IDENTIFICATION AND CHARACTERIZATION OF *tac5*, A TELOMERASE ACTIVATION MUTANT, CHARACTERIZATION OF DNA DAMAGE RESPONSES AND ASSESSMENT OF INTERACTIONS BETWEEN TELOMERE-RELATED PROTEINS IN *ARABIDOPSIS THALIANA*

A Thesis

by

MADHURI JASTI

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

MASTER OF SCIENCE

May 2007

Major Subject: Biochemistry

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Approved by:

Chair of Committee, Dorothy E. Shippen
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ABSTRACT

Identification and Characterization of *tac5*, a Telomerase Activation Mutant, Characterization of DNA Damage Responses and Assessment of Interactions Between Telomere-Related Proteins in *Arabidopsis thaliana*. (May 2007)

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Telomerase is a ribonucleoprotein complex that synthesizes telomeric DNA in most eukaryotic organisms. Telomerase expression is highly regulated and the enzyme activity is confined to germline and stem cells in mammals and plants. Utilizing a biochemical screening strategy (TRAP assay) to identify genes regulating telomerase expression in *Arabidopsis*, activation tagged lines were screened for mutants demonstrating inappropriate expression of telomerase in leaves. As described in chapter II, the mutant *tac5* was identified in this screening process and was characterized. Segregation analysis demonstrated that telomerase expression in *tac5* is dominant and linked to a complex T-DNA insertion. Plasmid rescue showed that *tac5* mapped to the right arm of chromosome 5. *AtTERT* was transcriptionally upregulated in *tac5* indicating that telomerase activation in *tac5* is either regulated at the transcriptional level or at the post-transcript stabilization level. RT-PCR on genes adjacent to the T-DNA insertion revealed that *NADH/Ubiquinone dehydrogenase*, which is right next to the T-DNA insertion, is transcriptionally upregulated. Overexpression of this gene in wild type plants conferred telomerase activation in leaves, confirming that *NADH/Ubiquinone dehydrogenase* is

responsible for telomere activation. In addition *tac5* showed sensitivity to hydrogen peroxide treatment, suggesting a novel role of telomerase in the mitochondrial environment.

Chapter III reports the role of PARP proteins in plant telomere biology. Both *AtPARP1* and *AtPARP2* are transcriptionally upregulated in response to DNA damage treatment or telomere dysfunction. However, in contrast to mammalian PARPs, the *Arabidopsis* proteins do not appear to have a function in telomere length maintenance as indicated by TRF analysis or in promoting genome stability maintenance as indicated by cytogenetic studies. Further analysis of PARP interactions at dysfunctional telomeres in the genetically tractable *Arabidopsis* model may provide insight into the cellular response to dysfunctional telomeres.

As explained in chapter IV, the yeast two-hybrid screen was utilized to confirm the interactions of ATR with AtPOT2 and Ku80 and to identify novel interacting partners of *Arabidopsis* telomere proteins. At2g04410 (Unknown protein) was identified as a direct interacting partner of AtPOT1. This interaction was confirmed in vitro by co-immunoprecipitation assay. Further analysis of the unknown protein may shed light on AtPOT1's function in telomere maintenance.

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Above all, my whole hearted prostrations to the “ALMIGHTY” for pouring his love upon me to be in this position.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Telomere structure and dynamics

Maintaining genomic integrity is fundamental for the life of a cell and the survival of a species. Telomeres are one of several key elements required for this purpose. Telomeres are specialized nucleoprotein structures that cap chromosome termini to protect them from enzymatic end degradation.

In most species, telomeres contain lengthy stretches of noncoding, tandemly repeated guanine-rich sequences. Typically, telomeric-DNA tracts consist of G-rich repeats that are six to eight nucleotides long (Blackburn, 1991), with the consensus $(TTAGGG)_n$ repeat in vertebrates (Moyzis *et al.,* 1988) and $(TTTAGGG)_n$ in *Arabidopsis thaliana* (Richard and Ausubel, 1988). The length of telomeres also varies in different species. Human telomeres are 8-14 kbp length, whereas in some ciliates, telomeres are as short as 36 bp (Kipling and Cooke, 1990). *Arabidopsis* has 2-5 kbp long telomeres (Richard and Ausubel, 1988; and Shakirov *et al.,* 2005), and in tobacco, telomeres range from 20 – 166 kbp (Suzuki *et al.,* 1994).

The guanine-rich strand (G-strand) of telomere extends beyond the complementary cytosine-rich strand (C-strand), creating a 3’ protrusion called the G-overhang. The G-overhang is required for the association of telomere specific proteins that form the cap to protect the chromosome ends (Henderson and Blackburn, 1989).

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The free 3' end of G-overhang is tucked back into the double-stranded DNA forming a loop structure called t-loop (Griffith *et al.,* 1999). In addition to chromosome stabilization, telomeres also play essential roles in the functional organization of chromosomes with in the nucleus during mitosis and meiosis (Franklin and Cande, 1999), and in regulating expression of telomere-proximal genes (Nautiyal *et al.,* 2002).

Due to the semi-conservative nature of DNA replication, the sequences at the ends of chromosomes will be lost. In addition, telomeres can also be shortened through exonucleolytic degradation (Hackett and Greider, 2003) and through recombinational mechanisms such as Telomere Rapid Deletion (TRD; Li and Lustig, 1996). Eukaryotic chromosomes require mechanisms in addition to the conventional DNA polymerases to complete the replication of their very extreme termini. Otherwise the attrition of sequences from ends of chromosomes would eventually lead to loss of genetic information. The discovery of telomerase provided a general solution to telomere shortening events that is utilized by almost all eukaryotes.

Telomerase structure

Telomerase was originally discovered by *in vitro* biochemical assays using *Tetrahymena* cell extracts (Greider and Blackburn, 1985). Later, it was identified in most eukaryotes. Telomerase is a ribonucleoprotein (RNP) complex that synthesizes the G-rich strand utilizing its RNA moiety as a template (Shippen-Lentz and Blackburn, 1990). Synthesis of the complementary C-strand is thought to occur by the normal cellular DNA replication machinery.

Telomerase consists of two essential components: one is the RNA moiety, which serves as a template for telomere synthesis (TR in mice or TERC in humans or TLC1 in yeast; Feng *et al.,* 1995; Lingner *et al.,* 1997), and the other is a catalytic subunit called Telomerase Reverse Transcriptase, TERT (Kilian *et al.,* 1997). In vitro reconstitution of telomerase activity with these two components indicates that TR and TERT are the minimal requirements necessary for telomerase activity (Weinrich *et al.,* 1997). The catalytic subunit of telomerase, *TERT* was identified in many eukaryotes including *Arabidopsis* (Fitzgerald *et al.,* 1999). However, telomerase action *in vivo* requires several other telomerase associated factors. In budding yeast, telomerase action requires at least four genes, *EST1*, *EST2*, *EST3*, and *CDC13* (Vega *et al.,* 2003). In *Arabidopsis*, the RNA subunit and other components of telomerase holoenzyme have not yet been identified.

Telomerase regulation

Although other mechanisms (primarily recombination) can maintain chromosome termini in the absence of telomerase (Nakamura et al., 1998; McEachern and Blackburn, 1996), telomerase plays an essential role in maintenance of telomere length. Telomerase activity is required for cell proliferation. Regulation of telomerase occurs at several different levels (Cong *et al.,* 2002; and Vega *et al.,* 2003). For *TERT*, these include regulation of transcription, mRNA splicing, and post-translational modifications. In addition, the RNP is subjected to regulation in transport and subcellular localization of each component, assembly and accessibility to telomeres.

In mammals, the telomerase RNA is constitutively expressed (Blasco *et al.,* 1995), whereas *TERT* is expressed only in proliferating cells, but not in most somatic

cells (Liu *et al.,* 2000). As a consequence, telomerase activity is absent in somatic cells but present in cells that are dividing. In *Arabidopsis* also, telomerase activity is limited to cells with high proliferation capacity and is undetectable in most somatic tissue (Fitzgerald *et al.,* 1999). These data indicate that telomerase activity is developmentally regulated both in mammals and in plants. The selective expression of telomerase in rapidly dividing plant cells strengthens the argument that telomerase activation is a conserved mechanism required for long-term proliferation capacity (Fitzgerald *et al.,* 1996).

Telomerase regulation at transcriptional level

The expression pattern of *TERT* indicates that it is the limiting component for telomerase activity and is under more rigorous transcriptional regulation than the RNA subunit. *hTERT* transcriptional activity is positively regulated by transcription factors like SP1 (Kyo *et al.,* 2000), c-Myc (Greenberg *et al.,* 1999), and also by the papillomavirus E6 protein (Klingelhutz *et al.,* 1996). c-Myc recognizes and binds to E-box containing promoters and activates expression of the target gene. Sequence analysis of the *hTERT* promoter region identified two E-boxes upstream of ATG start codon (Takakura *et al.,* 1999). Promoter analysis of *hTERT* also indicates that it lacks a TATA box, but it has five GC-boxes that are putative binding sites for SP1 transcription factor (Kyo *et al.,* 2000). Mad1 and p53 were identified as the negative regulators of *hTERT* transcription (Xu *et al.,* 2001; and Kanaya *et al.,* 2000). Besides these examples, there are many other factors that may repress the expression of *TERT*. These include E2F, histone deacetylases, and Rb family of proteins (Cong *et al.,* 2002; and Takakura *et al.,* 2001). In

Arabidopsis, TAC1, a zinc finger protein, induces transcriptional activation of *TERT* and thereby activates the telomerase in a phytohormone-dependent pathway. Reducing endogenous concentrations of auxin in the mutant blocks the ability of TAC1 to induce telomerase (Ren *et al.,* 2004).

With regard to splicing events, *hTERT* mRNA has at least six splice variants that appear to be developmentally regulated (Kilian *et al.,* 1997; Yi *et al.,* 2000). *Euplotes TERT* contains three splice variants and all these three *EcTERT* genes require +1 ribosomal frameshifting to generate catalytically active TERT protein (Karamysheva *et al.,* 2003). The expression profile of these three genes indicated that they are expressed at different stages of the ciliate cell cycle. In addition, the 5' part of *OsTERT* in rice is subject to alternative splicing events (Heller-Uszynska *et al.,* 2002). In contrast, *Arabidopsis TERT* does not have any alternative splice variants (Fitzgerald *et al.,* 1999).

Another step in regulation of telomerase at the post-transcriptional level is stabilization of RNA component hTR and its functional association with TERT. H/ACA proteins such as Dyskerin play an essential role in stabilization of hTR (Mitchell *et al.,* 1999). In addition, heterogeneous nuclear ribonucleoproteins like C1 and C2 (Ford *et al.,* 2000) and La antigen, which are the components of mature telomerase RNP, play a role in stabilization of hTR component either in its independent form or when complexed with hTERT (Ford *et al.,* 2001).

Telomerase regulation at the post-translational level

Post-translational modification of TERT is crucial for telomerase activity in human cells. Akt kinase and Protein Kinase C regulate telomerase activity by

phosphorylating TERT (Li *et al.,* 1998; Kharbanda *et al.,* 2000). A mechanism by which hTERT protein phosphorylation regulates telomerase activity has also been proposed, in which hTERT stays in the cytoplasm in an inactive, unphosphorylated form while the phosphorylated form translocates to the nucleus, thereby allowing for assembly of active telomerase (Liu *et al.,* 2001). Another post-translational modification, ADP-ribosylation of hTERT by PARPs (Poly (ADP-ribose) polymerases), regulates telomerase activity (Ghosh and Bhattacharya, 2005).

Localization of hTERT is regulated by several hTERT binding proteins. hTERT is found throughout the nucleoplasm in S phase, but is concentrated in nucleoli in the remaining phases of the cell cycle (Wong *et al.,* 2002; and Yang *et al.,* 2002). PinX1p, an inhibitor of telomerase, regulates telomerase by sequestering TERT into the nucleolus, thereby preventing the association of TERT with the RNA subunit (Lin and Blackburn, 2004). In a yeast two-hybrid screen, the 14-3-3 proteins were identified as hTERT binding proteins that regulate telomerase nuclear localization (Seimiya *et al.,* 2000).

Telomerase regulation by telomere binding proteins

The access of telomerase to the telomere is controlled through telomere binding proteins. Several non-nucleosomal proteins bind both double-stranded and single-stranded portions of telomeric DNA and regulate telomerase's accessibility. The major proteins that bind double-stranded telomeric DNA in humans are TRF1 (Zhong *et al.,* 1992) and TRF2 (Bilaud *et al.,* 1997). Rap1 and Taz1 are the double-stranded telomere binding proteins in yeast (Konig *et al.,* 1996; and Cooper *et al.,* 1997). In *Arabidopsis thaliana*, a family of double-stranded telomere binding proteins (TRF-like proteins) was

identified using the MYB DNA binding domain as a query (Karamysheva *et al.,* 2004). Characterization of TRFL proteins in *Arabidopsis* is currently ongoing in the Shippen lab.

Overexpression of TRF1 and TRF2 inhibits telomere elongation, indicating their role in negatively regulating telomerase (Smogorzewska *et al.,* 2000). TRF2 mediates the formation of t-loops *in vitro* (Griffith *et al.,* 1999), while TRF2 is reported to be a negative regulator of telomere length *in vivo* (Karlseder et al., 2002). The yeast Rap1 is also reported to negatively regulate telomerase access to the telomere (Ray and Runge, 1999). The C-terminal domain of RAP1 interacts with two other proteins Rif1 and Rif2, which contribute to telomere length control in yeast (Wotton and Shore, 1997).

The Ku70/80 heterodimer is another double-stranded telomeric DNA associated protein. In human cells, Ku acts as a negative regulator of telomerase-mediated telomere elongation (Hsu *et al.,* 2000), while in yeast Ku70 is a positive regulator and helps to recruit or activate telomerase through interaction with telomerase RNA subunit TLC1 (Boulton and Jackson, 1996; Peterson *et al.,* 2001). Recent evidence also shows that the human Ku heterodimer physically associates with the telomerase holoenzyme through its interaction with hTERT (Chai *et al.,* 2002). In *Arabidopsis*, as in mammalian cells, Ku70 acts as a negative regulator of telomere length (Riha *et al.,* 2002). As expected, analysis of mutants of *AtKu80* revealed the same result as with mutants of *AtKu70* (Gallego *et al.,* 2003).

In addition to these double-stranded telomere binding proteins, protection of G-overhangs and recruitment of telomerase are regulated by the single-stranded overhang binding proteins Cdc13p in yeast (Baumann and Cech, 2001) and POT1 (Protection of

telomeres) in other eukaryotes. Cdc13p contributes to both positive and negative regulation of telomerase by recruiting telomerase to the telomere through its direct association with the EST1 component of the telomerase RNP and by recruiting negative regulators such as STN1p and TEN1p to the telomere (Nugent *et al.,* 1996; and Chandra *et al.,* 2001). A role for *POT1* in chromosome end protection was first illustrated in *Schizosaccharomyces pombe* (Baumann and Cech, 2001). Knocking down the expression of *hPot1* in human cells causes apoptosis or senescence, as well as an increase in telomere-telomere associations and anaphase bridges, telltale signs of telomere instability (Veldman *et al.,* 2004). *Arabidopsis* encodes three POT1-like proteins. *AtPOT1* and *AtPOT2* play roles in telomere length homeostasis and chromosome end protection, respectively (Shakirov *et al.,* 2005). All of these telomere specific proteins form a complex at the telomere, called Shelterin, which acts together with several associated DNA repair factors to mediate structural change in telomeric DNA, allowing its regulation and protection of chromosome ends (de Lange, 2005). As the telomere terminus is expected to be the prime site for telomerase regulation by telomere binding proteins, it is necessary to study the role of telomere binding proteins to explore the mechanism of regulation of telomeres.

Consequences of telomerase inactivation

In cells lacking telomerase activity, telomeres gradually shorten primarily due to the end-replication problem. Once telomeres reach a critical telomere length, cells enter senescence and cease division. Suppression of telomerase expression is directly linked to a human disease Dyskeratosis congenital (DKC), which results from inactivation of

Dyskerin, a telomerase RNP component (Marciniak and Guarente, 2001). DKC cells have a lower level of telomerase RNA, produce lower levels of telomerase activity and have shorter telomeres than normal cells, which limits their proliferative capacity. In contrast, overexpression of *TERT* results in extended life span of human cells (Bondnar *et al.,* 1998). In addition, *TERT* exhibits neuroprotective properties in experimental models of neurodegenerative disorders suggesting that manipulations that induce telomerase in neurons may protect against age-related neurodegeneration (Mattson, 2000). In summary, telomerase is both an important target for the treatment of cancer and age-related disease.

Telomerase acts preferentially on shorter telomeres, with a gradual decline in activity as telomeres become elongated (Shakirov and Shippen 2004). The rate of shortening of telomeres in the absence of *TERT* ranges from 3-5bp/division in *S. cerevisiae* (Teng and Zakian, 1999) and 50-150bp/division in mammals (Harley *et al.,* 1990). In *Arabidopsis*, disruption of *TERT* leads to shortening of telomeres by 250-500bp/generation (Riha *et al.,* 2001).

Cells from mice in which the *mTERT* gene has been disrupted show progressive loss of telomere DNA, and by the sixth generation (G6), *mTERT*-deficient embryonic stem (ES) cells exhibit genomic instability, aneuploidy and telomeric fusions (Liu *et al.,* 2000). In contrast, *Arabidopsis* can survive ten generations without telomerase, but the last five generations undergo increased levels of cytogenetic damage and developmental anomalies (Riha *et al.,* 2001). The differences in plant and animal responses to dysfunctional telomeres suggest that cellular responses to telomere dysfunction are unique to plants and reflect the plasticity of their development and genome organization.

Telomeres and DNA damage

The crucial feature of telomeres is that they mask the ends of linear chromosomes from being recognized as double-strand breaks. Telomere dysfunction elicits a DNA damage response. hPOT1, a single stranded telomere binding protein protects telomere from a transient DNA damage response by determining the structure of 3' and 5' ends of the chromosomes (Hockemeyer *et al.,* 2005). In addition, mPOT1 deficiency leads to DNA damage checkpoint activation and aberrant homologous recombination at telomeres (Wu *et al.,* 2006). In mammals, removal of TRF2 from the telomere leads to a DNA damage response that results in cell-cycle arrest or apoptosis, depending on the cell type (van Steensel *et al.,* 1998). Moreover, the DNA damage responses in senescent and TRF2-inhibited cells appear to be strikingly similar (d'Adda di Fagagna et al. 2003; Takai *et al.,* 2003). Similarly, progressive telomere shortening caused by inactivation of telomerase in *S. cerevisiae* leads to the accumulation of cells that are unable to divide further and which display an activated DNA damage response, as determined by the phosphorylation of Rad53p (Enomoto *et al.,* 2002). Taken together, these results suggest that the loss of telomeric DNA is not detrimental per se, but it is the loss of telomere-associated factors that results in telomere deprotection and concomitant activation of the DNA damage responses.

Probably the best characterized and most highly evolutionarily conserved features of the DNA damage response are the recruitment of DNA-repair proteins to sites of DNA damage and the checkpoint events that slow down or arrest cell-cycle progression, thus delaying key cell-cycle transitions until the damage has been removed (Zhou and Elledge,

2000; Khanna and Jackson, 2001). The earliest well-characterized earliest responses to induction of double-strand breaks in humans are the phosphorylation of a histone variant H2AX (γ -H2AX) at the break site (Paull *et al.,* 2000) and transcriptional upregulation of PARPs (Poly ADP-Ribose Polymerases; Fernet *et al.,* 2000). γ-H2AX induces changes in local chromatin structure and facilitates the focal accumulation of DNA-repair and checkpoint proteins to the damaged regions. PARPs rescue cells from DNA damage by recruiting scaffold proteins to the damaged site and mediating repair. Inhibition of TRF2 showed the H2AX foci formation and checkpoint activation in human cells (Karlseder *et al.,* 1999).

Phosphorylated H2AX is recruited to dysfunctional telomeres in human cells (Takai *et al.,* 2003). To date, it has not been studied in plants whether telomere dysfunction elicits DNA damage response. However, ionizing radiation induces the formation of H2AX foci in *Arabidopsis* at the sites of DNA double-strand breaks and this H2AX activity is ATM (ataxia telangiectasia mutated kinase) dependent (Friesner *et al.,* 2005).

Transcriptional upregulation of PARPs in response to DNA damage by ATM kinase was reported in *Arabidopsis* (Culligan *et al.,* submitted). hPARP1 is a protein involved in the base excision repair process that occurs preferentially at eroded telomeres in response to DNA damage. hPARP1 protects the telomeres from end to end fusions, thereby maintaining the genomic stability (Gomez *et al.,* 2006). Phosphorylation of hTRF2 in response to the DNA damage provides an example of direct cross-talk via a signaling pathway between different cellular processes essential for genomic stability, telomere maintenance, and DNA repair (Tanaka *et al.,* 2005).

Many proteins involved in DNA repair are implicated in telomere maintenance. Mammalian cells with mutations in AT (Ataxia Telangiectasia), a key regulator of DNA damage response, displays accelerated telomere shortening and higher frequencies of end to end fusions (Metcalfe *et al.,* 1996). Similarly, yeast cells defective in Ku, a key component of the Non-homologous End Joining (NHEJ) pathway give rise to a similar phenotype (Boulton and Jackson, 1996). Deficiency of the DNA dependent protein kinase catalytic subunit (DNA-PKcs), an enzyme involved in NHEJ pathway for repair of double-strand breaks, leads to telomeric fusions and abnormal telomere elongation (Bailey *et al.,* 1999). Even in wild type cells, human telomeres are transiently recognized as DNA damage in G2 stage of the cell cycle and recruit MRN and ATM with a partial release of POT1 (Verdun *et al.,* 2005). These results suggest a role for DNA repair proteins in protecting chromosomal integrity by balancing repair activities and telomere maintenance.

As discussed above, *Arabidopsis* has an extreme tolerance to genomic instability. ATM and ATR, the key protein kinases in the DNA damage pathway, make essential contributions to chromosome end protection and telomere maintenance, respectively, in *Arabidopsis* (Vespa *et al.,* 2005). In addition, Ku, another DNA repair gene, is a negative regulator of telomere length maintenance in *Arabidopsis* (Riha and Shippen, 2003). Due to the critical role of both DNA damage repair and telomere homeostasis in maintaining genetic stability, the knowledge of their interconnections is essential for understanding key cellular controls.

PARP1 and PARP2

Poly ADP-ribosylation is an early DNA damage dependent post-translational modification of histones and other nuclear proteins that contributes to the survival of damaged cells. Poly(ADP-ribose) polymerases (PARPs) catalyze the synthesis of ADP-ribose polymers and attach them to specific target proteins. A superfamily of eighteen *PARP*s has been identified so far in humans. Several PARP family members associate with telomeres or telomerase (Smith *et al.,* 1998). Inhibition of PARP activity results in decreased telomerase activity in human cells, suggesting a role for PARPs in regulating telomerase activity (Ghosh and Bhattacharya, 2004).

PARP1, the founding family member, has been extensively studied in mammals. In response to DNA damage, PARPs utilize NAD^+ to synthesize a multibranched polymer of ADP-ribose on itself and on various acceptors (De Murcia *et al.,* 1994). *PARP1* deficient mice show hypersensitivity to ionizing radiation and various alkylating DNA damage agents (D'Amours *et al.,* 1999). *PARP1* deficient mice exhibit normal telomere length and chromosome end capping (Samper *et al.,* 2001). However hPARP1 is associated with TRF2, and appears preferentially at eroded telomeres after induction of DNA damage to protect telomeres against end to end fusions and genomic instability (Gomez *et al.,* 2006). These data suggest that PARPs play a key role at dysfunctional telomeres to maintain genomic stability.

PARP2 was identified as a result of the presence of residual DNA-dependent PARP activity in *PARP1* deficient mice (Shieh *et al.,* 1998). Like PARP1, PARP2 also interacts with TRF2 and regulates the telomeric binding activity of TRF2 through poly ADP-ribosylation (Dantzer *et al.,* 2004). In addition, *PARP2* deficient mice exhibit

normal telomere length and telomere capping, but display an increase in chromosome ends lacking detectable telomeric DNA (Dantzer *et al.,* 2004). Since the double mutant for *PARP1*/*PARP2* is lethal in mice (de Murcia *et al.,* 2003), investigation of PARPs in other systems is necessary to understand their role at telomeres. Tankyrase, another poly (ADP-ribose) polymerase, alters telomere homeostasis by affecting the localization of TRF1, a double-stranded telomere binding protein (Smith *et al.,* 1998). These results indicate that poly ADP-ribosylation is necessary to maintain the genomic integrity.

With regard to plants, PARP activity has been demonstrated in isolated nuclei from cultured tobacco cells (Willmitzer and Wagner, 1982). Later studies with maize, pea and wheat nuclei confirmed the presence of PARP activity in plants (Chen *et al.,* 1994). In *Arabidopsis*, PARP1 and PARP2 are induced in response to DNA damage (Doucet-Chabeaud *et al.,* 2001).

Thus, poly ADP-ribosylation plays an important role in the recovery of proliferating cells from certain types of DNA damage. However, it is not clear whether plant PARP genes have a similar function as animal PARPs in terms of maintenance of genomic stability. Therefore, studying the role of PARPs in plants with telomere dysfunction might shed light on their function in response to DNA damage.

Arabidopsis thaliana as a model plant for telomere biology

Arabidopsis thaliana is a small flowering plant and a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. *Arabidopsis* is not of major agronomic significance; however it offers advantages for basic research in genetics and molecular biology, because of its small, sequenced genome

(130Mb). In addition, *Arabidopsis* telomeres are short (2-5kb) and adjoined by unique sub-telomeric sequences on seven of the ten chromosome ends, making this plant a powerful tool for studying the dynamics of telomeres. Most importantly, *Arabidopsis* exhibits extreme tolerance to telomere dysfunction and its ability to withstand the loss of DNA damage proteins that are essential in other model systems makes it a good system to study the cellular responses to uncapped telomeres.

The main goals of my projects are to find how plants regulate telomerase expression and how plants respond to DNA damage using *Arabidopsis* as model system. In spite of the evolutionary and developmental divergence between *Arabidopsis* and humans, the overall pattern of telomerase expression is similar in these two organisms. However, telomerase regulation has not been studied in detail in *Arabidopsis* so far. Characterization of telomerase mutants should increase understanding of the pathways that regulate the expression and activity of this essential enzyme in plants. With respect to DNA damage responses, mechanisms of DNA repair process in plants are just beginning to be elucidated.

CHAPTER II

CHARACTERIZATION OF A TELOMERASE ACTIVATOR MUTANT (*tac5*) IN *ARABADOPSIS THALIANA*

Introduction

Telomeres are complex nucleo-protein structures at the ends of linear chromosomes and consist of six to eight tandem, G-rich nucleotide repeats (McClintock 1939; and Moyzis *et al.,* 1988). The telomere repeat sequence is similar in many species: TTAGGG in humans (Moyzis, 1988) and TTTAGGG in *Arabidopsis* (Richards and Ausubel, 1988). Telomeric DNA, along with telomere binding proteins form a protective t-loop, a cap structure that protects the chromosome ends from exonucleolytic degradation and terminal fusions (Van Steensel and de Lange, 1997; van Steensel *et al.,* 1998; and Griffith *et al.,* 1999).

Maintenance of telomeres is carried out by a telomere specific DNA polymerase called telomerase, a ribonucleoprotein (RNP) complex (Collins and Mitchell, 2002). Telomerase consists of a reverse transcriptase (TERT) subunit that catalyzes the addition of telomere repeats to the 3' G-overhang through reverse transcription of an intrinsic RNA (TR) moiety that carries a template complementary to the G-rich strand (Greider and Blackburn, 1985; and Shippen-Lentz and Blackburn, 1990). Absence of telomerase leads to an unsolved end-replication problem, thereby causing the progressive shortening of telomeres, which ultimately leads to instability of the genome typified by chromosome fusions (Riha *et al.,* 2001).

Expression of telomerase is developmentally regulated in higher eukaryotes in order to maintain the telomere length dynamics. In spite of the evolutionary and developmental divergence between humans and *Arabidopsis*, the overall pattern of telomerase expression in plants is similar to that in humans (Fitzgerald *et al.,* 1996). In humans, telomerase activity is abundant only in germ line cells, regenerating tissues and many types of cancer cells (Kim *et al.,* 1994), while in plants; telomerase activity is confined to reproductive and highly proliferative tissues and undetectable in vegetative tissues (Fitzgerald *et al.,* 1996; and Riha *et al.,* 1998). Telomerase activity in humans is primarily regulated by transcription of the *TERT* gene (Wu *et al.,* 1999 and Kyo *et al.,* 2000). h*TERT* promoter has been characterized to identify transcription factor binding sites for regulating the catalytic action of the enzyme (Grandori *et al.,* 2000). In addition to transcriptional regulation, post-transcriptional processing events also play a significant role in regulating telomerase activity (Kharbanda *et al.,* 2000).

Regulation of telomerase has not been studied in detail in *Arabidopsis*. However, telomerase activity in *Arabidopsis* correlates with *TERT* mRNA levels (Fitzgerald *et al.,* 1999), suggesting that as in humans, transcriptional regulation is the major means for regulating telomerase activity. TAC1, a zinc finger protein, has been shown to act synergistically with auxins and regulate telomerase activity transcriptionally (Ren *et al.,* 2004). Evidence indicates that telomerase activity in rice is also regulated by alternative splicing of *TERT* (Heller-Uszynska *et al.,* 2002). No such alternative splicing events have been reported in *Arabidopsis* (Fitzgerald *et al.,* 1999).

To further explicate the mechanisms of telomerase regulation in higher eukaryotes, we made an attempt to identify telomerase mutants with altered patterns of

telomerase expression. *Arabidopsis thaliana* is an ideal model for its relatively small genome size; availability of complete genome sequence and availability of mutants for functional genomic studies.

In an attempt to identify genes that regulate telomerase expression in *Arabidopsis*, we screened activation-tagged lines. Activation tagging utilizes a T-DNA sequence that contains four tandem copies of the cauliflower mosaic virus (CaMV) *35S* enhancer sequence, which enhances the expression of neighbouring genes on either side of the randomly integrated T-DNA tag, resulting in gain-of-function mutants (Weigel *et al.,* 2000). This technique has identified a number of genes fundamental to plant development (Boerjan *et al.,* 1995), disease resistance (Xia *et al.,* 2004), and telomerase regulation (Ren *et al.,* 2004).

The present study reports the identification and characterization of a telomerase mutant, *tac5*, recovered in an activation tag screening process. *tac5* mutant showed telomerase activity in leaves and also showed upregulation of *TERT* mRNA levels in *tac5* leaves. Our data reveal that the gene activated in *tac5* mutant is *NADH-Ubiquinone dehydrogenase*, which encodes a complex I subunit in mitochondria. In addition, *tac5* mutant also showed H_2O_2 sensitivity indicating that NADH-Ubiquinone dehydrogenase is part of a pathway linking oxidative stress signaling and telomerase activation.

Materials and methods

Plant materials and growth conditions

Activation-tagged lines of *Arabidopsis thaliana* from the Weigel collection CS23153, a set of sixty-two pools of 100 lines from ecotype Columbia Col-7, were

purchased from the *Arabidopsis* Biological Resource Center (Ohio State University). Each of the sixty-two pools of one hundred lines was divided among four flats of soil labeled A, B, C, and D, cold-treated overnight at 4°C, and placed in a growth chamber under continuous light at 23°C. Each plant was pulled from a flat and one leaf was collected and pooled in a 15mL Falcon tube, immediately frozen in liquid nitrogen, and stored at -80°C until use. Another leaf from each of the plants was collected and pooled in a 15mL Falcon tube, immediately frozen in liquid nitrogen, and stored at -80°C to reserve extra material.

Backcrosses were performed between *tac5* and wild type to generate F1 and F2 populations for segregation analysis of T-DNA insertions. Leaf samples from each plant were collected and frozen in liquid nitrogen and stored at -80^{o}C for later extraction of DNA, RNA and proteins.

Plant protein extraction

Leaf samples were ground in a mortar and pestle under liquid nitrogen and resuspended the tissue in a ratio of 4 mL per gram of tissue with buffer W (50 mM Tris-acetate, pH 7.5; 5 mM $MgCl_2$; 100 mM potassium glutamate; 20 mM EGTA; 1 mM DTT; 0.1 mM PMSF; 0.6 mM vanadyl ribonucloside complex; 1.5% (wt/vol) polyvinylpyrrolidone and 10% glycerol) at 4^{o}C. The samples in the buffer W were incubated for 10 min on a labquake and centrifuged for 15 min at 14K rpm at 4^{o}C. The supernatants were transferred to a new microfuge tube containing PEG800 to a final concentration of 10%, mixed on a labquake for 30 min at 4^{o}C and centrifuged at 14K rpm at 4^{o}C. The pellet was resuspended in 50 μL of buffer W and mixed on a labquake for 30

min at 4°C. These tubes were centrifuged for 2 min at 14K rpm at 4°C. The samples were frozen in liquid nitrogen and stored at -80°C until use.

Telomere Repeat Amplification Protocol (TRAP) assay

Telomerase activity in the leaves of activation tagged plants was detected by the TRAP protocol (Fitzgerald *et al.,* 1996). The following primers (Gene Technology Laboratory, Texas A&M University) were used in the TRAP assay: Forward primer (TRAPForG) 5'(CACTATCGACTACGCGATCAG)3' and Reverse primer (TRAP-RV) 5' (CCCTAAACCCTAAACCCTAAA) 3'. Reaction mixtures were prepared in a total volume of 50 µl :50 mM Tris-acetate, pH 8.3; 50 mM Potassium Glutamate; 10 mM EGTA; 5 mM $MgCl_2$; 0.1% Triton X-100; 1 mM spermidine; 1 mM DTT; 50 µM each dATP, dGTP, dTTP; 0.5 µl (α^{32}P) dGTP (3000 Ci/mmol; Amersham Biosciences); 100 ng/ µl BSA; 0.5 µl SSB; 0.5 units Taq polymerase (5U/ µl); 1 µl (10mM) TRAPForG; and 2 µl of plant extract containing ~ 0.1-0.2 µg of protein. The extension reaction by telomerase was allowed to proceed at 37 °C for 40 min prior to the addition of 50 µM of dCTP and 1 µl of TRAP-RV. The telomerase-added repeats to the forward primer were amplified by 34 cycles of PCR; denaturing at 94°C for 5 min and followed by 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min 30 sec and a final extension at 72°C for 5 min. The reaction was stopped by 60 µl of telomerase stop buffer (10 mM Tris-HCl, pH 7.5 and 21 mM EDTA) and the DNA was precipitated in ethanol following a phenol chloroform extraction. The samples were resolved on 6% sequencing gels. Extracts from *Arabidopsis* Wild type flowers and cauliflower were used as positive controls and *Arabidopsis* Wild

type leaves as a negative control. The gel was dried and exposed to a PhosphorImage screen.

Terminal Restriction Fragment (TRF) length analysis

For the extraction of genomic DNA, plant tissue was ground in liquid nitrogen, resuspended in CTAB buffer (140 mM Sorbitol; 220 mM Tris, pH 8.5; 22 mM EDTA; 800 mM NaCl; 1% Sarkosyl; and 0.8% CTAB) and incubated at 65^{o}C for one hour. Phenol:chloroform extraction was performed and DNA was precipitated in isopropanol. Three to five micrograms of genomic DNA from both Wild type and *tac5* plants was digested with *Tru*9I (Fermentas) for 12 h at 65^{o}C. The digested DNA was resolved on a 0.8% agarose gel and subjected to Southern blot. A telomeric oligo was used as a probe after labeling with (γ^{32}P) dATP by polynucleotide kinase. Radioactivity signal was detected by PhosphorImager analysis.

Determination of the number of T-DNA insertions in tac5 mutant

Three to five micrograms of genomic DNA from Wild type and *tac5* plants were digested with several restriction enzymes such as *Bam*HI, *Eco*RI and *Hind*III in a 100 µl reactions at 37 ^{o}C for 12 h. The digested DNA was resolved on a 0.8% agarose gel and subjected to Southern blot analysis. The probe for the Southern blotting was double stranded pBluescript plasmid digested with *Hind*III and labeled with (α^{32}P) dCTP by random priming. As an additional probe for confirmation, the *35S* enhancer sequence was released from the double stranded pCBKO5 vector by digestion with *Kpn*I and *Xho*I, and then labeled with (α^{32}P) dCTP by random priming.

Determination of the site of T-DNA insertion by plasmid-rescue

One microgram of genomic DNA was digested for 12 h with *Bam*H1 in a 100 µl reaction at 37 ^{o}C to rescue the plasmid containing the T-DNA along with part of plant genomic DNA at the left border (Weigel *et al.,* 2000). The digested DNA was subjected to phenol-chloroform extraction, ethanol precipitation and the pellet was resuspended in 50 µl of Tris-EDTA, pH 8.0. Ten µl of *Bam*HI digested DNA was ligated overnight at room temperature (23^{o}C) in a total volume of 200 µl, ethanol precipitated and resuspended in a final volume of 20 µl and 10 µl of which was used to transform *E. coli* competent cells. Ampicillin resistant transformants were sequenced by Big Dye sequencing method using primers for the T7 and T3 promoter on the pBSK^{+} (plasmid Bluescript) region of the activation-tag vector (pSKOI5).

Total RNA extraction and RT-PCR analysis

For extraction of total RNA, plant tissues from both Wild type and *tac5* plants were finely ground in liquid nitrogen and 1mL of Tri-reagent (Invitrogen) was added. The samples were incubated at room temperature (23 ^{o}C) for 10 min and centrifuged at 12K rpm for 10 min at 4^{o}C. The supernatants were transferred to new microfuge tubes with isopropanol and incubated at 4^{o}C for 10 min. The samples were centrifuged at 12K rpm for 10 min at 4^{o}C to pellet the RNA. The pellet was washed with 70% ethanol and air dried. The pellet was then dissolved in 20 µl DEPC water and incubated at 37^{o}C for 10 min. The RNA sample was treated with RQ1 DNase at 37^{o}C for 1 hr and inactivated for 15 min at 65^{o}C. Reverse transcription reactions were performed at 55^{o}C for 1 hr using Superscript III RT kit (Invitrogen) and oligo d(T). After the reverse transcription

reaction, RNA in the DNA:RNA hybrid was removed by RNaseH treatment for 30 min at 37^{o}C to yield the first strand cDNA. Amplification of *TERT* cDNA was performed by using the first strand cDNA in a 50 µl reaction. The following primers (Integrated DNA Technologies) were used for amplification of *TERT*: TERT5 5' (GCCCTTGATGGATATGTCC) 3', and TERT48 5' (CCAACTGCATGTTGTTC) 3'. The reaction mixture was amplified by 25 cycles of PCR at 94^{o}C for 3 min, 55^{o}C for 40 sec and 72^{o}C for 1 min 15 sec with a final extension time at 72^{o}C for 5 min. All the reaction volume was resolved on a 1 % agarose gel and subjected to Southern blot analysis with a *TERT* cDNA probe (TERT5 and TERT48 PCR product) labeled with (α^{32}P) dGTP. As a loading control, RT-PCR was performed with primers specific for Actin-2 gene (forward: 5' GTTGCACCACCTGAAAGGAA 3'and reverse: 5' TCATACTCGGCCTTGGAGATC 3'). In addition, to measure the steady state levels of transcripts from genes that are adjacent to the T-DNA insertion site, primers were designed for several genes on both sides of T-DNA insertion site and RT-PCR was performed.

Reverse northern analysis

BAC clones MPA22, T25O11 and K12B20, which spanned the T-DNA insertion region on chromosome 5 were obtained from ABRC (Ohio State University). BAC clones were digested for 12 h with several restriction enzymes (*Xho*I, *Hind*III and *Eco*RI) in a total volume of 200 µl. The digested BAC DNA was resolved on 1% agarose gel, and hybridized with radiolabeled RNA derived from wild type or *tac5* leaves. Five micrograms of total RNA from both Wild type and *tac5* leaves were used to generate

radiolabeled first strand cDNA by reverse transcription reaction with SuperscriptIII RT (Invitrogen). Radioactive signals were compared to identify genes that showed differential expression patterns.

Overexpression of NADH dehydrogenase

The NADH dehydrogenase genomic region was cloned into the pEARLYGATE vector immediately downstream of the CaMV *35S* promoter utilizing Gateway cloning technology (Invitrogen). These constructs were transformed into Wild type *Arabidopsis* plants by *Agrobacterium* mediated transformation. The transformants were selected on MS media with 50mg/L BASTA, and the survivors were transferred to soil and subjected to TRAP assay to analyze the telomerase activity in their leaves.

Results

Identification of telomerase activation mutant – tac5

To identify genes that upregulate telomerase activity, we screened pools of activation tagged lines for inappropriate expression of telomerase activity in leaves, using the TRAP assay. If the telomerase was active in leaves, it would add telomeric repeats to the forward primer. The extended primer would then be amplified by PCR with a reverse primer complementary to the telomeric repeat. The products can be visualized on PAGE. In this screening process, the starting population was sixty two pools of 100 activation tagged lines. Among these lines, we found one pool that showed ectopic activation of telomerase activity in leaves. Sub-pools of 10 plants were then screened again for telomerase activity in leaves to identify an individual mutant plant. An individual mutant

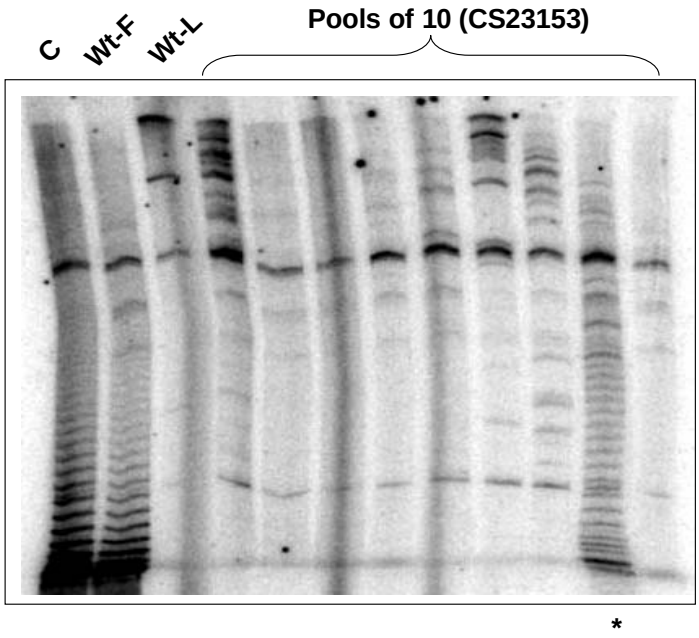

Figure 2.1. Identification of *tac5* from screening of activation-tagged lines by TRAP assay. Protein extracts from leaves of activation-tagged lines in pools of 10 from the Weigel collection CS23153 were used for this assay. Cauliflower floral extract (C) and wild type floral protein extracts (Wt-F) were used as positive controls and wild type leaf protein extract (Wt-L) was used as a negative control. The pool containing the telomerase mutant –*tac5* showing the ectopic activation of telomerase in leaves is indicated with an asterisk (*).

plant showing telomerase activity in leaves was then selected after screening the individual plants in the positive pools of 10.The screen resulted in the identification of *tac5*, a telomerase mutant, which exhibits telomerase activity in leaves (Figure 2.1).

Telomere length in tac5

Telomere length was analyzed in *tac5* by TRF analysis to check whether the ectopic activation of telomerase in leaves altered telomere length. TRF analysis showed that there is no dramatic alteration in telomere length in *tac5* relative to Wild type (Figure 2.2). This finding is consistent with studies in yeast and mammals, indicating that telomere length is primarily regulated by controlling the access of telomerase to telomeres by other factors like telomere binding proteins (Griffith *et al.,* 1999).

Correlation of ectopic telomerase activity in tac5 with TERT expression levels

In Wild type *Arabidopsis*, high levels of *TERT* mRNA correlate with high levels of telomerase activity present in reproductive tissues, like flowers, while low levels of *TERT* mRNA correlate with low levels of telomerase activity in leaves (Fitzgerald *et al.,* 1999). To investigate the mechanism of telomerase regulation in *tac5*, *TERT* mRNA levels were measured in different tissues of *tac5*, including flowers and leaves by RT-PCR (Figure 2.3). The results indicate that *TERT* mRNA levels in *tac5* leaves were higher than those in Wild type leaves, but not as high as the levels in Wild type flowers, suggesting that telomerase activation in *tac5* leaves is regulated at the transcriptional level.

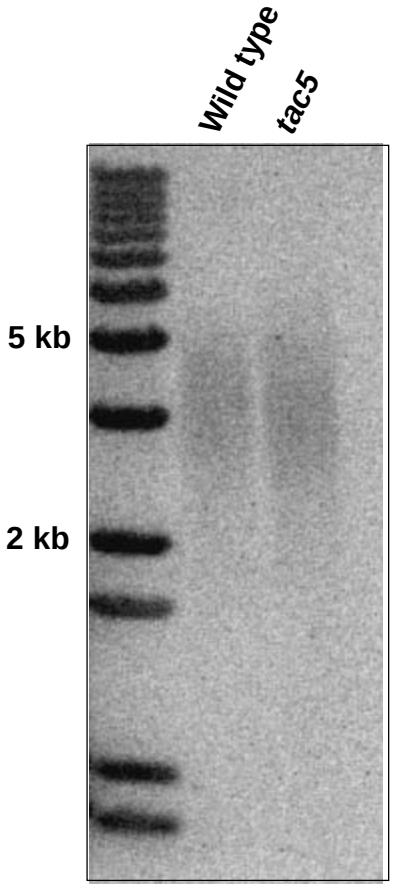

Figure 2.2. Characterization of telomere length in *tac5*. TRF analysis of *Tru9I* digested genomic DNA hybridized with a (γ^{32}P) dATP-labeled telomere probe. Telomere length in *tac5* is same as wild type (2-5 kb).

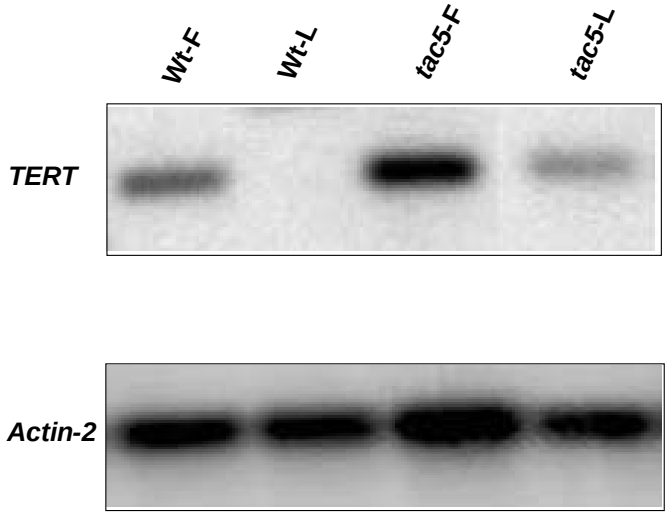

Figure 2.3. *TERT* mRNA levels in *tac5*. Hybridization of RT-PCR reactions with an (α^{32}P) dCTP-labeled probe specific to *TERT* to determine expression levels in the *tac5* mutant. *TERT* mRNA is upregulated in *tac5* leaves compared to wild type leaves. Actin-2 is shown as loading control.

Genetic analysis of the tac5 mutation

To analyze the nature of the *tac5* mutation, mutant plants were out-crossed to wild type and the resulting F1 population was analyzed for telomerase activity. All the individuals in the F1 population showed telomerase activity, indicating that the mutation in *tac5* is dominant (Figure 2.4). The segregation pattern of the F1 population ***i.e.,*** all the plants in F1 population showing the ectopic expression of telomerase in leaves, also suggested that the *tac5* mutant we identified is homozygous for the mutation. Since TRAP assay is a PCR based assay, depending upon the quantity of telomerase present in the protein extracts, the intensity of ladder for each sample changes.

Determining the number of T-DNA insertions in tac5

The activation tagging pools utilized for identification of *tac5* were originally created by transforming Wild type *Arabidopsis* plants with T-DNA vectors having four copies of *35S* enhancer sequence. These transformed lines contain T-DNAs integrated at random sites throughout the whole genome. Southern blot analysis was performed with two different probes specific to the T-DNA region to determine the number of T-DNA insertions in *tac5*. Genomic DNA from *tac5* was digested with several restriction enzymes, separated by electrophoresis and hybridized either with *35S* enhancer probe or with an oligonucleotide corresponding to a region of the pBluescript sequence located within the activation tag vector. The results indicated that *tac5* has multiple T-DNA insertions in its genome (Figure 2.5). The non-specific band detected in 2.5 kb range both in wild type and *tac5* might be due to the star activity of the enzymes used for digestion.

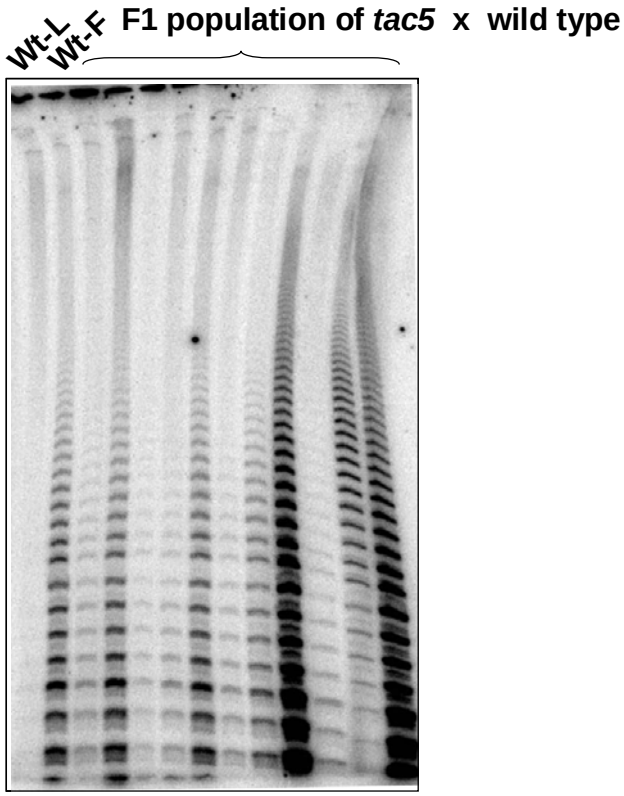

Figure 2.4. TRAP assay showing the genetic nature of mutation in *tac5*. *tac5* was crossed to Wild type to determine the nature of mutation in *tac5*. TRAP assay was performed on the F1 population and the result shown here indicated that mutation in *tac5* is dominant since all the F1 plant leaf extracts showed ectopic activation of telomerase. Variation in the intensity of signal among different lanes is due to the amount of protein extract used for this assay.

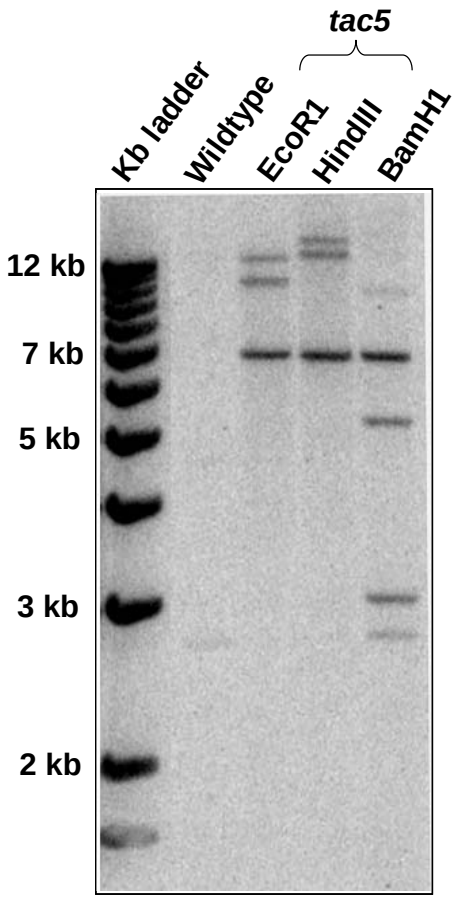

Figure 2.5. Number of T-DNA copies inserted in *tac5* genome. Southern blot analysis was performed on genomic DNA from *tac5* mutant digested with several restriction enzymes and hybridized with (α^{32}P) dCTP-labeled *35S* enhancer probe. Wild type control shown in lane 1 is digested with *Eco*RI. Results shown above indicating that *tac5* has multiple T-DNA insertions in its genome.

Segregation analysis of tac5 to determine, which T-DNA insertion is responsible for ectopic telomerase activation

Since the *tac5* genome showed multiple T-DNA copies, it was necessary to determine which T-DNA copy is linked to the *tac5* phenotype, ectopic activation of telomerase in leaves. *tac5* was out-crossed to wild type and the resulting F1 population was backcrossed to the wild type to segregate the T-DNA copies in the genome. The F2 population showed a segregation ratio of 1:1 indicating that half of the population contained the T-DNA insertion. TRAP assay and TRF blots on the F2 population showed that mutants with ectopically activated telomerase have multiple T-DNA insertions (Figure 2.6). Linked T-DNA insertions cannot segregate independently because they reside on the same chromosome at a single locus and are inherited as a unit. This result suggests that the multiple T-DNA insertions in *tac5* genome are at a single locus, which is why they cannot be segregated out by outcrossing.

Determination of T-DNA insertion site in tac5 genome

Plasmid-rescue was performed to determine the site of T-DNA insertion in the *tac5* genome. The pBKS+ sequence in T-DNA vector, pSKI015 that was used to transform *Arabidopsis* for generation of activation-tagged pools, can be used for rescue of T-DNA and adjacent plant sequences from transformed plants. Genomic DNA from *tac5* was digested with a restriction enzyme that cuts the T-DNA portion once and the digested DNA was then religated and used to transform competent bacterial cells. *Bam*HI digested and religated clones that have the T-DNA portion, which has ampicillin resistance marker and a part of the adjacent genomic DNA were selected on antibiotic

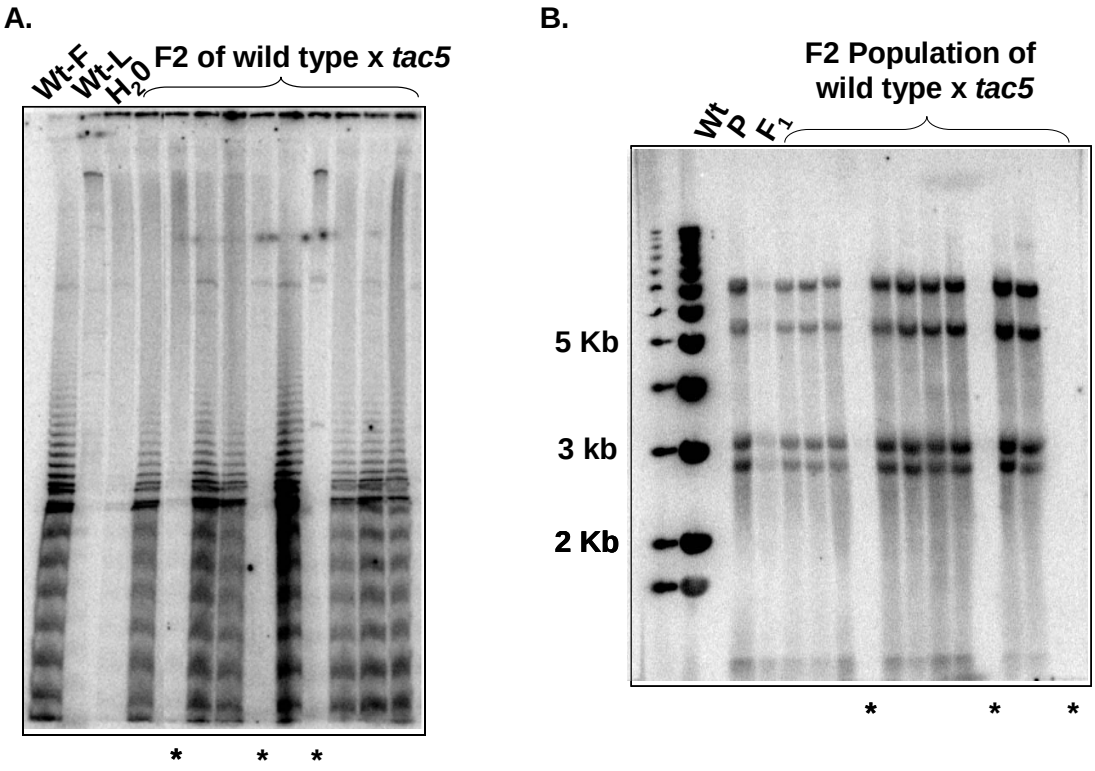

Figure 2.6. Linkage of T-DNA insertion to inappropriate telomerase expression. A. A 6% acrylamide gel showing the products of TRAP assay with leaf extracts from the F2 population of *tac5* crossed with wild type showing the segregation for ectopic telomerase activity. Wild type flower extract served as a positive control and leaf extract and water as negative controls. **B.** Southern blot analysis of *Bam*H1 digested genomic DNA from the F2 population of *tac5* crossed with wild type to determine the number of insertions and linkage of insertions to ectopic telomerase activity. Wt lane indicates the wild type control and lane P indicates the parent mutant (*tac5*) used to cross with wild type. Plants with ectopic telomerase activity have multiple T-DNA insertions. Plants lacking telomerase activity (lanes labeled with *) do not have T-DNA insertions.

selection plates and sequenced using primers specific to the promoter regions within the activation tag vector. BLAST analysis of the sequenced clones revealed that the T-DNA insertion was located on 5th chromosome in At5g37520, in a gene of unknown function (Figure 2.7).

Gene/genes activated in tac5 by the enhancer sequence of T-DNA

The plasmid-rescue experiment indicated the location of T-DNA insertion in the *tac5* mutant on 5th chromosome is in a gene of unknown function (At5g37520). The location of the T-DNA insertion on chromosome 5 relative to *TERT* is shown in a schematic diagram (Figure 2.7A). RT-PCR analysis for genes adjacent to At5g37520 on RNA from a *tac5* leaf showed that the *NADH dehydrogenase* (At5g37510) gene, which is next to the T-DNA insertion, was transcriptionally upregulated in *tac5* leaves compared to Wild type leaves (2.7A and 2.7B). As an alternative approach, reverse northern analysis was performed to investigate the genes that are activated in *tac5*. In this technique, restriction fragments from three BAC clones, T25O11, MPA22 and K12B20, which spanned a ~200 kb region around the T-DNA insertion, were hybridized with radiolabeled cDNA isolated from wild type and *tac5* leaves. We found that some fragments displayed increased hybridization to *tac5* RNA relative to Wild type RNA. These fragments carried different genes on chromosome 5 (data not shown). RT-PCR analysis for those genes tentatively identified by reverse northern did not show any difference in the expression level between Wild type leaves and *tac5* leaves (data not shown). Thus, *NADH dehydrogenase* is the only candidate gene we identified that may be responsible for ectopic activation of telomerase in *tac5* leaves.

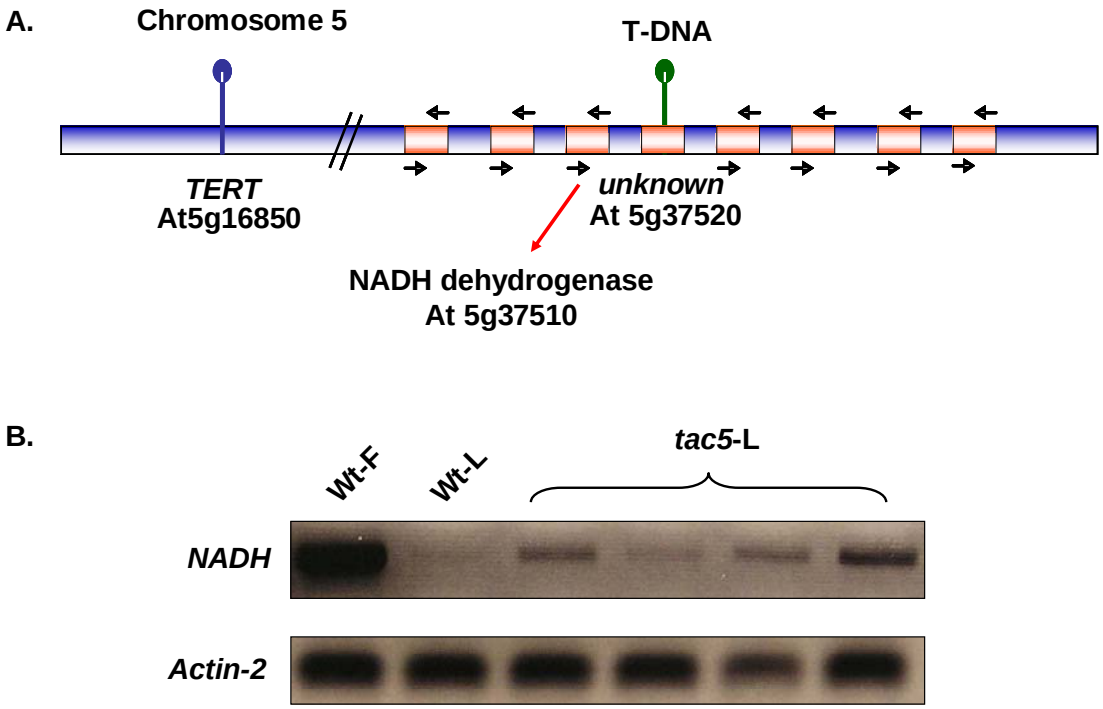

Figure 2.7. Transcriptional upregulation of *NADH dehydrogenase* in *tac5*. A. A schematic showing the location of the T-DNA insertion site relative to *TERT* on a small portion of chromosome 5. *TERT* is located 9 Mb away from the T-DNA insertion. The location of the *NADH dehydrogenase* near the insertion and primers used for RT-PCR on several genes near the insertion are also shown. **B.** RT-PCR analysis for *NADH dehydrogenase* gene is shown in the figure. Results showed that different individuals of *tac5* progeny have higher *NADH dehydrogenase* transcript levels in *tac5* leaves compared to Wild type leaves. *Actin-2* was used as a loading control.

Overexpression of NADH dehydrogenase to confirm its role in ectopic telomerase activation

RT-PCR data showed that *NADH dehydrogenase* is the only candidate gene that shows upregulation of its transcript levels in the *tac5* mutant. To confirm its role in ectopic activation of telomerase, a full length genomic construct of *NADH dehydrogenase* was prepared in pEARLYGATE (Invitrogen) vector, under the regulation of CaMV *35S* promoter and wild type plants were transformed with this construct through agrobacterium-mediated transformation. Transformants were selected on BASTA resistance plates and checked for telomerase activity in leaves by TRAP assay. RT-PCR data on these plants showed that *NADH dehydrogenase* is overexpressed in leaves of some of the transformants (Figure 2.8A) and the plants that overexpress *NADH dehydrogenase* also showed ectopic activation of telomerase in leaves as determined by TRAP assay (Figure 2.8B). This result confirms that *NADH dehydrogenase* is responsible for ecotpic activation of telomerase in *tac5* mutant leaves.

Sensitivity of tac5 to oxidative stress

In the process of mitochondrial electron transport, highly toxic reactive oxygen species (ROS) can be produced if single electrons are inappropriately transferred to oxygen, a phenomenon referred to as "electron leakage." The major sites of electron leakage have been proposed to be complex I (NADH dehydrogenase) (Balaban *et al.*, 2005). The *tac5* mutant showed the transcriptional activation of *NADH /Ubiquinone dehydrogenase* gene, suggesting that *tac5* might be involved in oxidative stress signaling.

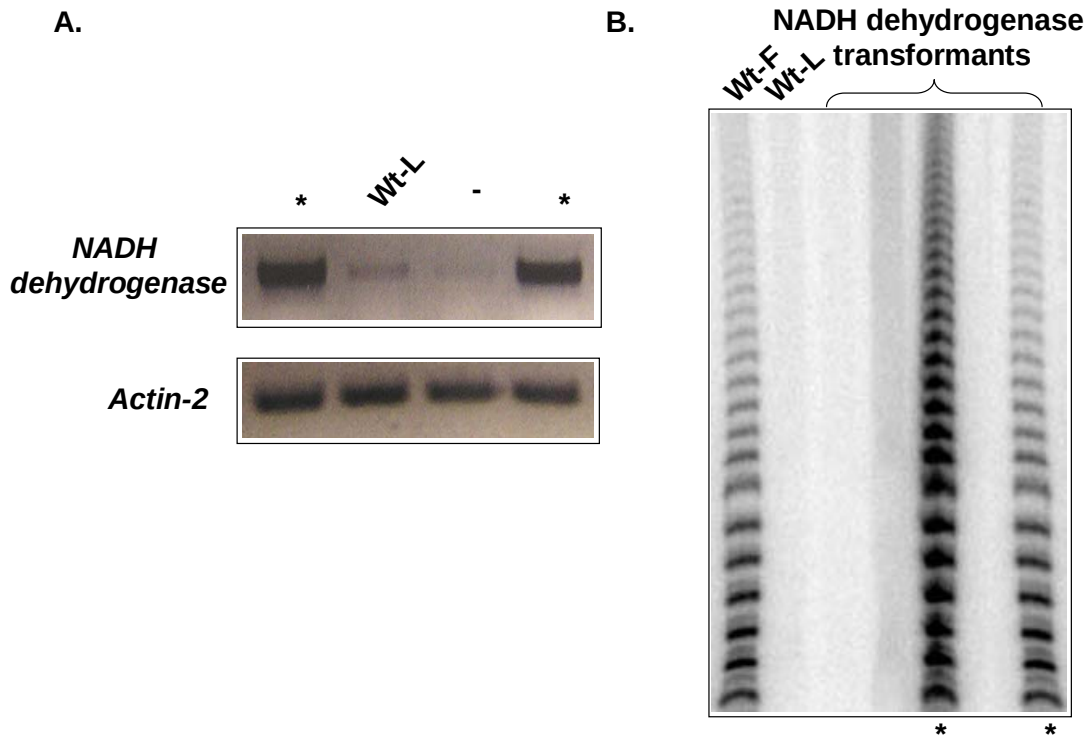

Figure 2.8. Overexpression of *NADH dehydrogenase* activates telomerase in leaves. **A.** RT-PCR analysis for *NADH dehydrogenase* gene on plants overexpressing *NADH dehydrogenase* gene (marked with *) is shown here. Wild type leaf RNA extract is used as a negative control. (-) lane indicates the plant that does not show any overexpression of this gene. *Actin-2* was used as a loading control. **B.** 6% acrylamide gel showing the products of the TRAP assay with leaf extracts from *NADH dehydrogenase* transformants selected on BASTA plate. Wild type flower extract served as a positive control and leaf extract as a negative control. Plants that overexpress *NADH dehydrogenase* (panel A, lane 1 and 4) showed telomerase activity in leaves (lanes labeled with *).

To find out whether the *tac5* mutant responds to oxidative stress or not, wild type and *tac5* seeds were grown on both complete MS media and MS media with 3 mM hydrogen peroxide for one week and oxidative stress sensitivity was determined. As a specificity control, AtKu70 was also utilized in addition to wild type, which did not show any oxidative stress sensitivity as wild type. The results indicated that *tac5* shows sensitivity to oxidative stress induced by hydrogen peroxide treatment (Figure 2.9), and this sensitivity is specific to telomerase activation mutant.

Discussion

Plant development differs in fundamental ways from human development. For example, many plant cells are totipotent and their germ-line is specified at the end of the life cycle as compared to mammalian cells, in which the germ-line is determined very early in development. Despite these differences, telomerase expression patterns are similar in both organisms. Telomerase is highly expressed in proliferative and undifferentiated cells in both plants and animals, suggesting a similar underlying regulatory mechanism. In this study, we report the characterization of *tac5*, a mutant that constitutively expresses the telomerase enzyme in leaves.

The activation tagging strategy we employed exploits an enhancer element from the cauliflower mosaic virus *35S* promoter to achieve the transcriptional activation of nearby genes (Weigel *et al.,* 2000). Thus activation tagging has identified a number of genes fundamental to plant development (Boerjan *et al.,* 1995), disease resistance (Xia *et al.,* 2004), anthocyanin production (Mol *et al.,* 1998), and flowering (Weigel *et al.,* 2000). Although this technique has been predominantly applied to *Arabidopsis*, recent

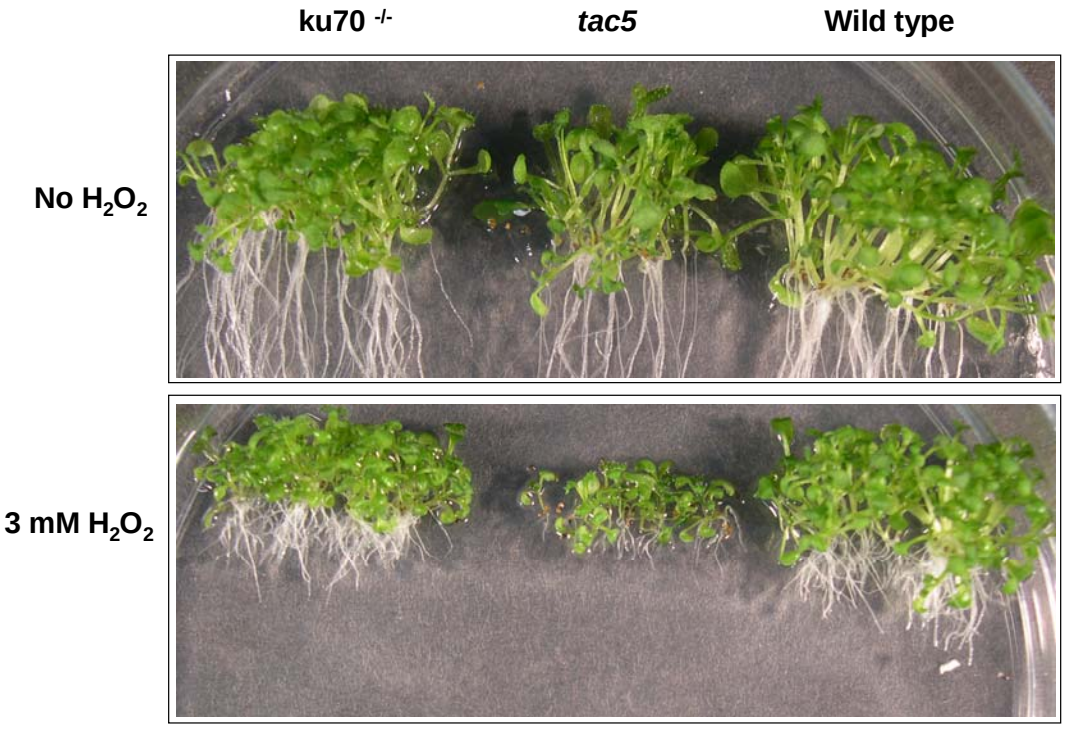

Figure 2.9. Sensitivity of *tac5* to hydrogen peroxide treatment. Wild type and *tac5* seeds were grown on complete MS media and MS media with 3 mM H_2O_2 for one week and sensitivity of seedlings by morphological appearance was observed. As a control, At*ku70* $^{-/-}$ was utilized. Upper panel shows the results for seeds that were grown without hydrogen peroxide treatment and lower panel shows the results for seeds treated with hydrogen peroxide.

studies reported the utilization of this strategy in other model plant species like tomato and rice (Jeong *et al.,* 2002).

Because the TRAP assay is extremely sensitive, it was possible to use this assay to identify mutants that show inappropriate expression of telomerase among individuals in large pools. The telomerase mutant, *tac5*, was found after screening 62 pools of 100 activation tagging lines and further investigation in sub-pools of 10 activation tag lines. To analyze the nature of the mutant, it was out-crossed to wild type and the resulting F1 progeny were analyzed for telomerase activity. All F1 plants showed telomerase activity, indicating that the mutation in *tac5* is dominant. Southern blotting showed that *tac5* has three T-DNA copies. Strikingly, all of the telomerase positive plants contained multiple copies of T-DNA, indicating that there is a complex insertion at a single locus and this complex T-DNA insertion is linked to the telomerase activity in the leaves. In addition, the progeny of *tac5* mutant self pollination retained telomerase activity for at least three generations, indicating that the mutant phenotype is stable.

Increasing evidence indicates that regulation of telomerase is a complex and dynamic process involving several steps at both post-transcriptional and post-translational levels. As in *tac1* mutants (Ren *et al.,* 2004), RT-PCR data from *tac5* showed that *TERT* mRNA is upregulated in leaves compared to wild type leaves. *TERT* transcriptional upregulation in *tac5* suggests that the ectopic expression of telomerase in *tac5* is due to the transcriptional upregulation of *TERT* in leaves. Interestingly, telomere length is in the wild type range (2-5 kb), as determined by TRF analysis. We speculate that telomere lengths remain stable in *tac5* despite increased telomerase activity because

telomerase access is controlled by a suite of telomere binding proteins (Vega *et al.,* 2003).

The location of the T-DNA insertion in *tac5* was determined by plasmid-rescue and shown to be on chromosome 5 in the 2nd intron of At5g37520, a gene with unknown function. Activation of genes near the T-DNA insertion was checked by RT-PCR and the *NADH* dehydrogenase (ubiquinone) gene (At5g 37510; *NADH*), which is next to the insertion site, was found to be transcriptionally upregulated. Genes further away from the T-DNA insertion were also checked for activation by the reverse northern technique (data not shown), but no additional activated genes were found. Thus, *NADH dehydrogenase* is the best candidate for upregulation of telomerase in the *tac5* mutant. To confirm that *NADH dehydrogenase* is responsible for ectopic activation of telomerase in *tac5*, a wild type genomic clone of *NADH dehydrogenase* was overexpressed using the CaMV *35S* promoter. TRAP assay results on plants overexpressing *NADH dehydrogenase* confirmed the ectopic activation of telomerase in leaves.

The finding of *NADH dehydrogenase* involvement in telomerase regulation was initially very perplexing as the enzyme acts in mitochondria. In addition, this gene was found as an interacting protein in the two-hybrid screens performed with TRFL9 (See Chapter IV for details), suggesting that it may play a role in telomere biology. In the process of mitochondrial electron transport, highly toxic ROS can be produced if single electrons are inappropriately transferred to oxygen, a phenomenon referred to as "electron leakage." The major sites of electron leakage have been proposed to be complex I (NADH dehydrogenase) (Balaban *et al.,* 2005). Recent studies in mammalian cells have uncovered a link between TERT and mitochondria. hTERT has a bonafide N-terminal

mitochondrial targeting sequence, and recent studies indicate that localization of hTERT to the mitochondria renders cells more susceptible to oxidative stress-induced mitochondrial DNA damage (Santos *et al.,* 2006). In addition, studies also showed that hTERT is involved in mitochondrial apoptosis induced by targeted inhibition of Bcl-2 (Del Bufalo *et al.,* 2005). Therefore, we asked whether *tac5* responds to oxidative stress by subjecting the mutants to hydrogen peroxide treatment. We found that *tac5* is more sensitive to oxidative stress compared to wild type plants. This result suggests that *NADH dehydrogenase* activation in *tac5* renders plants more susceptible to oxidative stress-induced damage and further, this stress signals the transcriptional activation of *TERT*.

In summary, our analysis of *tac5* and the demonstration of its sensitivity to oxidative stress points to a novel function of telomerase in the mitochondrial environment or of the NADH dehydrogenase in transcriptional control of a "moonlighting" protein. It is possible that mitochondrial hTERT might help to cull out cells with damaged mtDNA (due to oxidative stress) by inducing the apoptotic pathway. Further investigation is needed to demonstrate a biological role for mitochondrial telomerase in modulating the response of *Arabidopsis* to oxidative stress.

CHAPTER III

CHARACTERIZATION OF PARP1/PARP2 FOR THEIR ROLE AT TELOMERES

Introduction

Telomeres play a key protective role at chromosome ends. Chromosomal stability by telomeres is achieved through the protein complex shelterin composed of six proteins with high affinity for telomeric DNA and their interacting proteins (deLange, 2005). In addition to telomere-specific proteins, several DNA repair proteins and proteins involved in sensing DNA damage associate with telomeres. Moreover, alterations in many of DNA damage response proteins result in telomere dysfunction and subsequent chromosomal instability suggesting extensive functional interactions between telomere maintenance and DNA damage response mechanisms.

Cells with mutated AT (Ataxia Telangiectasia), a key regulator of DNA damage response, display accelerated telomere shortening and higher frequencies of end-to-end chromosome fusions (Metcalfe *et al.,* 1996). Similarly, yeast cells defective in Ku, a key component of the non-homologous end joining (NHEJ) pathway, give rise to a similar phenotype (Boulton and Jackson, 1996). Deficiency of the DNA dependent protein kinase catalytic subunit (DNA-PKcs), an enzyme involved in NHEJ pathway of repairing DNA double-strand breaks, also causes telomeric fusions and abnormal telomere elongation (Bailey *et al.,* 1999). Even in wild type cells, human telomeres are transiently recognized as DNA damage in G2 stage of the cell cycle and recruit MRN and ATM with a partial release of POT1 (Verdun *et al.,* 2005). These results suggest a role for DNA

repair proteins to protect the chromosomal integrity by balancing repair activities and telomere maintenance.

Poly ADP-ribosylation is an early DNA damage-dependent post-translational modification of histones and other nuclear proteins that contributes to the survival of damaged cells. Poly (ADP-ribose) polymerases (PARPs) catalyze the synthesis and attachment of ADP-ribose polymers to specific target proteins. A superfamily of eighteen *PARP*s has been identified so far in humans. Several PARP family members associate with telomeres or telomerase (Smith *et al.,* 1998). Inhibition of PARP activity results in decreased telomerase activity in human cells, suggesting a role of PARPs in regulating telomerase activity (Ghosh and Bhattacharya, 2004).

PARP1, the founding family member, has been extensively studied in mammals. *PARP1* deficient mice exhibit normal telomere length and chromosome end capping (Samper *et al.,* 2001). However hPARP1 is physically associated with TRF2, and appears preferentially at eroded telomeres after induction of DNA damage to protect telomeres against end to end fusions and genomic instability (Gomez *et al.,* 2006). These data suggest that PARP1 has a key role at the dysfunctional telomeres to maintain genomic stability. *PARP2* was identified as a result of the presence of residual DNA-dependent PARP activity in *PARP1* deficient mice (Shieh *et al.,* 1998). Like PARP1, PARP2 also interacts with TRF2 and regulates the telomeric binding activity of TRF2 through poly ADP-ribosylation (Dantzer *et al.,* 2004). In addition, *PARP2* deficient mice exhibit normal telomere length and telomere capping, but display an increase in chromosome ends lacking detectable telomeric DNA (Dantzer *et al.,* 2004). Since the double mutant

for *PARP1*/*PARP2* is lethal in mice (de Murcia *et al.,* 2003), the combined contribution of these proteins to telomere function is unknown.

Arabidopsis has an extreme tolerance to telomere dysfunction and an ability to withstand the loss of DNA damage repair proteins that are essential in other model systems. Thus, *Arabidopsis* is a good system to study the cellular responses to uncapped telomeres. Mutations in several telomere related genes and DNA repair genes are lethal in mammals (ATR - deKlein *et al.,* 2000, RAD50 - Luo *et al.,* 1999, and BRCA1 – Gowen *et al.,* 1996), but not in *Arabidopsis*. In addition, mammalian cells deficient in Ku, another DNA repair protein, display frequent end-to-end chromosome fusions (Samper *et al.,* 2000, d'Adda di Fagagna *et al.,* 2001), but its impact on telomere dynamics in mammals is still unclear as both telomere shortening and lengthening have been reported (Samper *et al.,* 2000, d'Adda di Fagagna *et al.,* 2001). In contrast to the situation in mammals, chromosome fusions are not associated with a Ku deficiency in *Arabidopsis*, and AtKu70 works as a negative regulator of telomere length (Riha *et al.,* 2002). These findings imply that DNA repair proteins may play different roles in capping of plant and animal telomeres.

Previous studies showed that *PARP1* and *PARP2* are induced in response to DNA damage in *Arabidopsis* (Doucet-Chabeaud *et al.,* 2001). In addition, transcriptional upregulation of *PARP*s in response to ionizing radiation was reported in *Arabidopsis* (Doucet-Chabeaud *et al.,* 2001). It is not clear whether these plant PARP genes have a similar function as animal PARPs in terms of maintenance of telomere length and genomic stability. Therefore, studying the role of PARPs in plants with telomere dysfunction might shed light on their function in response to DNA damage.

Here we explore the role of *AtPARP1* and *AtPARP2* at *Arabidopsis* telomeres. Unexpectedly we found that neither *PARP1* nor *PARP2* is individually required for telomere length homeostasis and chromosome end protection.

Material and methods

Mutant lines

The GABI-KAT and SALK T-DNA lines for At*PARP1* (GABI 380E06) and for At*PARP2* (SALK_ 140400) were obtained from *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. The collection was screened by genotyping with primers specific to T-DNA border sequences and gene-specific sequences. For genotyping, genomic DNA was extracted from flower buds using a high-throughput method (Xin *et al.*, 2003).

Double *parp1/parp2* mutants were made by crossing a plant homozygous for T-DNA insertion in *parp1* with a plant homozygous for T-DNA insertion in *parp2*. Double heterozygous F1 plants were identified by genotyping and then self-propagated to F2 to obtain double homozygous mutants. Plants were grown at 23^{o}C in an environmental chamber under a 16 h light/8h dark photoperiod. Triple mutants of *parp1/parp2/tert* were created by transforming *tert* mutants with *parp1/parp2* RNAi construct. RNAi construct for *parp1/parp2* was made utilizing gateway technology for the sequence suggested by CATMA web site. Transformants were selected on BASTA selection plates and transformants were analyzed for telomerase activity and telomere length by TRAP assay and TRF blot, respectively.

Total RNA extraction and RT-PCR analysis

To assay the DNA damage responses, plants were subjected to MMS (Methyl Methane Sulfonate) treatment. Five day old seedlings from wild type and *tert* were subjected to different concentrations of MMS (0, 25, 50 and 75ppm) for three days. MMS treated eight day old seedlings were finely ground in liquid nitrogen and 1 mL of Tri-reagent was added. The samples were incubated at room temperature for 10 min and centrifuged at 12K rpm for 10 min at 4^{o}C. The supernatants were transferred to new microfuge tubes with isopropanol and incubated at 4^{o}C for 10 min. The samples were centrifuged at 12K rpm for 10 min at 4^{o}C to pellet the RNA. The pellet was washed with 70% ethanol and air dried. The pellet was then dissolved in 20 µl DEPC water and incubated at 37^{o}C for 10 min. The RNA sample was treated with RQ1 DNase at 37^{o}C for 1 hr and inactivated for 15 min at 65^{o}C. Reverse transcription reactions were performed at 55^{o}C for 1 hr using Superscript III RT kit (Invitrogen) and oligo d(T).

DNA damage responses were measured in MMS treated wild type and *tert* $^{-/-}$ seedlings by checking the mRNA levels of *PARP2*. Amplification of *PARP2* mRNA was performed by using first strand cDNA in a 50 µl reaction. The reaction mixture was amplified by 20 cycles of PCR at 94^{o}C for 3 min, 55^{o}C for 40 sec and 72^{o}C for 1 min 15 sec with a final extension time at 72^{o}C for 5 min. All the reaction volume was resolved on a 1 % agarose gel and subjected to Southern blot with an *PARP2* cDNA probe labeled with (α^{32}P) dGTP. As a loading control, RT-PCR was performed with primers specific for Actin-2 gene (forward: 5' GTTGCACCACCTGAAAGGAA 3'and reverse: 5' TCATACTCGGCCTTGGAGATC 3'). RT-PCR for *PARP2* was performed on total RNA extracted from wild type flowers and *tert* flowers.

Terminal Restriction Fragment (TRF) length analysis

For the extraction of genomic DNA, plant tissue was ground in liquid nitrogen and resuspended in CTAB buffer (140 mM Sorbitol; 220 mM Tris, pH 8.5; 22 mM EDTA; 800 mM NaCl; 1% Sarkosyl; and 0.8% CTAB) and incubated at 65^{o}C for one hr. Phenol:Choloroform extraction was performed and DNA was precipitated in isopropanol. Three to five micrograms of genomic DNA from both Wild type and *tac5* mutant was digested with *Tru9I* (Fermentas) for overnight at 65^{o}C. The digested DNA was resolved on a 0.8% agarose gel and subjected to Southern blot. A telomeric oligo was used as a probe after labeling with (α^{32}P) dATP. The radioactivity signal was detected by PhosphorImager analysis.

Telomere Repeat Amplification Protocol (TRAP)

Telomerase activity in T-DNA insertion lines of PARPs was detected by TRAP (Fitzgerald *et al.,* 1996). The following primers (Gene Technology Laboratory, Texas A&M University) were used in the TRAP assay: Forward primer (TRAPForG) 5' (CACTATCGACTACGCGATCAG)3' and Reverse primer (TRAP-RV) 5' (CCCTAAACCCTAAACCCTAAA) 3'. Reaction mixtures were prepared in a total volume of 50 µl :50 mM Tris-acetate, pH 8.3; 50 mM potassium glutamate; 10 mM EGTA; 5 mM $MgCl_2$; 0.1% Triton X-100; 1 mM spermidine; 1 mM DTT; 50 µM each dATP, dGTP, dTTP; 0.5 µl (α^{32}P) dGTP (3000 Ci/mmol; Amersham Biosciences); 100 ng/µl BSA; 0.5 µl single-strand binding protein; 0.5 units Taq polymerase (5U/ µl); 1 µl (10 mM) TRAPForG; and 2 µl of plant extract containing ~ 0.1-0.2 µg of protein. The extension reaction by telomerase was allowed to proceed at 37^{o}C for 40 min prior to the

addition of 50 μM of dCTP and 1 μl of TRAP-RV. The telomere repeats added to the forward primer by telomerase were amplified by 34 cycles of PCR; denaturing at 94^{o}C for 5 min and followed by 94^{o}C for 30 sec, 65^{o}C for 30 sec, 72^{o}C for 1 min 30 sec and a final extension at 72^{o}C for 5 min. The reaction was stopped by 60 μl of telomerase stop buffer (10 mM TRIS-HCl, pH 7.5 and 21 mM EDTA) and the DNA was precipitated by ethanol following a phenol chloroform extraction. The samples were resolved on 6% sequencing gels. Extracts from *Arabidopsis* Wild type flowers was used as a positive control and *Arabidopsis* Wild type leaves as a negative control. The gel was dried and exposed to a PhosphorImage screen.

Cytogenetics

DAPI stained chromosome spreads were prepared from pistils as described (Riha et al., 2001). Anaphase spreads were prepared from pistils and stained with DAPI (4',6'-diamidino-2-phenylindole). DAPI stained cells were observed under a Zeiss microscope.

PETRA (Primer Extension Telomere Repeat Amplification)

PETRA was performed as described previously with some modifications (Heacock *et al.,* 2004). Terminal DNA sequences for eight *Arabidopsis* chromosome arms were identified in GenBank (1R, AC074299; 1L, AC007323 and AC074298; 2R, AC006072; 3L, AC067753; 3R, AL732522; 4R, AL035708 and Z12169; 5L, AB033277; 5R, AB033278). For PETRA, primer extension was performed using a primer bound to the telomeric G-overhang (PETRA-T 5' CTCTAGACTGTGAGACTTGGACTACCCTAAACCCT-3′), followed by PCR

amplification using a chromosome-specific subtelomeric primer (1R 5′-CTATTGCCAGAACCTTGATATTCAT-3′; 1L 5′-AGGACCATCCCATATCATTGAGAGA-3′; 2R 5′-CAACATGGCCCATTTAAGATTGAACGGG-3′; 3R 5′-CTGTTCTTGGAGCAAGTGACTGTGA-3′; 3L 5′-CATAATTCTCACAGCAGCACCGTAGA-3′; 4R 5′-TGGGTGATTGTCATGCTACATGGTA-3′; 5R 5′-CAGGACGTGTGAAACAGAAACTACA-3′; 5L 5′-AGGTAGAGTGAACCTAACACTTGGA-3′) and second primer (PETRA-A 5′-CTCTAGACTGTGAGACTTGGACTAC-3′) that recognizes sequence complementary to the 5′ non-telomeric sequence present on PETRA-T. Primer extension reactions (20 μl) included 1 × ExTaq buffer (TaKaRa), 250 μM dNTPs, 0.2 μM PETRA-T primer, 500 ng genomic DNA and 2 U ExTaq (TaKaRa). The reaction was incubated at 96°C for 5 min, 65°C for 1 min and 72°C for 10 min. After PETRA-T reaction, PCR amplification was performed for each chromosome arm utilizing the unique sub-telomeric sequence primers. Each chromosome-specific PCR reaction (20 μl) included 1 × ExTaq buffer (TaKaRa), 200 μM dNTPs, 0.4 μM PETRA-A primer, 0.4 μM subtelomeric primer, 1/10 of the DNA recovered from the primer extension and 2 U ExTaq (TaKaRa). Samples were incubated at 96°C for 5 min, followed by 20 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. A 10 μl aliquot of the PCR products was separated by electrophoresis through a 1% agarose gel, and transferred to a nylon membrane. Membranes were probed with a ^{32}P end-labeled telomeric oligonucleotide $(T_3AG_3)_4$. Hybridization was performed at 55°C overnight, in a buffer

consisting of 0.25 M sodium phosphate buffer (pH 7.5), 7% SDS and 1 mg/ml BSA. Filters were washed in 2 × SSC/0.1% SDS and 0.2 × SSC/0.1% SDS, twice each for 10 min at 55°C. Hybridization signals were detected using a STORM PhosphorImager (Molecular Dynamics) and the data were analyzed using IMAGEQUANT software (Molecular Dynamics).

Results

PARP1 and PARP2 are transcriptionally upregulated in response to DNA damage in Arabidopsis

To understand the DNA damage responses in *Arabidopsis* with respect to telomere dysfunction, wild type and G3 generation *tert* mutants were germinated on complete media (Murashige and Scoog, 1962), and five day old seedlings were transferred to complete media plates containing Methyl Methane Sulfonate (MMS), which is a DNA damage inducing drug. Three days after treatment with MMS, the seedlings were collected and total RNA was extracted and reverse transcribed for RT-PCR experiments. RT-PCR on those wild type and *tert* mutants showed that with increasing concentrations of MMS, *PARP2* mRNA levels were increased (Figure 3.1A). In addition, higher levels of both *PARP1* (Figure 3.1B) and *PARP2* mRNA (Figure 3.1A) were observed in *tert* mutants compared to wild type seedlings even without any DNA damage induction (compare 1st lane with 5th lane in Figure 3.1A). These data suggest that DNA damage responses are signaled in the absence of telomerase by transcriptionally upregulating the DNA repair genes *PARP1* and *PARP2*.

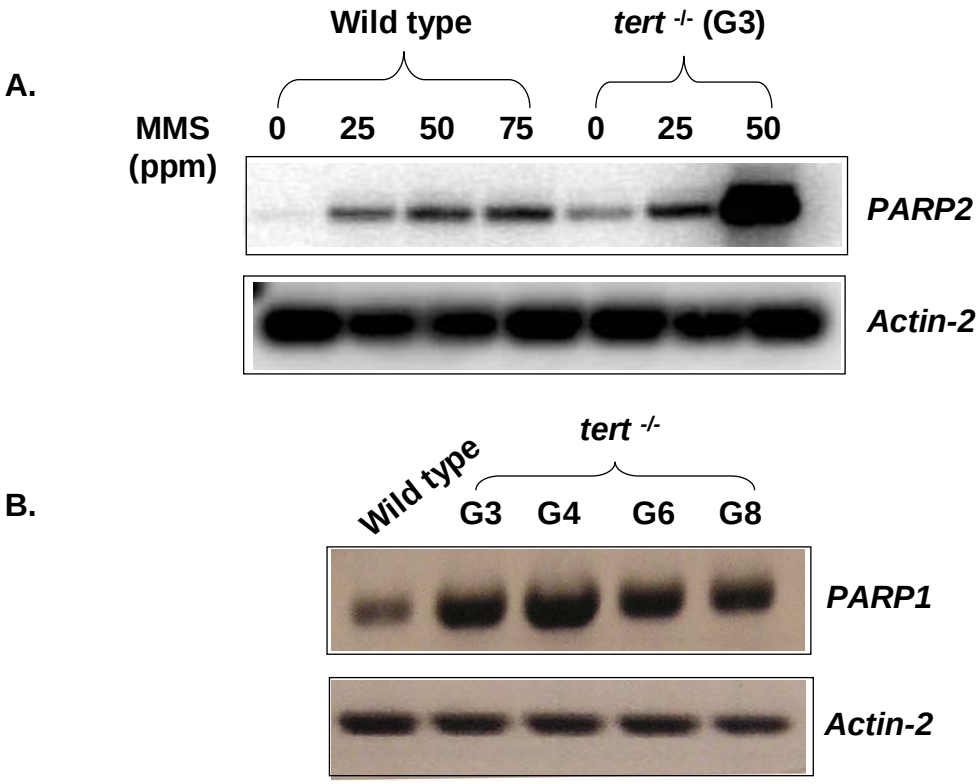

Figure 3.1. DNA damage upregulation in response to MMS treatment and telomerase deficiency. A. RT-PCR on wild type and *tert* mutants treated with MMS for three days showed that *PARP2* transcripts are upregulated in response to DNA damage induced by MMS and this upregulation is more evident even with no induction of DNA damage in *tert* mutants (compare lane 0 for wild type to lane 0 for *tert* $^{-/-}$). **B.** RT-PCR for *PARP1* in *tert* mutants showing *AtPARP1* mRNA upregulation in *tert* mutants. In both the panels, *Actin-2* was shown as a loading control.

Neither PARP1 nor PARP2 is required for telomere length maintenance in Arabidopsis

To examine the role of *PARP1* and *PARP2* in telomere length maintenance, we screened T-DNA collections from the University of Wisconsin *Arabidopsis* knock-out facility. SALK lines bearing a T-DNA insertion in 12th intron of PARP1 and 6th intron of PARP2 were identified by genotyping the collection.

T-DNA insertion lines were identified for both *PARP1* and *PARP2* and the location of T-DNA insertion for each gene is shown in the figures (Figure 3.2A and Figure 3.3A). *PARP1* and *PARP2* transcription was abolished in single mutants of each of those respective T-DNA insertion lines, as indicated by RT-PCR analysis (panel B in Figure 3.2 and 3.3 for *PARP1* and *PARP2* respectively). This data indicate that those mutants are null mutants for respective genes. We performed TRF analysis on genomic DNA extracted from *parp1* and *parp2* single mutants and the results showed that both *parp1* and *parp2* single mutants showed wild type telomere length (Figure 3.2 and 3.3, panel C).

In addition, we determined telomere length for individual chromosome arms using PETRA. In this assay, individual telomeres are amplified in a PCR reaction using primers specifically directed at the G-overhang and a primer directed at an unique sub-telomeric sequence. PETRA analysis of either *parp1* or *parp2* mutants did not show any changes in telomere length compared to wild type (Figure 3.2 and 3.3, panel D). These results indicate that neither *PARP1* nor *PARP2* is required for telomere length homeostasis in *Arabidopsis*.

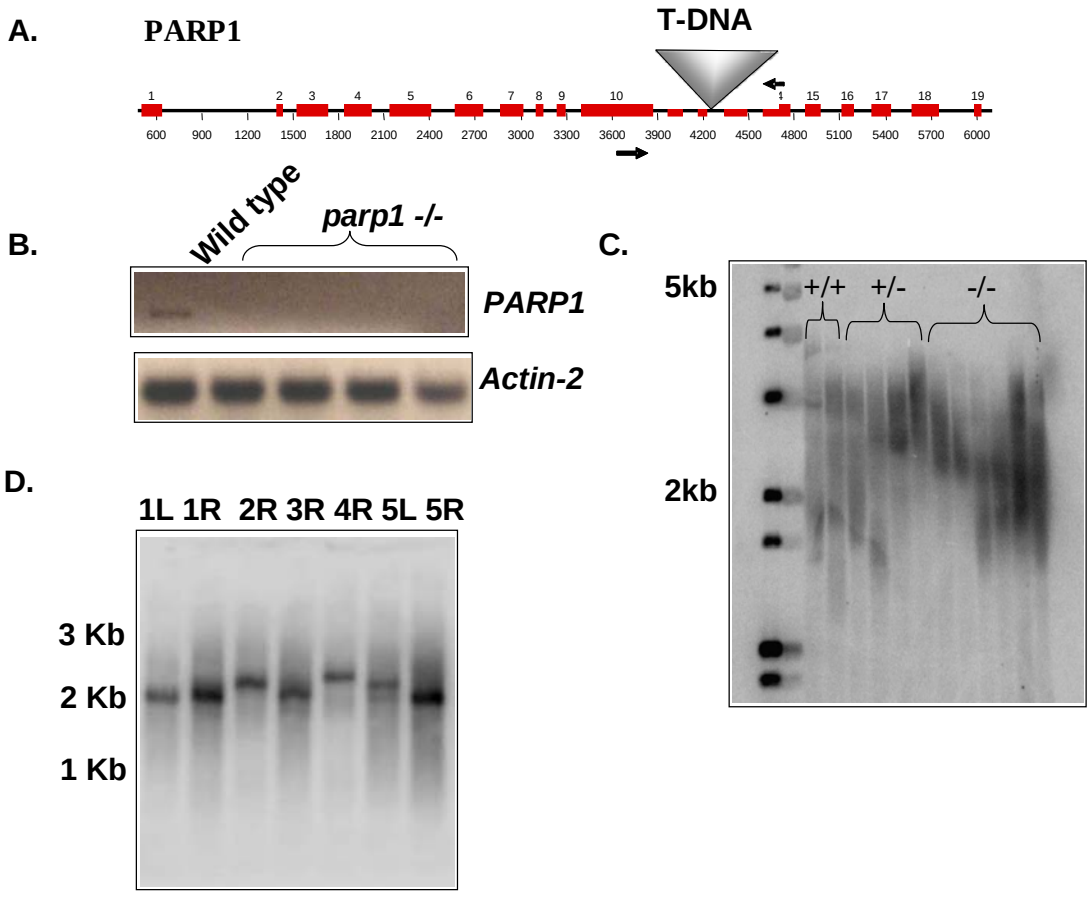

Figure 3.2. Characterization of T-DNA insertion line for *PARP1*. A. The location of T-DNA insertion in 12th intron of *PARP1* and primers used for RT-PCR are shown. **B.** RT-PCR analysis of *parp1*$^{-/-}$ showing the disruption of *PARP1* expression. Total RNA from wild type flowers was used as a control. *Actin-2* was used as a loading control. **C.** TRF blot showing the telomere length of *parp1*$^{-/-}$. +/+ indicates the wild type, +/- indicates heterozygous plants for *PARP1* and -/- indicates the homozygous nature of *parp1* mutant. **D.** PETRA results for a single *parp1* $^{-/-}$ plant. The range of telomere length is from 1.8 kb to 2.5 kb, which is in wild type telomere range (Heacock *et al.*, 2004). Marker sizes are indicated in the figure.

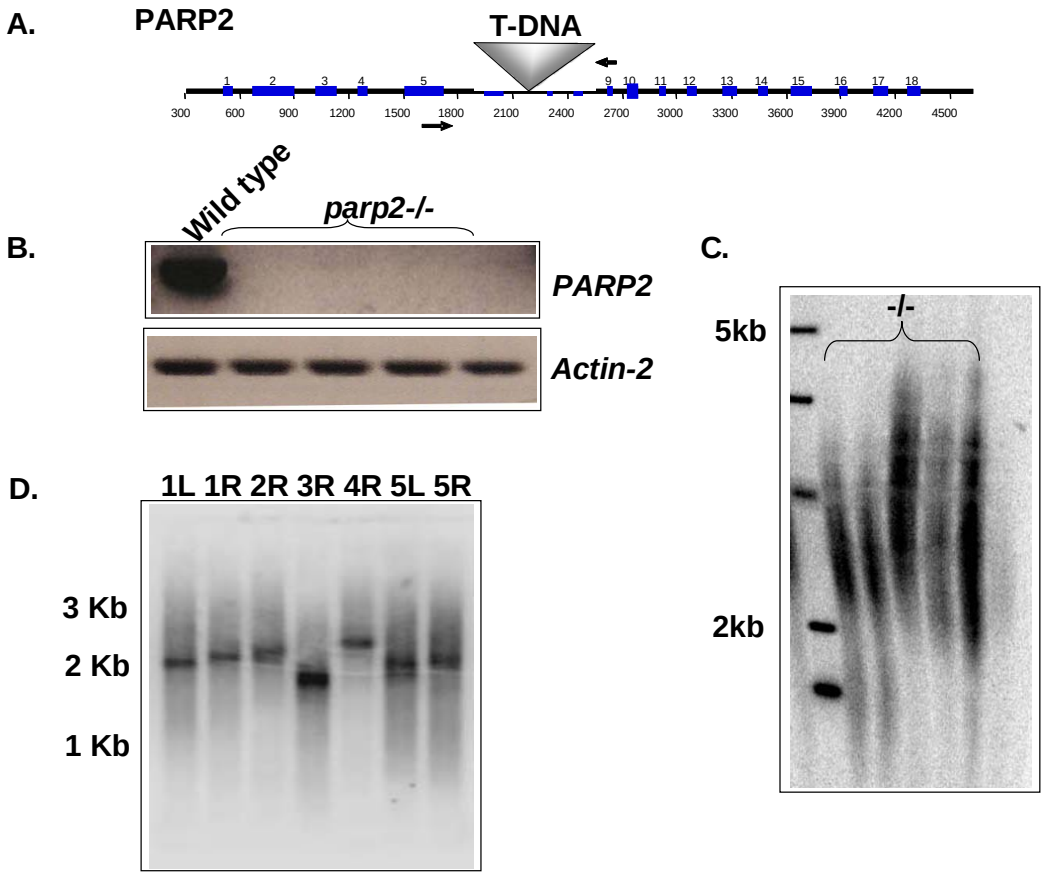

Figure 3.3. Characterization of T-DNA insertion line for *PARP2*. A. The location of T-DNA insertion in 6th intron of *PARP2* and primers used for RT-PCR are shown. **B.** RT-PCR analysis of *parp1*$^{-/-}$ showing the disruption of *PARP2* expression. Total RNA from wild type flowers was used as a control. *Actin-2* was used as a loading control. **C.** TRF blot showing the telomere length of *parp2*mutant. +/+ indicates the wild type, +/- indicates heterozygous plants for *PARP1* and -/- indicates the homozygous nature of *parp1* mutant. **D.** PETRA results for a single *parp2* $^{-/-}$ plant. The range of telomere length is from 1.8 kb to 2.5 kb, which is in the wild type telomere range (Heacock *et al.*, 2004). Marker sizes are indicated in the figures.

PARP2 might be a negative regulator of PARP1

Unexpectedly, RT-PCR data from plants deficient for *PARP2* showed that *PARP1* is transcriptionally upregulated (Figure 3.4). In contrast, *PARP2* is expressed at wild type levels in *PARP1* deficient plants. These data suggest that *PARP2* might be a negative regulator of *PARP1* in *Arabidopsis*. On the other hand, this transcriptional upregulation of *PARP1* might be happening as a DNA repair response to higher levels of induction of DNA damage in *parp2* deficient plants.

Neither PARP1 nor PARP2 is required for chromosome end protection and genome stability in Arabidopsis

To investigate the role of PARPs in chromosome end protection and genome stability, we performed cytogenetic analysis. Cytogenetic studies were conducted on dissected pistils from *parp1* and *parp2* single mutants. Anaphase bridges are the hallmark of dysfunctional telomeres, reflecting the formation of dicentric chromosomes resulting from the fusion of deprotected chromosome ends (Hande *et al.,* 1999).

No mitotic abnormalities were observed in either *parp1* or *parp2* single mutants (data not shown). A more sensitive telomere fusion PCR assay (Heacock *et al.,* 2004) also failed to detect any chromosome end joining products in mutants lacking either *PARP1* or *PARP2* (data not shown). These data indicate that deficiency of either *PARP1* or *PARP2* in *Arabidopsis* does not lead to gross chromosomal abnormalities. We conclude that neither *PARP1* nor *PARP2* plays an important role in chromosome end protection and the maintenance of genome stability.

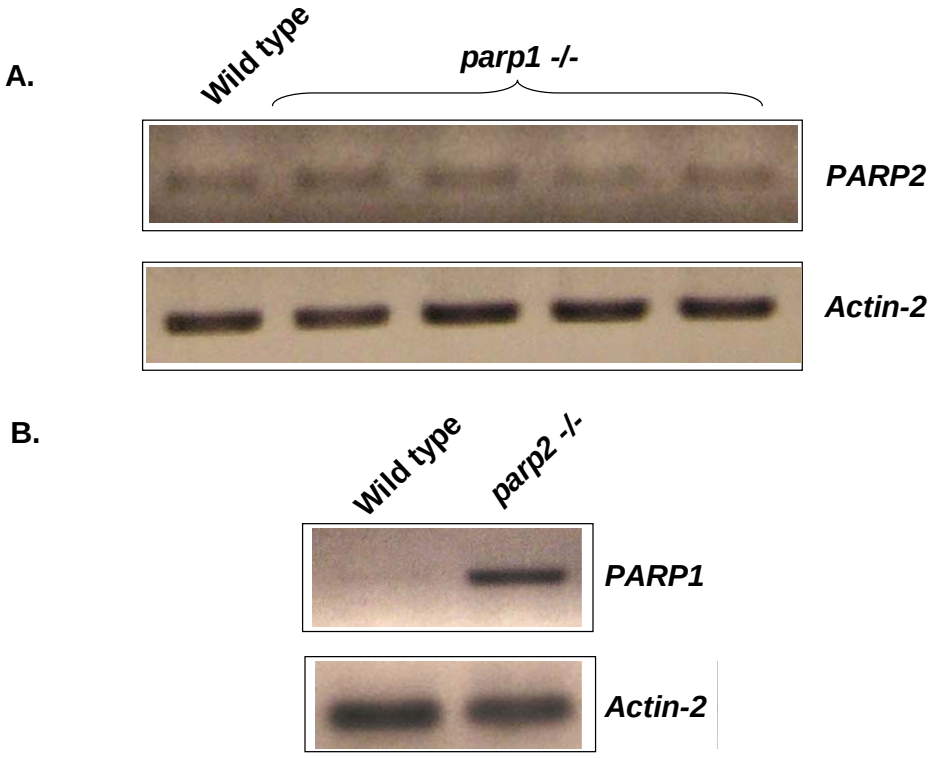

Figure 3.4. *AtPARP1* mRNA upregulation in parp2 mutants. A. RT-PCR analysis of plants deficient for *AtPARP1* showed that *AtPARP2* exhibits similar transcript levels as in wild type. **B.** In contrast, *AtPARP1* is upregulated transcriptionally in *PARP2* deficient plants. In both the cases, *Actin-2* was used as a loading control.

Telomere maintenance and genome stability do not rely on PARP1 and PARP2

The presence of residual DNA-dependent PARP activity was observed in *PARP1* deficient mice (Shieh *et al.,* 1998), indicating the redundancy in this pathway. Since the double mutant for *PARP1*/*PARP2* is lethal in mice (de Murcia *et al.,* 2003), we attempted to generate a double *parp1/parp2* $^{-/-}$ mutant in *Arabidopsis*, as a way of investigating the combined contribution of these genes in telomere biology. We crossed a homozygous *parp1* mutant plant to a homozygous *parp2* mutant. The F2 population of this cross was screened for a double mutant by genotyping. The F2 population showed a typical Mendelian segregation pattern of inheritance, and several homozygous mutants for both *parp1* and *parp2* were identified. In the double mutants expression of both *PARP1* and *PARP2* was abolished, as shown by RT-PCR (Figure 3.5A). TRF analysis was performed on genomic DNA extracted from the double *parp1/parp2* mutants. As shown by TRF analysis, telomeres from the double mutants exhibited the same telomere length as those from wild type plants (Figure 3.5B). PETRA analysis also indicated that individual telomeres fall within the wild type range (Figure 3.5C). In addition, telomerase activity can be detected in double mutants for PARPs (data not shown) indicating that ADP ribosylation is not required for telomerase activity as in humans (Ghosh and Bhattacharya, 2005).

To observe the status of chromosome end protection and genome stability in *parp1*/*parp2* double mutants, cytogenetic studies were performed on dissected pistils from the double mutants. No mitotic abnormalities were observed in *parp1*/*parp2* double mutants (data not shown). In addition, no products were generated in a PCR assay to

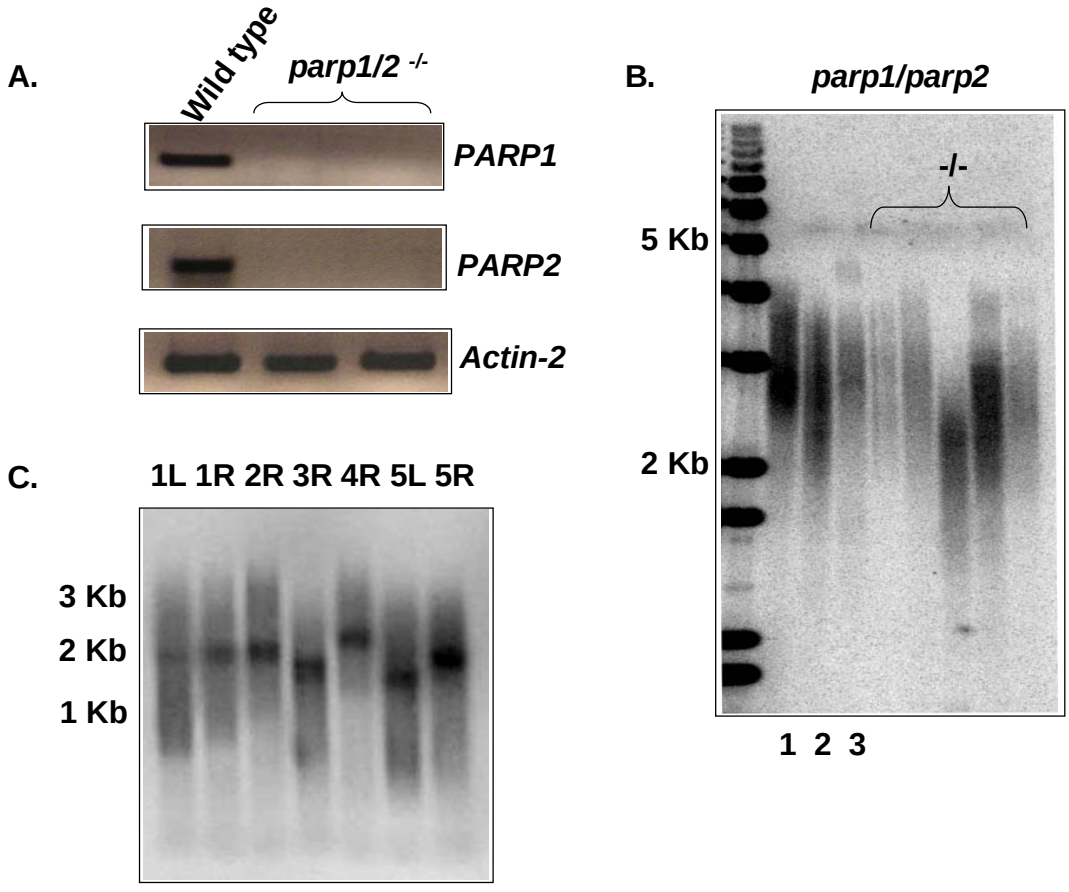

Figure 3.5. Characterization of double mutant for *parp1/parp2*. A. RT-PCR analysis of *parp1/parp2* $^{-/-}$ showing the disruption of both *PARP1* and *PARP2* expression. Total RNA from wild type flowers was used as a control. *Actin-2* was used as a loading control. **B.** TRF blot showing the telomere length of *parp1/parp2* double mutant. -/- indicates the homozygous nature of *parp1* mutant. Wild type is shown in lane 1, *parp1* and *parp2* single mutants are shown in lane 2 and 3, respectively. **C.** PETRA results for a single *parp1*/parp2 $^{-/-}$ plant. The range of telomere length is from 1.8 kb to 2.5 kb, which is in wild type telomere range (Heacock *et al.,* 2004). Marker sizes are indicated in the figure.

detect telomere specific fusions (data not shown). Taken together, these data indicate that deficiency of both *PARP1* and *PARP2* in *Arabidopsis* do not lead to any telomere length deregulation and genome instability.

Discussion

All living cells have the ability to detect and respond to the DNA damage to minimize the consequences of that damage. In eukaryotes, the initial stages of response are governed by a pair of protein kinases, ATM and ATR (Shechter *et al.,* 2004; Kurz and Lees-Miller, 2004). It is well established that treatment of *Arabidopsis* with DNA damaging agents induces a robust transcriptional upregulation of many DNA repair genes and ATM is required for this response (Garcia *et al.,* 2003). Interestingly, there is no consistency within the eukaryotic kingdoms with respect to transcriptional upregulation of DNA repair genes in response to DNA damage treatment. For example, BRCA1 is highly induced in response to ionizing radiation in plants but not in human cells (Rieger and Chu, 2004). Thus, different DNA repair genes may have different roles in response to DNA damage.

In order to understand the DNA damage responses in *Arabidopsis*, wild type seedlings were treated with a DNA damage inducing drug (MMS –Methyl Methane Sulfonate) and the transcription levels of PARPs were determined. Our results indicate that *PARP2* was transcriptionally upregulated in a dose-dependent manner with MMS treatment. In addition, telomerase-deficient plants showed upregulation of PARP transcripts even without DNA damage induction, arguing that telomere dysfunction is recognized as DNA damage.

To investigate the role of *Arabidopsis* PARPs in telomere length maintenance and genome stability, T-DNA insertion lines for both *PARP1* and *PARP2* were characterized. TRF analysis and cytogenetic analysis indicated that both PARPs are not essential either for telomere length maintenance or for genome stability maintenance. These data were supported by the observation that *Arabidopsis* shows much greater tolerance to telomere dysfunction and DNA damage than mammals. Notably, mutations in several telomere related genes and DNA repair genes are lethal in mammals (ATR - deKlein *et al.,* 2000, RAD50 - Luo *et al.,* 1999, and BRCA1 – Gowen *et al.,* 1996), but not in *Arabidopsis* (ATR – Vespa *et al.,* 2005, RAD50 – Gallego *et al.,* 2001, and BRCA1 – Jasti, M. and Shippen, D., unpublished data).

In mammals, *parp2* $^{-/-}$ cells display increased frequency of spontaneous chromosome and chromatid breaks and of ends lacking detectable $T_{(2)}AG_{(3)}$ repeats suggesting a functional role for PARP-2 activity in the maintenance of telomere integrity (Dantzer *et al.,* 2004). In contrast, cytogenetic analysis of single and double mutants for the *Arabidopsis* PARPs indicated that they do not have a significant role in chromosome end protection in plants. Since a significant part (60%, according to *Arabidopsis* Genome Initiative, 2000) of *Arabidopsis* genome was duplicated, even loss of some sequences by aneuploidy could be tolerated. Another important characteristic of plants is the totipotency of plant cells (basically each cell can give rise to all other cell types). One could imagine that cells with high levels of genome instability could be replaced by those with more stable genomes, and resulting in a selection against severe instability.

Due to the lethality of *PARP1/PARP2* double mutants in mammals (Shieh *et al.,* 1998), the contribution of PARPs to telomere biology has not been explored in higher

eukaryotes. Hence current study provides the first insight into the combined role of the two PARPs at higher eukaryotic telomeres. Plants and animals display remarkable differences in their tolerance to genome assault. While most of the cell cycle components and DNA damage signaling pathways are conserved in plants, p53 is absent in *Arabidopsis* genome, which may enable some proliferation in the face of genome instability. Thus, many questions remain unanswered: what are the molecular responses to telomere dysfunciton, and what are the contributing factors in sensing the damaged telomeres.

Although plants lacking ATM or ATR (key players in DNA damage response) display wild-type telomere length homeostasis, *atm tert Arabidopsis* experience an abrupt, early onset of genome instability, arguing that ATM is required for protection of short telomeres (Vespa *et al.,* 2005). ATR, by contrast, is required for maintenance of telomeric DNA as telomere shortening is dramatically accelerated in *atr tert* mutants relative to *tert* plants (Vespa *et al.,* 2005). Likewise, studying the role of PARPs at dysfunctional telomeres by analyzing the triple mutant of *parp1*/*parp2*/*tert* $^{-/-}$ might help determine the role of PARPs at dysfunctional telomeres, thereby helps to understand the contribution of DNA repair proteins at dysfunctional telomeres.

CHAPTER IV

INTERACTIONS BETWEEN TELOMERE RELATED PROTEINS

Introduction

The study of protein-protein interactions has been vital to the understanding of how proteins function within the cell. Often, the molecular basis for how a protein functions can be explained by its interacting partners.

Telomere binding proteins are essential to maintain the chromosome end structures and to regulate telomerase. The "Shelterin" complex, which contains six components, functions as a unit for chromosome end protection and telomere length regulation in mammals (de Lange, 2005). TRF1 and TRF2, the founding members of shelterin, were identified as double-strand telomere binding proteins (Zhong *et al.,* 1992; Bilaud *et al.,* 1997). Later, TIN2 and Rap1 were found as interacting partners in two-hybrid screens with hTRF1 and hTRF2, respectively (Kim et al. 1999; Li et al. 2000). TPP1 and PTOP were subsequently identified as interacting proteins with hTIN2 (Houghtaling *et al.,* 2004; Liu *et al.,* 2004). hTIN2 also connects TRF1 to TRF2 and this link contributes to the stabilization of TRF2 on telomeres (Liu *et al.,* 2004). hPOT1, a single stranded telomere binding protein, interacts with hTRF2 (Yang *et al.,* 2005).

Several double-strand telomeric DNA binding proteins (TRFL – TRF-like: TRP1, TRFL4, TRFL9, TBP1) were identified in *Arabidopsis* based on their homology to MYB DNA binding domain of hTRF1 and hTRF2 (Karamysheva *et al.,* 2004). *In vitro* co-immunoprecipitation experiments revealed that some of the recombinant TRFL proteins, formed homodimers and heterodimers *in vitro* (Karamysheva *et al.,* 2004). In contrast,

hTRF1 and hTRF2 formed homodimers but not heterodimers (Ye *et al.,* 2004). Currently, there is no direct evidence that TRFLs play any role in telomere biology, as the single, double and triple mutants do not show any telomere-specific phenotypes (Karamysheva, Z. and Vespa, L. and Shippen, D., unpublished data). Thus, the characteristic heterodimerization of TRFLs, which is distinct from human TRFs, and lack of striking telomere phenotypes for TRFL mutants suggest that identification of TRFL-interacting proteins in *Arabidopsis* might help unravel the role of these proteins at telomeres.

In addition to telomere-specific proteins, several DNA repair proteins and proteins involved in sensing DNA damage associate with telomeres. The phosphatidylinositol 3-kinase-like kinases (PIKKs) ATM and ATR activate a complex signaling network in response to diverse forms of DNA damage. ATM also protects against telomere fusions and participates in telomere length homeostasis as *atm/tel1* mutants display shorter telomeres and fused chromosome ends in both mammals and yeast (Lustig and Petes 1986; Smilenov *et al.,* 1997). The shelterin subunit TRF2 has a weak interaction with the ATM kinase (Karlseder *et al.,* 2004). In addition, localization of ATM at telomeres in the G2 phase of cell cycle suggests that localized DNA damage response at telomeres after replication is essential for recruiting the processing machinery that promotes formation of a chromosome end protection complex (Verdun *et al.,* 2005). The role of ATR is less defined, because null mutants are lethal in yeast and mammals (Brown and Baltimore, 2000, Zhao *et al.,* 2001). ATR is recruited by RPA, a single-stranded DNA binding protein, to stalled replication forks through its interaction partner ATRIP (ATR-interacting protein) (Shechter *et al.,* 2004). A large number of interaction

studies reveal that ATM has many interacting partners whereas ATR has only one identified so far, which is ATRIP.

Arabidopsis plants lacking ATM and ATR display Wild type telomere length homeostasis (Vespa *et al.,* 2005). However, *atm tert Arabidopsis* experience an abrupt, early onset of genome instability, arguing that ATM is required for protection of short telomeres. ATR, by contrast, is required for maintenance of telomeric DNA as telomere shortening is dramatically accelerated in *atr tert* mutants relative to *tert* plants. Thus, ATM and ATR make essential and distinct contributions to chromosome end protection and telomere maintenance in *Arabidopsis* (Vespa *et al.,* 2005). Thus, identification of interacting partners for ATM and ATR may provide insight into the function of these proteins at telomeres.

hPOT1 is a multifunctional telomeric DNA binding protein that interacts with the G-rich overhang by its oligonucleotide/oligosaccharide binding fold (Lei *et al.,* 2004). . Deletion of fission yeast *pot1* causes rapid and complete loss of telomeric DNA followed by chromosome end fusions (Baumann and Cech, 2001). Expression of DNA binding-defective mutants of hPot1 results in dramatic telomere elongation (Bunch *et al.,* 2005; Loayza and de Lange, 2003). Thus, the interaction between Pot1 and proteins bound along the length of the telomere (e.g., hTRF1) may be crucial to converting telomere length information into increased or decreased accessibility of the 3′ end to telomerase. TPP1, an hPOT1 interacting protein recruits POT1 to the TIN2/TRF1 complex and contributes to telomere length control (Ye *et al.,* 2004). The C-terminal half of hPOT1 binds directly to Ptop/Pip1/TPP1, an interaction that appears to be essential for the recruitment of hPOT1 to telomeres (Ye *et al.,* 2004). Recently, a direct interaction

between hPOT1 and TRF2 has also been reported (Yang *et al.,* 2005). The mouse genome contains two POT1 orthologs, *POT1a* and *POT1b*. *Pot1a* is crucial for maintenance of both telomere integrity and genome stability (Wu *et al.,* 2006). POT1b protects telomeres from end-to-end chromosomal fusions and aberrant homologous recombination (He *et al.,* 2006).

In contrast to humans and S. *pombe*, *Arabidopsis* has three POT like proteins, POT1, POT2 and POT3 (Shakirov *et al.,* 2006 and data unpublished). POT1 has a specialized function in positive regulation of telomere length by telomerase, but does not participate in the protection of telomeres from end fusions, whereas POT2 protects the telomeres from end fusions (Shakirov *et al.,* 2006). These multiple POT proteins in *Arabidopsis* might reflect subfunctionalization of a multifunctional ancestral *POT1* gene. The sequence divergence among different POT1 homologs from different plant species suggests that POT1 is evolving much faster than the other POTs (Shakirov, E., and Shippen, D., unpublished data). In addition there is no obvious homolog for TPP1 (PTOP) in *Arabidopsis*. The striking telomere phenotype of *pot1* mutant and the lack of an obvious homolog for TPP1 suggest that identification of POT1 interacting proteins in *Arabidopsis* might help provide the insights into the mechanism of POT proteins at telomeres.

Yeast two-hybrid screens enable the identification and characterization of protein-protein interaction partners. This approach also embodies the technological means to manipulate protein-protein interactions. In the present study, we performed a yeast two-hybrid screen to confirm some of the interactions between *Arabidopsis* telomere-related proteins that had previously been detected by the *in vitro* co-immunoprecipitation

method. We also conducted several screens with an *Arabidopsis* cDNA library to identify novel interacting partners for TRFL9, ATR and POT1.

Materials and methods

Cloning of candidate telomere-binding proteins for two-hybrid assay

Full-length cDNA sequences of TRFL9, TRFL4, and TRP1, different parts of ATR, Ku70, Ku80 and POT1 were cloned to analyze their interactions. cDNA sequences coding for all the proteins mentioned above were obtained using RT-PCR from total RNA isolated from *A. thaliana* seedlings and cloned into vectors pAS2.1 (bait vector having Gal4 DNA binding domain) and pACT-2 (prey vector having Gal4 activation domain). A customized normalized cDNA library in pACT-2 made from *Arabidopsis* seedlings was purchased from Virotech international, Inc. Prior to two-hybrid system screening, cloned constructs were checked for their correct reading frame by sequence analysis (Sanger, 1980).

Yeast media and reagents

For preparation of Synthetic Dropout media plates (SD), 6.7 g Difco Yeast Nitrogen Base (w/o amino acids) was mixed with 20 g glucose and synthetic complete drop-out Mix (as per the quantity indicated on the bottle- CLONTECH). One liter of distilled water was added to the above dropout mix and 20 g of Difco Bacto Agar was added (for solid medium). All the media and plates were prepared fresh whenever needed.

Yeast two-hybrid screen

The baits were introduced into yeast *S. cerevisiae* strain AH109 (*MATa, his3, trp1, leu2, his3* - Clontech) by a modified lithium acetate method (Clontech). The transformants were selected on the yeast synthetic media (media lacking Tryptophan) with 2% glucose and used as a host for transformation with the *Arabidopsis* cDNA library cloned in pACT2 vector. The cDNA library in pACT-2 (0.2 mg/library transformation) was sequentially transformed into the competent bait transformed yeast cells, and transformants were selected on the plates with synthetic medium lacking tryptophan (pAS2.1 selection marker), leucine (pACT-2 selection marker) containing 2% glucose. The transformation efficiency was calculated according to the method mentioned in the CLONTECH yeast two-hybrid manual. All transformants were pooled and respread on the synthetic medium lacking tryptophan, leucine and histidine (selection marker for protein-protein interactions) containing 2% glucose to confirm their growth in the absence of histidine. Cells growing on the -Trp-Leu-His plates were selected and tested for β-galactosidase activity, another reporter gene, by colony lift assay to check for protein-protein interactions.

Colony lift assay

To assay for β-galactosidase (LacZ) reporter gene expression, the colony lift assay was performed. In this assay, the colonies grown on the selection medium plates were reprinted onto a filter paper, transferred to liquid nitrogen to freeze the filter and then placed this frozen filter on a new filter soaked with Z buffer/X-gal solution (Z buffer: 16.1 g/L Na_2HPO_4 and 5.5 g/L NaH_2PO_4, 0.75g/L KCl and 0.246 g/L $MgSO_4.7H_20$)

with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside: 20 mg/Ml in N,N,-dimethyl formamide*)*. The colonies were incubated at 30^{o}C for 4 hrs and checked for the development of blue color. After identification of the interaction between telomere related proteins, the reciprocal experiments between the bait and prey vectors were carried out.

Plasmid-rescue from yeast cells

To identify the clones that interact with the bait protein, high efficiency yeast plamid rescue was performed. In this procedure, yeast minipreps were performed to extract the plasmid DNA from yeast cells. Five ml of culture was grown overnight and spun for 5 min at maximum speed at room temperature. The pellet was then resuspended in QIAGEN buffer P1. Two hundred mg of glass beads were added to the P1 resuspended pellet. The tubes were vortexed for five min and then plasmid DNA was isolated according to miniprep protocol from QAIGEN. The plasmid DNA extracted from the yeast cells was then transformed into *E. coli* competent cells. Plasmid extraction was carried out from *E. coli* cells (QIAGEN miniprep) and the plasmid was sequenced with primers specific to the target vector, pACT-2.

Co-immunoprecipitation

For each protein analyzed, two constructs were made, one with a T7 protein tag and one without. [^{35}S] methionine-labeled non-tagged proteins or T7-tagged unlabeled proteins were synthesized in a TNT-coupled rabbit reticulocyte lysate translation system following the manufacturer's recommendations (Promega). Translation of T7-tagged

proteins was verified in the presence of [^{35}S] methionine before performing co-immunoprecipitation experiments. T7-tagged and untagged radiolabeled proteins were combined and subjected to immunoprecipitation using agarose beads (Novagen) containing the T7 monoclonal antibody. Precipitate and supernatant fractions were analyzed by SDS-PAGE and autoradiography.

Results

Confirmation of in vitro interactions of telomere-related proteins in vivo

To identify interactions among candidate telomere-related proteins, the two-hybrid system in *Saccharomyces cerevisiae* was utilized. Target proteins were cross-tested with each other to assay all combinations in vectors pAS2.1 and pACT-2, so each protein was used as a bait or prey. Two proteins Ku70 and Ku80 from *A. thaliana* have been isolated on the basis of sequence similarity to the human protein hKu70 and hKu80. These proteins form a stable heterodimer (Tamura *et al.,* 2002). Therefore, heterodimer formation of *Arabidopsis* Ku70 and Ku80 was utilized as a positive control in all the assays performed in this study. The cDNAs of TRFL9, TRFL4, TRP1, different regions of ATR, Ku70, Ku80 and POT1 from *A. thaliana* were cloned in-frame with both Gal4DBD and Gal4AD for either bait or prey. Previous *in vitro* co-immunoprecipitation experiments showed that specific homomeric protein interactions were detected for several putative double-strand telomeric DNA binding proteins including TRP1, TRFL1, TRFL2, TRFL4, and TRFL9. It was also shown that TRP1 and TRFL1 can heterodimerize, as can TRP1 and TRFL9 (Karamysheva *et al.,* 2004).

To confirm these interactions *in vivo*, the yeast two-hybrid system was utilized. The 1500-bp full length *ADH1* promoter, that normally drives the expression of the metabolic enzyme alcohol dehydrogenase 1, leads to high-level expression of sequences under its control (Clontech). This promoter was used in the pAS2.1 plasmid for cloning the bait-fusion. It is also present in pACT-2 that is used to clone the cDNA library. The DNA-BD/bait plasmid was introduced through a small-scale transformation; selected transformants were then grown up and transformed with the AD fusion target construct through a sequential transformation. The two-hybrid technique exploits the fact that the DNA-binding domain of GAL4 is incapable of activating transcription unless physically, but not necessary covalently, associated with an activating domain (Figure 4.1). The colony lift assay was performed to estimate the strength of interaction between the testing partners.

As expected, yeast two-hybrid analysis confirmed that AtKu70 and AtKu80 form a heterodimer, while AtKu80 alone did not form a homodimer (Figure 4.2). Thus, these reactions were used as positive and negative controls, respectively. Two-hybrid data confirmed the homodimerization of TRP1 (Figure 4.2) and TRFL4 (data not shown) and also confirmed the heterodimer formation of TRP1 with TRFL9 and of TRP1with TRFL4 (Figure 4.2). These data are consistent with results for hTRF1 and hTRF2, as the homodimerization of hTRF1 and hTRF2 is required for association with telomeric DNA *in vivo*. These proteins cannot form heterodimers (Fairall *et al.,* 2001). Thus, the capacity to form both homodimers and heterodimers suggests that TRFL proteins could participate in complex structural and functional regulation *in vivo*. Since the single, double and triple mutants of TRFL proteins did not show any defects in telomere biology, we performed

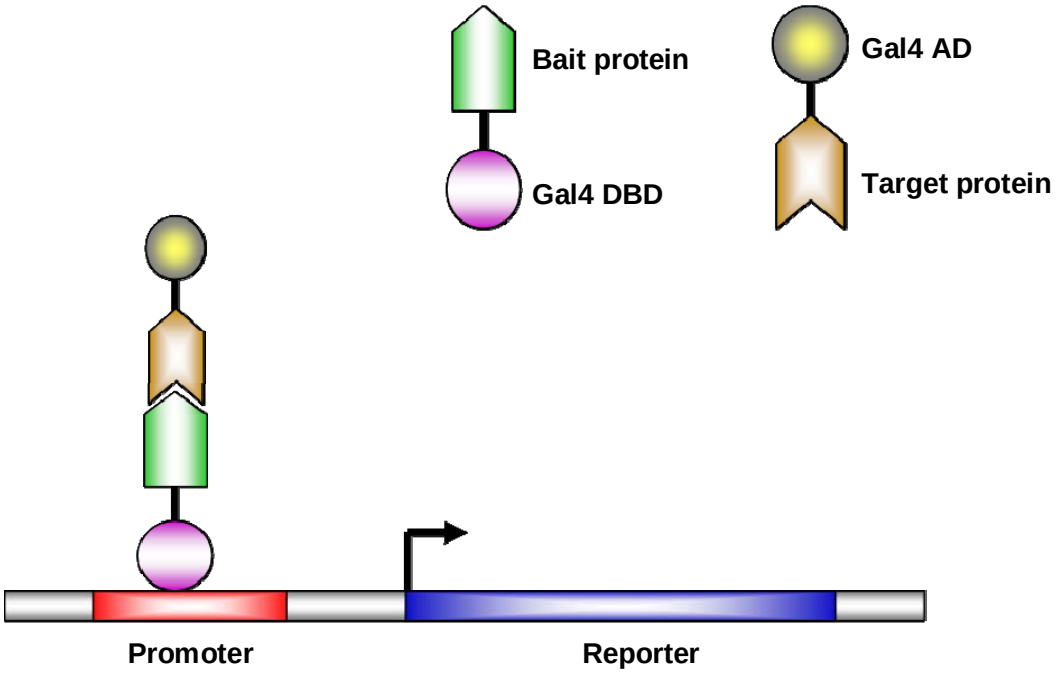

Figure 4.1. Principle of the yeast two-hybrid system. The yeast two-hybrid technique measures protein-protein interactions by measuring transcription of a reporter gene. If the bait protein and target protein interact, then their DNA-binding domain and activation domain will combine to form a functional transcriptional activator (TA). The TA will then activate transcription of the reporter gene.

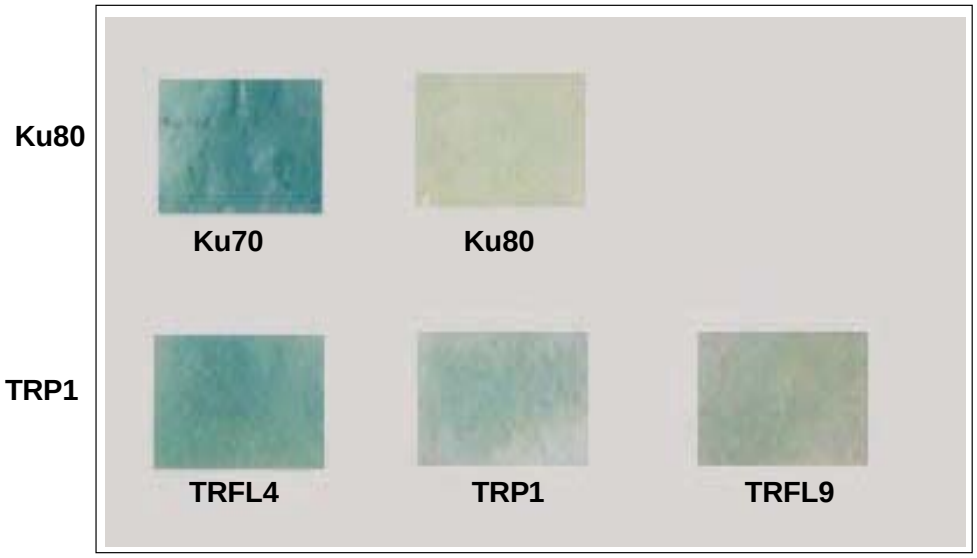

Figure 4.2. Interactions of telomere proteins by yeast two-hybrid assay. Gal4DBD-Ku80 and Gal4DBD-TRP1 were used as baits and Ku70, Ku80, TRFL4, TRP1 and TRFL9 were used as targets for this assay . Transformants with both bait and target proteins were selected on SD-Leu-Trp-His plates and were subjected to colony lift assay. The intensity of blue color, generated by the reporter β-galactosidase activity is indicative of the strength of interaction. Interaction of Ku80 with Ku70 is used as a positive control and Ku80 by itself is shown as a negative control.

the yeast two-hybrid screen to identify the interacting proteins for one of the TRFL proteins, TRFL9. Although most of the genes identified appear to be false positives (chloroplast specific or ribosomal subunit genes), one gene, *NADH dehydrogenase*, which was identified in this screen (data not shown), may play a role in telomere biology (See Chapter II).

ATR interactions with telomere-related proteins

The observation that deprotected telomeres activate the DNA damage response has been solidified by experiments in which DNA damage response factors were observed at telomeres (d'Adda di Fagagna *et al.,* 2003; Takai *et al.,* 2003). In *Arabidopsis*, ATR is required for maintenance of telomeric DNA as telomere shortening is dramatically accelerated in *atr tert* mutants relative to *tert* plants (Vespa *et al.,* 2005). Experiments to find interacting proteins of ATR might help shed light on how ATR facilitates telomere maintenance.

Co-immunoprecipitation experiments with *in vitro* translated ATR, AtPOT2 and AtKu80 proteins showed that ATR interacts with POT2 and Ku80 (Vespa, L. and Shippen, D., unpublished data). To confirm these interactions, the yeast two-hybrid system was utilized. Different regions of ATR (N, middle and C-terminus) cloned into the bait vector were transformed into the AH109 yeast strain. The transformants were selected on –Trp plates and sequentially transformed with POT2 and Ku80. Yeast two-hybrid data on ATR showed that middle portion of ATR interacts with both POT2 and Ku80 (Figure 4.3), a single-stranded telomere binding protein and a DNA repair

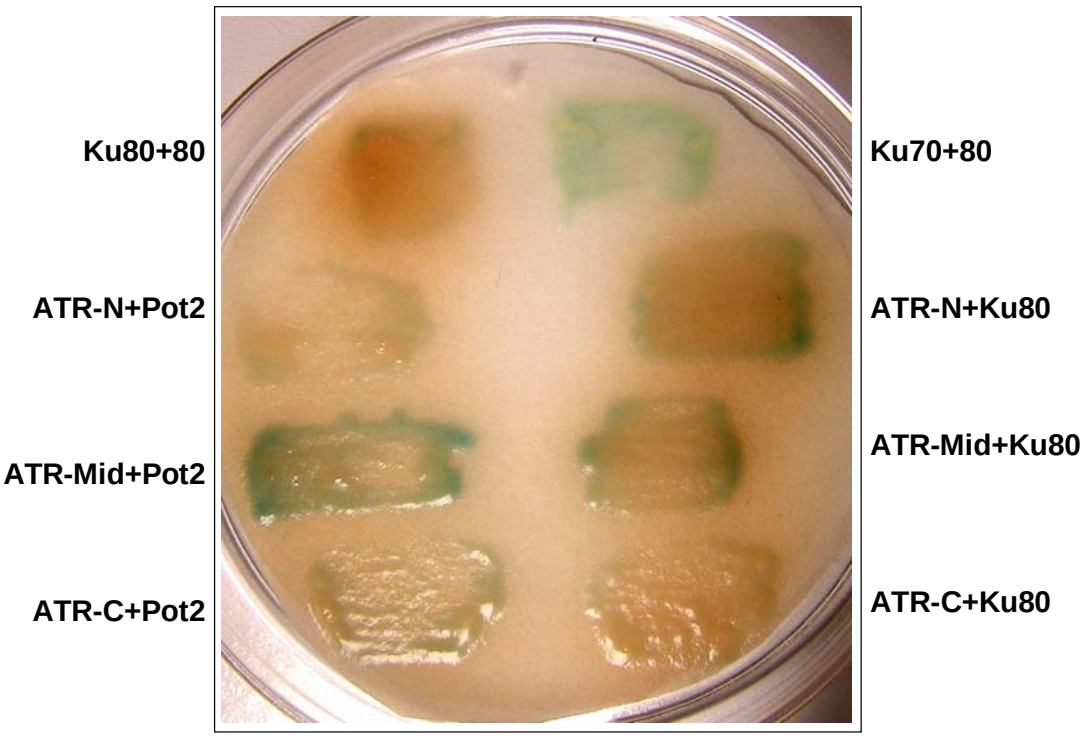

Figure 4.3. Interactions of ATR with POT2 and Ku80. Three different regions of ATR were utilized for this assay. N-terminus, middle portion and C-terminus of ATR cloned in fusion with Gal4DBD were used as baits, and Ku80 and POT2 were used as targets. Transformants with both bait and target proteins were selected on SD-Leu-Trp-His plates and were subjected to colony lift assay. β-galactosidase activity (formation of blue color) is shown for each interaction tested. The intensity of blue color is indicative of the strength of the interaction. Interaction of Ku80 with Ku70 is shown here as a positive control and Ku80 by itself is shown as a negative control.

protein, respectively. These interactions were specific, as ATR did not show any interaction with POT1 (data not shown). These data suggest a possible role of ATR at telomeres by regulating POT2 and Ku80, thereby interconnecting the telomere regulation with DNA repair pathway. Since the homolog of ATRIP was not found in *Arabidopsis* genome, we performed the yeast two-hybrid screen to identify the interacting proteins for ATR, especially the homolog for ATRIP. Unfortunately, the screen resulted in several false positives, which did not appear to be related to telomeres (data not shown).

POT1 interaction with TERT

POT (Protection of Telomeres) is a conserved single-stranded DNA binding protein with crucial functions in the protection of telomeres and maintenance of their length. POT1is required for telomerase-mediated telomere maintenance, as the *pot1* mutant showed progressive telomere shortening at a rate similar to *tert* mutants in *Arabidopsis* (Shakirov *et al.,* 2005). In order to investigate the mechanism of regulation of telomerase by POT1, the yeast two-hybrid assay was performed to assess the interaction between POT1 and TERT. Constructs containing different portions of *TERT* (N- and C-terminus) were transformed into the AH109 yeast strain and then a construct with *POT1* or *POT2* was sequentially introduced by transformation. A colony lift assay was performed on the colonies selected on the selection media (-Leu-Trp-His plates). Results showed that neither the N-terminus nor C-terminus of TERT interacted with either POT1 or POT2 *in vivo* (Figure 4.4). These data suggest that there is no direct interaction between TERT and the *Arabidopsis* POT proteins.

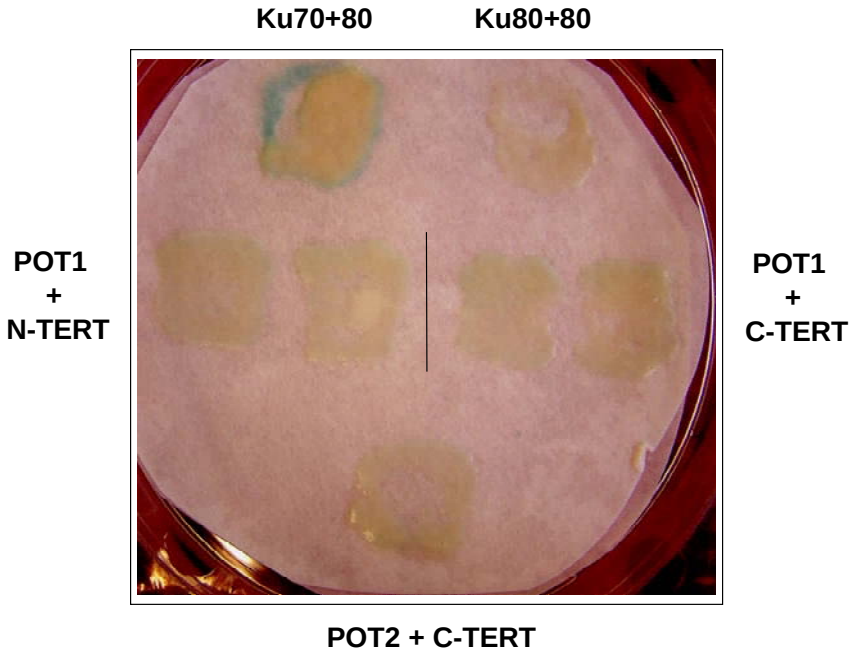

Figure 4.4. Interaction of POT1 and POT2 with TERT. Two different regions of TERT were utilized for this assay. The N-terminus, and C-terminus of TERT cloned in fusion with Gal4DBD were used as baits for this assay and were transformed into the AH109 yeast strain. Yeast clones containing these bait vectors were selected on SD-Trp plates and then sequentially transformed with target cDNA-containing vector POT1 or POT2. Transformants with both bait and target proteins were selected on SD-Leu-Trp-His plates and were subjected to a colony lift assay. β-galactosidase activity is shown for each interaction tested. Interaction of Ku80 with Ku70 is shown here as a positive control and Ku80 by itself is shown as a negative control.

Yeast two-hybrid screen for novel interacting partners of AtPOT1

To identify interacting partners for POT1, an *Arabidopsis* cDNA library was utilized in the yeast two-hybrid screens. Using the Gal4DBD POT1 as bait,
The cDNA library was screened as described under "Experimental Procedures." The bait did not have any intrinsic activity of transcriptional activation for the reporters (data not shown). The cDNA library in pACT-2 was transformed sequentially into AH109 that was already transformed with Gal4DBD-POT1. Transformation efficiency was checked by plating the transformation mixture on –Leu-Trp selection plates without selection for the reporter activity. POT1 expression in the yeast strain was checked by western blot utilizing both a peptide antibody and an antibody directed at full length POT1 protein, which are available in our lab. Western blot analysis showed that POT1 is expressed in yeast cells (data not shown).

Based on good transformation efficiency (approximately 0.2 X 10^6 independent transformants), transformants were pooled and respread on the selection media (-His-Trp-Leu plates). On the selection media, a total of 96 colonies were grown. Colony lift assay was performed on these colonies and 18 out of 96 clones showed β-galactosidase activity (Figure 4.5). The plasmids were rescued from yeast cells for DNA sequence analysis to identify the interacting proteins. Basically plasmid-rescue includes the extraction of plasmid by yeast miniprep, transforming the yeast mini prep into *E.coli* competent cells and then extracting the rescued plasmids from *E.coli* cells. The rescued plasmids were PCR-sequenced with primers derived from the vector pACT-2 to recognize the interacting partners. Sequencing the 18 clones that showed β-galactosidase activity

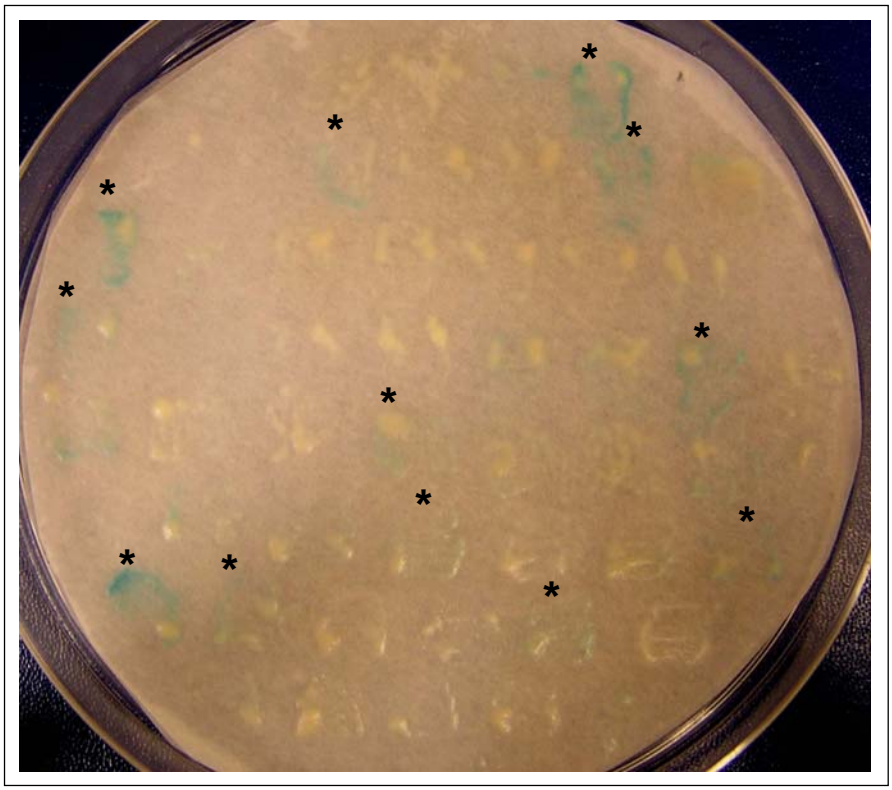

Figure 4.5. Yeast two-hybrid screen with POT1 to identify its partners. POT1 cloned in fusion with Gal4DBD was used as a bait to screen for interacting proteins in this assay. Ninety six transformants with both bait and target proteins were selected on SD-Leu-Trp-His plates and were subjected to colony lift assay. Out of 96 clones, 18 showed β-galactosidase activity as some of them were shown in the figure (indicated with an *). These colonies were selected for plasmid-rescue and rescued plasmids were sequenced to identify the interacting proteins.

revealed that many of them were false positives *i.e.,* chlorophyll a binding proteins, mitochondrial respiratory complex proteins and ribosomal proteins.

However, out of these 18 hits, two hits (RNA binding protein –At5g10350 and Unknown protein – At2g04410) were selected for further study. To confirm the interaction identified by yeast two-hybrid, co-immunoprecipitation experiments using *in vitro* translated proteins were performed on these two selected candidates. In this experiment untagged [^{35}S] methionine-labeled POT1 protein was subjected to immunoprecipitation in the presence of the corresponding unlabeled T7-tagged proteins (candidates from two-hybrid) using a T7 antibody. Following immunoprecipitation, an interaction with a tagged protein will allow a radiolabeled but untagged protein to precipitate with the beads. Control reactions performed in the absence of tagged proteins showed no interaction between the T7 antibody and untagged proteins (Figure 4.6). Ku70 interaction with Ku80 was used as a positive control and Ku70 homodimerization was used as a negative control in these experiments. These experiments demonstrated that At5g10350 did not interact with POT1 *in vitro*, whereas At2g04410 showed interaction with POT1 (Figure 4.6), suggesting the direct interaction of POT1 with this unknown protein. Further characterization of this novel unknown interacting partner of POT1 might help unravel the mechanism of telomere length regulation by POT1 in *Arabidopsis*.

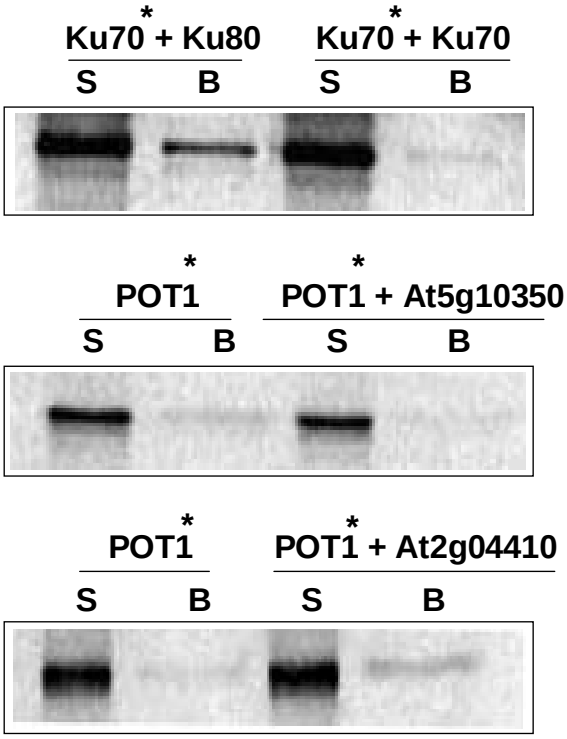

Figure 4.6. Confirmation of interactions identified by yeast two-hybrid screens. Co-immunoprecipitation experiments were conducted with full-length proteins expressed in rabbit reticulocyte lysate. In each experiment, one protein was labeled with [^{35}S] Met (*asterisk*), and the other was unlabeled but contained a T7 tag. Autoradiographed SDS-PAGE gel revealing the results for co-immunoprecipitation of POT1 with both At5g10350 and with At2g04410 were shown in the figure. Ku70 interaction with Ku80 used as a positive control and Ku70 homodimerization used as a negative control were also shown in the figure.

Discussion

In the present study, I made an attempt to identify interactions between telomere-related proteins. Several putative double-strand telomere binding proteins have been identified in *Arabidopsis*, but their functional significance at telomere is unknown (Karamysheva *et al.,* 2004). Homodimerization of hTRF1 and hTRF2 is required for association with telomeric DNA *in vivo*, but these proteins cannot form heterodimers (Fairall *et al.,* 2001). In contrast, *Arabidopsis* TRFL proteins form both homodimers and heterodimers *in vitro* (Karamysheva *et al.,* 2004). To check whether these proteins are interacting *in vivo*, the yeast two-hybrid system was utilized. Two-hybrid data confirmed the *in vitro* interactions of telomere binding proteins. TRP1 homodimerization, as well as TRP1 heterodimerization with both TRFL4 and TRFL9 were detected in two-hybrid experiments. Thus, the capacity of *Arabidopsis* TRFL proteins to form both homo- and heterodimers, unlike hTRF1 and hTRF2, suggests that these proteins could participate in complex structural and functional regulation *in vivo*. However, an attempt to identify the interacting proteins for one of the TRFL proteins, TRFL9 was unfruitful. Surprisingly, we identified *NADH dehydrogenase* as an interacting protein with TRFL9 in these screens. Further experiments should be performed to confirm this interaction. Work is in progress to examine the consequences of simultaneously inactivating all these TRFL genes. In the future, two-hybrid screens should be performed to identify interacting proteins for other TRFL proteins, which might help to understand their role at telomeres.

ATM and ATR kinases function by phosphorylating serine/threonine residues on protein targets immediately after activation (Shiloh, 2001). The role of ATR is less defined, because null mutants are lethal in yeast and mammals (Zhao *et al.,* 2001). ATR

is recruited by RPA, a single-stranded DNA binding protein to stalled replication forks through its interaction partner ATRIP (ATR-interacting protein) (Shechter *et al.,* 2004). Identification of interacting proteins for ATR will help unravel the mechanism by which ATR regulates the telomere length maintenance. *In vitro* co-immunoprecipitation experiments with *Arabidopsis* proteins translated in rabbit reticulocyte lysate showed that ATR interacts with POT2 and Ku80 but not with POT1 and Ku70 (Vespa *et al.*, unpublished data).

To confirm these interactions *in vivo*, yeast two-hybrid system was utilized. Yeast two-hybrid data showed that middle region of ATR interacts with both POT2 and Ku80, whereas the C-terminus did not show any interaction (Figure 4.2). These data suggest that ATR might be recruited to extended G-overhangs, which are produced either through the action of telomerase or through nucleolytic processing of the C-strand. After recruitment, ATR might help recruit or stabilize the binding of telomere protection proteins like POT2 and Ku. However, the attempt to find ATRIP homolog in *Arabidopsis* by yeast two-hybrid screens was unsuccessful.

POT1 is specialized to function with telomerase in telomere maintenance. It may recruit telomerase to telomeres, or activate it once it is associated with telomeres. Therefore, it is interesting to check the interaction of POT1 with telomerase components. Our two-hybrid data showed that neither POT1 nor POT2 interacted with TERT (Figure 4.3). It is possible that POT1 interacts with RNA subunit of telomerase. However, the RNA subunit from *Arabidopsis* has not been identified.

One of the most interesting features of POT1 proteins from vertebrates is that their localization at telomeres is primarily mediated through interactions with other

protein components of telomere complex (Lozyza and deLange, 2003). How the constituents of the telomere complex interact with each other to facilitate the chromosome end maintenance is unknown. In the present study I looked for the proteins that interact with POT1 by utilizing yeast two-hybrid screen. After screening 0.2 X 10^6 colonies, two possible candidates were identified. Out of these two candidates, At2g04410 (Unknown protein) showed interaction with POT1 by co-immunoprecipitation experiments performed *in vitro* utilizing rabbit reticulocyte lysate translated proteins.

These data suggest that this interaction between the unknown protein and POT1 is direct. Further studies are needed to determine the function of this POT1-interacting protein. Is it a component of telomerase RNP complex? Does it recruit AtPOT1 to telomeric DNA or tether POT1 with other telomere complex proteins? This novel protein might help us understand the mechanism of POT1 at telomeres, as POT1 has a key role at telomeres.

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

Telomeres are the nucleoprotein complexes that cap the end of all linear chromosomes that distinguish the ends from double-strand breaks. Paradoxically, telomere ends need to be recognized as DNA damage to complete end replication and to acquire a structure that is essential for function. For this purpose, several DNA repair proteins and proteins involved in sensing DNA damage also associate with telomeres. One key component of telomeres is the telomerase enzyme. Although plants are developmentally different from humans, the overall pattern of telomerase expression is similar. This finding not only indicates that telomerase regulation pathways might be conserved between plants and humans but also justifies the use of *Arabidopsis* as a model system to study the mechanism of telomerase regulation.

In the current study, we report the characterization of *tac5*, a mutant that constitutively expresses the telomerase enzyme. *tac5* was found after screening pools of activation tag lines (T-DNA tagged lines) by the highly sensitive TRAP assay. *tac5* showed ectopic activation of telomerase in leaves. *TERT* mRNA is upregulated in *tac5* leaves compared to wild type leaves, but telomere length is in the wild type range (2-5kb). Similar results were observed for *tac1* mutants (Ren *et al.,* 2004). Transcriptional upregulation of *TERT* in *tac5* suggests that the telomerase activation in *tac5* is due to the transcriptional upregulation of *TERT* in leaves. The fact that the telomere lengths remain stable in *tac5* inspite of increased telomerase activity is likely to be because telomerase access is regulated by a suite of telomere binding proteins.

The location of T-DNA insertion in *tac5* was determined by plasmid-rescue and shown to be on chromosome 5 in the 2nd intron of At5g37520, an unknown gene. The *NADH dehydrogenase (ubiquinone) gene* (At5g 37510), which is next to the T-DNA insertion site, is transcriptionally upregulated in *tac5* plant. Overexpression of *NADH dehydrogenase* in wild type plants leads to activation of telomerase in leaves, indicating that *NADH dehydrogenase* is responsible for ectopic activation of telomerase in *tac5* mutant. We found to our surprise that *tac5* is sensitive to oxidative stress, suggesting that *NADH dehydrogenase* activated in *tac5* renders plants more susceptible to oxidative stress induced damage and this stress signals the transcriptional activation of *TERT*. These data indicate a novel function of telomerase in mitochondrial environment.

Interestingly, hTERT has a bonafide N-terminal mitochondrial targeting sequence and recent studies indicate that localization of hTERT to the mitochondria renders cells more susceptible to oxidative stress-induced mitochondrial DNA damage (Santos *et al.,* 2006). In addition, exogenously as well as endogenously induced oxidative stress leads to translocation of endogenous as well as overexpressed human TERT from the nucleus into the cytosol (Haendeler *et al.,* 2003). Notably, *Arabidopsis* TERT also appears to have a mitochondrial localization signal (Santos *et al.,* 2006).

A number of studies have investigated the consequences of hTERT expression in response to various physiological processes, including: cell proliferation (Ramirez *et al*., 2002); apoptosis (Ramirez *et al*., 2003); and cancer (Hackett and Greider, 2003). Moreover, hTERT has been implicated in mitochondrial apoptosis induced by targeted inhibition of Bcl-2 (Del Bufalo *et al.,* 2005). Further experiments will be needed to

understand the correlation of oxidative stress and telomerase in mitochondrial environment in *Arabidopsis*. Some of these are outlined below.

In order to confirm that oxidative stress activates telomerase in *Arabidopsis*, wild type plants should be subjected to oxidative stress by hydrogen peroxide treatment and TRAP assay should be performed on leaf extracts from these plants. As shown in human cells (Santos *et al.,* 2006), we would expect to see activation of telomerase in response to oxidative stress. In addition, *tert* mutants and plants overexpressing *TERT* should be included in this hydrogen peroxide sensitivity assay to investigate the role of telomerase in determining oxidative stress response in mitochondria, as in human cells (Haendeler *et al.,* 2003). In addition, to determine whether TERT localizes to mitochondria or not, immunolocalization experiments can be performed. We predict to see re-localization of telomerase to mitochondria in response to oxidative stress, as *Arabidopsis* TERT also appears to have a mitochondrial localization signal (Santos et *al.,* 2004). Furthermore, a TUNEL (Terminal transferase dUTP nick end labeling) assay can be performed on wild type and *tac5* plants subjected to hydrogen peroxide treatment to investigate the role of *TERT* in mitochondrial apoptosis in response to oxidative stress. In addition, the identification of transcription factors that activate *TERT* transcription under stress conditions may be achieved utilizing microarray analysis on plants subjected to oxidative stress.

It may also be of interest to redesign activation tagging experiments to find new telomerase regulatory genes. One of the limitations of the current activation tagging strategy is the promoter preference or promoter selectivity of *35S* enhancers (Weigel *et al.,* 2000). Such enhancers activate only a subset of adjacent genes. A similar mechanism

of action has been shown in *Drosophila* embryos (Ohtsuki *et al.,* 1998). Thus, application of an alternative enhancer sequences may generate a different spectrum of activation tagged genes in *Arabidopsis*. Another limitation of the current procedure is the tissue specificity of the CaMV *35S* enhancer sequence. While this enhancer is shown to be active in leaves, it has poor activity in roots (Weigel *et al.,* 2000). Thus, future studies could be proposed in which the enhancer targets over expression of genes in specific tissues.

An additional limitation to the activation tagging method comes from the potential for instability of the mutant phenotypes. Selective inactivation of the *35S* enhancers by methylation can lead to silencing of the enhancing effect in future generations, causing reversion of the mutant phenotype.

A second line of investigation in this thesis was to study the DNA damage responses in *Arabidopsis* with regard to telomeres. In mammals, poly ADP-ribosylation by PARPs is an early DNA damage dependent post-translational modification of histones and other nuclear proteins that contributes to the survival of damaged cells. However, due to the lethality of *PARP1/PARP2* double deletion in mammals, the contribution of PARPs to telomere biology has not been explored in higher eukaryotes. Hence, an attempt was made to provide the first insight into the role of PARPs at higher eukaryotic telomeres.

Although it was found that both *PARP1* and *PARP2* are transcriptionally upregulated in response to both DNA damage treatment and with telomere dysfunction in *Arabidopsis*, no role for those proteins could be determined in telomere biology. Inactivation of *PARP1* and *PARP2* did not perturb telomere length maintenance or genome stability. Since a significant part (60%, according to *Arabidopsis* Genome

Initiative, 2000) of *Arabidopsis* genome has been duplicated, loss of some sequences by aneuploidy could be tolerated. Thus, the plant genome may tolerate DNA damage more readily than in other eukaryotes. Another important characteristic of plants is totipotency (basically each cell can give rise to all other cell types). The combined tolerance of aneuploidy and totipotency of cells could be responsible for maintaining a stable genome even in the absence of some of the DNA repair proteins. To investigate this idea further, DNA damage sensitivity of single and double mutants for PARPs should be assessed. In addition, the expression of other DNA repair genes should also be monitored to find out whether other redundant pathways of DNA repair are functioning in these mutants.

Plants and animals display remarkable differences in their tolerance to genome instability. While most of the cell cycle components and DNA damage signaling pathways are conserved in plants, p53 is absent from *Arabidopsis* genome, which might allow some degree of proliferation of cells with damaged genomes. However, several questions remain unanswered, most notable: what are the molecular responses to telomere dysfunction in plants, and what are the contributing factors involved in sensing damaged telomeres. Further analysis of PARP interactions at dysfunctional telomeres in the genetically tractable *Arabidopsis* model may help clarify the contribution of these proteins to chromosome end protection and telomere maintenance.

Finally, in the current study, an attempt was made to identify interacting proteins for telomere-related proteins using yeast two hybrid screens. The major focus was to find new telomere-specific proteins, since only a fraction of these proteins in the plant telomere complex have been identified. Little is known about double-strand telomere binding proteins in plants, although the TRFL family of proteins appear to be strong

candidates (Karamysheva *et al.*, 2004), as they contain a DNA binding domain conserved among other known double-strand telomere proteins. The capacity of these proteins to form both homodimers and heterodimers of TRFL proteins suggest that they could participate in complex structural and functional regulation *in vivo*. However, genetic analysis suggests that these genes are members of a large family with redundant function. Since the single, double and triple mutants of TRFL proteins did not show any defects in telomere length regulation, the yeast two-hybrid screen was performed to identify the interacting proteins for one of the TRFL protein, TRFL9. Interestingly, the *NADH dehydrogenase* gene identified as the factor that turns on telomerase in *tac5* mutant was identified as a binding partner for TRFL9 (data not shown). Further analysis is needed to verify this interaction and also further two-hybrid screens with other TRFL proteins, to identify their respective interacting proteins, which might give a clue about their function at telomeres.

Another target for the yeast two-hybrid screen was ATR. ATRIP is the only known binding partner for ATR in mammalian system. However, an ATRIP homolog cannot be discerned from the *Arabidopsis* genome sequence. Therefore, an attempt was made to identify the homolog of ATRIP in *Arabidopsis* by utilizing the two-hybrid screen, using different regions of ATR as bait. The screens did not result in any interesting interacting proteins for ATR. In the future, screens could be performed with full-length ATR, instead of different regions, which might eliminate the binding sites for its interacting proteins. In addition, other approaches like mass spectrometry could also be performed with the purified ATR from synchronized suspension cultures to find binding partners.

Although we were unable to identify a novel binding partner for ATR, *in vitro* co-immunoprecipitation experiments with recombinant ATR protein confirmed that it interacts with POT2 and Ku80. These results were further substantiated by *in vivo* yeast two-hybrid assays. The ATR-Ku80 interaction is exciting because plants lacking both ATM and TERT show the same accelerated rate of telomere shortening phenotype as *ku70/tert* mutants (Vespa *et al.,* 2005). Thus, ATR and Ku might play a similar role in telomere maintenance. To further study the significance of these interactions, the consequences of disrupting these interactions *in vivo* should be examined. In addition, whether ATR is involved in the recruitment of POT2 to telomeres should be tested. Furthermore, it should be determined whether disruption of ATR alters the localization of POT2 and Ku80 at telomeres.

Perhaps the most important result for the two-hybrid screen was the identification of At2g04410 (Unknown protein) as a direct interacting partner of POT1, a single-stranded telomeric DNA binding protein. This interaction was confirmed *in vitro* by co-immunoprecipitation experiments. The role of At2g04410 in telomere biology should be determined by characterizing a T-DNA insertion line for this gene to document its role in telomere length control. As POT1 is thought to be a component of telomerase RNP complex, further studies are needed to determine whether this novel protein is also a component of telomerase RNP or whether it helps to recruit POT1 to telomeric DNA or tethers POT1 with other telomere complex proteins (TRFL proteins). A double mutant for both POT1 and this novel protein should be generated to determine whether this novel protein is functioning in the same pathway as POT1 or not. These experiments should give new insight into the significance of its interaction with POT1 at telomeres.

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- Identified and characterized a telomerase activation mutant, *tac5*, in *Arabidopsis thaliana*.
- Characterized plant DNA damage responses.
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Research Associate, ARS, India. July 1997–Jan.2001

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