand breaks (DSBs). How the cell mitigates these two crises is complex and usually involves careful regulation of the telomerase enzyme.

One way eukaryotes evolved to manage these predicaments is through dynamic interactions of the protein capping complexes that bind telomeric DNA. These guard telomeres, suppressing DNA damage signaling and preventing nuclease degradation. Deprotected telomeres trigger a DNA damage response (DDR), nucleolytic resectioning, and chromosomal end-to-end fusions. Capping complexes bind double strand (ds) and single strand (ss) portions of the telomere. However, sequestering the ss G-overhang for protection is not conducive to telomere replication (Lei et al, 2004; Lei et al, 2005; Mitton-Fry et al, 2004). The proteins that contact G-overhangs are bound by proteins that control access to the telomerase enzyme responsible for maintaining telomere length

homeostasis.They repress telomerase access when telomeres are in a telomerase un-extendable state (Teixeira et al, 2004). As DNA replication commences in S phase, an orchestrated series of events "opens" the G-overhang to allow telomerase access to its substrate. After telomerase-mediated G-strand synthesis, the complementary C-strand is replicated by DNA polymerase α, followed by G-overhang processing events, and finally reversion back to the protected un-extendable telomere state (Blackburn, 2001; Jain & Cooper, 2010).

The two most well characterized telomere capping complexes are budding yeast Cdc13-Stn1-Ten1 (CST) and vertebrate shelterin. CST is a heterotrimeric complex that binds the ss G-overhang and defines the telomerase un-extendable state. Post-translational modifications on Cdc13 during S phase remove Stn1-Ten1, promoting binding of the telomerase accessory protein Est1 (Li et al, 2009; Liu et al, 2014). The Cdc13 interaction with Est1 is necessary for telomerase recruitment and accessibility to the chromosome end (Steiner et al, 1996; Wu & Zakian, 2011). In humans, the six membered shelterin complex consists of two ds telomere binding proteins TRF1 and TRF2 that are linked to the ss G-overhang binding protein POT1 through RAP1, TIN2, and TPP1 (de Lange, 2009). Like CST, shelterin prohibits DDR signaling and sequesters the telomere in an un-extendable state (de Lange, 2009; Jain & Cooper, 2010). Shelterin dynamics also facilitate telomere maintenance. TPP1 recruits telomerase by physically binding the telomerase catalytic subunit TERT and the telomere binding protein POT1 (Nandakumar et al, 2012; Nandakurnar & Cech, 2013). Furthermore, the POT1-TPP1 heterodimer stimulates the repeat addition processivity (RAP) property of telomerase by

enhancing its ability to associate with telomeric DNA (Latrick & Cech, 2010; Wang et al, 2007). Post-translational modifications on TPP1 also promote telomerase recruitment, but it is not clear if such events alter sub-complex formation as it has been shown in yeast (Zhang et al, 2013a).

A. thaliana similarly possesses a trimeric CST capping complex responsible for chromosome end-protection at G-overhangs (Leehy et al, 2013; Song et al, 2008; Surovtseva et al, 2009a). However, an interesting and unique feature of *Arabidopsis* telomeres is the presence of blunt-ended chromosomes (Kazda et al, 2012). Specifically, half the telomeres contain canonical G-rich overhangs, while the other half is blunt-ended. The Ku70/80 heterodimer maintains blunt end integrity in *Arabidopsis*. Ku is highly conserved with roles in telomere maintenance and DNA damage repair (Riha et al, 2006). Ku depletion in plants results in EXO1 re-sectioning as well as telomerase-dependent hyper-elongated telomeres, suggesting Ku may regulate the telomerase un-extendable state at blunt ends (Kazda et al, 2012; Riha et al, 2002). Ku binds ds DNA and inhibits nuclease access at budding yeast telomeres suggesting this end-protection role is conserved (Bonetti et al, 2010; Lopez et al, 2011; Pfingsten et al, 2012). Moreover, Ku represses recombination at *Arabidopsis* telomeres as *ku* mutants lead to increased formation of recombination based extra chromosomal telomeric circles (ECTC) (Zellinger et al, 2007). Therefore, Ku functions in telomere protection, but it is not clear whether it acts alone on blunt ends, or is part of a larger blunt end telomere capping complex.

Recent work in *Arabidopsis* revealed duplication of the telomerase RNA gene. TER1 and TER2 both assemble with TERT protein *in vivo*, but only the TER1 RNP maintains telomere tracts (Cifuentes-Rojas et al, 2011; Cifuentes-Rojas et al, 2012a). Interestingly, each RNA associates with a different set of telomere accessory proteins. The POT1 homolog, POT1a, binds TER1, promotes telomere extendibility, up regulates telomerase activity, and functions in the same genetic pathway as TERT (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007). In contrast, the POT1a paralog POT1b, binds TER2 and is implicated in chromosome-end protection (Cifuentes-Rojas et al, 2012a; Shakirov et al, 2005). Plants carrying a null mutation inTER2 do not display obvious defects in telomere length homeostasis, but do have constitutively increased telomerase activity *in vitro,* indicating that TER2 plays a role in negatively regulating telomerase activity (Cifuentes-Rojas et al, 2012a). Moreover, the DDR related BRCA1 and PARP1 transcripts are up-regulated in *ter2* mutants and are hypersensitive to zeocin-induced DSBs as evidenced by increased stem cell death in the root apical meristem (Cifuentes-Rojas et al, 2012a). Zeocin treatment increases TER2 expression, and causes telomerase activity levels to decline indicating TER2 may function to promote proper DSB repair by suppressing *de novo* telomere formation from telomerase. Lastly, TER2 has a direct interaction with Ku *in vitro*. The fact that TER2 and Ku physically interact, and both play roles in cellular DDR (Cifuentes-Rojas et al, 2012a; Riha et al, 2002), suggests that those functions might be coordinated at blunt ended telomeres.

The role of TER2 is still mystifying in *Arabidodsis*. Because *ter2* mutants appear phenotypically wild type, we took a *genetic* approach to ask if TER2 contributes

to telomere maintenance and if that role can be revealed in a "sensitized" background where telomerase is compromised. For these experiments, we examined the function of TER2 in plants lacking POT1a. Our results indicate a synergistic telomere maintenance defect. Specifically, we find that telomere shortening is increased in *pot1a ter2* relative to *pot1a* single mutants. We also observe an earlier onset of morphological abnormalities including altered phyllotaxy, vegetative arrest, and seed loss. These phenotypes can be attributed to telomere deprotection as they are observed in late generation *tert* mutants with critically shortened telomeres (Riha et al, 2001).Their early onset in *pot1a ter2* suggests a role for TER2 in telomere maintenance. Our data further suggest that TER2 and POT1a function in two related, but distinct genetic pathways at telomeres.

Materials and methods

Plant materials

Plants were grown in chambers with a 16 hr photoperiod at 22°C. *pot1a ter2* crosses were generated from homozygous parents. F1 progeny were planted for selection by genotyping. *ter2*, *pot1a-1,* and *pot1a ter2* mutants were genotyped using a combination of gene- and T-DNA specific primers as described (Cifuentes-Rojas et al, 2012a; Surovtseva et al, 2007).

Telomere assays

DNA from whole plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed using 50 µg of DNA digested with *Tru1*I (Fermentas) and

hybridized with a [^{32}P] 5' end–labeled $(TTTAGGG)_4$ probe (Fitzgerald et al, 1999). Blots were developed using a Pharos FX Plus Molecular Imager (Bio-Rad) and data were analyzed with Quantity One software (Bio-Rad). Primer extension telomere repeat amplification (PETRA) and Fusion PCR was performed as described (Heacock et al, 2004). 2 μg of DNA was used per reaction for telomere extension, followed by PCR amplification. PETRA products were separated on an agarose gel and subjected to Southern blotting using the same telomeric probe mentioned above (Heacock et al, 2004).

Seed loss assay

Green siliques were collected from the genotypes indicated at multiple positions of the stem, following by overnight incubation in an ethanol:acetic acid bleaching solution (3:1) to remove chlorophyll pigment. The bleached siliques were transferred to a container with 1N NaOH for repeated overnight incubation. The de-pigmented siliques were placed on slides and observed under a light microscope. The total number of siliques and the number of siliques with seed loss aberrancies were recorded.

Morphological scoring

Double *pot1a ter2* mutants were scored based upon their silique phenotypes and the extent of abnormal morphology. Specifically, mutants with numerous siliques and wild type-like development were classified as class I. Plants with very short siliques or no siliques, but still capable of bolting were scored as class II. Mutants that failed to bolt,

vegetatively arrested, and did not produce any seed bearing organs were deemed terminal (T).

qRT-PCR

RNA was extracted from flower tissues followed by DNase I digestion (Zymogen) for 30 min at RT. RNA was phenol:chloroform extracted followed by EtOH precipitation. 1ug of RNA was reverse transcribed (Quanta Supermix), then diluted 1:4 using diluted yeast tRNAs. 1 µl of cDNA was used for qRT-PCR using CFX Connect Real-Time System (Bio-Rad) in triplicate.

Results

Plants lacking both POT1a and TER2 have early onset morphological abnormalities

Homozygous null *pot1a* and *ter2* plants were crossed to generate the double heterozygous F1 progeny. These plants were selfed to generate segregants of the following progeny: *pot1a-/-*, *ter2-/-*, and *pot1a-/- ter2-/-* mutants identified by genotyping PCR (Fig.3- 1A). Early generation *pot1a* mutants showed no morphological phenotype (Fig. 3-1B), however the prolonged absence of POT1a over multiple plant generations led to severe morphological phenotypes due to proliferative defects from telomere erosion (Data not shown). The loss of TER2 resulted in wild type resembling plants that continued to propagate normally, as expected (Cifuentes-Rojas et al, 2012a). In contrast, F3 *pot1a ter2* mutants began to display early onset morphological phenotypes which were not associated with *pot1a* or *ter2* segregants (Fig.3-1B-D). There

was variable expressivity in the severity of these phenotypes, as is typical of telomere maintenance defects (Riha et al, 2001). These mutants were placed into three classes based on their ability to produce seed-bearing silique organs and the severity of their developmental defects (Fig. 3-1C-D). Class I had only mild phenotypes with slightly shorter silique length compared to wild type, suggesting there was a reduction in seed production. In constrast, class II mutants had very few siliques, smaller and rough textured rosette leaves, as well as severely altered phyllotaxy with weakly bolted shoots. Class T mutants were terminal and severely diminutive in stature and leaf shape, produced no siliques, and hence were incapable of further propagation.

Because of the wide variability in the phenotypes of *pot1a ter2* mutants, we examined the progeny of these mutants. If the phenotype is dependent on the progressive loss of telomeric DNA, we expect to observe an increase in the ratio of severely affected plants with each passing generation. Indeed, the ratio of these phenotypes changed significantly from F2 to F4 double mutants (Fig. 3-1E). Class I plants decreased in the population from ~50% to 6%, and terminal type progeny increased from ~15% to 59%. The fraction of class II mutants did not changed and remained around ~35%. Altogether we conclude *pot1a ter2* mutants exhibit an early onset of stem-cell related morphological defects that worsen in subsequent generations.

Reproductive defects in pot1a ter2 mutants

There was also a marked difference in the shape and size of silique organs. Siliques became smaller in length and width (Fig. 3-2A). Even class I plants seem to

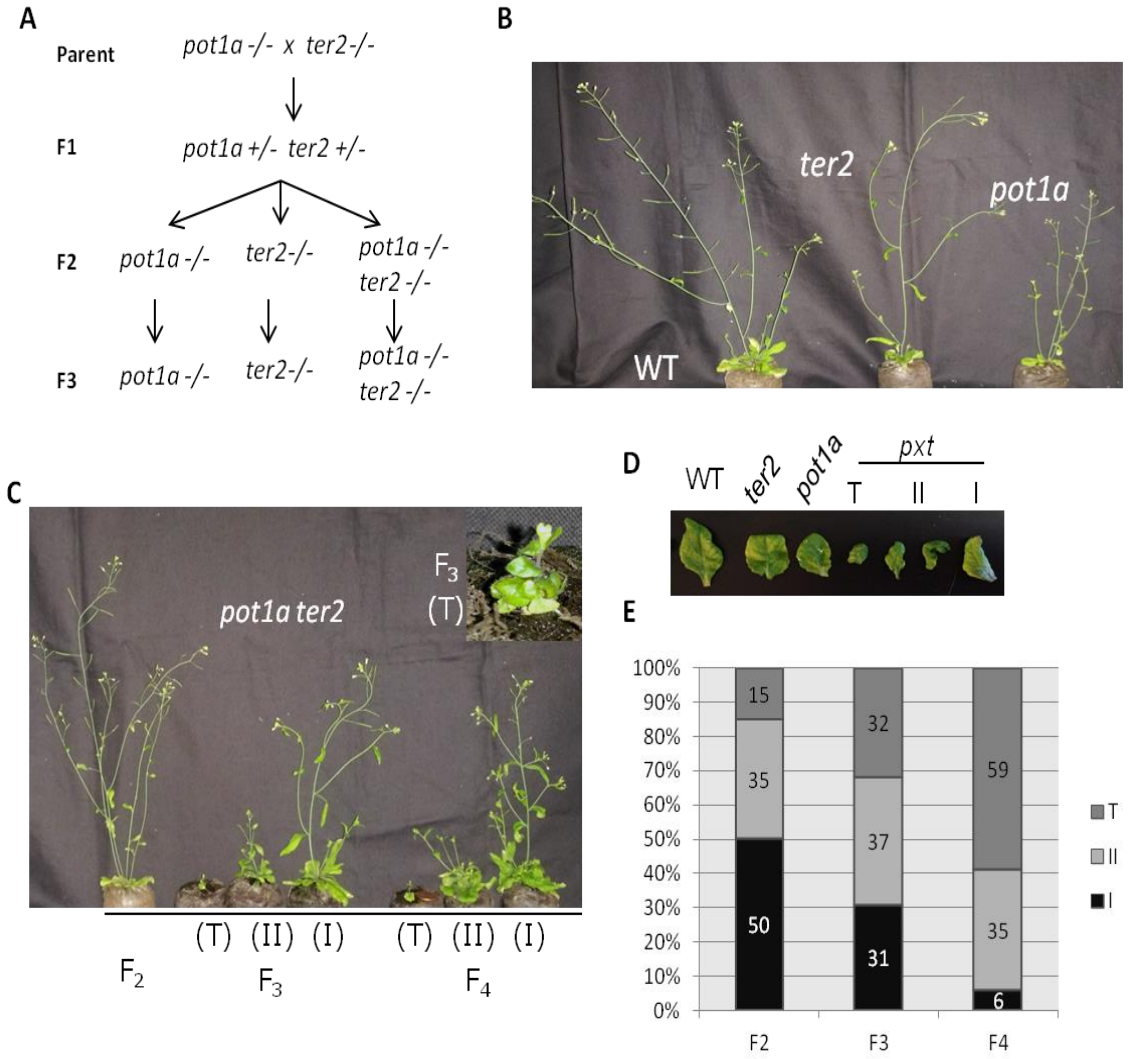


Figure 3-1. Plants lacking both POT1a and TER2 have early onset morphological abnormalities (A) Diagram of *pot1a ter2* crossing scheme. (B) Morphological comparison of F3 *pot1a* and *ter2* mutants with wild type. (B) Comparison of *pot1a ter2* mutant morphological between F2-F4 plants. Inset displays a class T F3 double mutant. (D) Comparison of rosette leaf development in F3 segregants. (E) Quantification of the ratios of each class of double mutant from the F2 to F4 generations.

produce noticeably smaller siliques despite their mostly wild type appearance (Fig. 3-2B). Many of these organs became unusually curled which could imply deficient seed production (Fig. 3-2A and B). Some mutants had exceptionally irregular numbers of increased silique offshoots (Fig.3- 2C and D). This is in odd contrast to other mutant siblings which are incapable of silique production (Fig.3-1A, terminal plants). Perhaps this is a compensatory mechanism for plants to increase their ability to produce offspring in the face of genomic crisis.

Because siliques are seed-generating organs, we examined these plants for abnormal seed production by visualizing the ratio of aberrant siliques. Siliques were chemically treated to remove chlorophyll pigment allowing us to to examine seed numbers and position. Interestingly, F3 *ter2* mutants as well as F3 and F4 *pot1a* plants had abnormally high amounts of seed loss (Fig. 3-3A and B). As expected, *pot1a* mutants progressively worsened likely due to telomere attrition similar to seed viability defects reported for *tert* mutants (Riha et al, 2001). Double *pot1a ter2* mutants had an exacerbated effect on the proportion of aberrant siliques (Fig. 3-3A and B). These plants produced significantly higher amounts of siliques with very limited seed production and gaps in seed position. Furthermore, class II plants had significantly decreased seed aberrancies than class I mutants. The levels of silique abnormalities seem to correspond to the severity of the overall morphology suggesting there may be a global effect on plant development and genome stability in these mutants.

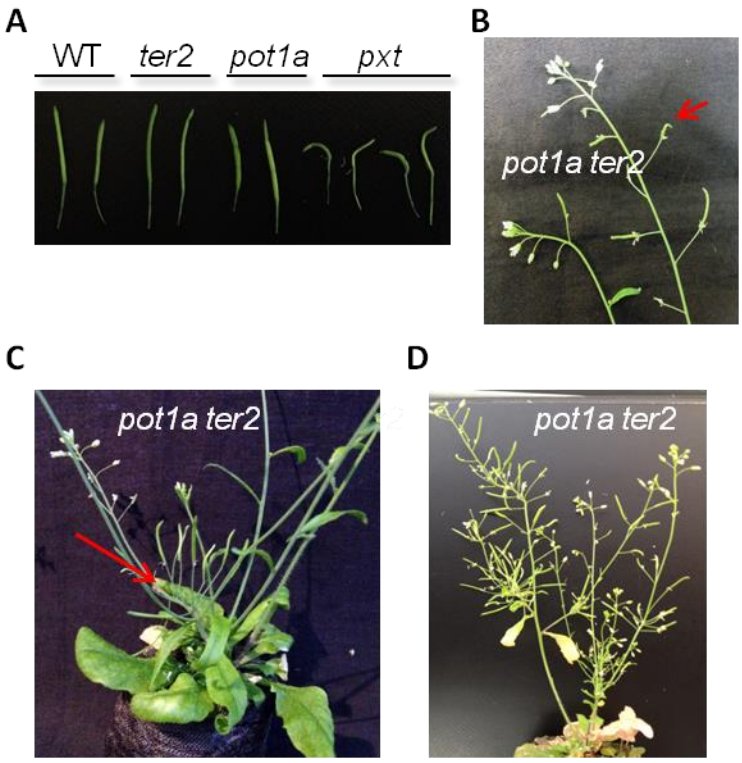
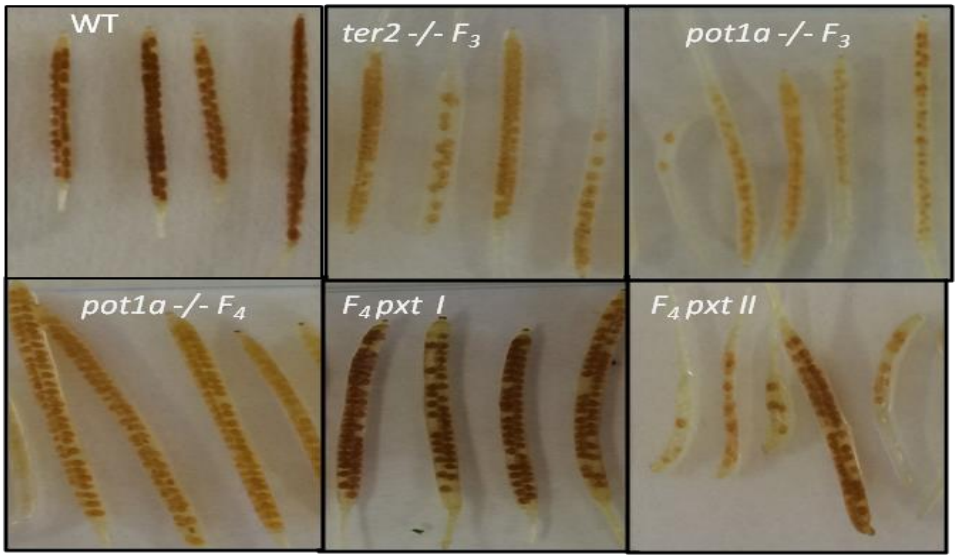


Figure 3-2. Reproductive defects in pot1a ter2 mutants. (A) Comparison of silique development in F3 segregants. (C) Example of Class I F3 double mutant silique phenotype. Arrow indicates a shorter, hooked, and curled silique (C) and (D) Examination of unusual silique phyllotaxy in F3 *pot1a ter2* mutant siblings.

A



B

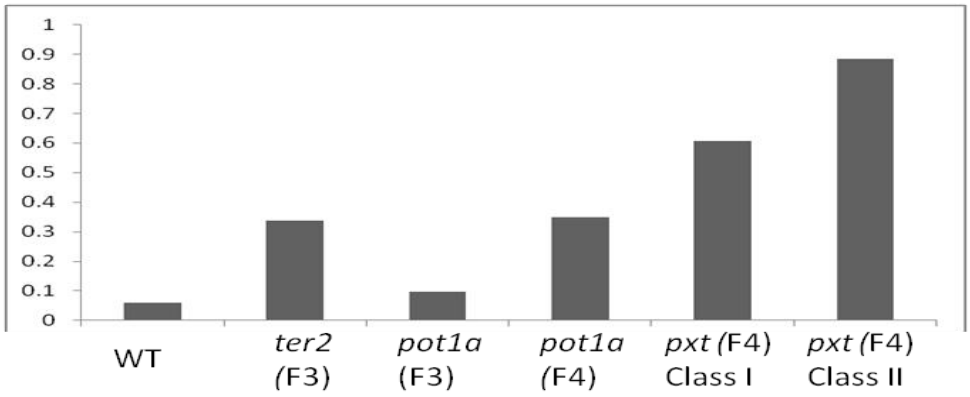


Figure 3-3. ***Loss of POT1a and TER2 results in abundant seed loss.*** (A and B) Analysis of the ratio of aberrant siliques in wild type, single and double mutant plants. Quantification represents the fraction of siliques that display aberrant seed loss from their total pool of siliques scored.

Accelerated telomere shortening in pot1a ter2 mutants

The early onset and more severe nature of *pot1a ter2* mutants suggested that their telomeres might be severely deregulated. Therefore we examined the telomere length profile of *pot1a ter2* mutants. Initial telomere length characterization of parental and progeny lines was performed using telomere restriction fragment (TRF) analysis (Fig. 3-4A). Parental *ter2* mutant telomeres appeared wild type, while G5 parental *pot1a* mutant telomere tracts were significantly eroded, as expected (Surovtseva et al, 2007). Relative to their F1 parents, F2 *ter2* mutant segregants showed no indication of telomere shortening, while F2 *pot1a* mutants had a slight decrease in telomere length as well as more homogeneous telomere tracts (Fig. 3-4). Telomere tracts in *pot1a ter2* mutants resembled F2 *pot1a* single mutants (Fig. 3-1B), but in F3 there was a significant difference in the amount of telomere length of F3 *pot1a* mutants compared to F3 *pot1a ter2* mutants (Fig. 3-4). Specifically, *pot1a* mutants displayed a decline of 0.3kb while *pot1a ter2* mutant telomeres shortened by more than twice that rate (~0.6-1.2kb). Interestingly, the *pot1a ter2* telomere tracts were still homogenous with sharper banding similar to *tert* or *pot1a* mutants, suggesting that they were not subjected to nuclease attack. Together these data indicate that POT1a and TER2 lie in separate genetic pathways, and simultaneous absence of both molecules leads to a synergistic acceleration in telomere shortening.

To directly ask how the morphological phenotypes were correlated with the rate and amount of telomere shortening, we examined F2, F3, and F4 class I, II, and T mutants by bulk telomere analysis (TRF, Fig. 3-5A), or individual chromosome arms

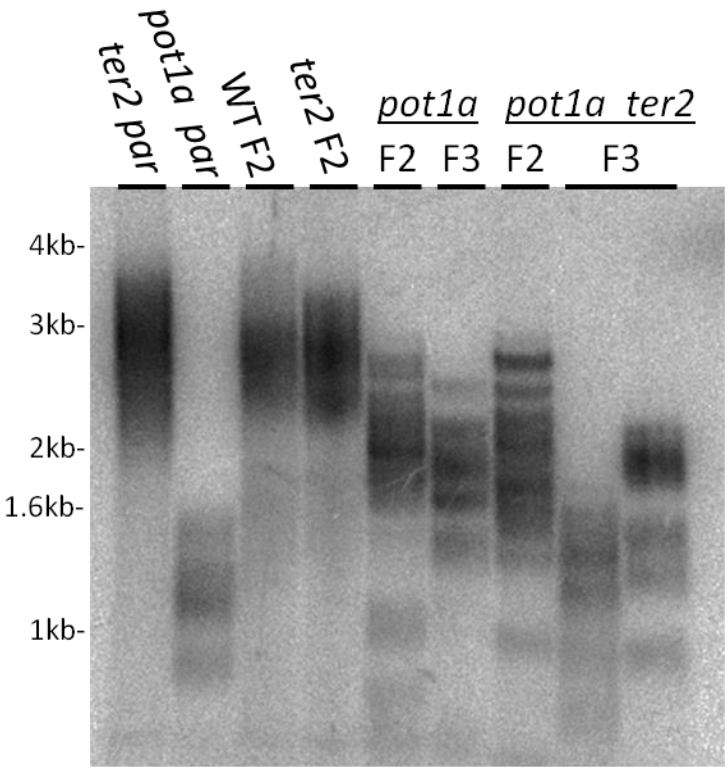


Figure 3-4. Accelerated telomere shortening in pot1a ter2 mutants. (A) Telomere length analysis by TRF of mutant parental and propagated lines. The *pot1a* mutant parent is a later generation (G5) plant *with* significant telomere shortening. The *ter2* parent generation is unknown.

using primer extension telomere repeat amplification (PETRA, Fig. 3-5B). Telomeres in class II plants were shorter than class I mutants (Fig. 3-5A). Moreover, in class II mutants a substantial fraction of telomeres were below the 1kb threshold previously shown to elicit chromosome end-to-end fusions (Heacock et al, 2004). In comparison to F2 and F3 generation *pot1a* mutants, the telomeres of *pot1a ter2* plants were much shorter, especially in the class II populations (Fig. 3-5A). PETRA analysis of individual chromosome arms revealed the extent of telomere shortening in class II plants was greater relative to class I (Fig. 3-5B). Interestingly, the absolute telomere length was more variable in class T mutants. Class I and class T telomeres also appeared sharper compared to the heterogenous and smeared class II telomere profiles. These profiles are somewhat perplexing, but may reflect differences in vegetative arrest compared to cells in the less severely affected class I and II mutants that could be able to continue to proliferate.

In an effort to quantify the rate of telomere attrition in class I and II mutants, five individual F3 plants from of each class and genotype were examined. The extent of telomere shortening in class I mutants was variable with telomeres in some individuals being much longer or shorter than telomeres of their own sibling genotype (Fig. 3-6A and B). This is typical of wild type plants and *pot1a* mutants as there is a variable range of telomere tract length (Surovtseva et al, 2007). The more variable telomeres were generally homogenous with more discretely banded profiles, suggesting that the variability is not due to massive nuclease attack, but rather reflects a telomere maintenance defect (Fitzgerald et al, 1999). In contrast, class II plants displayed more

homogeneous size and banding profiles relative to their siblings, but the telomeres themselves were more heterogeneous and smeared, consistent with the previous PETRA analysis (Fig. 3-6A and 3-5B). This result suggests deprotection of critically shortened telomeres followed by nuclease attack.

Quantification of telomere length revealed a correlation with their classification (Fig. 3-6B). Class II mutants on average were ~300bp shorter than their class I counterparts. Further, double mutants of the same generation were significantly shorter than *pot1a* siblings, and much shorter than wild type and *ter2* plants (Fig. 3-6B). Statistical significance will verify these trends, but this initial examination indicates a pattern of telomere shortening in *pot1a ter2* mutants that is correlated with plant developmental abnormalities.

Telomeres are not subjected to massive end-to-end fusions in plants lacking POT1a and TER2

Chromosomal fusions are typical in plants with significant loss of telomeric DNA (Riha et al, 2001; Song et al, 2008; Surovtseva et al, 2009a). Because *pot1a ter2* mutants have populations of telomeres below 1kb some telomeres may be subjected to end-to-end fusions. To test this, we employed telomere fusion PCR. In this assay, unique sub-telomeric primers are used to specifically amplify fused chromosome arms of two independent chromosomes. As a positive control, we monitored telomere fusions in plants lacking CTC1, a critical component of the CST telomere capping complex. (Surovtseva et al, 2009a)As expected *ctc1* and wild type plants showed the presence and

absence of chromosome fusions, respectively (Fig. 3-7A and B). Neither F2 nor F3 generation *pot1a* and *ter2* mutants displayed signs of fusions, suggesting telomere ends are protected in these backgrounds (Fig. 3-7A). However, in F3 and F4 class II double mutants we show only faint evidence of fusions (Fig. 3-7A and B). Surprisingly, class T mutants did display telomere fusions, but we were only able to evaluate two samples due to insufficient material (Fig. 3-7A). While sequence analysis is necessary to verify these are bone fide fusions in the the class II mutants, they are consistent with their very short heterogeneous telomere tracts.

DNA damage response signaling in pot1 ter2 mutants

The lack of robust fusion products is perplexing given the level of telomere erosion in class II mutants. We questioned whether *pot1a ter2* plants had a reduced response to DNA damage. We used quantitative PCR (qPCR) to examine the levels of DDR-related BRCA1 and RAD51 transcripts (Boltz et al, 2012; Lafarge & Montane, 2003; Wang et al, 2014). Total RNA from flower tissue of approximately the same developmental age was extracted and reverse transcribed. qPCR analysis revealed elevated levels of BRCA1 and RAD51 gene expression in all but wild type plants (Fig. 3-8A). As expected, *ter2* mutants have a slight increase in DDR transcript levels (Cifuentes-Rojas et al, 2012a). Unexpectedly, *pot1a* plants had an approximate two-fold increase in RAD51 transcripts and nearly a four-fold increase in BRCA1 levels. Double mutants from third and fourth generation plants of class I, II, and T had substantially elevated levels of each gene (Fig. 3-8A). The DDR transcript levels declined from class I

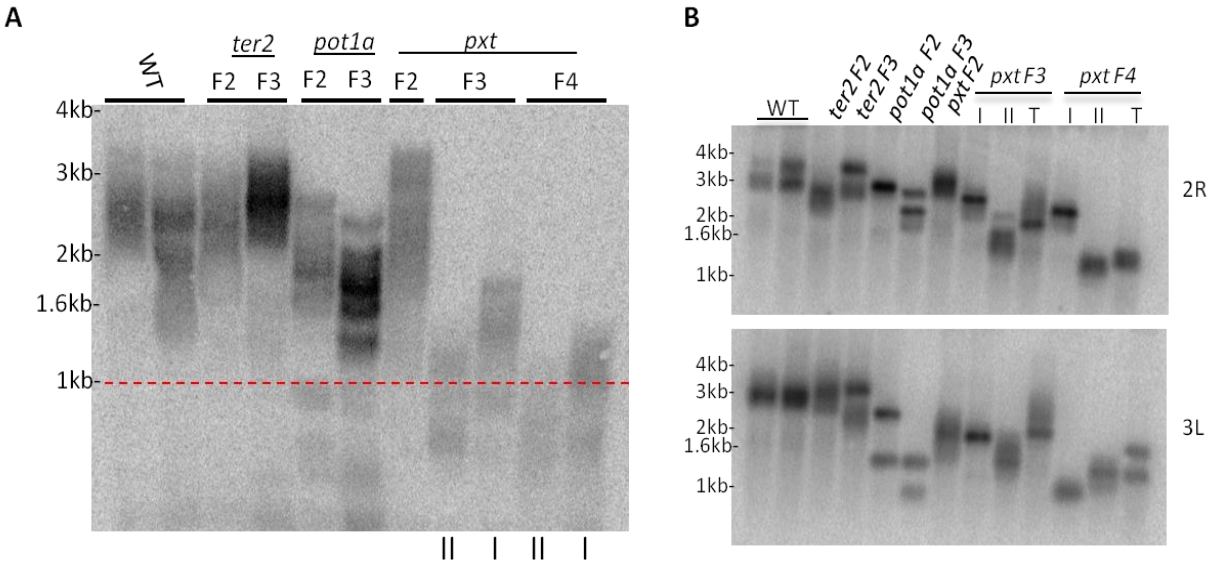


Figure 3-5. Morphological severity is correlated with the rate of telomere attrition. (A) Telomere length analysis of single and double mutants from F2 to F3/F4 by TRF. Class I and class II plants display different amounts of telomere shortening. Each genotype is represented by two biological replicates. (B) Telomere length analysis of individual chromosome arms by PETRA. The right arm of chromosome 2 (2R) and the left arm of chromosome 3 (3L) were examined.

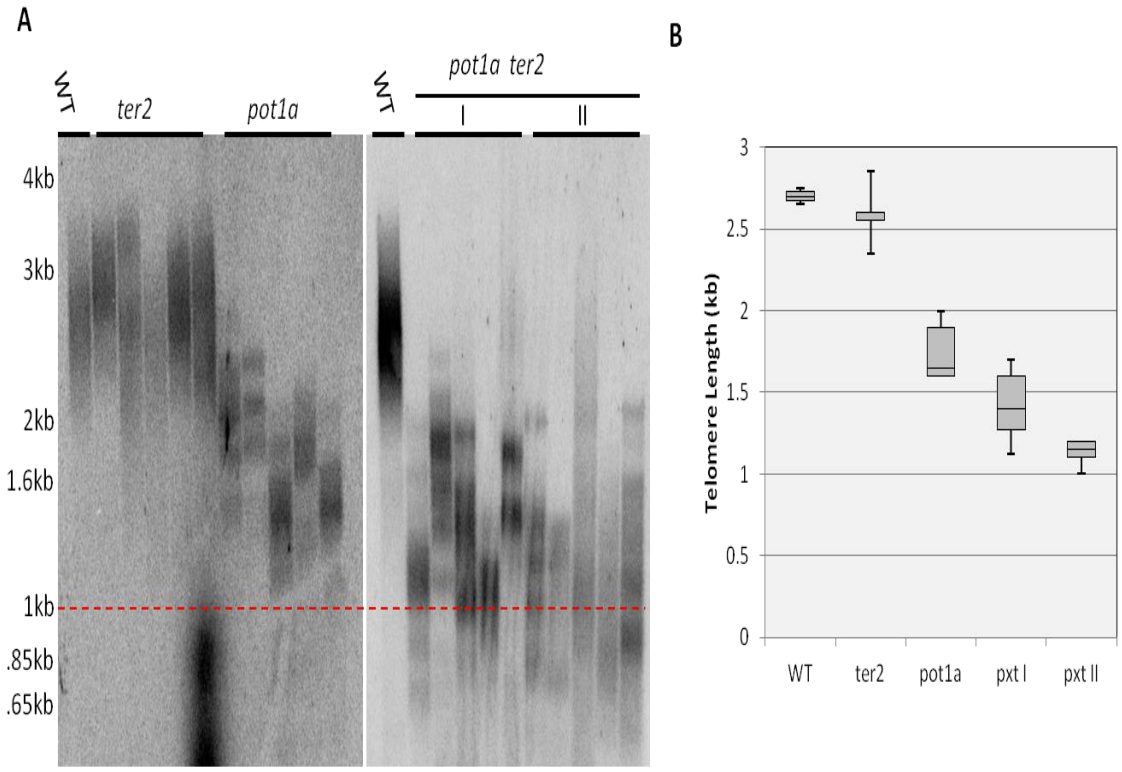


Figure 3-6. Quantitative examination of pot1a ter2 telomere length (A) TRF analysis of five independent F3 plants of the indicated genotype and class. (B) Quantitation of the range of telomere lengths via a box whisker plot. Center bars represent median values while whiskers represent the standard deviation from 5 biological replicates (WT=2).

to class II and even terminal mutants despite the opposite trend in their morphological and telomere severity. These pilot experiments require more biological replicates to verify their significance. However, the preliminary results are surprising given the lack of robust chromosome fusions displayed in *pot1a ter2* mutants.

Cell cycle gene expression is unchanged in pot1a ter2 mutants

One potential explanation for the accelerated rate of telomere depletion in *pot1a ter2* mutants is an increased rate of cell division. POT1a is a key regulator of telomerase and its absence disables telomere replication. If POT1a or TER2 affect cell cycle progression or checkpoint status, cells may divide more frequently in *pot1a ter2* mutants thus exacerbating the end-replication problem. To explore this idea, we examined transcript levels of two key mitotic cell cycle regulators, CDKA;1 and CDKB1;1 (Boudolf et al, 2004; Qi & John, 2007). These transcripts should be elevated in tissue with increased cell cycling. qPCR of CDKA;1 and CDKB1;1 transcripts in wild type, *ter2, pot1a,* or double *pot1a ter2* plants actively dividing flower tissues revealed a similar abundance relative to wild type (Fig. 3-8B). These findings indicate that the cell cycle is not grossly perturbed, and thus the *pot1a ter2* accelerated telomere shortening may not be caused by a check point defect.

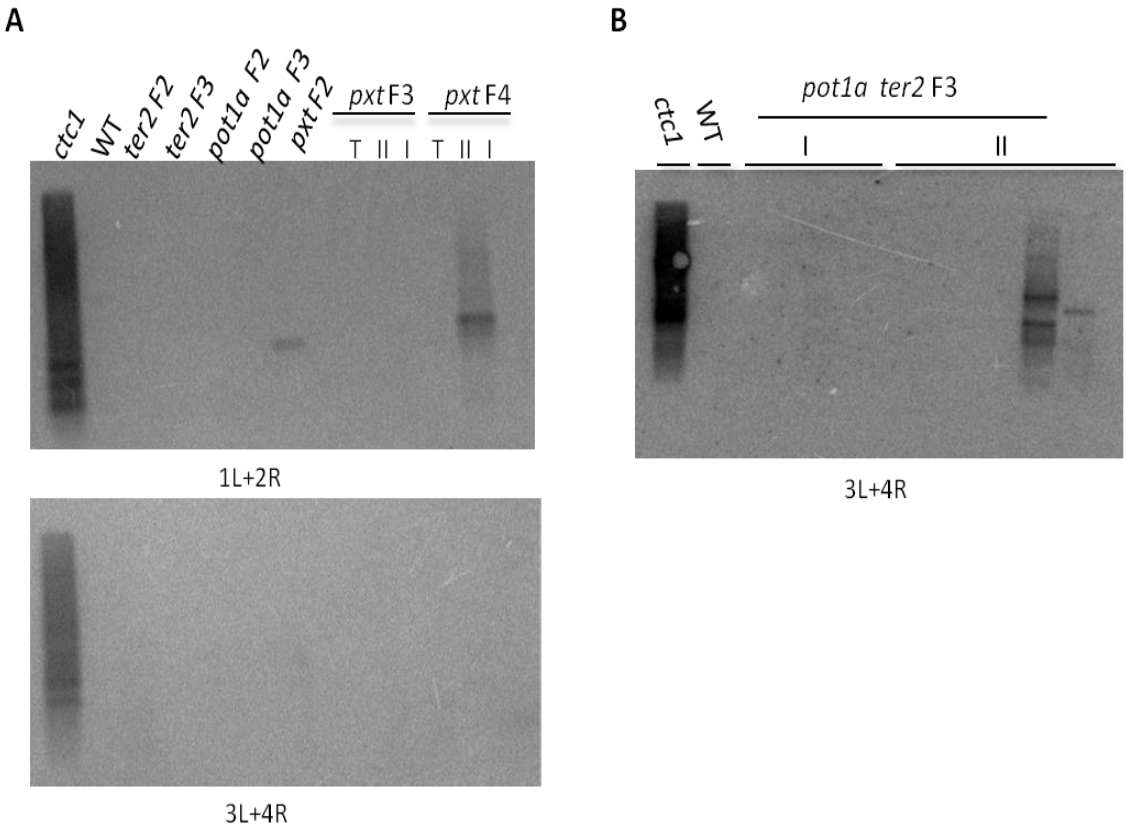


Figure 3-7. Telomeres are not subjected to massive end-to-end fusions in plants lacking POT1a and TER2. (A) Chromosome fusion PCR analysis of the indicated chromosome arms. Samples are from the same DNA extraction in Fig. 3-5B. (B) Chromosome fusion PCR analysis of the indicated chromosome arms. Samples are from the same DNA extraction in Fig. 3-6A.

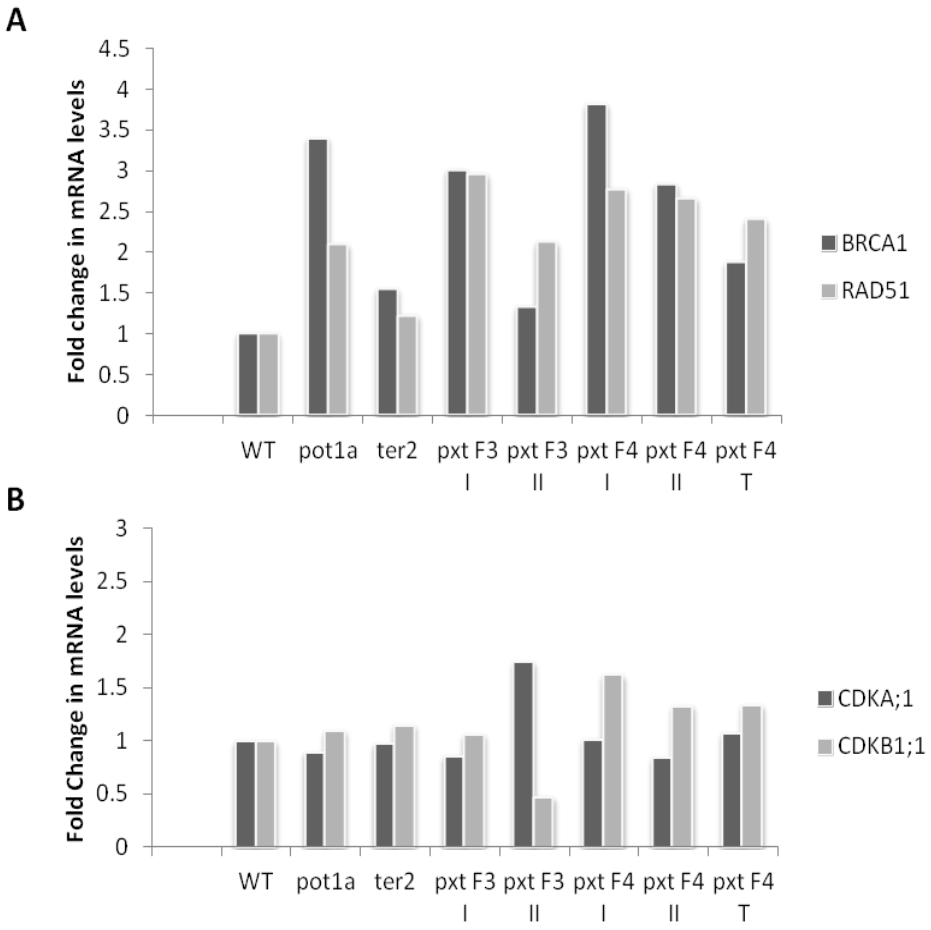


Figure 3-8. DDR and cell cycle related gene expression in pot1a ter2. (A) qRT-PCR analysis of BRCA1 and RAD51 transcripts of the indicated genotypes. Samples are normalized to the wild type sibling. Reactions were performed in triplicate from one independent biological sample. (B) qRT-PCR of CDKA;1 and CDKB1;1 transcripts of the indicated genotypes. Samples are normalized to the wild type sibling. Reactions were performed in triplicate from one independent biological sample.

Discussion

Telomere length homeostasis is a highly regulated process and each organism adheres to a specific telomere length set point. In *Arabidopsis* the set point is 2-5kb. Telomere homeostasis is esatablished and then maintained in actively dividing root and shoot meristems by telomerase and in organs where there is little cell division (e.g. leaves), telomere length is steady. Here we observed the consequences of abolishing two molecules that regulate telomerase in opposite manners, POT1a and TER2 (Cifuentes-Rojas et al, 2012a; Surovtseva et al, 2007).

While the role of POT1a has been well established in telomere length maintenance and telomerase regulation (Surovtseva et al, 2007), TER2 function has remained perplexing. We found that when TER2 is eliminated in a *pot1a* mutant background, double mutants exhibit early onset abnormal morphological defects unlike single *ter2* and *pot1a* plants. Some *pot1a ter2* plants seem to retain their wild type appearance, while others become diminutive, irregularly branched, and altered in rosette leaf shape and texture. As these mutants are propagated, a higher fraction of plants exhibit these phenotypes and many arrest in a vegetative state. This pattern is reminiscent of later generation *tert* mutants which have had no telomerase for 7-10 generations (Riha et al, 2001). These findings suggest that TER2 exacerbates the telomere dysfunction of not having telomerase.

The reproductive defects of *pot1a ter2* mutants are particularly striking. Siliques became shorter, hooked and curled. It is not clear if this is caused by a specific disruption in the mechanism of silique development, or by the high rates of seed loss.

Double *pot1a ter2* mutants had significantly higher incidence of seed absence compared to single *pot1a* or *ter2* mutants, and once again this phenotype worsens in subsequent generations. Other work from our lab has discovered *ter2* mutants have decreased pollen viability, indicating TER2 is needed for embryogenesis (Hengyi Xu, unpublished data). How this reproductive defect is linked to the vegetative developmental defects in *pot1a ter2* mutants is unclear at this time.

The early onset of severe morphological defects strongly correlated with telomere length. Telomeres in *pot1a ter2* mutants shortened at an increased rate relative to *pot1a* mutants (Surovtseva et al, 2007). Moreover, *pot1a ter2* telomeres resembled *pot1a* or *tert* telomeres in their homogeneity and sharply banded profile (Riha et al, 2001; Surovtseva et al, 2007). This is in contrast to mutants within the telomere capping pathway that undergo in rapid shortening, but possess heterogeneous telomere tracts indicating their erosion is due to nuclease attack (Leehy et al, 2013; Riha et al, 2001; Song et al, 2008; Surovtseva et al, 2009a). Thus, this reflects the exacerbated telomere shortening profile of *pot1a ter2* mutants may not initially be due to loss of telomere integrity via telomere deprotection, but rather an unknown mechanism pertaining to telomerase regulation or some other aspect of telomere replication. Ultimately, later generation *pot1a ter2* mutants did become more heterogeneous in their telomere profiles, suggesting cells may lose their ability to repress molecular insults such as nuclease degradation. Despite their critically shortened telomere, we saw very little evidence of chromosome fusions. The faint TF-PCR signals were only detected in a sub-population of very sick class II double mutants. Further analysis of later generation mutants will

determine if chromosome end-to-end fusions becomes more ubiquitous as these mutants worsen. However, one intriguing possibility is whether TER2 functions in eliciting the chromosome fusions.

We examined one potential mechanism for the accelerated telomere shortening phenotype by testing for irregularities in the cell cycle. Telomeres shorten in dividing cells with inactive telomerase. If the combination of eliminating POT1a and TER2 increases the number of cell divisions, this would enhance the amount of telomere shortening in the absence of telomere replication. Gene transcript levels of two critical cell cycle regulators, CDKA;1 and CDKB1;1, were similar relative to wild type plants. These experiments need to be validated by more biological replicates, but the preliminary results suggest the cell cycle is not gross deregulated in *pot1a ter2* mutants. Other experiments such as observing ploidy levels via FACS analysis or microscopically visualizing cell growth and shape in leaves and shoots may indicate cell cycle defects. This cell cycle hypothesis is interesting because this mechanism is not expected to affect telomere protection and thus could explain the homogeneous telomere tracts as seen in many of the *pot1a ter2* mutants.

Another interesting hypothesis is that TER2's role in the DDR exacerbates *pot1a* mutant severity. We found DDR related transcript levels of BRCA1 and RAD51 were elevated in *ter2* plants relative to wild type as expected (Cifuentes-Rojas et al, 2012a), but *pot1a* mutants also seemed to have an unusually high abundance. This analysis needs to be refined with more biological samples to gain statistical significance. It is possible that TER2 interacts with other telomere DDR related components like ATR or Ku to

influence telomere maintenance. ATR does promote telomere maintenance in conjunction with telomerase and double *atr tert* mutants resemble *pot1a ter2* plants morphologically and in their telomere shortening profile (Vespa et al, 2005). Ku, which we have shown to physically interact with TER2 (Cifuentes-Rojas et al, 2012a), has the distinct function of mediating blunt end telomere integrity in *Arabidopsis* (Kazda et al, 2012). Perhaps TER2 coordinates with Ku at blunt-ends and is important for telomere maintenance. This may explain why the loss of POT1a and TER2 would lead to synergistic phenotypes as POT1a is not expected to be involved in the blunt end pathway. There is also the possibility that TER2 acts as a molecular scaffold independent of its association with TERT. This idea is supported by the fact that loss of TERT, which eliminates TER1 and TER2 RNP production, leads to similar telomere shortening as *pot1a* mutants, unlike *pot1a ter2* plants (Fitzgerald et al, 1999; Riha et al, 2001; Surovtseva et al, 2007). We not that a scaffolding function has been ascribed to other long non-coding RNAs (lncRNA) (Wang & Chang, 2011). Telomerase RNA gene duplication is unreported in other model systems. This highlights the exciting possibility of a novel role for a long non-coding RNA involved in regulating aspects of an ancient cellular pathway in telomere biology.

CHAPTER IV

EVOLUTION OF THE TELOMERE ASSOCIATED PROTEIN POT1a IS CHARACTERIZED BY POSITIVE SELECTION TO REINFORCE PROTEIN-PROTEIN INTERACTION

Summary

The flowering plant *Arabidopsis thaliana* encodes two divergent Protection Of Telomeres (POT1) proteins termed AtPOT1a and AtPOT1b. Like the single-copy *POT1* genes in yeast and humans, *AtPOT1b* is implicated in chromosome end protection, but *AtPOT1a* encodes a telomerase RNP accessory factor required for enzyme activity *in vivo*. To explore the nature and origin of the *POT1* gene duplication in Arabidopsis, we analyzed *POT1* genes from species across Brassicaceae plus *Carica papaya* (Caricaceae: Brassicales) and *Gossypium hirsutum* (Malvaceae: Malvales). Both *POT1a* and *POT1b* orthologs were recovered from nearly all sampled species within Brassicaceae, however BLAST searches of the *C. papaya* and *C. hirsutum* yielded only a single copy. Phylogenetic analysis of aligned *POT1a*, *POT1b* and single copy *POT1* genes indicated that *POT1a* and *POT1b* are the products of a duplication event that likely occurred at the base of Brassicaceae. Tests for positive selection implemented in PAML revealed that the *POT1a* lineage, but not *POT1b*, experienced positive selection post-duplication. *In vivo* and *in vitro* analyses of sites responsible for the signature of positive selection indicated that they affect telomere length maintenance and the specificity of the interaction between POT1a and CTC1, which is known to stimulate telomerase activity and promote an extendible state at telomeres. Taken together, the data presented suggest

that post POT1 duplication, the Brassicaceae POT1a copy experienced positive selection that increased its affinity for CTC1. Moreover, this finding is an important empirical example that can help refine theories of duplicate gene retention since the outcome of positive selection here may be reinforcement of an ancestral function, rather than the evolution of a novel function.

Introduction

Publication of the Arabidopsis whole genome sequence more than a decade ago continues to provide biologists an important view of the composition and evolution of plant genomes, especially as compared to other eukaryotic lineages. One surprising finding is the evidence of widespread gene and genome duplications in Arabidopsis We now know that Arabidopsis is not unique among plants in having a genome characterized by duplication fueled gene expansion (Cui et al, 2006). In fact, hybridization and other genome duplication events have impacted lineage diversification (Beilstein et al, 2010) and may even have permitted some lineages to survive through mass extinction events (Fawcett et al, 2009).

As our appreciation for the extent of duplications increases, theories to explain the retention of duplicate genes have been proposed. These theories fall into three major categories: neofunctionalization (Ohno, 1970), subfunctionalization (Force et al, 1999), and maintenance of dosage balance (Birchler & Veitia, 2007). Since the outline of these alternatives, evolutionary biologists have sought empirical examples to strengthen theory. At the same time, theories that refine these major classes have emerged,

including escape from adaptive conflict (EAC) (Des Marais & Rausher, 2008) and positive dosage (reviewed in (Kondrashov et al, 2002)), among others (reviewed in (Innan & Kondrashov, 2010)).

Test of molecular evolution at the protein level permit the processes that underlie some of these theories to be examined. For example, in neofunctionalization, one of the duplicate copies evolves a new function not performed by its single copy ancestor. At the molecular level, this change at the protein level is described by a signature of positive Darwinian selection in which the non-synonymous substitution rate (dN) outpaces the synonymous substitution rate (dS), causing the ratio of the two values, ω, to exceed one (i.e., $dN/dS = \omega > 1$) ((Zhang et al, 2005). In contrast, subfunctionalization parses the functions of the ancestral single copy gene between the descendant copies and can be driven by changes in expression through differential degeneration of promoter regions. Such a process does not require changes to the protein coding region, and thus the descendant gene copies may lack evidence of positive selection. Finally, retention by dosage balance describes situations following whole genome duplication where stoichiometry in biochemical pathways must be maintained to achieve optimal function. Similar to subfunctionalization, changes to the protein coding region are not required, nor are changes to regulatory domains necessary, rather the expectation is that other members of a particular pathway will be represented in the genome by multiple copies. The framework for examining gene duplication events has become a powerful tool for understanding the evolution of protein function. Here we examined the duplication history of the POT1 (Protection Of Telomeres1) protein in the plant family Brassicaceae.

POT1 was first described as a single-strand telomeric DNA binding protein (Baumann & Cech, 2001a) that is a key component of the shelterin complex responsible for protecting telomeres (Hockemeyer et al, 2006), the ends of linear chromosomes in vertebrates and fission yeast. Telomeres are an ancient hallmark feature of most eukaryotic chromosomes and are essential for genome stability and long-term proliferative capacity of cells. The GT-rich sequence of telomeric DNA repeats is well conserved across eukaryotes (TTAGGG in vertebrates and TTTAGGG in plants), but composition of telomere proteins varies significantly between distant organisms. Interestingly, POT1 is one of the few telomere-associated proteins that is remarkably conserved across eukaryotes.

Most eukaryotes harbor a single POT1 gene (Baumann et al, 2002), but two or more POT1 paralogs have been reported in Arabidopsis (Shakirov et al, 2005), mouse (Hockemeyer et al, 2006; Wu et al, 2006), and some ciliates (Jacob et al, 2007). The POT1 paralogs of Arabidopsis have very low sequence similarity (49%), implying either an ancient duplication, rapid evolution or a combination of both processes. Previous work suggests that neither Arabidopsis POT1 paralog binds telomeric DNA (Shakirov et al, 2009a), setting them apart from their vertebrate, yeast and ciliate counterparts. In fact, the encoded proteins appear to have opposing functions; AtPOT1a is involved in a protein complex responsible for telomere extension (telomerase) ((Surovtseva et al, 2007)), while AtPOT1b is a component of a telomerase-like protein complex that is responsible for inhibiting telomerase activity during stress-induced DNA damage (Cifuentes-Rojas et al, 2012a).

To gain an understanding of the timing of the duplication event responsible for the two functionally divergent Arabidopsis POT1 proteins and to characterize the processes underlying retention of the duplicate copies we 1) inferred phylogeny for POT1 from Arabidopsis and its closes relatives, 2) tested whether neo- or sub-functionalization better describes the evolution of the genes post duplication, and 3) used complementation and *in vitro* binding assays to determine whether amino-acids with a significant signal of positive selection were important for POT1 function.

Materials and methods

Recovery of POT1 orthologs from sampled species

POT1 BLAST searches of the plant genomes were performed using the blastp or tblastn options available at the corresponding genome portals (http://asgpb.mhpcc.hawaii.edu/tools/tools.php, http://genome.jgi-psf.org/euk_cur1.html, http://www.appliedgenomics.org/blast) with Arabidopsis POT1 proteins as a query. BLAST searches with human or *S. pombe* POT1 proteins were attempted, but did not improve the outcome. For species in Brassicaceae lacking whole genome sequence, we designed degenerate primers to amplify either the POT1a or POT1b paralog using hiTAIL-PCR (Liu & Chen, 2007).

Phylogenetic and positive selection analyses

Translated amino acid sequences were aligned using ClustalW (Larkin et al, 2007). The resulting translated amino acid alignment was then used to correct the

nucleotide alignment using MacClade Vers. 4.08 (Maddison & Maddison, 2005), and subsequent alignment changes were made by eye. Phylogenetic trees were reconstructed using RAxML v7.0.4 (Stamatakis, 2006)using the GTRGAMMA option, which employs the general time reversible model with Γ distributed rate heterogeneity.

Using PAML4.0 (Yang, 1997), we implemented the branch-site model A test with the foreground branch represented by either the *Pot1a* lineage or the *Pot1b* lineage. In the null model of the branch-site test both the background and foreground branches consist of sites where $0 < \omega < 1$ or $\omega = 1$ (Zhang et al, 2005). In the alternative model, the designated foreground branch is permitted two additional site classes in which $\omega > 1$ (Zhang et al, 2005). The site classes on the foreground branch that represent $\omega > 1$ may be derived from sites in the background lineages that are in either the site class of $0 < \omega < 1$ (purifying selection) or $\omega = 1$ (neutrally evolving sites) (Zhang et al, 2005).

Plant growth and transformation procedures

Arabidopsis 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. *pot1a-1*, *ku70*, and *pot1a-1*$^{-/-}$*ku70*$^{+/-}$ mutants were described previously (Riha et al, 2002; Surovtseva et al, 2007). For complementation experiments, POT1 cDNAs were subcloned into the pCBK05 binary vector carrying the *bar* gene as a selectable marker (Riha et al, 2002) under the control of *AtPOT1a* native promoter (a 1.5 kb region immediately upstream of the start codon). Complementation constructs were introduced into the *Agrobacterium tumefaciens* GV3101 strain, which was used to transform *pot1a-*

1$^{-/-}$*ku70*$^{+/-}$ plants by the modified *in planta* method (Bechtold & Pelletier, 1998). T1 primary transformants were selected on 0.5 Murashige and Skoog basal medium supplemented with 2mg/liter of phosphinothricine (BASTA) (Crescent Chemical, Islandia, New York) and genotyped by PCR to identify *pot1a-1*$^{-/-}$*ku70*$^{-/-}$ plants expressing the transgene. PCR genotyping was also used to identify their siblings without the transgene.

Telomere length analysis and quantification

DNA from individual whole plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed with DNA digested with *Tru1*I (Fermentas, Hanover, MD) restriction enzyme. ^{32}P 5' end–labeled $(T_3AG_3)_4$ oligonucleotide was used as a probe (Fitzgerald et al, 1999). Radioactive signals were scanned by a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the data were analyzed by IMAGEQUANT software (Molecular Dynamics). The average telomere length (L) was measured using Telometric-1.2 program (Grant et al, 2001). The average telomere lengths of untransformed *pot1a ku70* mutants, transformants expressing wild-type *AtPOT1a* and other *POT1* constructs were designated as L_0, L_1, and L_x, respectively. We set the complementation level of wild-type *AtPOT1a* transformants (positive control) as one, and that of untransformed *pot1a ku70* mutants (negative control) as zero. The complementation efficiency (*E*) of each *POT1* construct was calculated as: $E = (L_x - L_0) / (L_1 - L_0) * 100\%$. At least three individual transformants for each construct were analyzed for statistical support.

POT1a protein interactions

Proteins were expressed from Rabbit Reticulocyte Lysate according to the manufacturer's instructions (Promega). Constructs were expressed with a T7 tag in the pET28a vector (Novagen) or without a tag in the pCITE4a vector (Novagen). Proteins without a tag were incorporated with [S35] methionine to detect the amount of co-precipitated protein. T7 tagged proteins were verified for expression by labeling an aliquot of the expression master mix. The Co-IP procedure was carried out as described (Karamysheva et al, 2004). Binding quantification was performed using Quantity One software (Bio-Rad). The bound signal was represented as a fraction of the bound+unbound total signal followed by subtracting the amount of background noise (beads control). Samples were then normalized to wild type POT1a binding to CTC1 or STN1. Error bars represent the standard of the mean from three independent replicates.

Nucleotide sequence accession numbers

Accession numbers for *AtPOT1a* (AY884593) and *AtPOT1b* (AY884594) were reported previously (Shakirov et al, 2005). The following plant POT1 proteins were deposited into the GenBank: AlPOT1a (EU880293), AlPOT1b (EU880294), BoPOT1a (EU880299), BoPOT1b (EU880300), GhPOT1 (EU880305), CpPOT1 (EU887728).

Results

POT1 phylogeny and duplication

There are several well documented genome duplication events that have occurred during the evolution of land plants. To explore the origin of the Arabidopsis POT1 gene duplication, we analyzed POT1 sequences from 14 species within the plant family Brassicaceae, to which Arabidopsis belongs, as well as *Carica papaya* (papaya, order Brassicales) and *Gossypium hirsutum* (cotton, order Malvales). Together this sampling represents approximately 100 million years of plant evolution (Beilstein et al, 2010). Our bioinformatics and phylogenetic analyses indicated that the duplication giving rise to *AtPOT1a* and *AtPOT1b* occurred near the origin of the Brassicaceae (Figure 4-1), and furthermore that both *C. papaya* and *G. hirsutum* contain only a single POT1 ortholog. The two Arabidopsis POT1 gene copies are found in regions on chromosomes 2 and 5, which do not belong to the set of canonical duplicate chromosomes that share large stretches of co-linearity (e.g, chr. 2 and 4) (Vandepoele et al, 2002). Given the inferred POT1 gene tree and since only a single POT1 gene was recovered in the whole genome sequences of papaya and cotton, we conclude that the POT1 gene duplication arose in the lineage leading to Arabidopsis after its divergence from the last common ancestor with papaya ~90 mya (Beilstein et al, 2010).

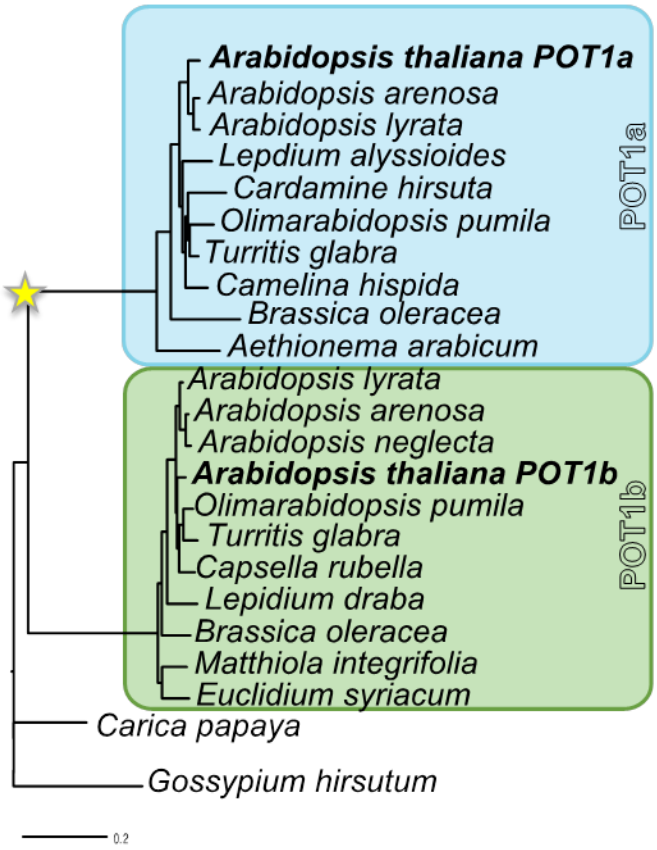


Figure 4-1 Results from the branch-sites test in PAML. *POT1* phylogeny inferred using a maximum likelihood approach from full length *POT1* genes for 14 species spanning the family Brassicaceae and its close relatives. The GTR + Γ model of evolution was implemented in RAxML v 7.0.4. Duplication event is denoted by a star, *POT1a* and *POT1b* lineages are indicated by colored and labeled boxes.

Evolution of Brassicaceae POT1 proteins

Given the previously documented functional differences between the duplicated POT1 paralogs in Arabidopsis, we hypothesized that either *POT1a* or *POT1b* had undergone adaptive evolution and that the corresponding amino acid substitutions would be correlated with functional diversification. To test these hypotheses we asked if sites in the *POT1a* or *POT1b* lineage experienced positive selection. Specifically, we examined the ratio (ω = dN/dS) of non-synonymous (dN) to synonymous (dS) changes along the branches leading to either *POT1a* or *POT1b*. We used the branch-site test in PAML (Zhang et al, 2005) with the foreground branch represented by either the *POT1a* lineage or the *POT1b* lineage. Background branches consist of site classes whose ω values are not permitted to exceed 1. In contrast, the foreground branch contains additional site classes where $\omega > 1$. This analysis resulted in a significant difference between the null model (background and foreground branches are evolving under the same rates) vs. the alternative model (some sites in the foreground branch have $\omega > 1$), $p = 0.00014$, when the POT1a branch, but not the POT1b branch was tested (Table 4-1). Bayes Empirical Bayes (BEB) was used to calculate the posterior probability of sites coming from the site class with $\omega > 1$. Three sites were identified (Table 4-2), and those sites with posterior probability > .90 were treated as important with potentially adaptive roles in the function of POT1a. We selected three positively selected sites, E35, S212 and E293 (Figure 4-2A, B) for functional tests *in vivo*.

Table 1.

Lineage	**Log-Likelihood** (lnL) Null Model	**Log-Likelihood** (lnL) Alternative Model	**Likelihood ratio test** (2×|Lnl null – Lnl alternative|), X^2 p-value
POT1a	-16958.41	-16951.01	14.8, p=0.00014
POT1b	-16959.68	-16958.81	1.7, p=0.187

Table 4-1. Results of the branch-sites test in PAML. POT1a but not the POT1b lineage is under positive slection post duplication.

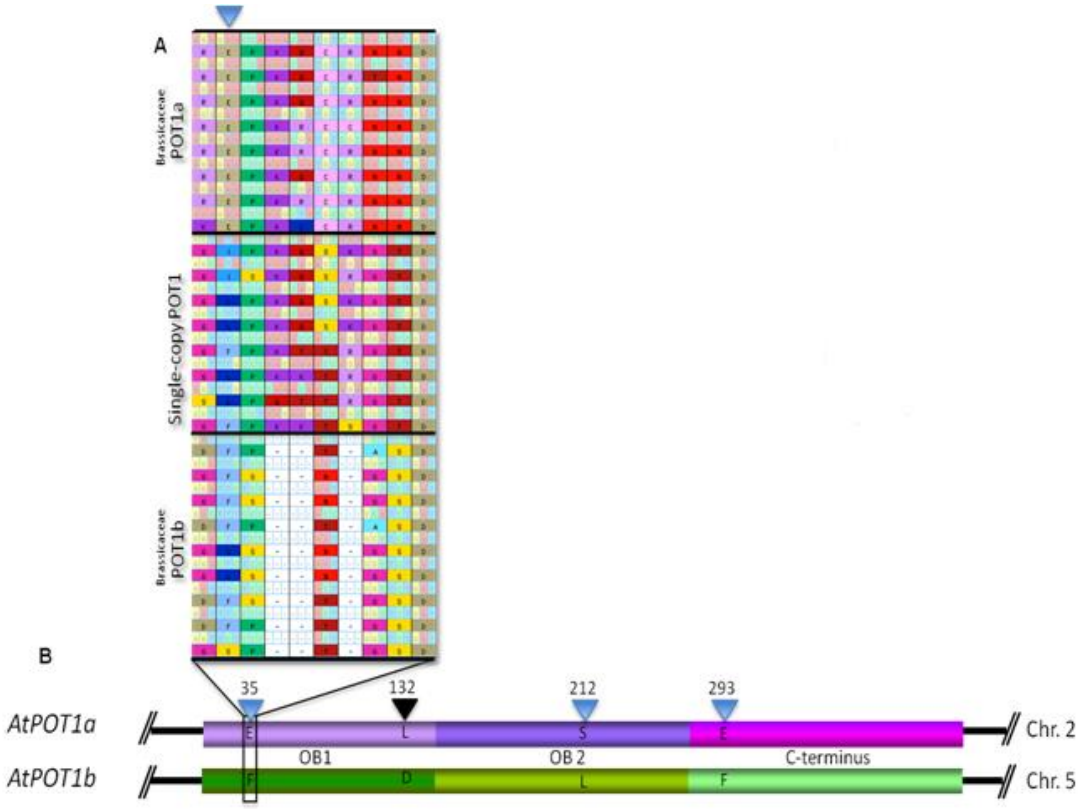


Figure 4-2. Sites under positive selection in *POT1a.* A. Schematic of amino acid alignment position 35 showing glutamic acid (E) encoded in Brassicaceae *POT1a* sequences and alternative amino acids encoded in Brassicaceae *POT1b* and species with single copy *POT1* genes. For complementation experiments, E35 was mutated to phenylalanine (F), the amino acid encoded in Arabidopsis *POT1b*. **B.** The relative position of sites under selection are shown along with amino acid mutations made. Po sitions of the OB-fold domains were predicted by threading Arabidopsis POT1a and POT1b sequences on the human POT1 structure.

Table 2.

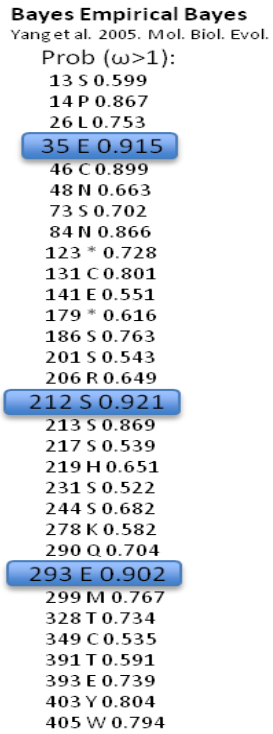


Table 4-2. Sites predicted to be under positive selection post POT1 duplication at the base of Brassicaceae. Numbers in the left column are amino acid alignment positions and numbers in the far right column are Bayes empirical Bayes (BEB) values generated in PAML. Letters in the center column are amino acids encoded in POT1a at the indicated alignment position. The three sites with BEB values exceeding the .90 threshold were chosen for further analysis (blue boxes).

Sites of positive selection are required for AtPOT1a function in vivo

To test the functional importance of positively selected sites *in vivo*, we developed a novel *AtPOT1a* genetic complementation assay (Figure 4-3A-D). If the ancestor of *AtPOT1a* was indeed subjected to an extensive evolutionary sweep, substituting the identified positively selected amino acids with residues found in the *AtPOT1b* copy would be expected to decrease the ability of AtPOT1a to complement the *Atpot1a* null mutant since the function of the two *POT1* paralogs differs. The three positive selection sites fall in three different domains of AtPOT1a when the protein is threaded on the human POT1a structure (Figure 4-2B).

Telomere elongation in transgenic AtPOT1a knockout plants expressing exogenous AtPOT1aE35F was reduced to 57% (Figure 4-4A, B) of the wild-type AtPOT1a levels. Telomere elongation in transgenic plants expressing AtPOT1aS212L was 44% (Figure 4-4A, B) of the wild-type AtPOT1a levels. Transgenic plants expressing AtPOT1aE293F showed 88% elongation compared with AtPOT1a wild-type levels (Figure 4-4A, B). In contrast, AtPOT1aL132D, a site not predicted to be under positive selection had 98% telomere elongation compared with wild-type AtPOT1a. These results demonstrate that positively selected E35, S212, and E293 sites are indeed important for AtPOT1a function *in vivo*, while sites without lacking a signature of positive selection did not affect function.

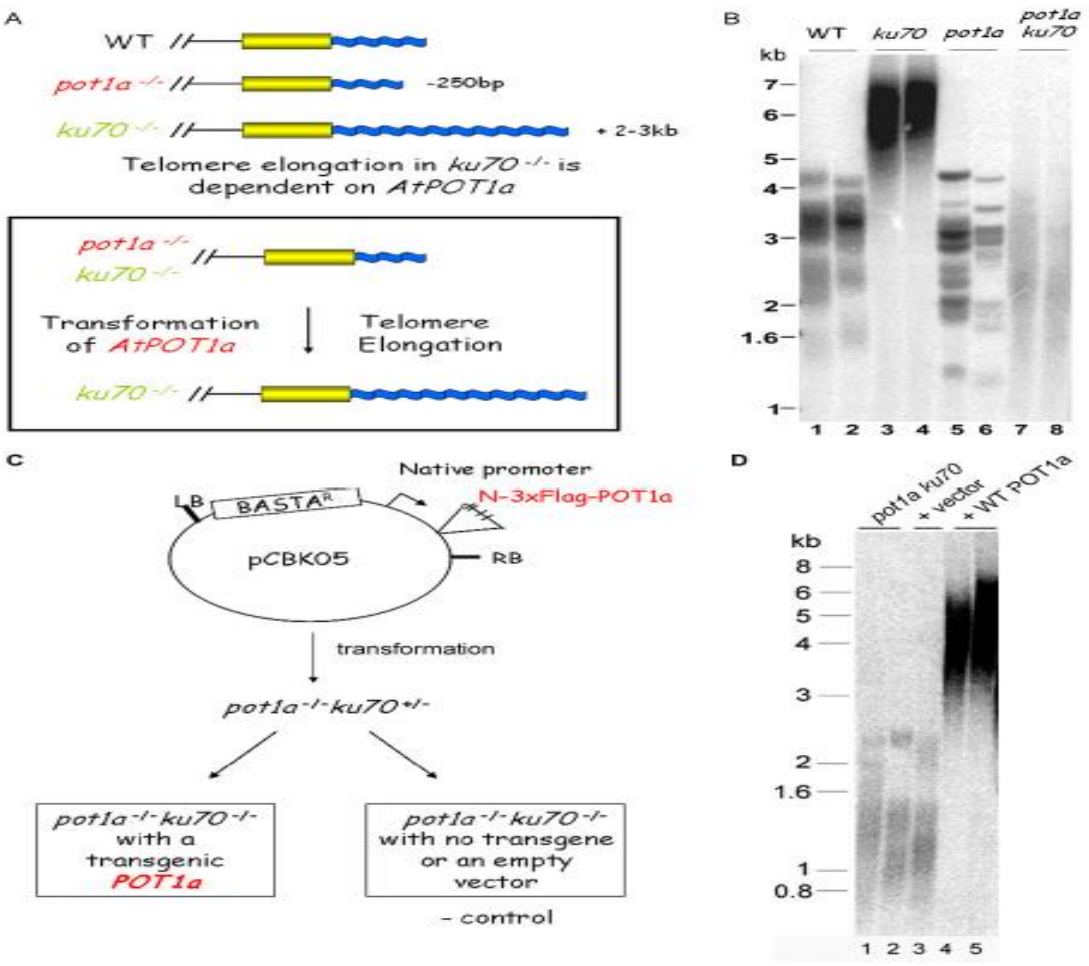


Figure 4-3. Genetic complementation system for *POT1a*. (**A**) A complementation systemfor *POT1a* was set up in a *pot1a ku70* background. (**B**) TRF analysis of WT, *pot1a*, *ku70*, and *pot1a ku70* mutants (Surovtseva et al, 2007). In the absence of *POT1a*, telomeres are progressively shortened by ~ 250 bp per plant generation (lanes 5 and 6). In *ku70* mutants, telomeres are elongated by ~ 2-3 kb per plant generation (lanes 3 and 4). Telomeres remain short in *pot1a ku70* mutants (lanes 7 and 8). (**C**) POT1a transgenes were driven by its putative native promoter. Telomere length of *pot1a ku70* transformants was analyzed to calculate complementation efficiency. (**D**) TRF results of untransformed *pot1a ku70* mutants (lanes 1 and 2), transforments with an empty vector (lane 3), and mutants with a transgenic copy of WT POT1a (lanes 4 and 5).

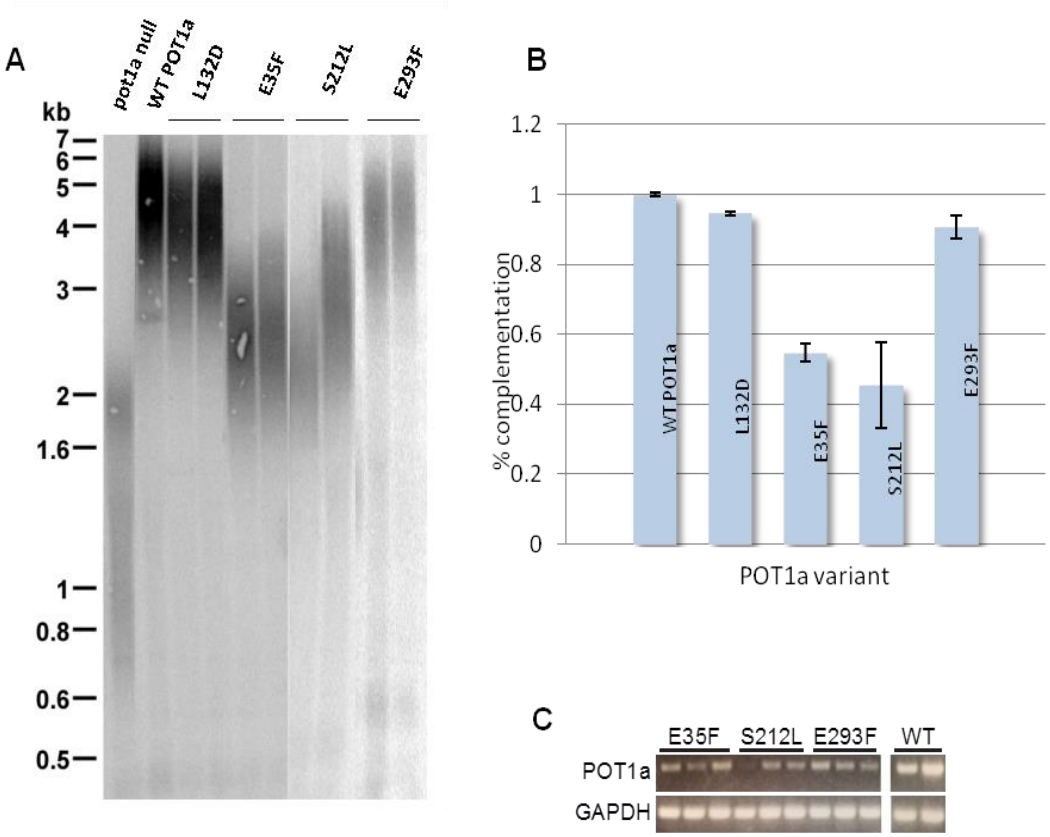


Figure 4-4. Sites under positive selection show reduced ability to complement the *pot1a* null mutation. A. Terminal restriction fragment length analysis western blot showing telomere length for pot1a null mutant (lane 1), and then the null complemented with wild type Pot1a (lane 2), the L132D mutant (lane 3 & 4, not under positive selection), and the three single site mutants: E35F (lanes 5 & 6), S212L (lanes 7 & 8), E293F (lanes 9 & 10). **B.** Quantification of telomere length. Error bars represent standard error from three independent replicates. **C.** RT-pcr results showing that each of the site mutants not achieving full complementation is transcribed.

Sites of positive selection affect the binding affinity of POT1a and CTC1 in vitro

Recent work has revealed AtPOT1a interacts with the telomere capping protein CTC1 *in vitro* and *in vivo*. This interaction is necessary to promote telomere maintenance by augmenting the telomeres into a telomerase extendable state (Renfrew et al 2014). Moreover, other studies have shown the mouse POT1 paralog POT1b has evolved an interaction for CTC1 as well suggesting this interface may have arose convergently. We questioned whether AtPOT1a positive selection site mutants altered the level of POT1a-CTC1 binding *in vitro.* Co-immunoprecipitation assays were employed from Rabbit Reticulocyte Lysate (RRL) expressed proteins. Due to insolubility of full length CTC1, we used an amino terminal truncation construct used previously to verify the POT1a-CTC1 interaction as well as binding between CTC1 and the POLα subunit ICU2 (Price et al, 2010), Renfrew et al 2014) The ability of POT1a or the positive selection variants to co-precipitate CTC1 was visualized and quantified from the amount of [35S] methionine labeled CTC1 that was pulled down in the reaction. As expected, wild type POT1a protein bound CTC1 efficiently (Figure 4-5A). However, all three positive selection site mutants had reduced affinity for CTC1 *in vitro* including the triple mutant protein (Figure 4-5 A,B). The L132D mutation did not perturb the CTC1 interaction, which is consistent with its ability to fully complement telomere length (Figure 4-5 and Figure 4-4 A). Moreover, POT1b had similar levels of binding as positive selection site mutants for CTC1 supporting the hypothesis that POT1a is evolving an enhanced association with CTC1 (Figure 4-5 A,B).

To verify the positive selection site mutations were not generally abrogating POT1a protein folding rather than specifically disrupting its interface with CTC1, we repeated these Co-IPs with another POT1a binding partner STN1 (Renfrew et al 2014). Interestingly, all three amino acid substitutions had no influence on POT1a-STN1 binding (Figure 4-6A,B) relative to wild type POT1a suggesting POT1a is not undergoing global misfolding. Furthermore, POT1b had normal levels of STN1 binding indicating this particular interaction may not be under the influence of positive selection in POT1a.

Rapid evolution of Brassicaceae POT1a proteins

Most of the sites under positive selection with high ω values in AtPOT1a are similar or identical to sites in POT1a protein from *Arabidopsis lyrata*, a closely related species that shared the last common ancestor with *A. thaliana* ~5.2 mya (Koch et al, 2000). As expected, cross-species complementation using AlPOT1a fully rescued *AtPOT1a* deficiency phenotype (Figure 4-7A, lanes 3 and 4, and Figure 4-3D). On the other hand, *A. thaliana* and cauliflower diverged ~ 20 mya (Koch et al, 2000; Yang et al, 1999), and BoPOT1a protein exhibits only 74% similarity to AtPOT1a overall. Strikingly, BoPOT1a displayed less than 20% of complementation efficiency relative to wild-type AtPOT1a (Figure 4-7B, lanes 3 and 4), indicating that Brassicacea POT1a is evolving at a rapid rate.

The neo-functionalization model of gene evolution predicts that if the *POT1a* genes from Brassicaceae species have acquired a novel function, a single-copy *POT1*

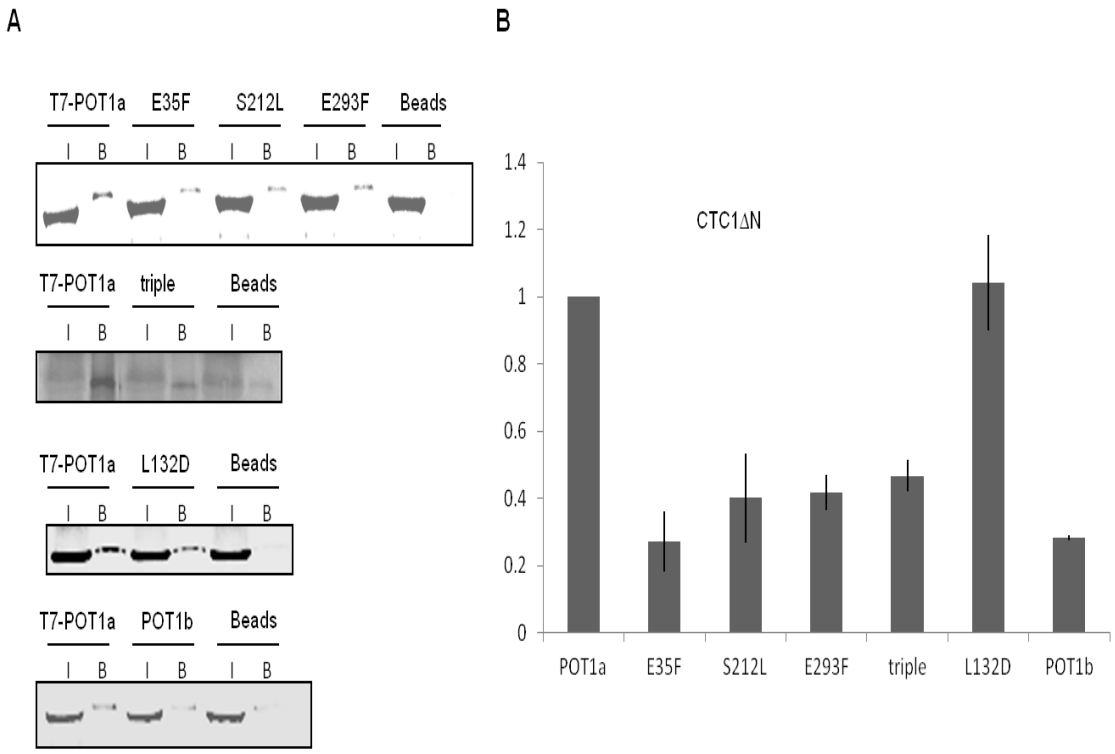


Figure 4-5. ***Positive selection site mutations reduce POT1a affinity for CTC1. A*** In vitro co-IP assays with recombinant RRL-expressed POT1a, mutants, and POT1b with [S35] methionine labeled CTC1ΔN. **B** Quantification of binding relative to wild type POT1a protein. Binding was calculated as a fraction of B/B+I followed by substraction of the background signal (beads) and normalized to wild type POT1a signal. Error bars represent standard error of the mean from three independent reactions.

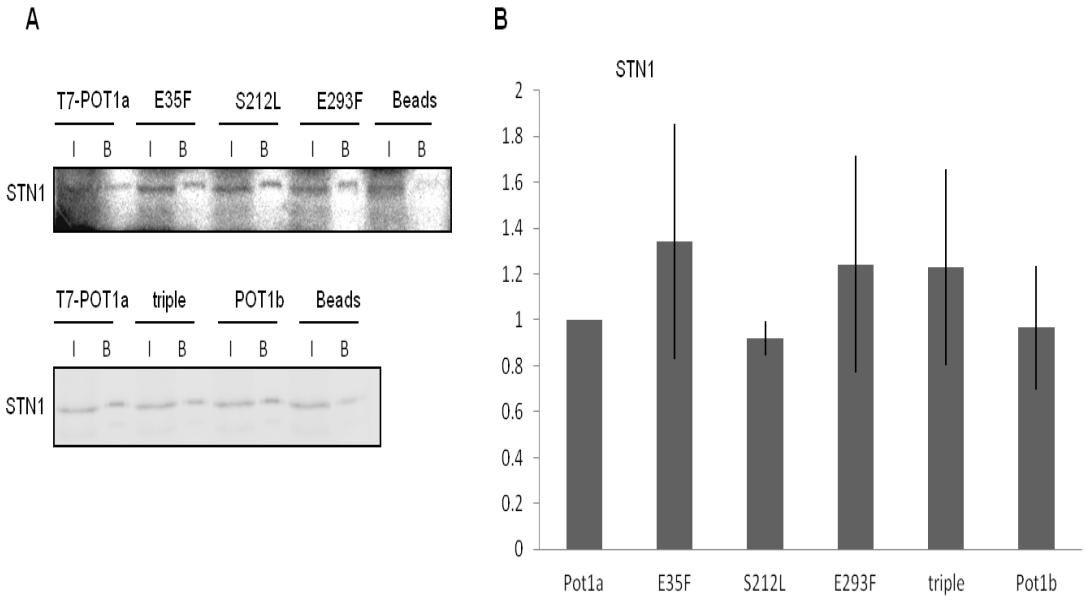


Figure 4-6. ***Positive selection site mutations do not alter POT1a affinity for STN1. A*** In vitro co-IP assays with recombinant RRL-expressed POT1a, mutants, and POT1b with [S35] methionine labeled STN1. **B** Quantification of binding relative to wild type POT1a protein. Binding was calculated as a fraction of B/B+I followed by substraction of the background signal (beads) and normalized to wild type POT1a signal. Error bars represent standard error of the mean from three independent reactions.

gene from non-Brassicaeae plants will fail to complement *AtPOT1a* deficiency. Consistent with this prediction, the single copy POT1 genes from cotton and poplar, which shared the last common ancestor with Arabidopsis 85 and 100 mya, respectively (Wikstrom et al, 2001), failed to complement *AtPOT1a* deficiency (Figure 4-7C, lanes 7 and 8). Next we asked whether the duplication of POT1 genes in maize, which occurred independently of the Brassicacea POT1 duplication, led to the evolution of functions similar to those associated with AtPOT1a. This was not the case as transgenic plants expressing ZmPOT1a or ZmPOT1b failed to complement AtPOT1a deficiency (Figure 4-7D, lanes 3 and 4). Finally, to determine whether there is a complete separation of function between Brassicaceae POT1a and POT1b lineages, we asked whether over-expression of AtPOT1b, AlPOT1b or BoPOT1b from the 35S CaMV promoter would rescue AtPOT1a deficiency. In all cases, less than 10% complementation efficiency was observed (Figure 4-7C, lanes 3-6), indicating that AtPOT1a has evolved functions distinct from AtPOT1b. Taken together, our findings argue that Brassicaceae POT1a proteins are evolving at an extraordinarily rapid rate and in the last 20 my years these proteins have been subjected to a strong evolutionary sweep that resulted in novel telomere functions.

Discussion

Implications of positive selection for POT1a function

Here we employed a combination of evolutionary, molecular genetic, and biochemical approaches to examine the evolution and function of *POT1* genes in the

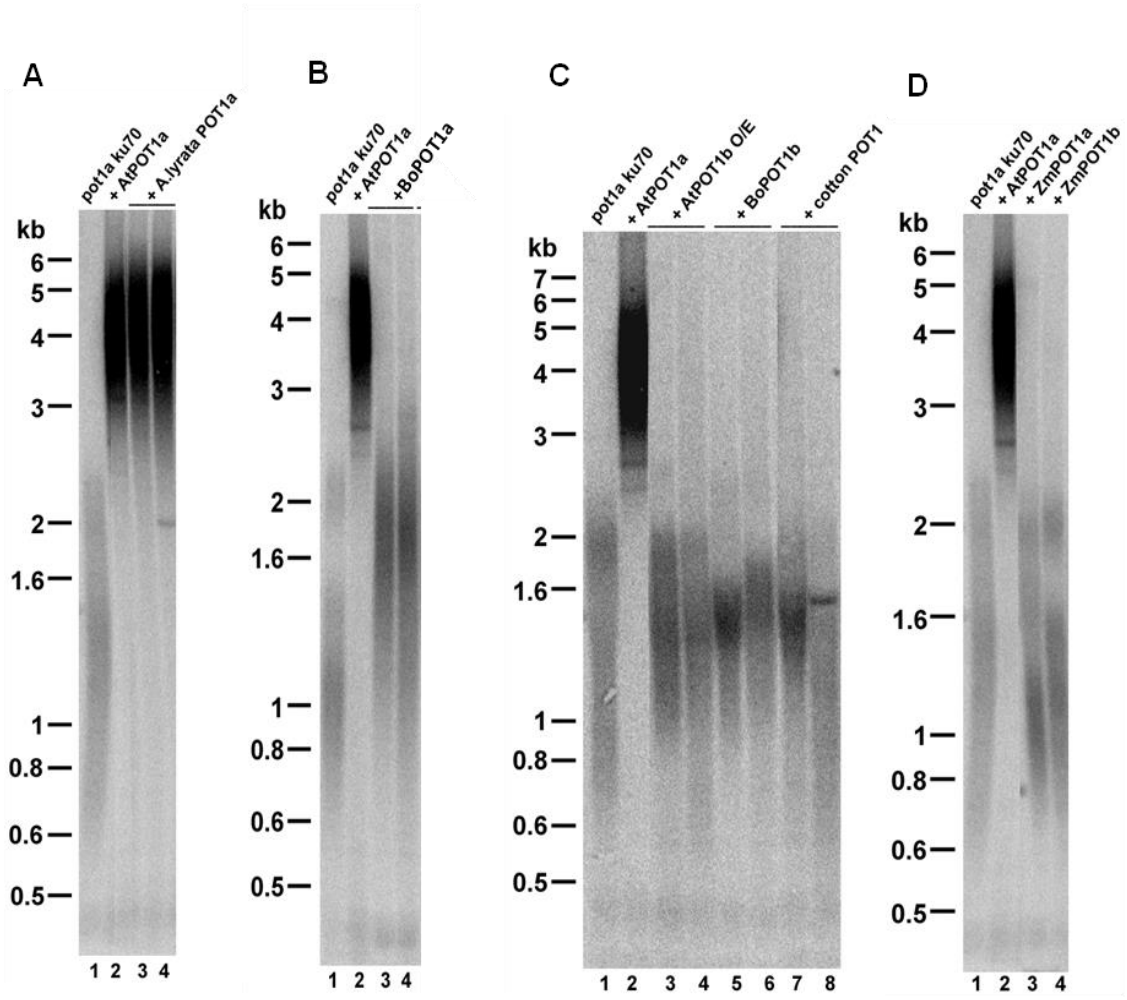


Figure 4-7. Cross-species complementation analysis of *AtPOT1a* deficiency. A-D TRF analysis of transformants expressing POT1 proteins from other plant species. **A** Lane 1, untransformed *pot1a ku70* plant; lanes 2-4, *pot1a ku70* plants expressing WT AtPOT1a (lane 2), and A. lyrata POT1a (lanes 3,4). **B** Lane 1, untransformed *pot1a ku70*; lanes2-4, *pot1a ku70* mutants expressing WT AtPOT1a (lane 2) or *Brassica oleracea* (Bo) POT1a (lanes 3, 4). **C** Lane 1, untransformed *pot1a ku70* plant; lanes 2-8, *pot1a ku70* plants expressing WT AtPOT1a (lane 2), over-expressing AtPOT1b (lanes 3,4), expressing cauliflower BoPOT1b (lanes 5,6), and cotton GhPOT1 (lanes 7,8). **D** Lane 1, untransformed *pot1a ku70* plant; lanes 2-4, *pot1a ku70* plants expressing WT AtPOT1a (lane 2), maize ZmPOT1a (lane 3), and maize ZmPOT1b (lane 4). (lanes 7,8).

plant family Brassicaceae. To investigate the functional contribution of specific residues within AtPOt1a *in vivo*, we developed a genetic complementation system that provides a quantitative read-out of POT1a activity. We obtained POT1 sequences from 14 different plants, representing ~100 million years of evolution. The identification of a large number of plant POT1 sequences provided an opportunity to search for evidence of selective pressure on the duplicated POT1 proteins of Brassicaceae. BEB implemented in the branch-sites test of PAML identified a number of positively selected sites in *POT1a*, which were prioritized on the basis of BEB-assigned statistical values. We developed a complementation assay to determine the effect of these sites on telomere length extension, the previously characterized function of AtPOT1a. In addition, we used the known interaction between POT1 and CTC1 as the basis of inquiry to explore whether sites under positive selection affect the ability of these two proteins to interact. Our results indicate that POT1a experienced a period of positive selection following the duplication event that marks its origin. Moreover, the amino acids responsible for the significant signature of selection affect the ability of AtPOT1a to extend telomere ends in vivo, and the affinity of AtPOT1a for CTC1. Taken together these results suggest that post-duplication, POT1a evolved a more highly specific interaction with CTC1 as compared to POT1b and that this interaction is critical for the extension of the ends of telomeres.

POT1a evolved under positive selection but may have retained the function of its single copy ancestor

Gene duplication is a major source of evolutionary novelty (Gilbert et al, 1997). By far, the most common outcome of gene duplication is the accumulation of deleterious mutations in one member of the pair, followed by subsequent silencing and eventual gene loss (Moore & Purugganan, 2003). Despite multiple ancient whole-genome polyploidization events in vertebrates, yeast and plants (Cui et al, 2006; Dehal & Boore, 2005; Kellis et al, 2004), *POT1* remains single-copy in most eukaryotic genomes. Thus, the duplication in *POT1* that characterizes genomes in the Brassicaceae appears to be an unusual event in *POT1* evolution.

In the neo-functionalization model for the retention of duplicated genes, one gene copy acquires a novel, beneficial function, while the other copy retains most of the ancestral gene functions (Lynch & Conery, 2000). At the molecular level, the gene copy that acquires a novel function is expected to undergo a period of positive Darwinian evolution following the duplication event that marks its origin (Lynch & Conery, 2000). Our data indicate that POT1a, but not POT1b, experienced positive selection post duplication. Indeed, AtPOT1b failed to complement the loss of AtPOT1a. To this end, we might expect that the AtPOT1a association with telomerase (Cifuentes-Rojas et al, 2011; Surovtseva et al, 2007), role in telomere length homeostasis (Shakirov et al, 2005), and interaction with CTC1 (Renfrew et al, 2014) are functions that were acquired post duplication. In agreement with this, more distantly related plant POT1 copies were

unable to compensate the loss of AtPOT1a, while the close relative *Arabidopsis lyrata* POT1a nearly completely complemented the telomere length defect of *pot1a* mutants.

POT1 function in Arabidopsis, and possibly in other plants, differs from that observed outside land plants. For example, in fission yeast and vertebrates, POT1 functions as a component of Shelterin, a protein complex essential for telomere end protection (de Lange, 2009). In this role, POT1 binds single stranded telomeric DNA and along with other members of the complex forms a bridge between double stranded and single stranded regions of the telomere (Liu et al, 2004a). In contrast, aside from POT1 Arabidopsis lacks other members of the Shelterin complex and neither AtPOT1a nor AtPOT1b have been shown to bind the telomeric repeat sequence (Shakirov et al, 2005). In fact, in a survey of the ability of POT1 to bind single stranded telomeric repeats in a variety of land plants, binding has only been observed in *Physcomitrella patens* (moss) (Shakirov et al, 2010) and *Asparagus officinale* (asparagus) (Shakirov et al, 2009b). Hence, while AtPOT1a is important for length homeostasis its mode of action appears to be through interaction with telomerase rather than as a member of a telomeric DNA binding complex. The likely loss of telomeric DNA binding early in the evolution of land plants suggests that an alternative function for POT1 explains its retention in plant genomes.

What other functions and interactions might explain POT1 retention in plant genomes? More recently, POT1 in mouse was shown to interact with CTC1, a component of the mammalian CST complex (Ctc1, Stn1, Ten1)(Wu et al, 2012). In this role, POT1 binds CST and extends the C-rich strand of telomeric DNA following its

resection by the enzyme Exo1 (Wu et al, 2012). CST is also present in Arabidopsis (Surovtseva et al, 2009a). Here we show that AtPOT1a binds CTC1, and Renfrew et al. (in press) finds evidence that the AtPOT1a-CST interaction is important for the extension of the G-rich strand of telomeric DNA. Thus, the CTC1-POT1 interaction may be conserved between plants and animals, although whether POT1 bound CST serves to extend the C-strand versus G-strand of telomeric DNA appears to be lineage specific. Our findings have important implications for the understanding of duplicate gene retention. We show that *POT1a* is the subject of positive selection post duplication. However, rather than driving POT1a toward a novel function, the selective pressure may have reinforced the affinity of POT1a for CTC1, thereby specifying POT1a rather than POT1b in the ancestral POT1-CST role of telomere extension. In contrast, POT1b did not experience a period of positive selection post duplication, appears to have lost the ability to bind CST, and may have acquired a function in a ribonucleoprotein complex that negatively regulates telomerase (Cifuentes-Rojas et al, 2012a). One caveat to this scenario for POT1b is that data detailing the function of POT1 in species with only a single copy of the gene are lacking, and thus it is impossible to know whether a role in the negative regulation of telomerase also predates the Brassicaceae *POT1* duplication. Such a result would indicate that subfunctionalization better describes the retention of *POT1a* and *POT1b*, although the signature of positive selection in only one of the descendant copies is inconsistent with current paradigms of the processes underlying duplicate gene retention (Innan & Kondrashov, 2010). Taken together, our findings

indicate that careful analysis of post duplication function is required to adequately assess the evolution of paralogous genes.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Telomeres are dynamic structures that have evolved to mitigate two cellular crises: end-replication and end-protection. Elucidating the full suite of telomere components and the global aspects of telomere regulation has been a major goal for numerous labs over the last two decades. There are many unanswered questions in part due to limited conservation of telomeric proteins which are best studied in different model systems. Despite this, the underlying principles of telomere protection and replication are beginning to be elucidated and are surprisingly conserved. Chromosome ends exist in a bimodal state that is defined by their accessibility to telomerase. There is wide scale conservation of telomere sequence, structure, and the telomerase enzyme itself, and the mechanisms used to to facilitate telomerase accessibility are similar, even though many of the actual molecular components are not conserved.

In this dissertation I have examined aspects of telomere dynamics in *Arabidopsis thaliana*, primarily through the telomerase regulatory protein POT1a. The POT1 gene has undergone duplication in the lineage leading up to *A. thaliana,* which has allowed Darwinian selection on the POT1a locus. Consequently this has led POT1a to lose its ability to function in chromosome end-protection, its ancestral function, and serve as a mediator of the telomerase RNP where it is a required factor for telomere maintenance. In my dissertation research, I have examined mechanistic underpinnings of how POT1a influences telomere maintenance through its interactions with the telomere capping

complex CST as well as telomerase. I have also explored the relationship of POT1a with the negative telomerase regulatory components TEN1 and TER2, and how they contribute to telomere dynamics, and examined their contributions to telomere dynamics.

Examining telomerase recruitment in *Arabidopsis*

In Chapter II, I examined how POT1a promotes telomere maintenance. Given the similarity of POT1a with ScEst1 in binding TER and CST, and its role in promoting telomere synthesis, I hypothesized a functionally analogous role for POT1a in telomerase recruitment. Surprisingly this was not the case as TERT chromatin immunoprecipitation assays revealed no decrease in telomere-bound telomerase in *pot1a,* and *pot1a ter2* mutants relative to wild type. One critical aspect of *Arabidopsis* telomere biology that remains unknown is the recruitment mechanism for telomerase. Recently, mammalian protein homeobox telomere binding protein 1 (HOT1) was identified as a novel recruitment protein independent of the shelterin capping complex (Kappei et al, 2013). BLAST searching revealed a putative homolog in *Arabidopsis*. T-DNA insertion lines at this locus should be examined. If HOT1 protein is a primary recruitment factor, homozygous mutants would be expected to display an ever shorter telomere phenotype due to the lack telomere maintenance. It is possible there is redundancy in the recruitment pathway, in which case a more refined examination using ChIP and/or cytology may reveal quantitative discrepancies in telomere localization.

Another approach is to identify a recruitment factor is the use of forward genetics. Many telomere proteins over the years have been identified using brute-force biochemistry. Biochemical purification of proteins such as TERT, POT1a, Ku, or even CST components followed by mass spectrometry analysis for associated proteins could reveal new candidates involved in recruitment. Affinity purification requires the creation of transgenic lines overexpressing affinity tagged proteins. Co-purifying would be identified on denaturing gels and secondary bands could be analyzed by proteomic analysis. Alternatively, the same process could be used for more evolutionarily distant organisms such as *Physcomitrella patens* that could identify previously unknown candidates. The advantage here is that *Physcomitrella* has been pioneered as a model system that is related and divergent enough from *A. thaliana* to possibly gap the evolutionary divide between higher plants and vertebrates. This may lead us to shelterin components that are conserved enough between moss and humans which may allow us to bootstrap back to *A. thaliana*. Another approach is trying to fish out shelterin components in *P. patens* using yeast-2-hybrid. Some of these screens are currently underway by Xintao She in our lab. One final approach to detect recruitment factors in *Arabidopsis* is the use of EMS mutagenized populations and scouring through candidates by brute force using TRF to find those that display telomere maintenance defects. Positive candidates could then be mapped to reveal the locus harboring mutation. Of course it may be that some proteins could have dual functions in recruitment and end-protection like ScCdc13 or human TPP1. In this situation, phenotypes resembling telomere deprotection resulting in excessive telomere erosion would be visualized. An

alternative screening method such as quantitative TRAP assays may reveal candidates that affect telomerase enzymology, and perhaps telomerase recruitment.

POT1a is an activator of telomerase

One of the important discoveries from my work is that POT1a is not a telomerase recruitment factor. POT1a is an activator of telomerase at the telomeres. I found that *pot1a* mutant extracts display altered telomerase activity profiles *in vitro*. To test whether this defect was caused by altered RAP, I modified an assay used for human telomerase to study biochemical properties of the plant enzyme (see below). In the absence of POT1a, I found that telomerase was unable to efficiently synthesize long telomere repeat arrays. This finding is indicative of a deficiency in the repeat addition processivity property of telomerase enzyme. Moreover, *pot1a ter2* double mutant extracts had reduced TP-TRAP profiles almost similar to *pot1a* mutants, suggesting POT1a modulates the TER1 RNP specifically. Lastly, I demonstrated that *pot1a* mutants do have altered gene expression of TERT, TER1, TER2, or TEN1 transcripts, which all influence telomerase activity. Therefore, POT1a positively regulates telomerase enzymology to promote efficient telomere synthesis. Recent work in yeast, humans and *Tetrahymena* has revealed that accessory proteins are important modulators of telomerase activity. My work uncovered the first plant protein that affects telomerase RAP and offers a comparative model for telomerase regulation in other eukaryotes.

I also examined the role of CST in telomere maintenance by analyzing *ctc1* or *stn1* mutants crossed with *tert*. These doubly deficient plants were unlike their single

mutant segregant siblings. The loss of TERT caused further telomere shortening compared to *ctc1* or *stn1* mutants, suggesting telomerase could still localize to telomeres and partially rescue telomere dysfunction in this background. Moreover, double mutants were severely developmentally retarded as they remained vegetatively arrested in a diminutive state. Significantly, this was dependent on POT1a as *pot1a stn1* plants were identical to *tert stn1* mutants in telomere length and morphology. Together these data established POT1a is critical for telomere-bound telomerase to partially rescue plants deficient in telomere capping, and the data also indicate that POT1a is an activator of telomerase-mediated telomere maintenance *in vivo*.

While TP-TRAP gives an indication of alteration in telomerase RAP qualitatively, a direct telomerase primer extension assay is the gold-standard methodology for measuring RAP in telomere biology. This technique requires robust telomerase activity that is visualized directly on gels due to the incorporation of [P32] dGTP radioisotope into telomerase products. To dissect the mechanism of telomerase *in vitro,* reconstitution with recombinant components is optimal. Typically TERT and TER, the core telomerase subunits, are expressed in rabbit reticulocyte lysate (RRL), assembled *in vitro,* and used for primer extension assays. An alternative approach is to purify telomerase from cell extracts for enzymatic assays. Although our lab has had modest success with *in vitro* reconstitution (Cifuentes-Rojas et al, 2011), the assay is not reliable. In addition, telomerase is in very low abundance in *Arabidopsis* making the purification method unfeasible. Perhaps the core TERT and TER components lack the robust activity needed for RRL based assays possibly due to low processivity, or lack of

proper RNA folding/RNP assembly. *Tetrahymena* telomerase requires the addition of exogenous Teb1 protein to fully stimulate telomerase activity *in vitro* (Min & Collins, 2009). AtPOT1a may to enhance *Arabidopsis* telomerase activity *in vitro*. Recent work by Dr. Amit Arora found that E. coli POT1a OB1 stimulated telomerase activity in conventional TRAP assays (personal communication). Addition of exogenous POT1a OB1 may allow the development of a direct assay for *Arabidopsis* and should be tested to see if this overcomes the previous limitations in the technique.

Examining CST and POT1a dynamics

Recent studies have reported interactions between POT1 and CST and their importance in regulating telomere replication and processing in vertebrates (Chen et al, 2012; Wu et al, 2010). Similarly, I found that POT1a directly binds CTC1 and STN1 *in vitro* using recombinant RRL-expressed protein. Importantly, POT1a did not interact with TEN1. Because TEN1 negatively regulates telomerase RAP, I asked if POT1a and TEN1 compete for STN1 binding. *In vitro* pull down assays revealed that POT1a OB1 in 10-fold molar excess of TEN1 could sequester STN1 away from TEN1. These data are indicative of mutually exclusive binding at STN1 between POT1a and TEN1. Intriguingly, both STN1 and CTC1 could immunoprecipitate active telomerase *in vivo* similar to POT1a. However, TEN1 is not associated with active telomerase. Altogether, these data suggest POT1a and TEN1 dynamically mediate the nature of the CST complex (Fig. 5-2).

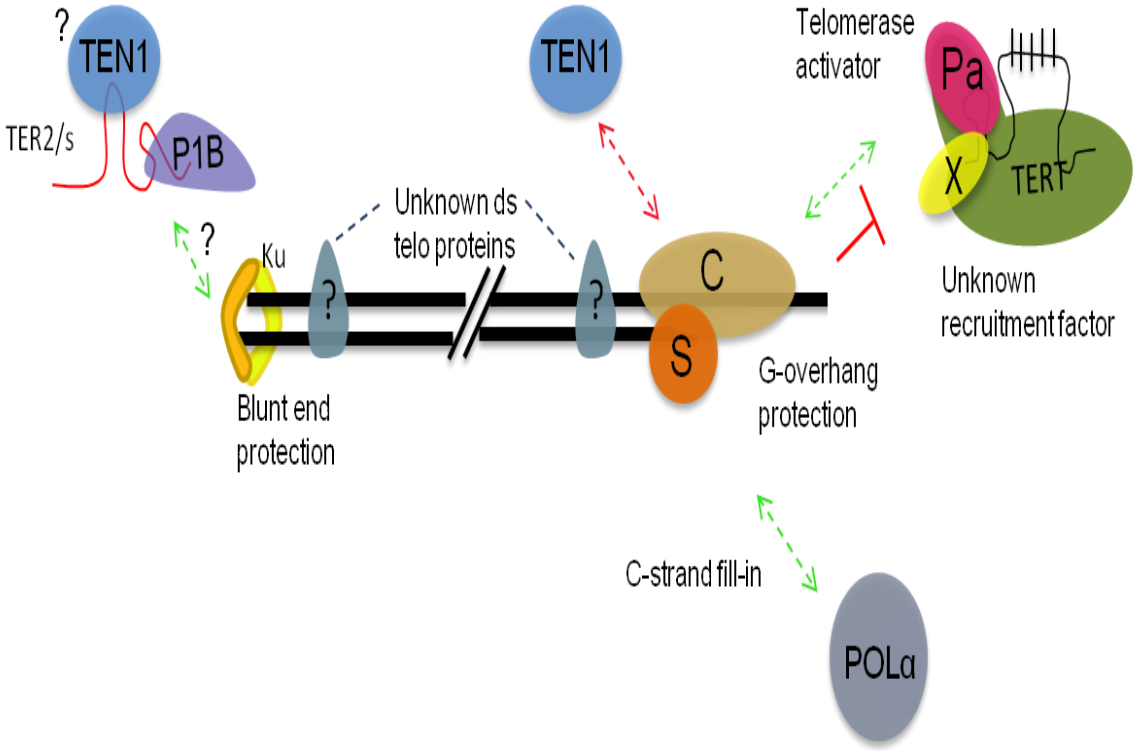


Figure 5-2. *Arabidopsis* telomere dynamics. A proposed model for global telomere dynamics in *A. thaliana. At G-overhangs*, telomeres are sequestered by CST (or CS) and thus telomerase inaccessible. Unknown recruitment proteins mediate telomerase localization at S phase to telomeres while POT1a activates/stimulates telomerase during replication. POL α is exchanged for telomerase to perform C-strand replication. TEN1 transiently localizes to telomeres to mediate end-protection and telomerase regulation (termination?). *At blunt ends,* Ku is bound and mediates blunt end integrity. A potential TER2/s-POT1b-TEN1 RNP associates with Ku/blunt ends through Ku-TER2 binding. The significance of these interactions is unknown. Lastly, unidentified ds telomere binding proteins provide new possibilities for undiscovered telomere transactions.

The fact that CTC1 and STN1 associate with active telomerase is surprising because yeast and human CST function in repressing telomerase accessibility or terminating telomerase activity, respectively (Chen et al, 2012; Lei et al, 2004). Therefore, my work highlights an interesting divergence in CST evolution in *Arabidopsis*. Another surprising functional divergence in an AtCST component is the protein chaperone function of TEN1 (Dr. Jung Ro Lee, unpublished data). CTC1 is a chaperone target for TEN1 during heat-shock induced stress, thus preventing large-scale telomere erosion during periods of instability. It is not clear if this function is unique to AtTEN1, but Xintao She in the Shippen lab is testing the ancestral moss species *Physcomitrella patens* for conserved TEN1 chaperone functions. However, the ability of TEN1 to negatively regulate telomerase activity levels *in vitro* (Leehy et al, 2013), suggest it may possess some similarity to human CST, a telomerase negative regulatory complex (Fig. 5-2; Chen et al, 2012).

One important prediction from my work is the existence of a cooperative complex between CTC1, STN1, and POT1a. This hypothesis is supported by their *in vitro* interactions, as well as their ability to immunoprecipitate active telomerase or TERT protein with CTC1, STN1, and POT1a antibodies *in vivo*. Preliminary data from RRL-expressed proteins suggests that titration of POT1a into pre-bound CTC1-STN1 complexes actually stabilizes the CTC1-STN1 interaction (K. Renfrew, unpublished data). A more definitive analysis is required to understand the dynamics of CST-POT1a interactions which necessitates soluble, purified *E. coli* proteins for each component. Due to the propensity of these proteins to aggregate, even for the relatively small

molecular weight STN1, careful dissection of functional soluble interacting domains for each protein is required. Alternatively, large solubility tags or careful troubleshooting of expression conditions will have to be performed to solubilize these proteins. If successful, I propose *in vitro* pull down assays and gel filtration experiments to verify the nature of the complexes being formed between. TEN1, which has already been purified to homogeneity from *E. coli*, can be added to these complexes to see how this shifts binding dynamics. Based upon competition assays from Chapter II, a likely outcome is that TEN1 would out-compete POT1a for STN1. Unexpectedly, preliminary data from RRL-expressed proteins did not show evidence of CST heterotrimer complex formation. This is in marked contrast to yeast and vertebrate CST (Price et al, 2010). Is it possible TEN1 forms a stable sub complex with STN1 off the telomere rather than the trimeric CST in plants? More *in vitro* based assays are needed to dissect the stability and dynamics of CST-POT1a subcomplexes.

Analysis of the CST-POT1a interaction needs to be pursued *in vivo* as well. Because we have strong antibodies for TEN1 and STN1, it is possible to pull these proteins down and detect them from cell extracts. To visualize CTC1 or POT1a, it may be necessary to use transgenic tagged lines. Furthermore, because the *in vitro* analysis above would require dissection of respective binding domains, genetic complementation experiments with these truncated constructs could be performed to verify their functionality, and the consequences of disrupting their interactions. In the future, it will be important to establish the biological details of the complex network of telomere protein interactions (Fig. 5-2).

Separation-of-function analysis of CTC1-STN1-POT1a interfaces

Some of the earliest analyses of yeast Cdc13 revealed its bipartite role in telomere replication and protection (Lin & Zakian, 1996). Two different Cdc13 alleles had dramatically different telomere phenotypes, showing Cdc13 to be a central player in coordinating telomerase accessibility. My data indicate that although STN1 is much smaller than CTC1, *Arabidopsis* STN1 is a molecular hub like ScCDC13, with separate interactions involving CTC1, POT1a, and TEN1, each with distinct biological relevance. As alluded to earlier, I hypothesize CTC1-STN1-POT1a form a cooperative subcomplex that facilitates telomere replication by making G-overhangs accessible to telomerase. I predict that disrupting STN1-POT1a would lead to a maintenance defect similar to *pot1a* mutants. Because POT1a may also interact with CTC1 as well as STN1 simultaneously, it may be necessary to abolish its interaction with CTC1 as well as STN1 to see a maintenance defect. One important question is whether disrupting POT1a-STN1/CTC1 interactions affects the telomere localization of TEN1. Because POT1a binding to STN1 is mutually exclusive of STN1-TEN1 binding, POT1a may be necessary to remove TEN1 from STN1 in S phase. I propose TEN1 ChIP experiments to examine this question which would provide mechanistic insight for potential telomere maintenance defect phenotypes.

These same questions could be addressed for the CTC1-STN1 interface (Fig. 5-1). It is possible that this interface coordinates C-strand fill-in or G-overhang processing. In that case we might expect to see long G-overhangs, but possibly normal telomere

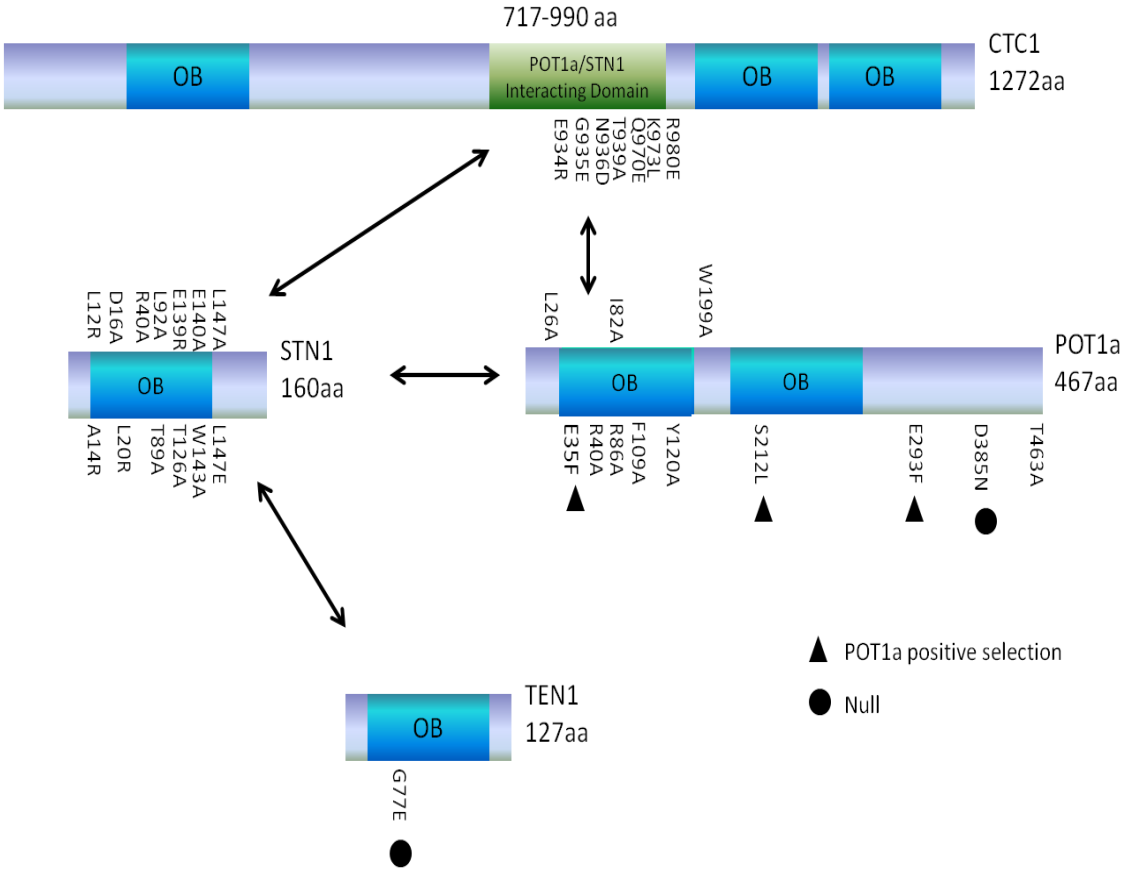


Figure 5-1. Site-directed mutagenesis library in *Arabidopsis* telomere proteins. Collections of site-directed mutagenesis protein candidates have been created and are ready to be screened for separation-of -function analysis. Some mutations have previously been analyzed phenotypically such as POT1a positive selection site mutants or null alleles in POT1a and TEN1 (D385N and G77E).

length overall. The function of POT1a may be dependent on CTC1-STN1 binding, in which binding mutants might be similar to POT1a null mutants. Many members of our lab have spent considerable time generating large collections of site-directed mutants in POT1a, STN1, and CTC1. These mutations will be a great resource to dissect the individual CST-POT1a interfaces.

The role of TEN1 in telomerase regulation

TEN1 is a perplexing component of telomeres in *Arabidopsis*. A null mutation leads to deprotection phenotypes similar to *ctc1* and *stn1* mutants, but contrastingly, *ten1* mutants have increased telomerase RAP (Leehy et al, 2013). Thus, TEN1 uniquely regulates telomerase enzyme activity. Moreover, I find that TEN1 does not associate with active telomerase like CTC1 and STN1. Because TEN1 transiently associates with telomeres and competes with POT1a for STN1 binding, all of these findings suggest TEN1 is a regulatory molecule that opposes the function of POT1a. Why do *ten1* plants exhibit telomere deprotection when TEN1 is not a constitutive member of CST? One hypothesis is that TEN1 is only needed transiently to maintain telomere integrity at a particular step of telomere replication. TEN1 could be important for terminating telomerase post-replication and leading the hand-off to C-strand fill-in (Fig. 5-2). A terminating role for CST components has already been proposed in humans (Chen et al, 2012).

To determine where TEN1 acts in the cell cycle, it will be helpful to disrupt the strong STN1-TEN1 interaction (Fig. 5-1). Preliminary data from STN1 point mutants

that disrupt the STN1-TEN1 interaction *in vitro* show that the mutant alleles can complement telomere length defects of a *stn1* mutant, but not chromosomal fusions. These data suggest TEN1 may mitigate DDR through its interaction with STN1. These mutants should also be tested for their effect on telomerase regulation. Because STN1 associates with enzymatically active telomerase *in vivo* and TEN1 does not, perhaps the interaction of TEN1 with STN1 functions to turn off telomerase-mediated replication. Conceivably, the ability of TEN1 to regulate telomerase is beyond its STN1 interaction. Work from Drs. Katie Leehy and Jung Ro Lee has shown an *in vitro* association of TEN1 with TER2, which also negatively regulates telomerase activity (unpublished data). Mutations that disrupt this interaction may lead to similar telomerase activity profiles as *ten1* nulls. One interesting possibility is that TEN1 associates with TER2 in the absence of TERT, which could explain why TEN1 IPs do not display telomerase activity or TERT protein. This may be important for regulation at the blunt-end of telomeres and may account for the telomere deprotection phenotype of *ten1* mutants (Fig. 5-2; see below).

Examining posttranslational modifications of CST/POT1a proteins

Many recent reports have indicated that post-translational modifications play a significant role in telomere biology. Cdk1 phosphorylation of Cdc13 in yeast augments its binding from Stn1-Ten1 to Est1 thereby promoting telomerase recruitment (Li et al, 2009). It is likely similar events occur in *Arabidopsis*. Potential targets for modification are CST components and POT1a. PhosPhAt 4.0 software, an *Arabidopsis* specific

phosphorylation predictor, indicates the likelihood of serine/threonine/tyrosine modification (http://phosphat.uni-hohenheim.de/). I used this software to screen for potential target residues in POT1a (Fig. 5-3). Alanine mutagenesis can also be performed followed by genetic complementation to see if potential modification sites are important for telomere integrity or maintenance. Confirmation of modification could be achieved by performing phospo-western blotting or mass spec analysis, comparing wild type and mutated proteins from *in vivo* immunoprecipitations. One candidate is a highly conserved threonine residue in the extreme C-terminus of POT1a, T463. Despite its low prediction for phosphorylation by PhosPhAt 4.0, T463A mutants are generally defective in complementing the telomere length defect of *pot1a* null plants (Dr. Xiangyu Song, unpublished data). My i*n vitro* binding studies revealed CTC1 and STN1 bound POT1a T463A at similar levels to wild type, but T463A mutants should be further examined for telomerase RNA binding defects (Kyle Renfrew, unpublished data).

Synergistic function of POT1a and TER2 as distinct regulatory molecules

Chapter III presents evidence that POT1a and TER2 are important regulators of telomere maintenance and integrity, but for different reasons. Initial analysis of *pot1a ter2* double mutants revealed a diverse array of morphological phenotypes. Some plants were relatively wild type in stature and development while others had severe growth retardation, and displayed either minimal or no germline tissue. Specifically, double mutant plants had decreased seed production, and irregularly shaped siliques. Interestingly, recent work by fellow graduate student in out lab showed that the pollen

Y75	0.2365
S82	0.947
S90	0.1218
T113	0.6958
Y136	0.4463
Y166	0.1598
T205	0.1021
T252	0.1016
T265	1.0103
Y267	0.9854
T273	0.6827
S303	0.0181
Y311	0.3278
Y312	0.8361
T357	0.2504
S361	0.193
S365	0.1651
S367	0.2863

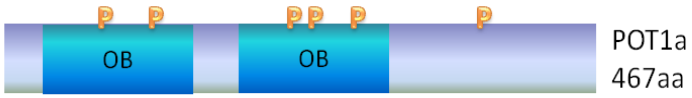


Figure 5-3. Phosphorylation prediction for AtPOT1a. Kinase hotspot prediction by PhosPhAt 4.0 software (http://phosphat.uni-hohenheim.de). The six highest predicted sites are highlighted in red and indicated on POT1a. Phosphorylation sites are targets for mutagenesis and can be tested for *in vitro* binding disruption (CTC1/STN1/TER1) or *in vivo* functional perturbations (telomere maintenance/telomerase RAP).

produced by *ter2* mutants is defective, suggesting why these phenotypes may be exacerbated in the presence of POT1a mutation (Hengyi Xu, unpublished data). My work showed a drastic decrease in telomere length in *pot1a ter2* double mutants, a phenotype unlike either single mutant. Early generation mutants retained a discrete homogeneous telomere banding profile reminiscent of *tert* or *pot1a* mutants. Altogether, these initial data indicate POT1a and TER2 lie in distinct genetic pathways and have unique contributions in telomere biology. The big question with respect to TER2 is what is its actual mechanistic contribution to telomere biology in *Arabidopsis?* Given that TER2 is up-regulated in response to DSBs, and is coincident with a down-regulation in telomerase activity (Cifuentes-Rojas et al, 2012a), this suggests TER2 may be needed to repress *de novo* telomere formation by telomerase at DSBs. However, this does not imply TER2 may not be needed at telomeres given the synergistic telomere shortening phenotypes associated with *pot1a ter2* mutants. More ideas for TER2 function are proposed below.

What is the contribution of POT1a in *pot1a ter2* mutants?

In Chapter II I revealed that TERT is still localized to telomeres at wild type levels in *pot1a ter2* mutants. Therefore, it is important to determine whether the *pot1a* ter2 telomere phenotype is due to a general telomere maintenance defect, or something that is specific to POT1a. Examining *tert ter2* mutants should address this question. If *tert ter2* mutants have similar accelerated telomere shortening, this suggests the lack of telomere maintenance is the factor that reveals the *ter2* defect. If *tert ter2* mutants do not

resemble *pot1a ter2* mutants, this suggests POT1a makes some unique unknown contribution to telomere homeostasis. Recent work in our lab has revealed that the OB1 domain of POT1a specifically binds telomeric ss DNA (Dr. Amit Arora, personal communication) as well as TER1. Perhaps the loss of this DNA binding function in *pot1a ter2* mutants contributes to the *pot1a ter2* phenotype.

A potential role for TER2 in the DDR pathway

A more detailed characterization of the different morphological classes of *pot1a ter2* mutants revealed that the extent of their telomere shortening directly correlated with the severity of their developmental defects. Moreover, the later generation, more severe mutants exhibited smeary and heterogeneous telomere tracts, suggesting their telomeres had become deprotected. Furthermore, I find later generation double mutants with telomeres that fell below the critical threshold of 1kb only displayed mild evidence of fusions., In comparison to telomere capping *ctc1* mutants, *pot1a ter2* chromosomal fusions were not as pronounced, suggesting TER2 may be necessary for eliciting a strong DDR to promote NHEJ, a novel role for telomerase RNAs.

One intriguing aspect of the *pot1a ter2* phenotypes is their resemblance to *atr tert* double mutants which also have accelerated telomere shortening as well as gross developmental defects (Vespa et al, 2005). Like *ter2* mutants, *atr* mutants show no defects in telomere maintenance. Only when combined with a telomerase deficiency, accelerated telomere shortening is observed. Is it possible TER2 similarly has a genetic interaction with ATR? Both molecules are associated with DDR, with ATR being a

primary watchdog against accumulating ss DNA in the cell and TER2 upregulated in response to genotoxic stress. Also, previous work in the Shippen Lab revealed a physical interaction between ATR and POT1b, which also binds TER2 (M. Jasti and D. Shippen, Unpublished data).

To investigate a potential mechanistic link between ATR and TER2 pathways *atr ter2* double mutants should be examined. I expect that the double mutants will have a wild type telomere profile, but the meiotic defects seen in *ter2* mutants may be worse since *atr* mutants have reduced fertility (Boltz et al, 2012; Vespa et al, 2005). It may be necessary to use a "sensitized" background similar to the *pot1a ter2* analysis to specify the contributions of ATR and TER2 in telomere maintenance. If viable, triple *atr ter2 tert/pot1a* mutants could reveal if simultaneous depletion of ATR and TER2 leads to even further telomere shortening versus *pot1a ter2* mutants. Similarly, it would be interesting to compare *atr ctc1/stn1* plants to *ter2 ctc1/stn1* mutants as ATR can suppresses chromosome fusions in CST mutants (Boltz et al, 2012), yet our data indicate TER2 may promote them (Chapter III). Furthermore, *atr* mutants have decreasing telomerase activity beyond the first generation in contrast to *ter2* mutants which display elevated telomerase activity. Perhaps ATR and TER2 contribute to telomerase regulation in different contexts such as at DNA damage or normal telomere maintenance. These results may clarify whether there are intersecting genetic pathways between TER2 and ATR. Biochemical analysis could provide some mechanistic insight as well. Our preliminary data indicate that ATR can immunoprecipitate telomerase activity, however it is not clear if that is the TER1 or TER2 RNP. Repeating this immunopulldown in a

ter2 mutant may abolish that result. Moreover, ATR IPs can be examined by RT-PCR to reveal which TER is enriched. Altogether, these experiments will further our understanding of the telomere interactome in *Arabidopsis*.

Ku, TER2, and blunt-ended telomeres

Another hypothesis that has emerged from recent analysis of TER2 is the idea that TER2 mediates blunt end telomere integrity in concert with Ku. TER2 and Ku interact directly *in vitro* and both are associated with cellular DDR. Ku also represses telomerase-mediated telomere extension as *ku* mutants have telomerase-dependent hyper-elongated telomeres (Riha & Shippen, 2003). An intriguing possibility is that TER2 acts as an RNA scaffold. Because *tert* mutants themselves have more gradual telomere shortening unlike *pot1a ter2* mutants, this suggests that the contributions of TER2 may not be in the context of a telomerase RNP as the *tert* mutation would simultaneously eliminate the TER1 and TER2 RNP pathways. Perhaps TER2 helps Ku binding to telomeres by preventing the Ku clamp from sliding down the chromosome. Loss of TER2 or Ku leads to elongated G-overhangs supporting the idea they may be coordinated at telomeres (Riha & Shippen, 2003). Examining *ku ter2* doubles will assess their genetic interactions. Doubly deficient plants may lead to even further elongated telomeres. Ku may also recruit TER2 to block normal telomere processing events such G-overhang formation (Fig. 5-2). This may explain why *ku* mutants have different telomere profiles than *ter2* mutants as the presence of Ku may mediate blunt end protection even in the absence of TER2. Perhaps TER2 is only needed transiently at

blunt ends. One key experiment is to test cytologically whether the TER2 molecule indeed co-localizes with telomeric DNA and if this is dependent on the Ku-TER2 interaction.

A role for POT1a and TER2 in cell cycle progression

To explain the accelerated telomere shortening of *pot1a ter2* mutants, we asked if the cell cycle was deregulated in this background. Because *pot1a* mutants are incapable of telomere maintenance, it is possible that increasing cell divisions make it appear that the rate of telomere shortening is increased. Initial qPCR analysis of two significant cell cycle mediators, CDKA;1 and CDKB1;1, revealed only slightly different transcript levels in later generation double mutants relative to wild type plants. To more definitively test this idea, further experiments are needed. One indicator of a cell cycle defect could be altered ploidy as endoduplication is disrupted in plants with irregular cycle activity (Dewitte et al, 2003). Double mutants should be examined by FACS analysis in which there might be decreased ploidy in *pot1a ter2* mutants compared to wildtype. It would also be interesting to examine *ter2* single mutants as maybe these plants constitutively have this phenotype, but it is undetected when telomerase can still maintain telomere length homeostasis. Another experiment would be to cross a known cell cycle disrupting mutant with *tert* plants to test whether a cell cycle phenotype is exacerbated. Other indicators of cell cycle defects include changes in cell size or cell number in leaf organs which could also be examined. Plants have surprising plasticity and they can compensate for proliferative defects by altering these properties to maintain

overall leaf shape and size (Riha 2001). Thus subtle changes in leaf morphology might belie important change in cell cycle regulation. Finally, the thymidine analog EdU has been used to examine root meristems undergoing DNA replication. We could employ this technique to test if there are an increasing proportion of cells in S phase relative to wild type. This would be indicative of a population of cells that are dividing more rapidly.

Examining the fate of POT1b post duplication

Because of the explosion of genome sequencing in plants, particularly *Arabidopsis* and its relatives, it is possible to use an evolutionary approach to study the functions of the POT1 gene family. Therefore, I examined the affect of Darwinian selection within the POT1 loci. In other systems such as S. pombe and humans, POT1 is a single copy gene that functions in chromosome-end protection through its association with shelterin and its ability to bind ss G-overhangs. However, in the lineage leading up to Brassicaceae, the POT1 locus underwent duplication resulting in two full length paralagous genes, POT1a and POT1b. Duplication events provide an opportunity for new gene function (neofunctionalization) and separation of function (sub functionalization). They also provide an opportunity for scientists to monitor selection pressures by examining the duplicated genes for signatures of evolutionary forces. We inspected nearly 100 million years worth of evolution using sequences from 14 different plants within Brassicaceae as well as more ancestral single copy plants *Carica papaya* and *Gossypium hirsute.* Branch-site tests using PAML and validation with Bayes

Empirical Bayes (BEB) revealed three divergent amino acid residues (E35, S212, and E293) in POT1a that were indicative of positive selection. Mutations reverting these amino acids back to their ancestral residue (POT1b amino acid) reduced POT1a mediated telomere length complementation and POT1a affinity for CTC1. Thus, POT1a is undergoing selective pressure to enhance its interface with CTC1 and to promote telomere maintenance. This work is a different approach to ascertaining gene function that provides a framework for the evolution and divergence of telomere proteins over the course of millions of years of selection.

Over-expression of a dominant negative allele of POT1b causes abrupt telomere shortening (Shakirov 2005). This could indicate POT1b retained an ancestral function in telomere protection, but it may also be an artifact of over-expression. Another interesting result is the finding that POT1b binds TER2 (Cifuentes 2012). Furthermore, *pot1b* mutants do not disrupt telomerase RAP similar to *pot1a* plants (Kyle Renfrew, unpublished data). Perhaps POT1b, similar to TER2, must be placed in a sensitized genetic background to reveal its function. Mutants of *pot1b* and *pot1a/tert* could have the same phenotype as *pot1a ter2* mutants. If such genetic experiments uncover a POT1b function, it would be important to assess whether that is dependent on the TER2 interaction. Another interesting experiment is testing the DNA binding affinity of POT1b versus POT1a. Recent work by Dr. Amit Arora in our lab has demonstrated POT1a OB1 specifically binds telomeric DNA. This was unexpected as all previous attempts to assess POT1a DNA binding failed to show this property. If POT1b retains some properties of end-protection (Shakirov et al, 2005), it could be predicted that it

retains DNA binding affinity as well. Similarly, it would be interesting to assess how the nucleic acid binding properties of POT1b diverged from other plant species harboring POT1 gene duplications. Such studies may help explain why POT1b has been retained in the genome post-duplication as gene loss is a common consequence of gene duplication (Moore & Purugganan, 2003).

From moss to *Arabidopsis*: How is telomere protection evolving?

Cross species complementation experiments using POT1 genes from *A. thaliana* relatives revealed rapid diversification of POT1a function. Moreover, the ancestral moss *Physcomitrella patens* possesses a single copy POT1 gene that is more similar in function to homologs in vertebrates than plants. PpPOT1 binds ss telomeric DNA and a null mutation leads to shortened telomere tracts, increased G-overhangs, and end-to-end fusions indicating its ancestral role in telomere protection (Shakirov et al, 2010). The POT1 gene duplication provides a plausible scenario that explains how POT1a could have undergone neofunctionalization and function in telomerase regulation. However, the remaining question is what took the place of POT1 in maintaining telomere integrity and when did this occur? The CST complex in *Arabidopsis* displays all the hallmarks of a protective capping complex, but how did it evolve into that role? *Physcomitrella* is a key model system that allows us to test some of these hypotheses. It is possible post duplication, POT1a and POT1b retained end-protection but as POT1a evolved a role off the telomere, CST was simultaneously selected to replace POT1a and POT1b at telomeres. CST counterparts in *Physcomitrella* have been identified. Gene knockouts

and subsequent phenotypic analysis will reveal if CST already possessed properties of end protection similar to PpPOT1 (Shakirov 2010), or evolved these functions in the lineage leading up to *A. thaliana.* One could predict that CST did not possess an end-protection function because there appears to be no redundancy in end-protection from *pot1* knockouts (Shakirov 2010). Reports of CST function in telomere replication have increased over the last decade from yeast to humans. Perhaps PpCST components retain a role in C-strand fill-in or G-overhang processing. One prediction would be that CST knockouts would lead to extended G-overhangs.

Cross-species complementation experiments of more evolutionarily distant CST genes in *Arabidopsis* CST mutants could provide new insights in CST divergence. Because the telomeric DNA sequence is conserved from *Arabidopsis* to *Physcomitrella*, presumably CST proteins from other species could be able to bind *Arabidopsis* telomeres. Previous cross-species complementation analysis of *Brassica Oleracea (Bo)* POT1a only rescued 15% of telomere length while more evolutionarily related *Arabidopsis lyrata* POT1a achieved near full complementation in *A. thaliana pot1a* mutants (Chapter IV). *B. olracea* and *A. thaliana* diverged ~40 million years ago (mya) suggesting this may represent a period when POT1a and CST could have began swapping roles in end-protection. This could be tested by performing a genetic complementation experiment with BoCST proteins in *Arabidopsis* CST mutants. Another prediction is that CST evolved tighter and more specific ss telomeric DNA binding affinity as its role in end protection increased. Cloning CST proteins and

examining their *in vitro* propensity to bind telomeric DNA may provide a pattern of evolutionary selection from moss to *A. thaliana*.

What is the role of POT1a DNA binding in telomerase regulation

Initially, AtPOT1 proteins were not found to bind telomeric DNA, but new data has indicated the first OB fold domain of POT1a binds specifically to ss telomere DNA *in vitro* (Dr. Amit Arora, unpublished data). Why then has POT1a retained telomere binding? It is possible that POT1a OB1 does not reflect the nature of binding with full length POT1a, which needs to be more accurately tested. However, an interesting hypothesis is that POT1a utilizes this property to promote telomerase repeat addition processivity. POT1a may stabilize telomerase-telomere association or facilitate telomerase translocation similar to reported findings for human POT1 (Latrick & Cech, 2010). Mutations in POT1a that specifically disrupt telomeric DNA binding but not CTC1 or STN1are needed to test this hypothesis. These mutants could be used to genetically complement *pot1a* mutants and examined for alterations in telomerase RAP using our TP-TRAP assay. One particular mutant already generated, POT1a F65A, is conserved and was based on the crystal structure of human POT1 binding to ss telomeric DNA (Lei et al, 2004). POT1a F65a mutants have significantly reduced ability to complement telomere length defects in *pot1a* mutants (Dr. Xiangyu Song, personal communication). This mutant protein needs to be verified for loss of DNA binding biochemically. One possibility is that F65A perturbs TER1 binding. Whichever interface is lost (DNA or RNA), complementation lines should be examined to see if these

interfaces are necessary for POT1a's ability to regulate telomerase, and more specifically, telomerase RAP.

Conclusions

In summary, by taking advantage of the benefits of the model system *Arabidopsis thaliana* and the use of genetics and biochemistry, this work has provided new insights into telomere dynamics in higher plants. The interplay of POT1a with the CST capping complex as well as the TER2 RNA was examined and important information was obtained concerning the mechanism of telomere maintenance and protection. Moreover, the unique evolution of POT1 in land plants was studied and the results raised new questions about telomere and telomerase regulation. These studies offer a launching pad for new hypotheses that can refine our understanding of plant telomere biology (Fig. 5-2). Importantly, these analyses will be a significant comparative assessment for vertebrate and yeast model systems and consequently will serve as a basis for revealing unifying principles of telomeres and their dynamics.

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APPENDIX I

MERISTEM DISORGANIZATION 1 ENCODES TEN1, AN ESSENTIAL TELOMERE PROTEIN THAT MODULATES TELOMERASE PROCESSIVITY IN ARABIDOPSIS*

Summary

Telomeres protect chromosome ends from being recognized as DNA damage, and they facilitate the complete replication of linear chromosomes. CST (CTC1(Cdc13)/STN1/TEN1) is a trimeric chromosome end binding complex implicated in both aspects of telomere function. Here we characterize TEN1 in the flowering plant *Arabidopsis thaliana*. We report that *TEN1* is encoded by a previously characterized gene, *Meristem Disorganization 1 (MDO1). A* point mutation in *MDO1*, *mdo1-1/ten1-3* (G77E), triggers stem cell differentiation and death, and a constitutive DNA damage response. We provide biochemical and genetic evidence that *ten1-3* is likely to be a null mutation. As with *ctc1* and *stn1* null mutants, telomere tracts in *ten1-3* are shorter and more heterogeneous than wild type. Mutants also exhibit frequent telomere fusions, increased single-strand telomeric DNA and telomeric circles. However, unlike *stn1* or *ctc1* mutants, telomerase enzyme activity is elevated in *ten1-3* mutants due to an

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increase in repeat addition processivity. In addition, TEN1 is detected at a significantly smaller fraction of telomeres than CTC1. These data indicate that TEN1 is critical for telomere stability and also has a novel role in modulating telomerase enzyme activity.

Introduction

Telomeres are essential for chromosome integrity and consist of tandem arrays simple G-rich repeats terminating in a single-strand 3' extension termed the G-overhang. The telomere tract is bound by proteins that protect the terminus from nucleolytic degradation, inappropriate recombination and activation of a DNA damage response. Telomeres also promote replication of the chromosome terminus through recruitment of telomerase and lagging strand replication machinery. Mammalian telomeres are capped by a six member protein complex called shelterin (de Lange, 2005), while the telomere ends in budding yeast are bound by the trimeric RPA-like CST (Cdc13;Stn1;Ten1) complex (Giraud-Panis et al, 2010).

All three of the yeast CST genes are essential (Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997; Nugent et al, 1996). CST binds the G-overhang primarily through interactions with Cdc13 (Nugent et al, 1996). Cdc13 plays a central role in coordinating telomeric DNA replication by promoting G-strand synthesis via an interaction with the Est1 component of the telomerase RNP, and C-strand synthesis through its association with DNA polymerase □/primase (Chandra et al, 2001; Evans & Lundblad, 1999; Qi & Zakian, 2000; Wu & Zakian, 2011). Mutation of any of the CST

components triggers nucleolytic degradation of the telomeric C-strand, leading to gross extension of the G-overhang (Garvik et al, 1995; Grandin et al, 2001; Grandin et al, 1997).

In budding yeast Ten1 and Stn1 are proposed to regulate telomerase. Telomeres in temperature-sensitive *ten1* and *stn1* mutants elongate in a telomerase-dependent manner (Grandin et al, 2001; Grandin et al, 1997). Furthermore, Stn1 appears to block the binding of Est1 to Cdc13, preventing telomerase action on the telomere (Chandra et al, 2001). Ten1 may act in concert with Stn1 since a Stn1-Ten1 fusion protein rescues the telomere lengthening phenotype of *stn1* (Grandin et al, 2001). Ten1 could also regulate telomerase via a separate mechanism as over-expression of *TEN1* partially rescues telomere defects in *stn1* mutants, hence its discovery as a partial suppressor of *stn1* (Grandin et al, 2001).

The consequences of CST depletion are much less severe in *Candida albicans.* Null mutations in *STN1 or TEN1* are not lethal, and cells do not accumulate single-stranded G-rich telomere DNA (Sun et al, 2009), implying that the essential contributions of CST components are not universally conserved. CST orthologs (CTC1/STN1/TEN1) have been reported in vertebrates and plants (Miyake et al, 2009; Nakaoka et al, 2012; Song et al, 2008; Surovtseva et al, 2009). Although CTC1 shares no sequence similarity with Cdc13 from budding yeast, CST complexes appear to function in similar capacities across eukaryotes.

As in yeast, vertebrate and plant CST interact with pol □ (pol □) (Casteel et al, 2009; Huang et al, 2012; Nakaoka et al, 2012; Price et al, 2010). Indeed, mammalian CTC1 and STN1 were first identified as pol □ accessory factors (AAF) (Casteel et al, 2009). Biochemical studies reveal that vertebrate CST stimulates pol □/primase activity on telomeric substrates (Dai et al, 2010; Huang et al, 2012; Nakaoka et al, 2012), and promotes new origin firing at non-telomeric sites (Stewart et al, 2012b). CST also interacts with the shelterin components TPP1 in human cells (Wan et al, 2009) and POT1b in mice (Wu et al, 2012). Moreover, recent data suggest that human CST modulates telomerase enzyme activity through primer sequestration (Chen et al, 2012). These findings argue that CST coordinates replication of telomeric C and G-strands via dynamic interactions with shelterin, pol α and telomerase.

Despite these biochemical findings, the *in vivo* function of vertebrate CST remains poorly understood. A conditional CTC1 knock-out in mice triggers telomere loss, increased G-overhangs and ultimately activation of an ATR-dependent DNA damage response, primarily in highly proliferating tissues (Gu et al, 2012). Knock-down of CTC1 in HeLa and MCF7 human cell lines leads to similar phenotypes (Surovtseva et al, 2009). More recent studies of CTC1 knock-down in other human cancer lines revealed telomere elongation in one case (Chen et al, 2012), and no significant change in telomere length in another (Wu et al, 2012).

The first reports of CTC1 and STN1 in multicellular eukaryotes came from studies in *Arabidopsis thaliana*. A null mutation in either *AtCTC1* or *AtSTN1* profoundly affects telomere integrity and stem cell proliferation. Although mutant plants are viable,

they exhibit dramatic morphological phenotypes, including abnormally small leaves, irregular phyllotaxy, fasciated stems and reduced fertility (Song et al, 2008; Surovtseva et al, 2009). In addition, telomere tracts are drastically shorter and plants display abundant end-to-end chromosome fusions, enhanced G-overhang signals and telomeric circles (Song et al, 2008; Surovtseva et al, 2009).

Recently, CST was shown to work in concert with the Ku70/80 heterodimer to promote telomere integrity in Arabidopsis (Kazda et al, 2012). Unlike other model organisms, half of the chromosome ends in Arabidopsis, presumably those replicated by the leading strand machinery, are blunt-ended and protected by Ku. The remaining telomeres, replicated by the lagging strand mechanism, possess a canonical G-overhang bound by CST (Kazda et al, 2012). Plants encode only a subset of the vertebrate shelterin components. This observation coupled with the unusual architecture of plant telomeres suggests that CST evolved a more pivotal role than its vertebrate counterparts in protecting chromosome ends throughout the cell cycle (Nelson & Shippen, 2012).
In this study, we examine the contribution of TEN1 in Arabidopsis. We show that *AtTEN1* is encoded by *Meristem Disorganization 1*, a gene recently discovered by Hashimura and Ueguchi (2011) that is crucial for stem cell viability. A point mutation in *MDO1* (*mdo1-1/ten1-3*) causes severe shoot apical meristem aberrations including stem cell death or differentiation, developmental defects and a constitutive DNA damage response. Here we demonstrate that the defects associated with *mdo1-1/ten1-3* result from severe telomere dysfunction. Although most of the mutant phenotypes closely parallel those in plants lacking CTC1 or STN1, an unexpected role for TEN1 in the

negative regulation of telomerase repeat addition processivity was uncovered. Thus, in conjunction with its essential function in telomere protection/replication as a component of CST, TEN1 plays an additional novel role in modulating telomerase activity.

Materials and methods

Plant materials and plasmids

The *ten1-1* mutant was obtained from ABRC. Plants were genotyped using TEN1-1 F and TEN1-1 R (Table 3-1). The *ten1-2AS* lines were created utilizing the Gateway vector pB7WG2 with a 35S promoter (Karimi et al, 2002); two separate constructs were created targeting two separate regions of *TEN1* (Figureure 3-1A, Table 3-1). Antisense constructs were introduced using *Agrobacterium*-mediated transformation (Zhang et al, 2006). Transformed plants were selected on MS (Murashige and Skoog) + Basta plates. The *ten1-3* mutant and the complementation line were obtained from the Ueguchi lab at the Bioscience and Biotechnology Center, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan. Plants were genotyped as previously described (Hashimura & Ueguchi, 2011). Plants were grown in the conditions described (Surovtseva et al, 2007).

Cytology, immunofluorescence and FISH

To examine anaphase bridge formations flower pistils were prepared and analyzed as described (Riha et al, 2001; Song et al, 2008). Immunolocalization and FISH were performed on Arabidopsis suspension cells, MM2d (Menges & Murray, 2002), that were grown under continuous darkness at 130 rpm and a temperature of 25°C. Nuclei

were extracted from one week old cells (Song et al, 2008). The TEN1 was detected with Rabbit anti-TEN1 (1:200) antibody and the signal was amplified using FITC Donkey anti-Rabbit antibody (1:200, Jackson ImmunoResearch). FISH was performed as described (Armstrong et al, 2009).

Antibody preparation, protein extraction and western blot analysis

200 ug *E. coli* expressed AtTEN1 and adjuvant were mixed and injected into rabbits. Blood was collected from the central ear artery and clotted blood was clarified by centrifugation at 2,500 rpm for 20 min. Protein A purification was performed to purify anti-TEN1 antibody. To determine a suitable dilution of the antibody, immunoblotting was conducted with serial dilutions of antigen and stored at -20$_{o}$C. To analyze the expression of TEN1, protein was extracted from wild type and mutant seedlings using CelLytic P protein extraction buffer (Sigma). 45 µg of each protein was used for SDS-PAGE, followed by western blotting. The PVDF membrane was blocked in 6% (w/v) non-fat dry milk in 1X TBST buffer for 2h at RT. The membrane was incubated with anti-TEN1 antibody (1:7,500) in 6% (w/v) non-fat dry milk in 1X TBST buffer for 2h at RT. Anti-rabbit-HRP secondary antibody (1:6,667 of 0.4mg/ml in 50% glycerol, Jackson ImmunoResearch) in the same conditions using ECL prime western blotting detection kit (GE Healthcare).

Transient expression of Flag-HA-TEN1 was performed as described (Zhu et al, 2011) with the following modifications. Leaves were collected 20h after agroinfiltration, ground in liquid nitrogen and resuspended in 1XSDS loading dye (1 ml/g of tissue). 25ul of *N. benthamiana* and 45ug of Arabidopsis total protein were run

on an SDS-PAGE gel. Western blotting with anti-HA was performed with anti-HA antibody (1:3,000; Sigma) and anti-mouse-HRP secondary (1:3,000; GE). Membrane was stripped and probed with anti-TEN1 antibody.

Protein interaction assays

STN1, TEN1, Ku70 and Ku80 cDNA were cloned into pET28a (T7-tag fusion) and pCITE4a vectors (Novagen). Proteins were expressed in RRL (Promega) according to manufacturer's instructions with [^{35}S]-methionine (Perkin Elmer) to label the protein expressed from pCITE4a, and in some cases pET28a. Co-immunoprecipitation was performed as described (Karamysheva et al, 2004). Quantification was preformed by calculating the ratio of TEN1: STN1 signal and comparing, with the wild type interaction set to 100%. For yeast two-hyrbid assays, GAL4-AD or GAL4-BD constructs of TEN1 and STN1 cDNA were transformed and expressed in yeast strain PJ69-4A. To eliminate false positives, the yeast two-hybrid assay was conducted under stringent media conditions consisting of synthetic drop-out (SD)/-Leu/-Trp/-His/-Ade selection medium with 50 mM 3-aminotriazole (3-AT). To confirm positive interactions, we switched inserts from the GAL4-AD vector to the GAL4-BD vector and repeated the assay.

RNA analysis

Total RNA was extracted from plant tissue using a plant RNA extraction kit (OMEGA). Reverse transcription was performed with cDNA Supermix (Quanta) per manufacturer’s instructions. TEN1 mRNA levels were measured by Q RT-PCR with Primers TEN1 Q RT-PCR 1F and TEN1 Q RT-PCR 1R (Table 3-1) using Sso Fast Eva

Green Supermix (BioRad) in accordance with manufacturer's specifications. mRNA levels were normalized to GAPDH and TIP41L mRNA levels in corresponding samples.

TRF, PETRA, and TF-PCR

DNA from individual plants was extracted as described (Cocciolone & Cone, 1993). TRF analysis was performed with 50 □g of DNA as previously described (Fitzgerald et al, 1999). For Bal31 assay 200ug of DNA was incubated with 65 units of *Bal*31 (New England Biolabs) in 1x *Bal*31 reaction buffer. Equal amounts of sample were taken out at 15 or 30 minute intervals for 60 or 90 minutes. Phenol:choloroform extraction was performed followed by isopropanol precipitation. Resuspended DNA was digested by *Tru1I* following the TRF protocol. PETRA and TF-PCR were performed as described (Heacock et al, 2004).

In-gel hybridization and telomere circle amplification

In-gel hybridization was performed as described with the following modifications (Heacock et al, 2007). Exonuclease treatment was performed with T4 DNA polymerase (New England Biolabs) at 37□C for 10 min. Prior to drying the gel the lower portion containing the interstitial telomere repeats was removed and a Southern blot was performed using a [^{32}P] 5' end labeled $(T_3AG_3)_4$ oligonucleotide probe. The relative amount of single-strand G-overhang was calculated by quantifying the hybridization signal obtained from the native gel and then normalizing this value with

the loading control of either interstitial telomere signal from the Southern blot or ethidium bromide staining. The value for wild type was set to one. TCA was performed as previously described (Zellinger et al, 2007).

Telomerase activity assays

Protein for TRAP assays was extracted from flowers and reactions were conducted as described (Fitzgerald et al, 1996). Q-TRAP was performed as discussed in (Kannan et al, 2008). pTRAP protocol was adapted from (Szatmari & Aradi, 2001). Telomerase extension was performed with reaction mix containing 1xGo Taq MasterMix, colorless (Promega), 50 ng protein extract, 0.1□M TRAP forward (Table 3-1), ~5uCi [α-^{32}P]dGTP (PerkinElmer). After telomerase extension, 0.04□M 1RPgg (Table 3-1) was added to the reaction followed by two PCR cycles (95^{o}C for 30s, 60^{o}C for 1m, and 72^{o}C for 45s). After addition of 0.1□M 2RP (Table 3-1) 33 PCR cycles were conducted (95^{o}C for 30s, 64^{o}C for 30s, and 72^{o}C for 1m). Products were ethanol precipitated and resolved on 6% denaturing PAGE, followed by autoradiography. PrimerSequence 5'-3'MDO1-GFGCG AAG CAC GAT TCA AAC CCT TTT CGTMDO1-GRGGT TCG ACA CCA AAC ATC GAG TCC TMDO1-1FGTG TTA TTG AAG ATG GAG GCA GAA GTC TCMDO1-1RCTA AGA TGC TGA ACC TAC ATC GTC TTG AGTEN1 Q RT-PCR 1LCCG TCC ACA TTT CTT CCT GTTEN1 Q RT-PCR 1RTGG AGG CAG AAG TCT CAA AATEN1 G77EGGC TCT ATT TAT CAG TTT ATT GAA GAG CTT CAC ATT GAA CAA CCTEN1 G77E_antisenseGGT TGT TCA ATG TGA AGC TCT TCA ATA AAC TGA TAA ATA GAG CCTEN1 Antisense Const. 1 FCAC CCG CCT CAT TAT TGG GTT GTTTEN1 Antisense Const.

1 RTTG AAC CTG GTG TTC CCA TTTEN1 Antisense Const. 2 FCAC CGA CCA AAA CAT ATC CAC CAT CCTEN1 Antisense Const. 2 RGGC TCG AAC AGG AAG AAA TGTEN1-1 FCAC CCA AAA CTG TCA TCA TTG CTT CATEN1-1 RGCC ATG GCG GCG GTG CAG TTT TTG TAG TTC CAA CAA AGLBa1TGG TTC ACG TAG TGG GCC ATC GTRAP ForwardCAC TAT CGA CTA CGC GAT G1RPgg TAG AGC ACA GCC TGT CCG TGC TAA ACC CTA AAC CCT AAA CCC TAA ACC GG2RPTAG AGC ACA GCC TGT CCG TGPT3AGC ATC CGT CGA GCA GAG TTA GGG TTT AGG GTT TAG GGT TTA GPT6AGC ATC CGT CGA GCA GAG TTA GGG TTT AGG GTT TAG GGT TTA GGG TTT AGG GTT TAG GGT TTA G

Table 3-1. Primers

Results

Identification of Arabidopsis TEN1

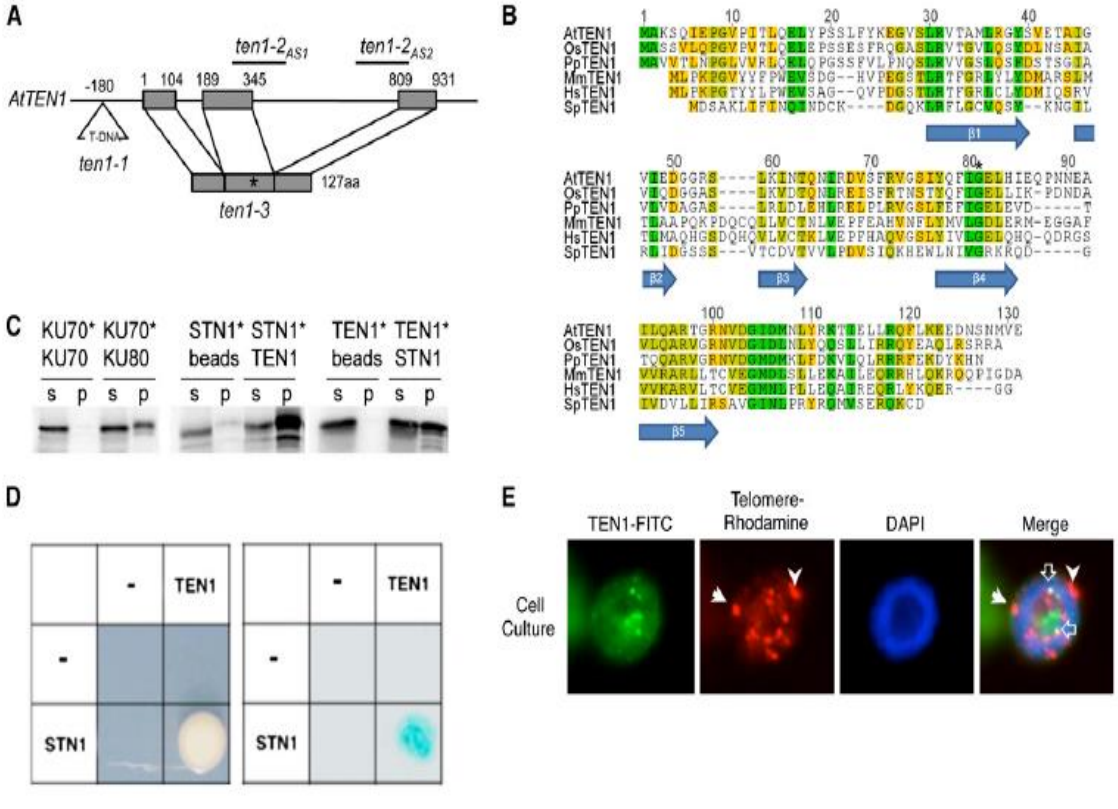
In the second iteration of PSI-BLAST using human TEN1 (Miyake et al, 2009) as a query we retrieved a single hit: NP_176022.2 (E-value=2e-07). This gene, At1g56260, corresponds to *MDO1* (Hashimura & Ueguchi, 2011), and hereafter is termed *AtTEN1*. *AtTEN1* is a single copy gene with one open reading frame interrupted by two introns (Figure A1-1A). The ORF encodes a 127 amino acid protein with 23% identity/48% similarity to human TEN1. Secondary structure prediction by PSIPRED (McGuffin et al, 2000) revealed a single OB-fold that shares significant similarity TEN1 in other plants, as well as in *S. pombe* and humans (Figure A1-1B). RT-PCR amplified a

single *AtTEN1* mRNA species that was expressed widely in Arabidopsis tissues (Figure A1-2A)

A hallmark of TEN1 is its ability to interact with STN1 (Grandin et al, 2001; Martin et al, 2007; Miyake et al, 2009; Nakaoka et al, 2012). To assay for TEN1-STN1 interaction, recombinant proteins were expressed in rabbit reticulocyte lysate (RRL) and labeled with [^{35}S] methionine. One of the proteins was expressed as T7-tagged fusion. Reciprocal co-immunoprecipitation (co-IP) experiments with T7 antibody showed a direct interaction between AtSTN1 and AtTEN1 (Figure A1-1C). Yeast-two-hybrid analysis confirmed this association (Figure A1-1D).

We next asked whether AtTEN1 co-localizes with telomeres using a polyclonal antibody raised specifically against recombinant AtTEN1 (see methods). Immunolocalization was performed with the TEN1 antibody on the nuclei of asynchronously dividing Arabidopsis suspension cell culture. Fluorescence in situ hybridization (FISH) using a rhodamine-labeled telomere probe was used to identify telomeres. AtTEN1 appeared as punctate spots in the nucleus (Figure A1-1E). A merged image of the TEN1 and telomere signals revealed that AtTEN1 co-localized with 13% (15/114) of the telomeres examined (Figure A1-1E, top). A similar value, 12.1% (18/149) was obtained with Arabidopsis seedlings. These observations were unexpected, as previous experiments with transgenic plants expressing a tagged version of CTC1 found that it associated with approximately 50% of the chromosome ends in cycling cells and cells arrested in G1 (Surovtseva et al, 2009). Thus, our data suggest that TEN1 association with telomeres may be more transient than STN1 or CTC1. Moreover,

Figure A1-1. Arabidopsis TEN1 is a member of a CST complex
(A) Schematic of AtTEN1 gene structure. The T-DNA insertion in *ten1-1* is illustrated, along with the position of two anti-sense constructs and the point mutation responsible for the G77E mutation in ten1-3. (B) Alignment of TEN1 proteins from different eukaryotes. At, *Arabidopsis thaliana*; Os, *Oryza sativa* (rice); Pt, *Populus trichocarpa* (poplar); Mm, *Mus musculus*; Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*. The positions of beta-strands of the OB-fold are indicated below the alignment. (C) TEN1 interacts with STN1 in vitro. Results of co-immunoprecipitation performed with recombinant proteins. One protein is [35S] methionine-labeled (asterisk) and the other is T7-tagged and unlabeled. S, supernatant; P, pellet. Results for the positive (KU70/KU80) and negative (KU70/KU70) controls are shown. (D) Yeast-two hybrid assay results for AtSTN1 and AtTEN1. The two proteins fused to GAL4-AD and GAL4-BD were co-expressed and grown on selection plates for His auxotrophy (left) or assayed to detect β-galactosidase activity of positive transformants (right). (-) indicates empty vector. (E) Nuclear localization of TEN1 in purified nuclei. TEN1 was detected by anti-TEN1 antibody in hexaploid Arabidopsis suspension cell culture, Telomeres were labeled by FISH using a rhodamine-labeled telomere probe. DAPI stained nuclei are shown. In the merge, filled white arrows denote sub-centromeric stretches of telomeric DNA on chromosome 1. TEN1 co-localization with telomeres is indicated by the open white arrow.


A
ten1-2AS1
ten1-2AS2
AtTEN1
-180
1 104 189 345
809 931
T-DNA
ten1-1
127aa
ten1-3
B
AtTEN1
OsTEN1
PpTEN1
MmTEN1
HsTEN1
SpTEN1
β1
β2
β3
β4
β5
C
KU70+ KU70
KU70+ KU80
STN1+ beads
STN1+ TEN1
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D
-
TEN1
STN1
E
TEN1-FITC
Telomere-Rhodamine
DAPI
Merge
Cell Culture

because some of the punctate spots recognized by the TEN1 antibody do not co-localize with telomeres, TEN1 may have extra chromosomal functions.

To test whether increased TEN1 expression would drive telomere localization, immunolocalization was conducted on seedling nuclei from a genetic complementation line in which *TEN1* was expressed from its native promoter in a *ten1-3* background (Hashimura & Ueguchi, 2011). In this line the level of TEN1 mRNA was three to ten-fold higher than in wild type plants (Figure A1-2C). The number of telomeres bound by TEN1 increased slightly to 26/127 (20.45%), implying that the low frequency of TEN1-telomere association is not due to coincident overlap or inaccessibility of telomere bound TEN1 to antibody. Instead, the data indicate that TEN1 is associated with a substantially smaller fraction of telomeres than CTC1.

The ten1-3 *mutation causes profound defects in plant development and fertility*

To examine the function of AtTEN1 *in vivo* we initially characterized a T-DNA insertion line (*ten1-1*) that contains a disruption in the 5' UTR of *AtTEN1*, 180bp upstream of the start codon (Figure A1-1A). Quantitative RT-PCR showed ~50% reduction in TEN1 mRNA in homozygous *ten1-1* mutants (Figure A1-2B). In an attempt to achieve a greater TEN1 knockdown, we targeted two regions of *AtTEN1* with anti-sense RNA (TEN1-2$_{AS1}$ and TEN1-2$_{AS2}$)(Figure A1-1A). Quantitative RT-PCR revealed a wide range of TEN1 mRNA depletion. For example, *ten1-2$_{AS1-15}$* showed an 82% reduction in TEN1 mRNA, while TEN1 mRNA levels were reduced by only 10% in *ten1-2$_{AS1-8}$* (Figure A1-2B). Similar results were obtained for *ten1-2$_{AS2}$* knock-down lines.

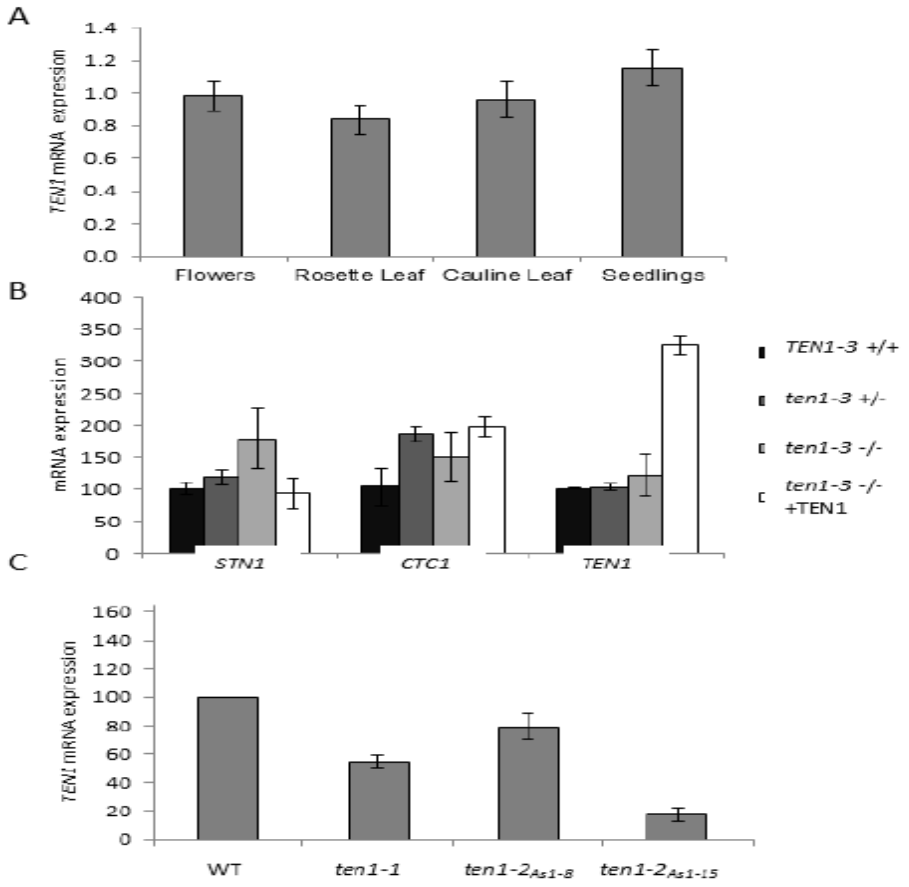


Figure A1-2. *TEN1* mRNA expression levels monitored by quantitative real time PCR. (A) TEN1 mRNA expression in different plant tissues. RNA was analyzed from four week-old wild type (WT) Arabidopsis leaves and flowers and from two week-old seedlings. QRT-PCR was performed with TEN1 specific primers, as well as TIP41L and GAPDH for normalization of cDNA levels. Experiments were performed in triplicate. (B) CST mRNA expression in the *ten1-3* line for whole plant tissue. Results are shown for sibling offspring of *ten1-3* heterozygotes as indicated. Also shown are data for the Native::TEN1 complementation line. Expression data were normalized to GAPDH and compared to expression in wild type siblings. Each bars represent standard deviation for 4 biological individuals, each sample done in triplcate. (C) TEN1 mRNA levels in anti-sense knockdown lines. mRNA was isolated from flowers, data were normalized for GAPDH expression, and values for each experiment compared to wild type. Experiments were performed in quadruplicate

Strikingly, despite the significant reduction of TEN1 mRNA in *ten1-2*$_{AS1-15}$, mutant plants were in marked contrast to *ten1-1* and *ten1-2*$_{AS}$ lines, plants harboring the *mdo1-1* mutation, exhibited severe growth and developmental defects (Hashimura & Ueguchi, 2011) (Figure A1-3), much like those described for *stn1 and ctc1* null mutants (Song et al, 2008; Surovtseva et al, 2009). Hereafter we term the *mdo1-1* mutation, *ten1-3*. Homozygous *ten1-3* mutants showed a lack of apical dominance. Many plants had fused stems, smaller leaves and irregular siliques (Figure A1-3A, middle). Seeds collected from first generation (G1) *ten1-3* mutants displayed remarkably variable germination rates spanning 30-90%. Compared to their parents, second generation (G2) *ten1-3* mutant developed even more slowly and the majority had more severe morphological defects, including the complete absence of apical meristems and exceptionally short roots (Figure A1-3C). As reported earlier (Hashimura & Ueguchi, 2011), over-expression of wild type *AtTEN1* rescued these morphological phenotypes (Figure A1-3A, right), confirming that the *ten1-3* mutation is responsible for the developmental abnormalities.

The *ten1-3* mutation is caused by a glycine to glutamic acid amino acid substitution at position 77 (Figure A1-1A) (Hashimura & Ueguchi, 2011). As expected, the level of *TEN1* mRNA was essentially wild type in *ten1-3* plants (Figure A1-2C). Western blotting was performed to assess TEN1 protein expression. To confirm the specificity of the TEN1 antibody, FLAG-HA-tagged TEN1 was transiently expressed in tobacco leaves and the extracted proteins were used for Western blot analysis with anti-HA and anti-AtTEN1 antibodies. A single band corresponding to the fusion protein was

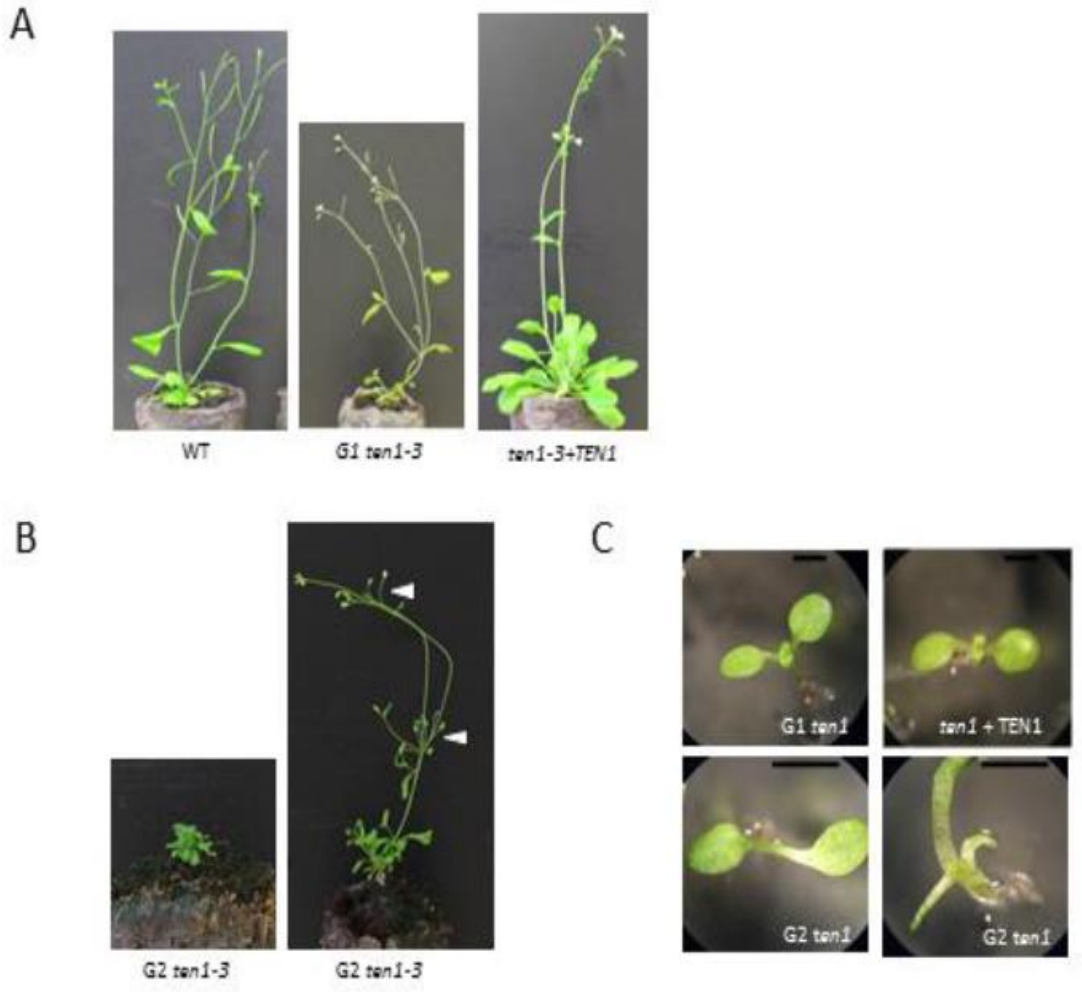


Figure A1-3. The *ten1-3* mutation causes severe morphological defects.
(A) First generation (G1) *ten1-3* mutants are smaller in stature than wild type (WT) and harbor smaller leaves, fused stems and irregular phyllotaxy (middle panel). These phenotypes are rescued by expression of a wild type copy of TEN1 (right panel). (B) Second generation (G2) *ten1-3* mutants display more severe growth phenotype than G1 mutants, and are infertile. Arrowhead denotes aborted siliques. (C) Two week-old seedlings of the genotypes indicated were grown on MS without selection. G2 *ten1-3* mutants exhibit shoot apical meristem abnormalities and fail to produce true leaves. G2 mutants are shown in a 2x zoom to show abnormal apical meristem.

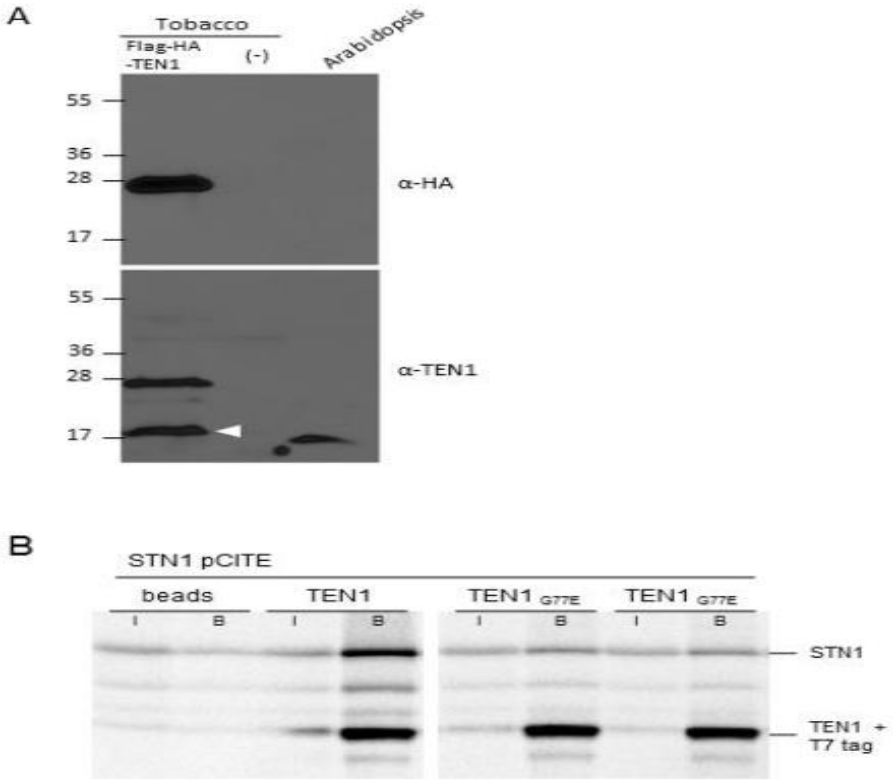


Figure A1-4. G77E mutation alters TEN1 expression *in vivo* and ability to bind STN1 in vitro. (A) TEN1 antibody recognizes transiently expressed Flag-HA-TEN1 in tobacco and endogenous TEN1 in Arabidopsis. The western blot was probed with anti-HA, then stripped and re-probed with polyclonal antibody raised against Arabidopsis TEN1. White arrow indicates lost of tag from transiently expressed TEN1 protein. (B) Pull –down of STN1 by a T7 tagged TEN1 in vitro from RRL expressed proteins by T7 conjugated agarose beads. Proteins are labeled with [^{35}S]-methionine to visualize and run on a 15% gel. This is an example of the data used to calculate the binding in Figure A1C. (I) Input, (B) Beads.

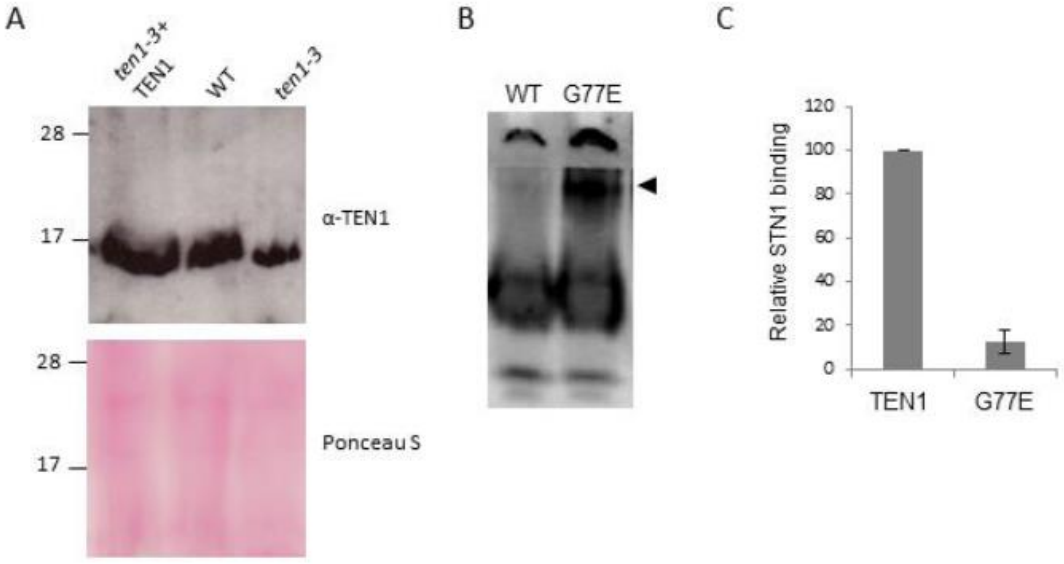


Figure A1-5. The TEN1^{G77E} mutant protein is unstable and does not interact with STN1 in vitro. (A) Western blot results for wild type (WT), *ten1-3* and *the TEN1 complementation line (TEN1-3 + TEN1) are shown*. Ponceau S stain loading controls included. Molecular weight size markers in kD are on the left. The blot was probed with a polyclonal antibody raised against Arabidopsis TEN1. (B) Native PAGE results for recombinant WT TEN1 or TEN1^{G77E} protein expressed in rabbit reticulocyte lysate. Arrow indicates a higher molecular weight polypeptide in the TEN1^{G77E} protein sample. (C) Quantification of recombinant TEN1 protein binding to STN1. Shown are results of co-immunoprecipitation experiments with recombinant WT TEN1 and TEN1^{G77E}. The interaction for WT TEN1-STN1 was set to 100%. An example of raw data is shown in Figure 4B.

detected with anti-HA antibody (Figure A1-4A). The same size product was detected with the anti-AtTEN1 antibody, along with another band of 17 kDa that may represent partial loss of the tags (Figure A1-4A). As expected, a single band corresponding to the predicted size of TEN1 (16 kDa) was observed in wild type Arabidopsis (Figure A1-4A). We found a slight increase in TEN1 abundance in the TEN1 complementation line, and decreased TEN1 in *ten1-3* plants (Figure A1-5A). Notably, attempts to express recombinant TEN1$_{G77E}$ in *E. coli* resulted in significantly lower protein yields than wild type TEN1, arguing that the mutation reduced TEN1 stability.

Although recombinant TEN1$_{G77E}$ expressed in rabbit reticulocyte lysate was soluble, a large fraction of the protein migrated much more slowly than wild type TEN1 on a non-denaturing gel (Figure3-5B), consistent with significant structural perturbation. In addition, co-IP experiments showed that the G77E mutation abolished the interaction of TEN1 with STN1 in vitro (Figure3-5C, Figure3-4B). Since the G77 residue on TEN1 is not predicted to lie within the AtSTN1 binding interface based on the Stn1-Ten1 crystal structure from *S. pombe* (Sun et al, 2009), we conclude that the *ten1-3* mutation profoundly disrupts TEN1 structure and stability. Thus, *ten1-3* mutants can be classified as null or nearly null for TEN1.

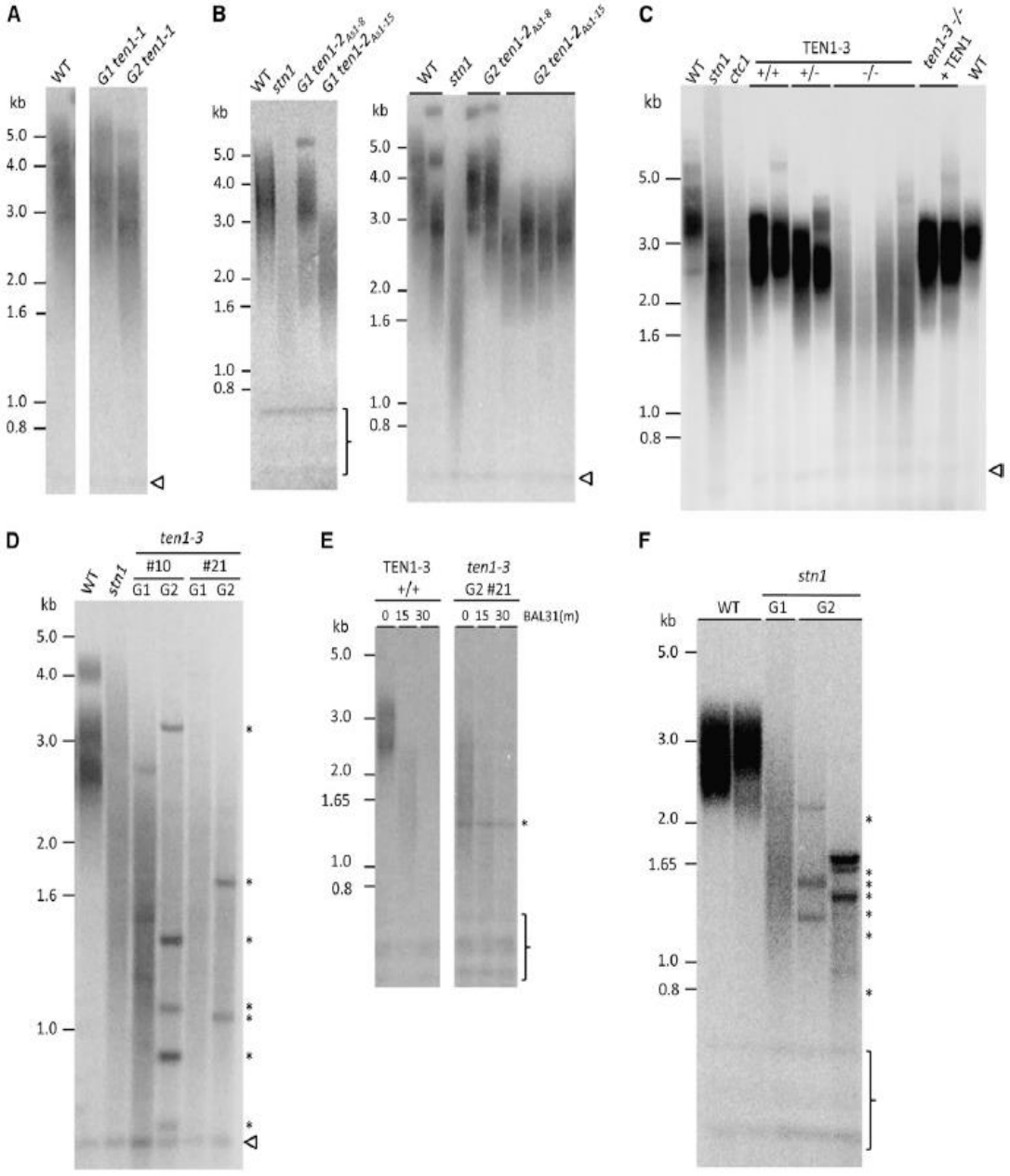
AtTEN1 is required for telomere length maintenance

Terminal restriction fragment (TRF) analysis was performed to analyze bulk telomere length in *ten1* mutants. Although there was no obvious change in telomere length in *ten1-1* or in the *ten1-2$_{AS1-8}$* antisense line relative to wild type (Figure3-6A and B), the telomere profile was altered in *ten1-2$_{AS1-15}$* mutants (Figure A1-6B), where TEN1

mRNA is decreased by 82%. The size range of telomeres contracted and longer telomeres were absent. Stronger evidence that TEN1 is important for telomere length maintenance came from analysis of *ten1-3* mutants. Telomere tracts were more heterogeneous, and significantly shorter overall than wild type or any of the *ten1-2AS* lines, and closely resembled *ctc1* and *stn1* mutants (Figure A1-6C). Primer Extension Telomere Repeat Assay (PETRA), which measures telomere length on individual chromosome arms, confirmed telomere shortening on all arms tested (Figure3-7A). As expected, telomeres in the TEN1 complementation line were wild type (Figure A1-6C), verifying that the *ten1-3* mutation is responsible for the defects in telomere length maintenance.

Unlike *tert* mutants that suffer progressive telomere shortening in subsequent plant generations (Riha et al, 2001), bulk telomeres did not shorten further in second generation (G2) *ten1-3* mutants (Figure A1-6D). However, TRF analysis revealed a new profile of products, consisting of heterogeneous telomere tracts punctuated by multiple discrete bands (Figure A1-6D). G2 *ten1-3* DNA was digested with BAL31 exonuclease prior to TRF analysis to determine whether the bands correspond to terminal DNA sequences (Figure A1-6E, Figure A1-7B). Although bulk telomeric DNA was completely degraded within 30 minutes, the sharp bands were insensitive to exonuclease treatment. Because this banding profile was not observed in G1 *ctc1* or G1 *stn1* mutants (Song et al, 2008; Surovtseva et al, 2009), we asked whether it represented a general response to the prolonged absence of CST. TRF analysis performed with DNA from G2 *stn1* mutants also revealed sharp bands (Figure A1-6F). We hypothesize that these

Figure A1-6. TEN1 is important for telomere length regulation and genome maintenance. TRF analysis of *ten1* mutants**.** Blots were hybridized with a radiolabeled G-rich telomeric probe.(A) Results for first (G1) and second (G2) generation *ten1-1* are shown relative to wild type (WT). (B) Telomere length in first (left) and second (right) generations of two anti-sense knock-down lines of TEN1. For comparison results are shown with first generation *stn1-1* mutants. (C) TRF analysis of *ten1-3* mutants. Results for offspring of *ten1-3* heterozygous plants are analyzed. (D) Parent-progeny analysis for two different *ten1-3* mutants. (E) BAL31 time course of DNA with WT and a G2 *ten1-3* mutant. (F) Telomere profile of G1 and G2 *stn1-1* mutants. Asterisks indicated abnormally sharp TRF bands. Interstitial telomeric DNA repeats are denoted by the bracket, or arrowhead.


A
WT
G1 tlc1-1
G2 tlc1-1
kb
5.0
4.0
3.0
2.0
1.6
1.0
0.8
B
WT
stn1
G1 tlc1-2Δ1-8
G1 tlc1-2Δ1-15
G2 tlc1-2Δ1-8
G2 tlc1-2Δ1-15
C
WT
stn1
ctc1
TEN1-3
+/+
+/-
-/-
tlc1-3 -/- + TEN1
WT
D
ten1-3
#10
#21
G1
G2
E
TEN1-3 +/+
ten1-3 G2 #21
0 15 30
BAL31(m)
F
stn1
WT
G1
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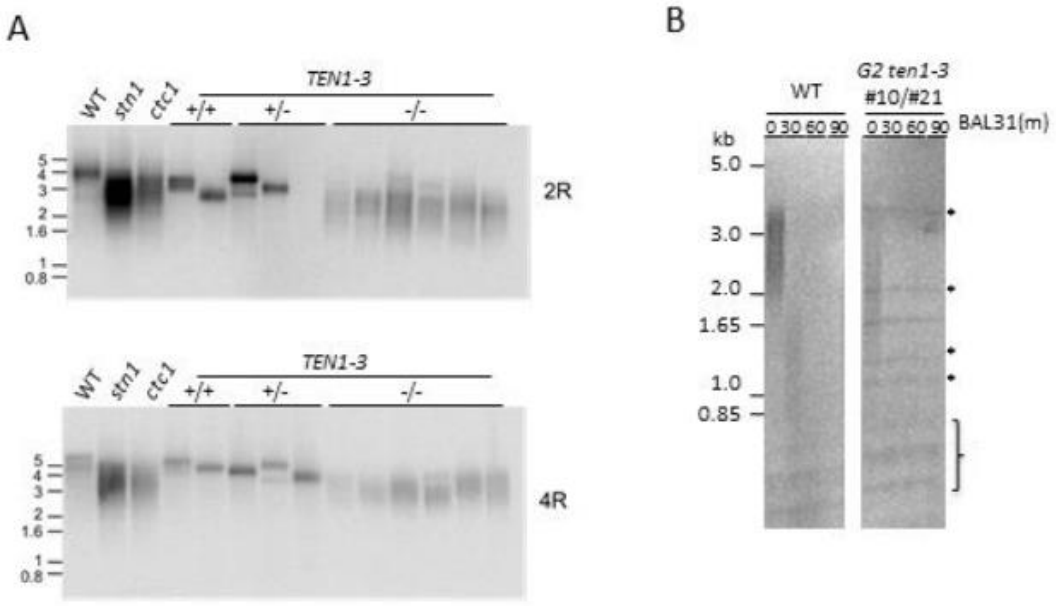

Figure A1-7. Telomere shortening and length deregulation in *ten1-3* mutants. (A) Results of PETRA using subtelomeric primers for 2R and 4R are shown. (B) BAL 31 nuclease time course experiment. DNA from WT and two G2 *ten1-3* mutant plants were treated with BAL 31 for the times indicated followed by digestion with Tru1I nuclease. Asterisk indicates BAL31 resistant digestion products. Previously characterized interstitial telomeric bands are indicated by the bracket. Blots were hybridized with a labeled G-rich telomeric probe. Molecular weight makers are shown.

products reflect gross rearrangements of telomeric DNA, resulting from chromosome end de-protection and multiple rounds of the breakage-fusion-bridge cycle (see below).

AtTEN1 promotes telomere integrity

Telomere Fusion PCR (TF-PCR) was employed to ask if TEN1 is needed to prevent end-to-end chromosome fusions. As for *ctc1* and *stn1* mutants, abundant TF-PCR products were generated in reactions with *ten1-3*, but not wild type DNA samples (Figure A1-8A). Cloning and sequence analysis showed evidence of extensive nucleolytic resection prior to chromosome end-joining (Table 3-2). To obtain a quantitative estimate of telomere fusion events, mitotic chromosomes were examined for evidence of anaphase bridges (Figure A1-8B). 30-50% of anaphases examined in *ten1-3* mutants contained bridged chromosomes, with several involving multiple chromosomes. Notably, this value is significantly higher than number of bridges found in *ctc1* or *stn1* mutants (18-30%).

In-gel hybridization was performed to determine if TEN1 modulates G-overhang architecture. Compared to wild type siblings, *ten1-3* mutants displayed a two-fold increase in G-overhang signal, similar to *stn1* and *ctc1* mutants (Figure A1-8C and D) (Song et al, 2008; Surovtseva et al, 2009). Exonuclease controls confirmed that the hybridization signal was derived from terminal single-stranded telomeric DNA (Figure A1-9A). As expected, the enhanced G-overhang signal was absent in the TEN1 complementation line (Figure A1-8C).

One other hallmark of telomere instability is an increase in the frequency of telomere recombination. The telomere circle assay (TCA) (Zellinger et al, 2007) was

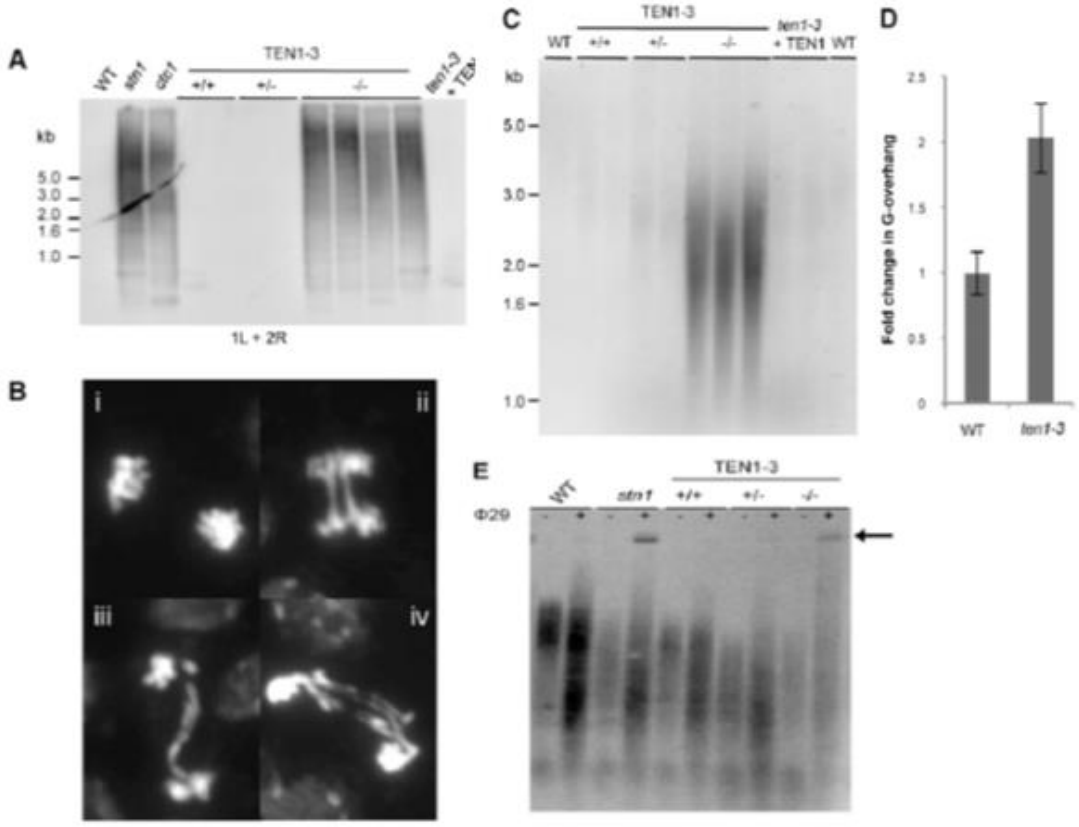

Figure A1-8. TEN1 prevents end-to-end chromosome fusions and promotes proper telomere architecture. (A) Telomere fusion PCR products obtained from wild type (WT) and ten1-3 mutants are shown. Primer pairs used to amplify specific subtelomeric regions are indicated. (B) Cytology of mitotic chromosomes in WT (i) and ten1-3 mutants (ii-iv) are shown. DAPI-stained chromosome spreads were prepared from pistils. (C) In-gel hybridization analysis of DNA isolated from WT and ten1-3 mutants using a C-strand telomeric probe under native conditions. (D) Quantification of the G-overhang signal for ten1-3 mutants. A Southern blot of interstitial telomere DNA or ethidium bromide staining of DNA was used as a DNA loading control for quantification of G-overhang signal. Data represent 7 individual biological replicates of ten1-3. (E) Telomeric circle amplification (TCA) was performed with WT, ten1-3 +/- offspring, and stn1-1 DNA in the presence (+) or absence (-) of phi (□) 29 polymerase. Arrow indicates extra-chromosomal telomere circles.

Type of Fusion	G2 *ten1-1* (n=15)	G1 *ten1-3* (n=12)	G1 *stn1*[1] (n=32)	G1 *ctc1*[2] (n=27)
Telomere-Telomere	13.0%	41.7%	0.0%	0.0%
Telomere-Subtelomere	87.0%	41.7%	21.0%	14.0%
Subtelomere-Subtelomere	0.0%	16.6%	79.0%	86.0%
Features of Fusion Junctions				
Deletion of subtelomereic sequence (avg)	130 bp	213 bp	870 bp	N.A.
Telomeric repeat retained at the fusion junction (avg)	280 bp	103 bp	30 bp	N.A.

[1]Data reported in Song *et al* 2008

[2]Data reported in Surovtseva *et al* 2009

Table 3-2. Sequence analysis of *ten1* mutant telomere fusion PCR products.

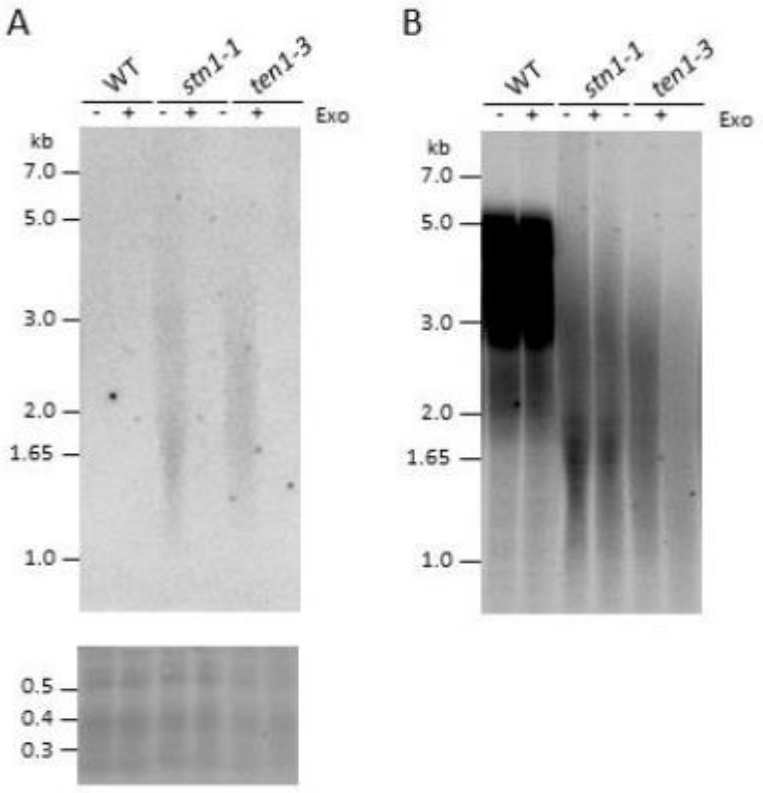

Figure A1-9. Increased G-overhang signals in *ten1-3* mutants in exonuclease sensitive. In gel hybridization results are shown for *ten1-3* and *stn1-1* mutants. Samples were treated with exonuclease as indicated. (A) Products from native in-gel hybridization of G-overhangs (top), Southern blot of interstitial telomeric DNA (bottom). (Right) Denatured gel showing telomere signal.

used to monitor telomere recombination events. As in *stn1* and *ctc1* mutants, *ten1-3* plants displayed increased production of extra-chromosomal telomere circles (Figure A1-8E). We conclude that TEN1, like the other components of CST, promotes telomere integrity by maintaining telomere length and proper architecture of the chromosome terminus.

TEN1 negatively regulates telomerase repeat addition processivity

Plants lacking STN1 or CTC1 exhibit no change in telomerase activity levels (Song et al, 2008; Surovtseva et al, 2009). Therefore, we were surprised to find that telomerase activity was elevated in *ten1-3* mutants (Figure A1-10A). Quantitative telomere repeat amplification protocol (Q-TRAP) revealed, on average, a two-fold increase in enzyme activity in *ten1-3* mutants compared with wild type siblings. The alteration in enzyme activity was somewhat variable with some plants showing only slightly increased activity, while others showed a three-fold increase (Figure A1-11A). When products of the TRAP reaction were resolved by denaturing PAGE, it was evident that *ten1-3* mutants generated substantially more of the longer telomere repeat arrays than wild type (Figure A1-10B). Importantly, the ratio of shorter products generated with *ten1-3* versus wild type was slightly less than 1:1, but increased to more than 10:1 for longer products. This skewed ratio indicates that the *ten1-3* extracts exhibit qualitatively different telomerase activity. Specifically, the findings indicate that telomere repeat addition processivity (RAP) was increased in the absence of TEN1.

A direct, non-PCR based telomerase activity assay is not available for Arabidopsis. Therefore, we modified the processivity TRAP (pTRAP) assay devised for

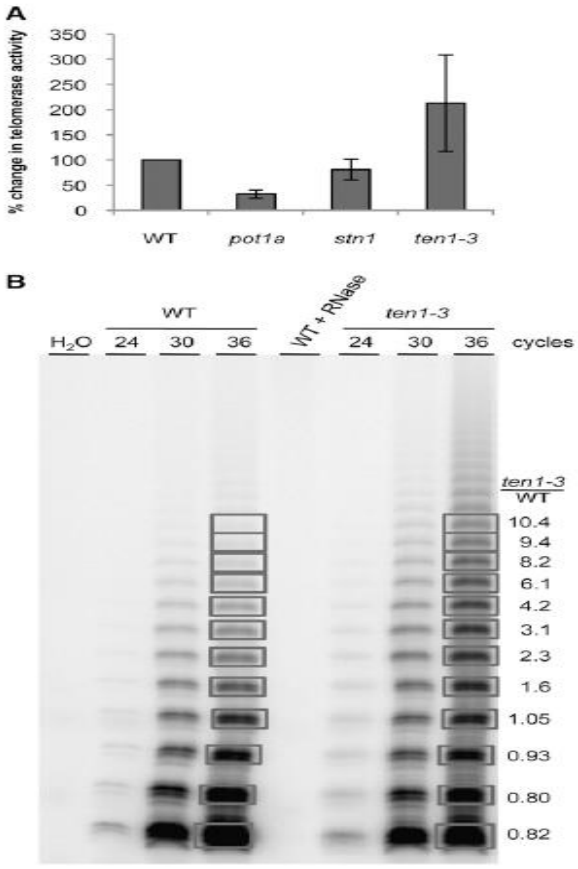

Figure 3-10. TEN1 is a negative regulator of telomerase activity.
(A) Telomerase activity in flowers measured by Q-TRAP. Data are normalized to wild type (WT); each data point represents two or three biological replicates, with three technical replicates. Standard deviation represents the deviation between biological replicates. (B) TRAP products from WT and *ten1-3* mutants at 24, 30 and 36 cycles of PCR resolved by PAGE. Quantification (right) represents the signal for the corresponding bands of *ten1-3* divided by WT for the 36 cycle PCR reaction.

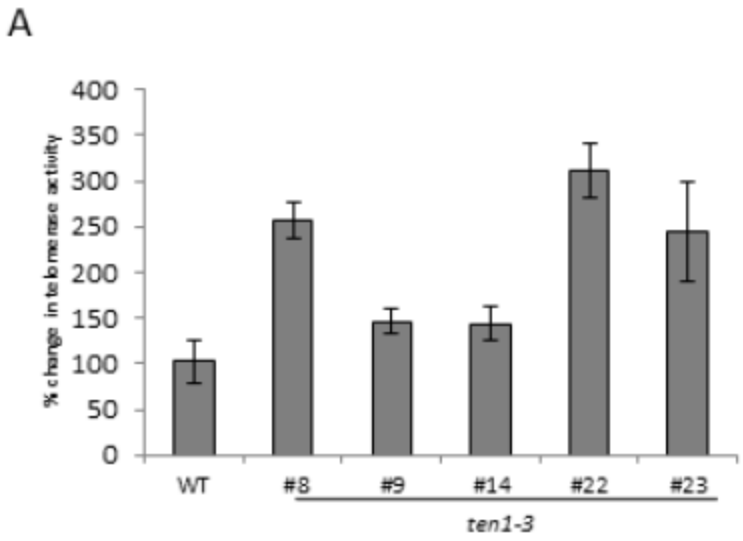

Figure 3-11. Analysis of telomerase enzyme activity in *ten1-3* mutants. (A) Q-TRAP results for flowers from individual *ten1-3* plants. Each data point represents three technical replicates. Error bars represent the standard deviation in technical replicates.

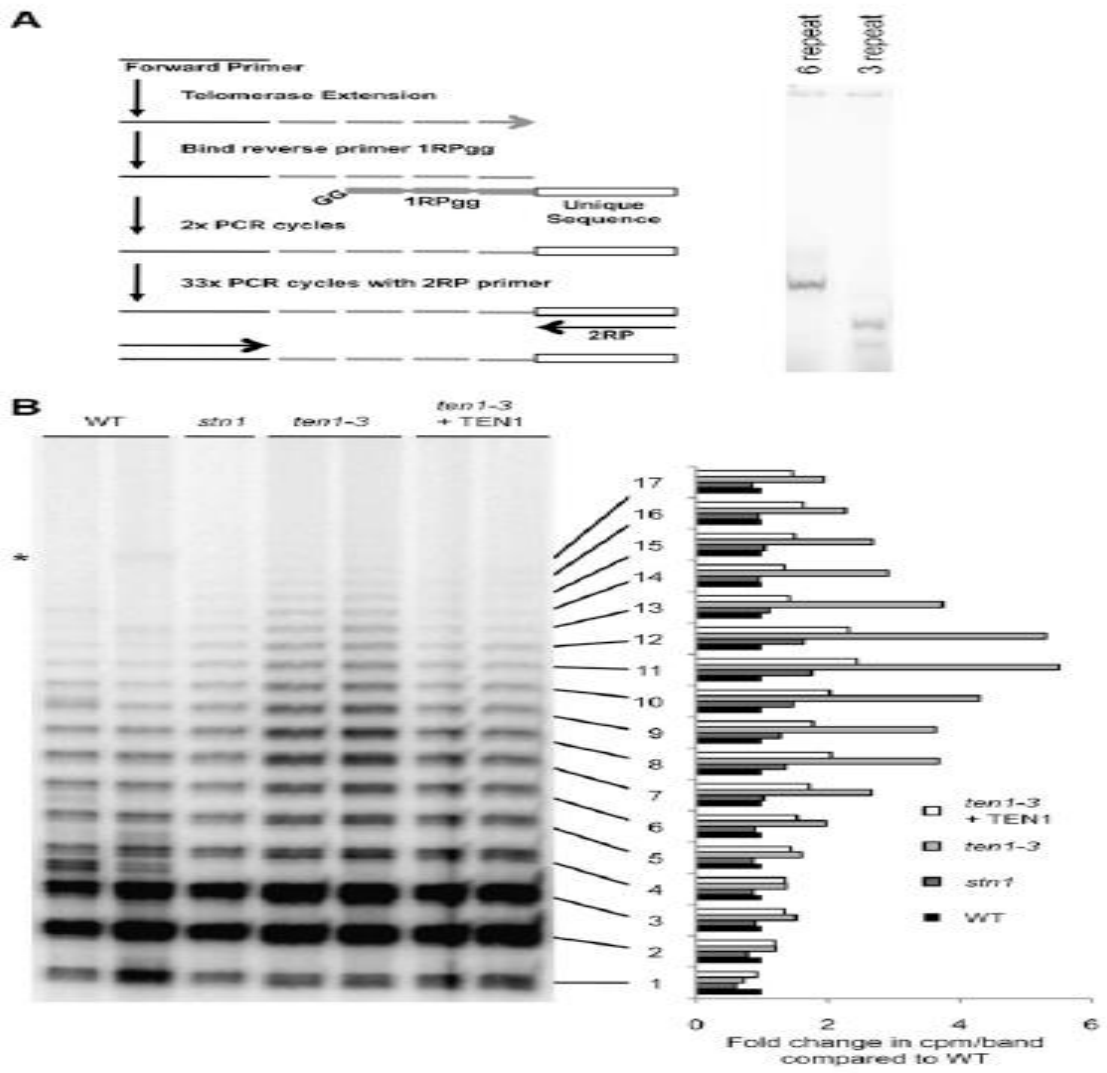

Figure 3-12. TEN1 decreases telomerase repeat addition processivity.
(A) (Left) Schematic of processivity TRAP (Szatmari & Aradi, 2001). (1) Telomerase extends a forward primer substrate. (2-3) Binding of reverse primer 1RPgg, which contains a unique sequence tag on the 5' end, and terminates in two 3' non-complementary G nucleotides that precisely position the primer at the terminus of the extension product. Two cycles of PCR are used to tag the telomerase product with the unique sequence tag. (4) 33 cycles of PCR using the forward primer and 2RP, a reverse primer complementary to the unique sequence tag. Right, Results for control reactions with oligonucleotides containing three (PT3) or six (PT6) telomere repeats (Table 3-1) subjected to steps 2-4 of processivity TRAP. (B) Results of processivity TRAP for floral extracts from wild type (WT), *stn1* and *ten1-3* mutants. Left, telomerase extension products displayed by PAGE. Asterisk denotes non-specific PCR amplification products. Right, quantification of processivity TRAP. Signal was quantified for the individual bands indicated. The average signal was calculated for each genotype (except *stn1*) and that average was compared to the average for WT.

human telomerase (Szatmari & Aradi, 2001) to assess RAP in extracts from *ten1-3* mutants. In this assay telomerase extension products are tagged with a unique sequence that is used for amplification in conventional PCR (Figure A1-12A). Control reactions with synthetic oligonucleotide substrates bearing either three or six telomere repeats yielded the expected products (Figure A1-12A). Examination of pTRAP products showed that the abundance of short products was essentially the same for wild type, *ten1-3* and *stn1-1* mutants. However, the longer products were strongly over-represented in reactions with *ten1-3* (Figure A1-12B). Although the TEN1 complementation line had a slightly elevated processivity, the product profile more closely resembled that of wild type than the mutant (Figure A1-12B). We conclude that TEN1 negatively regulates telomerase enzyme activity by decreasing RAP.

Discussion

Telomere integrity is critical for genome stability and the long-term proliferative capacity of stem cell pools. The *MDO1/TEN1* gene was originally identified in a forward genetic screen for defects in meristem maintenance (Hashimura & Ueguchi, 2011). Here we demonstrate that the molecular basis for stem cell failure is telomere dysfunction. Plants lacking TEN1 harbor short, highly heterogeneous telomere tracts with aberrant G-overhangs that are subjected to inappropriate recombination including massive end-to-end chromosome fusions. These phenotypes are strikingly similar to those of *ctc1* and *stn1* null mutants (Song et al, 2008; Surovtseva et al, 2009), and together with biochemical data showing that AtTEN1 physically interacts with AtSTN1, our findings

argue that TEN1 is a key component of the Arabidopsis CST complex required for genome integrity.

A critical role for CST in cell proliferation in humans is highlighted by a spate of genetic studies demonstrating that compound heterozygous mutations in CTC1 underlie the stem cell disorders Coats plus and Dyskeratosis congenita (Anderson et al, 2012; Keller et al, 2012; Mangino et al, 2012; Polvi et al, 2012). Interestingly, only a subset of patients with CTC1 mutations exhibit telomere shortening (Walne et al, 2012), consistent with the prevailing model that mammalian CTC1 is a multifunctional protein that contributes to different facets of DNA metabolism. We found that only a small amount of TEN1 is sufficient in Arabidopsis, suggesting that conflicting reports pertaining to CST deficiency in mammalian cell lines (Chen et al, 2012; Gu et al, 2012; Surovtseva et al, 2009; Wu et al, 2012) could reflect different levels of depletion. Studies to elucidate how CST contributes to human disease are in their infancy, and thus far no mutations in TEN1 or STN1 have been reported. Relative to CTC1, these genes are much smaller targets for mutation, and given the essential role of STN1 and TEN1 in plants (Hashimura & Ueguchi, 2011; Song et al, 2008); this study), disease-related mutations in their human counterparts may ultimately be recovered.

Several lines of evidence indicate that TEN1 does not always function in concert with CTC1 and STN1. Purification of AAF from human cells revealed the presence of CTC1 and STN1, but not TEN1 (Casteel et al, 2009). In addition, we found that TEN1 associates with a smaller fraction of Arabidopsis telomeres than CTC1. The current model for telomere protection in Arabidopsis proposes that CST is bound to half of the

chromosome ends (Kazda et al, 2012). Consistent with this model, CTC1 co-localizes with approximately 50% of the telomeres (Surovtseva et al, 2009). In contrast, we found only 13-20% of telomeres were bound by TEN1. These results are particularly striking, given that the same relatively low fraction of mammalian telomeres are bound by CST (13-20%) (Miyake et al, 2009). Unlike Arabidopsis CST, mammalian CST plays no significant role in chromosome end protection. Therefore an intriguing possibility is that Arabidopsis TEN1, unlike CTC1 and perhaps STN1, promotes telomere integrity through transient interactions with the chromosome terminus.

The most compelling argument that TEN1 makes a unique contribution outside the context of the trimeric CST complex comes from the unexpected observation that telomerase enzyme activity is elevated in plants lacking TEN1, but not CTC1 or STN1 (Song et al, 2008; Surovtseva et al, 2009); this study). In both conventional TRAP and pTRAP assays significantly longer telomere repeat arrays were generated in *ten1-3* reactions than with *stn1* or wild type extracts, indicating that TEN1 negatively regulates telomerase activity by controlling RAP. Studies in other model systems have uncovered telomerase-associated OB-fold proteins that stimulate RAP. These include p82 (Teb1), a stable component of the *Tetrahymena* telomerase RNP (Min & Collins, 2009), and the mammalian shelterin components TPP1/POT1 (de Lange, 2009). TPP1 stimulates the DNA binding ability of POT1 and the heterodimer then provides a bridge linking telomerase to the telomere (Wang et al, 2007; Xin et al, 2007). We were unable to detect telomerase activity in TEN1 immunoprecipitates (Dr. J.R. Lee and D. Shippen, unpublished data). This result may reflect the transient nature of the TEN1-telomerase

interaction, or the association of TEN1 with telomerase may result in non-processive elongation (e.g. the addition of less than one full telomere repeat). Such products would not be readily detected by TRAP.

It is noteworthy that studies in budding yeast (Grandin et al, 2001) and more recently in human cancer cells (Chen et al, 2012) suggest that CST contributes to the negative regulation of telomerase at chromosome ends. Although we did not observe telomere elongation in *ten1-3* mutants, enhanced telomerase action at chromosome ends would be masked by profound telomere de-protection in this background. Our data are thus consistent with yeast and mammalian CST studies, and go a step further by directly implicating TEN1 in telomerase regulation.