# *TELOMERASE ACTIVATOR 1*: A ZINC-FINGER PROTEIN THAT ACTS SYNERGISTICALLY WITH AUXIN TO CONTROL TELOMERASE EXPRESSION IN *ARABIDOPSIS THALIANA*

A Dissertation

by

SHUXIN REN

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

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Genetics

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# ABSTRACT

*Telomerase Activator 1*: A Zinc-Finger Protein That Acts Synergistically with Auxin to Control Telomerase Expression in *Arabidopsis thaliana*. (August 2003)

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Telomerase is the key enzyme synthesizing telomeric DNA in most eukaryotic organisms. In mammals, telomerase expression is abundant in the germline cells but is undetectable in most other differentiated organs. Intensive studies of telomerase have focused on human cancerous cells, where over 90% of all cancerous tissues examined have telomerase activity. In wild-type *Arabidopsis*, telomerase expression is abundant in reproductive organs and dedifferentiated tissues such as flowers, siliques and calli but barely detectable in vegetative tissues (both rosette and cauline leaves). In this study, a biochemical screen strategy was developed for isolation of telomerase activating mutants in *Arabidopsis thaliana.* Through screening of *Arabidopsis* activation-tagged lines by a PCR-based TRAP assay, two *tac* (for *telomerase activator*) mutants were isolated. RT-PCR analysis of *AtTERT* expression revealed that different mechanisms are involved in alternating telomerase activity in *tac1* and *tac2*.

We cloned and characterized the *TAC1* gene. *TAC1* encodes a single zinc finger protein and acts synergistically with auxin to induce telomerase expression without altering cell cycles. Telomere length was unperturbed in the mutant, but other phenotypes, such as altered root development and the ability of cells to grow in culture

without exogenous auxin, indicated that *TAC1* not only is part of the previously reported link between auxin and telomerase expression, but also potentiates other classic responses to this phytohormone.

DNA microarrays were used to analyze the expression profile of the *tac1* mutant and revealed that several drought-induced genes were up-regulated 3 to 10 fold in the *tac1-1D* mutant. RT-PCR analysis further confirmed this up-regulation for five of these genes. Investigation of root growth also indicated that *tac1-1D* roots were ~20% longer relative to wild-type. Further experiments demonstrated that over-expression of *TAC1* does confer drought tolerance, but not salt tolerance. In addition, our preliminary result showed that treatment with a low concentration of IAA could induce drought tolerance in wild-type *Arabidopsis*. Although plants with constitutive expression of telomerase have no practical utility, the ability of *TAC1* to confer drought tolerance could have significant agricultural applications.

# DEDICATION

To my parents, Yuhong Ren and Chunrong Xu, for their support, love and patience

To my parents-in-law, Changping Li and Xiumin Liang, for their encouragement

To my lovely wife, Haiwen Li, for her love and encouragement

To my adorable son, Kevin Ren, with love and love and love

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## BACKGROUND

Telomeres are unique DNA-protein structures present at the physical ends of linear eukaryotic chromosomes. In most species, telomeres are composed of tandem repeated guanine-rich sequences that are most commonly six to eight nucleotides long (Blackburn, 1991), for example, TTAGGG in human cells (Moyzis et al., 1988) and TTTAGGG in *Arabidopsis thaliana* (Richards and Ausubel, 1988). The length of telomere repeats among different organisms can be highly variable, ranging from 42 base pairs in some ciliates to over 150 kilobase pairs in mice (Kipling and Cooke, 1990). In plants, this telomere length variation also exists both among and within species. For instance, *Arabidopsis* telomeres are about 2-4 kilobase pairs long for the Columbia ecotype (Richards et al., 1992), whereas tobacco telomeres are from 20 kilobase pairs to over 166 kilobase pairs (Suzuki et al., 1994). In maize, telomere length varies over 20-fold among different varieties (Burr et al., 1992).

The G-rich strand of the telomere is longer than the C-rich strand and forms a 3' extension, called the G-overhang. This G-overhang is highly conserved in diverse species, including plants (Riha et al., 2000). Recent studies in mammals, ciliates and

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This dissertation follows the style and format of The Plant Cell.

protozoa discovered that telomeres could form a higher order chromatin structure that physically protects the 3'-end from exonucleolytic activities. This protective structure is provided by the ability of the G-overhang to fold back and invade the double-stranded region of the telomere, creating a t-loop (Griffith et al., 1999; Murti and Prescott, 1999; Munoz-Jordan et al., 2001).

Telomeric DNA, together with non-histone, telomere-specific binding proteins, plays an essential role in stabilizing chromosome ends by preventing end-to-end fusions and protecting chromosome DNA ends against uncontrolled nucleolytic degradation (Blasco et al., 1997; Sandell & Zakian, 1993; Kurenova and Mason, 1997; van Steensel et al., 1998). This capping function of telomeres was first observed and described by Muller (1938) and McClintock (1941, 1942) over 60 years ago using Drosophila, an organism without typical telomere structure, and maize, respectively. More recent studies on chromosome ends have further confirmed these early observations (Sandell and Zakian, 1993; Garvik et al., 1995; Riha et al., 2001).

Other important functions of the telomere are to compensate for the incomplete replication of linear chromosome ends by DNA-dependent DNA polymerases and to serve as a reservoir of disposable DNA (Prescott and Blackburn, 2000). The chromosome end that is replicated by lagging-strand synthesis cannot be fully duplicated because of the requirement for an RNA primer to initiate DNA synthesis. This process will lead to degradation and hence shorting of the chromosome by several nucleotides in each cell division. The solution to this end-replication problem that has been adopted by most eukaryotic organisms is to use a telomere-specific DNA polymerase called telomerase to extend the 3'end of the template DNA, which can then be copied by DNA-dependent DNA polymerases. With the presence of telomeres, repetitive telomeric DNA is lost instead of those DNA sequences carrying crucial genetic information.

In addition to chromosome stabilization and solving the end-replication problem, telomeres also play important roles in the functional organization of chromosomes within the nucleus during mitosis and meiosis (reviewed by Franklin and Cande, 1999; McKnight et al., 2002), and in regulating expression of telomere-proximal genes (Nautiyal et al., 2002).

Although a couple of mechanisms for maintaining telomere length have been uncovered, the primary one for adding telomeric DNA to chromosome ends is through the action of an enzyme called telomerase. Telomerase is a ribonucleoprotein with reverse transcriptase activity (Greider & Blackburn, 1985). The holoenzyme probably has other subunits (Gandhi and Collins, 1998; Greene and Shippen, 1998), however, the core enzyme with telomerase activity *in vitro* requires only the RNA subunit and the catalytic subunit. The RNA component carries a sequence complementary to the G-rich telomeric DNA strand and serves as the template for addition of telomere G-rich repeats (Shippen-Lentz and Blackburn, 1990; Yu et al., 1990; Autexier and Greider, 1994; Singer and Gottschling, 1994), while the catalytic component is an actual reverse transcriptase that adds telomeric DNA repeats onto the 3' end of chromosomes (Feng et al., 1995; Weinrich et al., 1997).

Telomerase activity is developmentally regulated among multi-cellular eukaryotes. In humans, telomerase activity is detected only in regenerating tissues, germline cells, and most cancer cells (Kim et al., 1994; Wright et al., 1996; Holt and Shay, 1999). Similarly, in plants, telomerase activity is detected in reproductive and proliferating cells, but not in most vegetative tissues (Fitzgerald et al., 1996; Riha et al., 1998). In the absence of telomerase, the consequences of DNA-dependent DNA replication result in the successive shortening of telomeres with each cell division, and this shortening eventually causes genome instability and cellular senescence (Shay et al., 2001; Forsyth et al., 2002; Lee et al., 1998; Riha et al., 2001; Artandi et al., 2000).

Since it was first identified biochemically 17 years ago (Greider and Blackburn, 1985), telomerase has become a hot area of research. Although insight into mechanisms that specifically control telomerase activity is still incomplete, such research efforts are continuing to help us understand how telomerase is regulated. The aim of this review is to summarize the current data regarding the mechanisms of telomerase regulation in unicellular organisms, mammals, and flowering plants.

## Telomerase regulation in unicellular organisms

Although other mechanisms can maintain chromosome termini in the absence of telomerase (Bryan et al., 1995; Sheen and Levis, 1994; Teng and Zakian, 1999; Nakamura et al., 1998; McEachern & Blackburn, 1996), telomerase itself plays an essential role in the maintenance of telomere length in most eukaryotes. In unicellular organisms, such as ciliates and yeast, telomerase is expressed throughout their life cycles, although the level of enzyme activity and/or abundance of TERT expression varies developmentally. A major mechanism of telomerase regulation is controlling access of the enzyme to telomeric DNA. During the last one and half decades, studies in unicellular organisms have identified many factors that regulate telomerase, both positively and negatively.

### *Negative regulation of telomerase*

Regarding negative regulation of telomerase, one of the earliest and best-developed models is the Rap1p counting model developed in budding yeast (Marcand et al., 1997). *Rap1* encodes a duplex telomeric-DNA-binding protein that binds with high affinity to tandem GGTGT sites through two Myb-like domains (Buchman et al., 1988; Konig et al., 1996). Targeting additional copies of the Rap1p C-terminus to an individual telomere results in shortening of that telomere (Marcand et al., 1997; Ray & Runge, 1999). Furthermore, this shortening is roughly proportional to the number of targeted Rap1p molecules. These experiments demonstrate that the total number of Rap1p molecules bound to the telomere, but not necessarily to G-rich repeats, is used to control telomere length through regulating access of telomerase to telomeres. Assembly of Rap1p to yeast telomeres is mediated in part through the C-terminal domain of Rap1p. This C-terminal domain can interact with two other proteins, Rif1p and Rif2p, and hence they also contribute to telomere length control in yeast (Hardy et al., 1992; Wotton & Shore, 1997).

An alternative version of this counting model was proposed by Krauskopf and Blackburn (1996). When mutations in the telomeric DNA that reduce Rap1p binding affinity were introduced into the yeast *Kluveryomyces lactis*, this change resulted in increased telomere length, and the degree of the effect corresponded to the degree of loss of Rap1p binding *in vitro* (Krauskopf & Blackburn, 1996). The loss of telomere length regulation was observed even when mutant repeat sequences were added only to the very end of the telomere. These observations demonstrated that the Rap1p complex assembled on the terminal repeats, but not internal repeats, was essential for the telomere length control.

### *CDC13 acts as both negative and positive regulator of telomerase in yeast*

The best characterized protein responsible for telomeric end protection in *Saccharomyces cerevisiae* is encoded by *CDC13*. Cdc13p binds specifically to single-stranded telomeric DNA *in vitro* and affects telomere behavior *in vivo* (Nugent et al., 1996; Lin & Zakian, 1996). A yeast strain deficient in *CDC13* function shows an extended single G-strand of the telomere in a telomerase-independent manner, which induces cell-cycle arrest in the G2 phase (Garvik et al., 1995; Qi & Zakian, 2000). Since G-strand telomeric DNA is a substrate for the telomerase holoenzyme, when Cdc13p binds to the end of the telomere, it limits extension of the G-strand by telomerase. In addition, CDC13p also recruits negative regulators such as STN1p and TEN1p to telomeres. From this point of view, *CDC13* acts as a negative regulator of telomerase.

However, a phenotype showed by a yeast mutant deficient in *Cdc13* defines it also as a positive regulator of telomerase (Nugent et al., 1996). A specific missense mutation, *cdc13-2*$^{est}$, has a defect in telomere replication even through telomerase activity is not affected (Nugent et al., 1996; Lingner et al., 1997a). These data suggest that Cdc13p is required to recruit telomerase to the telomere through a direct association with the enzyme, and this activity is eliminated by the *cdc13-2*$^{est}$ mutation. This dual function makes *CDC13* unique in yeast in regulating telomerase access to the telomere (Chandra et al., 2001). First, Cdc13p recruits telomerase to the telomere, and then it acts to limit the extension of the G-strand by telomerase.

### *Positive regulation of telomerase*

As mentioned above, *CDC13* serves as a positive regulator to help recruit telomerase to the telomere (Nugent et al., 1996). Several other proteins also act as positive regulators of the enzyme. In budding yeast, telomerase action requires at least five genes, *EST1, EST2, EST3, TLC1* and *CDC13*. Deficiencies in any or all of these genes result in the same *ever-shorter-telomere* phenotype. Among these, *EST2* and *TLC1* encode the reverse transcriptase and RNA subunit, respectively, which are required for the catalytic core of the telomerase enzyme (Counter et al., 1997; Lingner et al., 1997b). However, telomerase action *in vivo* also requires Est1p, Est3p and Cdc13p. Biochemical analysis has shown that Est1p and Est3p function in telomere replication as subunits of the telomerase holoenzyme (Steiner et al., 1996; Hughes et al., 2000). Although the specific function of the Est3p has not yet been determined, studies have shown that Est1p, as one of the components of telomerase, functions as co-mediator, together with Cdc13p, of access of telomerase to telomere. As mentioned above, the *cdc13-2*$^{est}$ mutant shows a telomere replication defect. However, this defect can be bypassed when the DNA binding domain of Cdc13p is fused directly to either Est1p or Est3p (Evans & Lundbald, 1999). In addition, telomere replication is restored when a *cdc13-2*$^{est}$ strain carries Est2p (the telomerase reverse transcriptase) fused to the CDC13 DNA binding domain. Over-expression of the wild-type Est1p protein partially suppresses the telomere replication defect of the *cdc13-2*$^{est}$ mutant (Nugent et al., 1996). Furthermore, Est1p and Cdc13p can be co-immunoprecipitated when both are over-expressed in yeast (Qi & Zakian, 2000). In another study, Evans and Lundblad (1999) demonstrate that a fusion between Cdc13p and the Est2p allows telomeres to be stably maintained in the absence of Est1p. Recently, Taggart et al. (2002) proposed that Est1p actually is a cell cycle regulated activator of telomerase that binds to an inactive, Est2p-TLC1 RNA complex in late S phase and then interacts with Cdc13p molecules arrayed on the G-strand overhang. This interaction changes the state of Est2p and results in activation of telomerase for synthesis. All these data suggest that Est1p serves as a bridging molecule, in collaboration with Cdc13p, that mediates access of the telomerase to telomere.

The Ku70/80 heterodimer, which plays an essential role in non-homologous end joining, also serves as a positive regulator of telomerase in yeast. The absence of either subunit of the Ku70/80 heterodimer, results in a shorter, but stable telomere (Boulton & Jackson, 1996, 1998; Porter et al., 1996; Kironmai & Muniyappa, 1997). In budding yeast, deletion of *Est2* and either *Ku70* or *Ku80* causes lethality (Gravel et al., 1998; Nugent et al., 1998). However, in fission yeast, this double mutant only results in accelerated cell senescence, implying a synergistic interaction between Ku and telomerase (Baumann & Cech, 2000). Further evidence suggests that the yeast Ku heterodimer helps to recruit or activate telomerase at telomeres through interaction with a stem-loop structure of telomerase RNA subunit *TLC1* (Peterson et al., 2001) or through promotion of *Cdc13*-mediated telomerase recruitment (Grandin et al., 2000). Collectively, these data define the yeast Ku heterodimer as a positive regulator of telomerase in contrast to its homologs in higher eukaryotes (Hsu et al., 2000; Samper et al., 2000; Riha et al., 2002; Riha & Shippen, 2003; Gallego et al., 2003).

### *Identification of TERT gene family in ciliate* Euplotes *reveals a novel mechanism to control telomerase in unicellular organisms*

Ciliates are unique organisms. They contain two nuclei: one is a diploid, transcriptionally silent germline micronucleus, and the other is a highly polyploid, transcriptionally active macronucleus. During the sexual stage of the life cycle, the old macronucleus is destroyed and new one is generated by copying from the micronucleus. To generate the macronucleus, site-specific chromosome fragmentation is required to release gene-size DNA molecules followed by *de novo* formation of telomeres via telomerase action (Fan & Yao, 1996).

Previous studies showed that telomerase from vegetative growing *Euplotes crassus* exists as a 280-400kD particle and can recognize only telomeric DNA substrates (Greene & Shippen, 1998). However, during macronucleus development, telomerase RNP architecture and substrate recognition are dramatically changed to fulfill its role in *de novo* telomere formation *in vivo* (Greene & Shippen, 1998).

In a recent study conducted by Karamysheva et al. (2003), through cloning and sequencing of RT-PCR products for *EcTERT* (Wang et al., 2002), the authors unexpectedly uncovered two new versions of *EcTERT,* that differed in their nucleotide and predicted amino acid sequences relative to each other and to the originally identified *EcTERT* gene. Further study demonstrated that all three *EcTERT* genes require +1 ribosomal frameshifting to generate catalytically active TERT protein. The expression profiles for these three genes showed that they are expressed at different stages of the ciliate life cycle; expression of *EcTERT-1* and *EcTERT-3* correlate with telomere maintenance, while expression of *EcTERT-2* correlates with *de novo* telomere formation. Following *de novo* telomere formation, DNA for *EcTERT-2* is apparently eliminated from the genome, a novel mechanism for silencing gene expression (Karamysheva et al., 2003).

## Telomerase regulation in humans

Telomerase activity in humans is also developmentally regulated (Reviewed by Forsyth et al., 2002). In humans, telomerase expression is abundant in the germline cells and developing embryonic tissues, but it is undetectable in most somatic tissues (Kim et al., 1994; Wright et al., 1998). On the other hand, telomerase is highly expressed in more than 85% of all human tumors but not in adjacent normal cells (Kim et al., 1994; Shay et al., 2001). Because of this apparent relationship between telomerase activity and cancer, intensive research has been focused on telomerase regulation in human. These studies indicate that telomerase is regulated at various levels, including transcription, post-transcription and post-translation levels. Additional key stages for regulating telomerase include assembling active holoenzyme and binding to telomeres.

### *Telomerase regulation at transcriptional level*

Because other core components of human telomerase are usually expressed ubiquitously, only the catalytic component hTERT appears to be the limiting determinant of telomerase activity. Telomerase activity is extinguished in many tissues during embryonic development, and the correlation between *hTERT* mRNA and telomerase activity is well established. A substantial number of experiments demonstrate that transcriptional regulation of *hTERT* is a primary mechanism of telomerase regulation in most human cells (Cong et al., 1999; Horikawa et *al*., 1999; Meyerson et al., 1997).

The *hTERT* promoter has been well characterized, and several binding sites have been identified for many transcription factors that may be involved in its regulation. The abundance of these potential transcription-factor binding sites suggests that regulation of *hTERT* may be subject to multiple levels of control by different factors in different tissues. Several such transcription factors are well studied for their role in *hTERT* expression through either activating or repressing the *hTERT* promoter, including *c-Myc*, *Sp1*, *p53*, *Mad1*, and many others.

*c-Myc* activates telomerase by control of *hTERT* transcription. *c-Myc* is a well-known oncogene, and is involved in a wide range of cellular processes including proliferation, growth, differentiation, and tumorigenesis (Grandori et al., 2000). The *c-Myc* gene family represents a group of transcriptional factors, which contain activation domains at their N termini and a bHLHZ domain at their C termini. This group of transcription factors recognizes and binds to the E-box-containing promoters and activates the target gene expression. It is actively expressed in most human cancers (DePinho et al., 1991), and it is upregulated in highly proliferative and immortal cells.

Wang et al. (1998) demonstrated that *c-Myc* could induce *hTERT* expression and telomerase activity in normal human mammary epithelial cells and primary fibroblasts. Sequence analysis of the *hTERT* promoter region identified two E-boxes, one at –34 and the other at –242 nucleotides upstream of the ATG start codon (Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999; Wick et al., 1999). These data indicate that the *hTERT* promoter may be a direct target of the c-Myc proteins. Actually, over-expression of c-Myc enhances *hTERT* promoter activity, and deletion of either or both E-boxes prevents induction by c-Myc (Greenberg et al., 1999; Gunes et al., 2000; Kyo et al., 2000). Gel shift, and chromatin immunoprecipitations revealed that c-Myc/Max heterodimers interact directly with the *hTERT* promoter (Wu et al., 1999; Xu et al, 2001). These results further confirmed that *hTERT* is a direct target of the c-Myc family of proteins (Cong et al., 2002).

*Sp1* is another activator of the *hTERT* gene. *Sp1* is a general transcriptional factor that binds to GC-boxes of promoters and enhancers to regulate a large number of genes. It interacts with components of the general transcription machinery to help initiate transcription of TATA-less promoters (Emili et al., 1994; Hoey et al., 1993). Sequence analysis of the *hTERT* promoter region indicated that it lacks a TATA box, but it contains five GC-boxes in its core promoter region that are putative binding sites for *Sp1* transcription factor. Mutations in these sites abolish *hTERT* promoter activity (Kyo et al., 2000). These data revealed that *Sp1* binding sites are absolutely required for *hTERT* promoter activity. The exact mechanisms by which *Sp1* contributes to *hTERT* transcription is not known. However, *Sp1* does cooperate with *c-Myc* to activate *hTERT* transcription in a cell type-specific manner (Kyo et al., 2000).

Besides these two transcription factors that activate hTERT transcription, other factors, such as the E6 protein from human papillomavirus 16 (Klingelhutz et al., 1996) and some steroid hormones (Kyo et al.,1999; Misiti et al., 2000; Wang et al., 2000) may also activate *hTERT* transcription. Although how these factors regulate telomerase activity is not yet known, the fact that steroid sex hormones regulate telomerase activity in hormone-sensitive tissues may provide insight into the new molecular mechanisms of hormone-induced telomerase activity in mammals.

Repression of *hTERT* transcription is also of importance in telomerase regulation. *Mad1*, as a competitor of *c-Myc*, also binds to the E-box region of promoters and acts as a negative regulator. The members of the *c-Myc/Max/Mad* network are essential to control normal cell growth and development (Grandori et al., 2000). *Mad1* was originally identified in a screen for *hTERT* transcriptional repressors by the expression cloning approach with a cDNA library prepared from normal human kidney cells (Oh et al., 2000). Over-expression of Mad1 decreased *hTERT* promoter activity. This repression relied on the E-boxes present in the *hTERT* promoter and was counteracted by ectopic expression of *c-Myc* (Gunes et al., 2000). Mad1 and c-Myc proteins have an antagonistic effect on regulation of *hTERT* transcription (Xu et al., 2001). Both Mad1 and c-Myc can dimerize with the ubiquitously expressed Max protein. c-Myc/Max heterodimers bound to E-boxes activate gene expression, while Mad1/Max heterodimers compete for binding to E-boxes and repress the *hTERT* transcription. The switches between c-Myc/Max and Mad/Max may play a direct role in determining either activation or repression of *hTERT* transcription.

Similar to the c-Myc/Mad system, the competitor of Sp1 in *hTERT* promoter regulation is p53. *p53* is a tumor suppressor protein that inhibits tumor formation by inducing cell-cycle arrest or apoptosis in response to a number of cellular damages (Levine 1997). However, independent of its role in cell cycle arrest, p53 also functions in inhibition of telomerase activity through transcriptional repression of *hTERT* (Kanaya et al., 2000; Kusumoto et al., 1999). This inhibition requires the transcription factor Sp1 (Xu et al., 2000). Mutation in Sp1 sites in the core promoter of *hTERT* abolished repression by p53. One possible explanation is that p53 interacts with Sp1 and prevents Sp1 from binding to the *hTERT* promoter. On the other hand, the complex of Sp1 and p53 may impede the binding of other transcription activators to the *hTERT* promoter. Instead, it may bring repressor complexes, such as histone deacetylase (HDAC), to the promoter (Cong et al., 2002; Mergny et al., 2002). Through these interactions, p53 may represent an important block to the activation of human telomerase.

*WT-1* is another gene potentially involved in the transcriptional repression of *hTERT*. Similar to *p53*, *WT-1* is also a tumor repressor (Oh et al., 1999). It interacts directly with the *hTERT* promoter. Mutations in the *WT-1* binding site, which is located at –352 upstream of the initiation codon in the *hTERT* gene, increase *hTERT* promoter activity in human cell line 293. Overexpression of *WT-1* significantly reduces *hTERT* mRNA expression and telomerase activity (Oh et al., 1999). This regulation of *hTERT* transcription is cell type specific because *WT-1* is only expressed certain cell types.

Besides the examples discussed above, there are many other factors that may repress the expression of *hTERT*. These include E2F, histone deacetylases (HDAC), MZf-2, Rb family of proteins, p16INK4A and some differentiation agents (Cong et al., 2002; Mergny et al., 2002; Maida et al., 2002; Takakura et al., 2001; Hou et al., 2002; Crowe & Nguyen, 2001; Fujimoto et al., 2000). However, some studies yield contradictory conclusions. For example, Crowe and Nguyen (2001) reported that Rb can regulate telomerase activity by repressing *hTERT* transcription. However, Garcia-Can et al. (2002) demonstrated that loss-of-function mutations for *RB1*, *RBL1* and *RBL2* or double or triple deficiencies in these genes do elongate telomere length, but do not increase telomerase activity. Nevertheless, the fact that multiple factors are involved in control of *hTERT* transcription indicates that the transcriptional regulation of telomerase activity is likely to be complex and involve different mechanisms.

### *Telomerase regulation at post-transcriptional level in humans*

In humans, telomerase expression is also regulated at post-transcriptional levels. In fact, several labs have found that the *hTERT* mRNA has at least six splice variants (Kilian et al., 1997; Ulaner et al., 1998; Wick et al., 1999; Colgin et al., 2000; Yi et al., 2000) that appear to be tissue-specific and developmentally regulated. In addition, various tumor cell lines have shown considerable differences in their splicing pattern of *hTERT* mRNA (Villa et al., 2001). All alternatively spliced forms of hTERT are inactive. However, these alternative forms may have some biological purpose. Possibly these forms play a role in fine-tuning the levels of active enzyme in cells by shifting the balance between the alternative forms and the full-length hTERT protein (Colgin et al., 2000; Yi et al., 2000).

Another major step in control of telomerase activity at the post-transcriptional level is stablization of the RNA component hTR and/or its functional association with hTERT. Many factors that bind to hTR have been discovered, and some are likely to play a role in the stabilization of hTR either in its independent form or when complexed with hTERT (Le et al., 2000; Ford et al., 2001; Mitchell et al., 1999). For instance, H/ACA proteins such as dyskerin play a fundamental role in stabilization of hTR. These proteins may serve to stabilize hTR only until it forms a stable complex with hTERT;

alternatively they may remain stably associated as part of the telomerase holoenzyme as dyskerin does (Mitchell et al., 1999).

### *Telomerase regulation in human at the post-translational level*

Without doubt, the regulation of *hTERT* transcripts is the primary mechanism in controlling telomerase activity in human cells. However, several studies have discovered that in some samples, there exist apparently full-length *hTERT* mRNA but no detectable telomerase activity (Ulaner et al., 2000; Rohde et al., 2000; Klapper et al., 2001). An early study also showed that *hTERT* mRNAs are present at similar levels in human lymphocytes, tonsils, and peripheral blood T and B cells, but the status of telomerase activity is different (Liu et al., 1999). All these data suggest that production of *hTERT* mRNA is not sufficient to produce active telomerase in certain cell types, and that post-translational modifications of hTERT protein may provide an additional step in telomerase activity control.

Reversible protein phosphorylation represents the most important mechanism in regulating enzyme activity, structure and localization. Increasing evidence indicates that telomerase activity can be regulated by hTERT phosphorylation. For example, in peripheral blood cells, telomerase activity is enhanced by the protein kinase C activator phorbol myristate acetate, and this activation is inhibited by the PKC inhibitor bisindolylmaeimide I (Bodnar et al., 1996). Also, in breast cancer cells, phosphatase 2A-treated nuclear extracts decrease telomerase activity, while the phosphatase 2A inhibitor okadaic acid prevents this activity from decreasing in vitro and stimulates telomerase activity in vivo (Li et al., 1997). These studies demonstrate that human telomerase activity can be regulated by protein phosphorylation without affecting hTERT transcription. However, whether these regulatory steps are at the level of hTERT phosphorylation itself has not been clear since these inhibitors may function upstream of hTERT and act to alter telomerase activity indirectly.

Recently, Kharbanda et al. (2000) has convincingly demonstrated that hTERT itself is phosphorylated by the tyrosine kinase c-Abl, both in vitro and in vivo, in response to ionizing radiation. In contrast to PKC, phosphorylation of hTERT by c-Abl inhibits telomerase activity. Over-expressing the *c-Abl* gene in 293T cells represses telomerase activity, and a null mutant of *c-Abl* shows increased telomerase activity and telomere length. This discovery of hTERT phosphorylation by c-Abl is the first study that both shows phosphorylation at a specific site in hTERT full-length protein (308-PSTSRPPRP-316) and establishes its functional significance.

A mechanism by which hTERT protein phosphorylation controls telomerase activity was recently proposed. Liu et al. (2001), studied T-lymphocyte cells, and found that un-stimulated lymphocytes do not have telomerase activity. However, they do have detectable hTERT protein. On the other hand, stimulation of the same cells produced a dramatic increase in hTERT phosphorylation. Also they found that telomerase activity is correlated with an alteration in the subcellular distribution of hTERT proteins. The authors proposed that hTERT exists in the cytoplasm in an inactive, unphosphorylated form in unstimulated cells, and upon stimulation, hTERT is phosphorylated and translocated from the cytoplasm to the nucleus, thereby allowing for assembly of active telomerase and function on telomeres. Similar observations from another study using vascular smooth muscle cells also confirmed this mechanism (Minamino et al., 2001).

### *Telomerase regulation by controlling access to telomeres*

The regulation of telomerase is a complex and dynamic process involving many steps, eventually leading to the ability of telomerase to locate, access and function on telomeres. Among these, controlling access of the telomerase holoenzyme to the telomere is one of the last steps required for telomerase function. During this step, although it is yet not fully understood, the telomeric DNA-protein complex plays a major role in regulating accessibility to telomeres. It is proposed that the telomeric DNA-protein complex might switch stochastically between capped and uncapped states (Blackburn, 2001). In vitro studies suggest that de novo telomere synthesis by telomerase requires an accessible G-strand overhang (Lingner & Cech, 1996; Wang & Blachburn, 1997). However, the G-overhang can be inserted into the telomeric DNA complex to form a t-loop in vitro (Griffith *et al*., 1999). This structure can stabilize and protect the chromosome ends. On the other hand, it prevents telomerase from accessing to telomere. This paradox could be solved by dynamic switches between an accessible G-overhang and an inaccessible t-loop structure (Riha et al., 2000; Griffith et al., 1999).

Telomere binding proteins and their interacting partners can regulate telomerase accessibility in either positive or negative ways (Evans & Lundblad, 2000). In humans, the first identified telomeric proteins are TRF1 and TRF2 (van Steensel & de Lange, 1997). These two proteins specifically bind to double stranded telomeric DNA regions and are involved in t-loop formation (Griffith et al., 1999). Over-expression of TRF1 or TRF2 inhibits telomere elongation in telomerase-positive cells (Smogorzewska et al., 2000) indicating that TRF1 and TRF2 serve as telomerase negative regulators.

Consistent with its role in inhibiting telomere elongation by telomerase, TRF1 binds to duplex telomeric DNA and inhibits C-strand DNA synthesis by DNA polymerase in vitro (Smucker & Turchi, 2001). When over-expressed as a dominant negative protein, TRF1 results in telomere lengthening in telomerase positive cells but not in telomerase-negative cells (Karlseder et al., 2002). These data suggest that TRF1 may inhibit the access of telomerase to telomeres. Considering that longer telomeres should bind more TRF1 molecules, this may create a negative feedback that controls telomere length. In addition, TIN2 and tankyrase, another two proteins associate with telomeres through TRF1, also join in this negative feedback control of telomerase access to telomeres (Cook et al., 2002; Kim et al., 1999; Simth et al., 1998). Another TRF1 associated protein, PinX1, directly interacts with hTERT protein as a potential telomerase inhibitor (Zhou & Lu, 2001).

Similarly, TRF2 was also identified as a double-stranded telomeric DNA binding protein (van Steensel & de Lange, 1997). Recent studies have demonstrated that TRF2 can also bind to the triple-stranded t-loop region and hence help form the loop (Griffith *et al.*, 1999; Karlseder et al., 1999). When TRF2 is over-expressed, it shortens telomere length in both telomerase positive and negative cells (Smogorzewska et al., 2000; Karlseder et al., 2002), possibly by denying telomerase access to telomeres.

Recently, Li et al. (2000) identified a human homolog of the yeast Rap1p. hRap1 binds to the telomere region through the interaction with TRF2. Limited studies demonstrate that, similar to its yeast homolog, hRap1 also functions as a negative regulator of telomere length (Li et al., 2000).

The Ku70/80 heterodimer is another telomere-associated protein. It was originally identified as a core component of the nonhomologous end-joining (NHEJ) DNA repair pathway and binds to double-strand breaks to help their alignment and subsequent ligation (Critchlow & Jackson, 1998; Featherstone & Jackson, 1999). Later studies demonstrated that the Ku70/80 heterodimer also localized to telomeres (Gravel et al., 1998; Hsu et al., 1999). However, the function of Ku70/80 in controlling telomere metabolism varies dramatically among different organisms. In contrast to yeast, mouse cells lacking Ku protein showed a moderate increase in telomere length (Hsu et al., 2000; Bailey et al., 1999; Samper et al., 2000). Furthermore, knocking-out *Ku80* led to telomere elongation only in normal mice but not in a telomerase-deficient background (Espejel et al., 2002). Recent evidence (Chai et al., 2002) shows that the human Ku70/80 heterodimer physically associates with the telomerase complex through interaction with *hTERT*. These data strongly suggest that Ku acts as a negative regulator of telomerase-mediated telomere elongation, and it may function in controlling the action of telomerase (Hsu et al., 2000; Song et al., 2000).

Besides these telomere-associated proteins, some hTERT-associated proteins also control telomerase function. A 14-3-3 protein is one such protein. The 14-3-3 family of proteins play a regulatory role in signal transduction and cell cycle checkpoints (Muslin & Xing, 2000). However, 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). In the same study, the authors showed that the C-terminal region of hTERT can specifically interact with the C-terminal region of 14-3-3 proteins both in vitro and in vivo, but this interaction is not required for telomerase activity. Since the telomerase complex must be assembled in and function in the nucleus, shuttling in and out of the nucleus by 14-3-3 family proteins represents another layer of regulation to control telomerase access to telomeres. In addition, the hsp90 chaperone complex also interacts with human telomerase, both physically and functionally, to assist in proper ribonucleoprotein assembly and the formation of active telomerase enzyme (Holt et al., 1999).

## Telomerase regulation in flowering plants

The overall pattern of telomerase expression in plants is similar to that in humans. In plants, telomerase activity is abundant in reproductive organs, embryos, plant tumors, and other rapidly dividing dedifferentiated cells, but undetectable in most of vegetative tissues (Fitzgerald et al., 1996; Fajkus et al., 1996; Heller et al., 1996). On the other hand, plants are evolutionarily and developmentally different than humans. One such difference, for example, is that many of plant cells are totipotent and they produce new organs throughout their lifespan. Because plants specify a germline late in their development, telomerase must be induced during the transition from the vegetative phase to the reproductive phase. Although telomere function was first defined in plants over 60 years ago (McClintock, 1942), mechanisms of telomerase regulation in plants are only beginning to be elucidated just now. Limited references indicate that telomerase regulation in plants, similar to that in humans, is controlled at several different levels.

In plants, telomerase activity is developmentally regulated. Using a modified version of telomere repeat amplification protocol (TRAP) assay, telomerase activity could be detected in proliferating organs, such as flowers, roots and callus; but low or no activity was detectable in vegetative organs, such as leaves and stems (Fitzgerald et al., 1996; Heller et al., 1996; Kilian et al., 1998; Riha et al., 1998). In barley and maize plants, telomerase was highly active in young, immature embryos, while dramatically less activity was identified in young endosperms (Kilian et al., 1998). Telomerase activity in these tissues decreased in the process of seed development, and the average telomere length shortened from ~80 kb in very young embryos to ~30 kb in old embryos (Kilian et al., 1995). Such a developmental expression pattern of telomerase activity in plants is similar to that of humans.

Telomerase regulation at the transcriptional level has not been studied in plants. However, telomerase activity in *Arabidopsis* does correlate with the level of *AtTERT* mRNA (Fitzgerald et al., 1999). Telomerase activity is absent from leaves but present in calli. However, *AtTERT* mRNA is detected in both leaves and calli with tenfold lower expression in leaves than that of calli (Fitzgerald et al., 1999). In an independent study, Oguchi et al., (1999) did not find either *AtTERT* transcripts or telomerase activity in rosette leaves, while both *AtTERT* mRNA and telomerase activity are detectable in cultured cells and shoot apical meristems. Nevertheless, both experiments indicate that *AtTERT* mRNA abundance is linked to telomerase activity. This correlation between telomerase activity and *AtTERT* expression indicates that, as in humans, transcriptional regulation of *AtTERT* expression is also a major level of control for telomerase activity in plants. However, new evidence from rice (Heller-Uszynska *et* al., 2002) shows that steady-state levels of transcript from the *OsTERT* gene do not seem to correlate with enzyme activity. Additionally, alternative splicing, which will be addressed below, seems to play an important role in telomerase regulation in rice. Further attention needs to be given to the transcriptional level regulation of telomerase activity in different plant species to examine whether this layer of regulation is different from species to species within the plant kingdom.

Alternative splicing is a common mechanism for gene regulation in higher eukaryotes (Adams et al., 1996). A specific pattern of *hTERT* mRNA variants affecting telomere length and telomerase activity has been found in humans (Ulaner et al., 2001). The existence of alternative splicing of the *TERT* gene in plants has also been addressed. In *Arabidopsis*, Fitzgerald et al. (1999) did not find any alternative splicing variants of *TERT* transcript. However, after cloning of the rice TERT gene, Heller-Uszynska et al. (2002) identified a number of differentially spliced transcripts both in telomerase-positive and telomerase-negative tissues indicating a possible mechanism for telomerase regulation by alternative splicing in plants. In comparison with *hTERT*, where alternative splicing events occurred only at the 3’ part of *hTERT* (Kilian et al., 1997; Villa et al., 2001), the 5’ part of *OsTERT* is subject to more alternative splicing events than the 3’ part of the gene (Heller-Uszynska et al., 2002). This difference could indicate that alternative splicing events at the 5’ part of *OsTERT* gene could be as biologically important as that arising from *hTERT* 3’ part of the gene. This new discovery also questions the results from *Arabidopsis*. Since Fitzgerald et al. (1999) only checked alternative splicing status at the 3’part of the *AtTERT* gene, where alternative splicing transcripts were found in humans, the possibility could not be ruled out that in *Arabidopsis*, similar to rice, alternative splicing events occur mostly in the 5’ part of *AtTERT* gene.

Using synchronized tobacco BY-2 cells, Tamura et al. (1999) demonstrated that plant telomerase activity is coordinated with the cell cycle. In their experiments, the amount of telomerase activity is significantly increased in S-phase, and this S-phase specific expression of telomerase is further induced by supplementing the culture with auxin, but not cytokinin (Tamura et al., 1999). Further studies showed that the plant hormones auxin and abscisic acid are necessarily involved in the cell cycle-dependent modulation of telomerase activity in tobacco cells (Yang et al., 2002). Based on this model, auxin enhances the level of telomerase activity whereas abscisic acid inhibits auxin- and cell cycle-dependent telomerase activity.

Evidence showed that telomerase regulation in plants is also controlled at post-translational levels. Yang et al. (2002) demonstrated that treatment of synchronized BY-

2 cells with the protein kinase inhibitors staurosporine or H-7 effectively prevented the S-phase-specific activation of telomerase activity. On the other hand, treatment with protein phosphatase 2A (PP2A) inhibitors, such as okadaic acid and cantharidin, resulted in continuous expression of telomerase activity in the cell cycle (Yang et al., 2002). These data strongly suggest that telomerase activity in tobacco BY-2 cells is regulated by protein phosphorylation.

Telomere-binding proteins may also regulate telomerase activity in plants (Fulnechova and Fajkus, 2000). Several genes encoding putative telomere-binding proteins have been recently identified, but nothing is known about their role in telomerase regulation (Kim et al., 1998; Chen et al., 2001; Yang et al., 2003). However, based on homologs in other organisms, one can predict that those telomere-binding proteins, such as *NgTRF1* (Yang et al., 2003), similar to its human homolog *TRF1*, will be involved in telomerase regulation.

The role of the Ku70/80 heterodimer in telomerase regulation in plants has also been addressed by several groups (Riha et al., 2002; Bundock et al., 2002; Gallego et al., 2003). *Arabidopsis Ku70/80* genes are ubiquitously expressed, and their products form a stable heterodimer in vitro (Riha et al., 2002; Tamura et al., 2002; West et al., 2002). In two independent studies, Riha et al. (2002) and Bundock et al. (2002) identified an *Arabidopsis* T-DNA line with a deficiency in the *Ku70* gene. Analysis of this mutant indicates that lack of Ku70 results in a dramatic deregulation of telomere length control with mutant telomeres expanding to more than twice the size of wild-type by the second generation (Riha et al., 2002). However, analysis of *AtTERT ku70* double mutants indicates that, rather than increasing in telomere length, ku70 deficiency in a TERT null background results in accelerating in loss of telomeres (Riha & Shippen, 2003). These data demonstrate that telomere lengthening in the *ku70* mutant is telomerase dependent and Ku70 serves as a negative regulator of telomere length. In another study, Gallego *et al*. (2003) identified an *Arabidopsis* mutant harboring a T-DNA in exon 10 of the *Arabidopsis Ku80* gene. Studies for this mutant line indicate that *Ku80* deficiency results in the lengthening of telomeres, the same phenotype seen in the Arabidopsis *Ku70* deficiency line, and this telomere lengthening is dependent on the presence of telomerase (Gallego et al., 2003). Similar to humans, this Ku70/80 heterodimer could act through direct interaction with telomerase (Grandin et al., 2000; Peterson et al., 2001; Chai et al., 2002) or telomere-binding proteins such as TRF1 and TRF2 (Hsu et al., 2000; Smogorzewska et al., 2000; Song et al., 2000).

### ***Arabidopsis* serves as a model for telomere biology study**

The small mustard weed *Arabidopsis thaliana* has been known to botanists for at least four centuries, and has been adopted as a model organism by thousands of biologists during the past 20 years (Somerville & Koornneef, 2002). Although not well recognized in the early stages, *Arabidopsis* has many advantages and serves as an experimental system for the study of plant biology. These include a small plant size, a short life cycle with 5-6 weeks per generation, and high fecundity. *Arabidopsis* also has the smallest known plant genome size (125 Mb), with fewer repetitive sequences than any other higher plant, which greatly facilitates molecular studies and map-based cloning. Recent completion of the entire *Arabidopsis* genome sequence and a simple high-efficiency *Agrobacterium*-mediated gene transformation technique make it even more powerful for plant biology studies.

One example of the power of the *Arabidopsis* system in particular, is its use in studying plant development by Meyerowitz and his colleagues, who developed the ABC model of floral morphogenesis (Weigel & Meyerowitz, 1994). These studies not only resulted in the model for understanding floral morphogenesis, but also set up an early example of how a developmental process could be analyzed and understood in plants.

The first investigation of telomere biology in the plant kingdom was reported over 60 years ago by McClintock (1941) using maize as experimental system. Little progress was made through the following half century. After uncovering mechanisms of DNA replication, scientists started to realize the end replication problem that was faced by conventional DNA replication mechanism and began to investigate how eukaryotes avoid loss of genetic material at the ends of chromosomes. These investigations identified telomere repeats and the enzyme for adding telomeres in unicellular organism *Tetrahymena* (Greider and Blackburn, 1985).

Plant telomere DNA was first isolated in *Arabidopsis* by Richards and Ausubel in 1988, and telomerase activity was detected in the mid 1990s (Fitzigerald et al., 1996). Since then, the powerful tools developed for *Arabidopsis* have allowed rapid progress in elucidating plant telomere biology. As detailed in the last section, many studies have focused on mechanisms of telomere regulation in *Arabiodopsis*. These findings demonstrated the basic similarities in terms of telomere biology and the difference between plants and other organisms. The preliminary studies also demonstrated that *Arabidopsis* could be a good model for telomere biology studies.

Telomerase regulation is a fundamental problem in multicellular eukaryotes. Massive research has been done in other organisms, however, mechanisms of telomerase regulation in plants are just beginning to be elucidated. The main goal of this project is to find how plants regulate telomerase expression, using *Arabidopsis* as model system. In this study, a biochemical screening strategy for identifying telomerase activator (*tac*) mutants was developed and two independent *tac* mutants were isolated. Studies of *tac1* have demonstrated that *TAC1* encodes a single zinc-finger protein, and it acts synergistically with auxin to induce telomerase in *Arabidopsis* fully differentiated leaves without activating cell cycle. This *tac1* mutant also confers drought tolerance, apparently a non-telomerase phenotype, which will be described in Chapter IV.

# CHAPTER II

# IDENTIFICATION AND CHROMOSOMAL LOCALIZATION OF TELOMERASE ACTIVATOR MUTANTS *tac1* AND *tac2* IN *ARABIDOPSIS THALIANA*

## OVERVIEW

Telomerase is a ribonucleoprotein complex that synthesizes telomeric DNA in most eukaryotic organisms. In wild-type *Arabidopsis*, telomerase expression is abundant in reproductive organs such as flowers and siliques and dedifferentiated tissues such as callus, but is barely detectable in most vegetative tissues, including rosette and cauline leaves. Here, we developed a biochemical strategy to screen for telomerase activating mutants of *Arabidopsis thaliana.* Screening of *Arabidopsis* activation-tagged lines by the PCR-based Telomere Repeat Amplification Protocol (TRAP) assay resulted in isolation of two telomerase activator (*tac*) mutants. Plasmid rescue indicated that *tac1* maps to the left arm of chromosome 3, while the *tac2* mutant harbored two independent T-DNAs; one on chromosome 1, the other on chromosome 5. Segregation analysis demonstrated that telomerase expression in *tac1* is dominant and linked to the T-DNA insertion. RT-PCR analysis of the gene encoding the telomerase reverse transcriptase (*AtTERT*) revealed that different mechanisms are involved in regulating telomerase activity in *tac1* and *tac2*.

## INTRODUCTION

Telomeres are unique structures at the ends of linear chromosomes, and in most eukaryotes they contain tandem arrays of GT-rich repeat sequence, 5'TTTAGGG3' in most plant species (Richards and Ausubel 1988; Fajkus et al. 1995) and 5'TTAGGG3' in onion family (Weiss and Scherthan 2002). Telomeric DNA, together with telomere binding proteins (van Steensel & de Lange, 1997; Cook et al., 2002; Simth et al., 1998; Chen et al., 2001; Yang et al., 2003) have an essential role in stabilizing chromosome ends by forming a cap structure that protects chromosome ends from exonucleolytic degradation and terminal fusions. In addition, telomeres also play important roles in the nuclear architecture and chromosome organization in mitosis and meiosis (reviewed in McKnight et al., 2002).

Telomerase, a ribonucleoprotein complex with reverse transcriptase activity, is the key enzyme for synthesizing and maintaining telomeric DNA in most eukaryotes (Collins and Mitchell, 2002). In the absence of telomerase, the end-replication problem leads to telomere shortening and eventual instability of the genome typified by chromosome fusions (Lee et al., 1998; Riha et al., 2001). In mammals, telomerase activity is undetectable in most normal somatic tissues, but it is strongly expressed in germline cells and reactivated in over 90% of all highly proliferating cancerous tissues. The regulation of telomerase activity is mainly controlled by transcription of the *telomerase reverse transcriptase* (*TERT*) gene, which is mediated by a number of molecules such as c-Myc and Sp1 (Wu et al., 1999; Kyo et al., 2000; Ducrest et al., 2002). In addition, post-transcriptional processing events may play a significant role in controlling enzyme activity (Aisner et al., 2002; Kharbanda et al., 2000).

The overall pattern of telomerase expression in plants is similar to that in mammals. Telomerase activity is abundant in reproductive organs, embryos, plant tumors, and other rapidly dividing dedifferentiated cells, but undetectable in most vegetative organs (Fitzgerald et al., 1996). Studies on mechanisms of telomerase regulation in plants are just beginning. However, in *Arabidopsis*, telomerase activity in various organs is correlated with the level of *AtTERT* transcripts (Fitzgerald et al., 1999). In addition, the phytohormone auxin can rapidly induce telomerase activity in plant cells and tissues during S-phase (Tamura et al., 1999), and this process appears, at least partly, to be regulated by protein phosphorylation (Yang et al., 2002). Evidence in rice showed that telomerase activity may also be controlled by alternative splicing of *OsTERT* (Heller-Uszynska et al., 2002).

To further elucidate mechanisms that specifically control telomerase expression in higher eukaryotes, we wanted to identify mutants with altered patterns of telomerase expression. *Arabidopsis thaliana* is a small mustard weed. Its relatively small genome size, a complete genome sequence, a high efficiency of transformation, and most importantly, hundreds of thousands of available mutants, make it perfect for large-scale, functional genomic studies. In this chapter, we describe the isolation and mapping of two telomerase activating mutants, *tac1* and *tac2,* through screening of *Arabidopsis* activation-tagged lines by a modified version of the telomere repeat amplification protocol (TRAP) (Fitzgerald et al., 1996).

# MATERIALS AND METHODS

## Plant materials and growth conditions

*Arabidopsis* activation tagged lines, together with the parental line, ecotype Columbia Col-7, were purchased from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). Activation tagged lines, grown in pools of 10 or 20 lines in soil, were cold-treated overnight at 4ºC, then placed in the growth chamber under continuous light at 23ºC. For the pools of 10 independent activation tagged lines, a minimum of 30 plants were grown for each pool, while for pools of 20, at least 60 plants were grown to provide a 95% probability that each individual line will be present in the samples. One leaf from each individual plant in the population was collected, immediately frozen in liquid nitrogen, and stored at –80ºC until use.

## Plant extracts preparation

Pooled leaf samples were ground to fine powder in a mortar and pestle under liquid nitrogen, then suspended in buffer W [50mM Tris acetate, pH 7.5; 5mM $MgCl_2$; 100mM potassium glutamate; 20mM EGTA; 1.0mM DTT; 0.1mM PMSF; 0.6mM vanadyl ribonucleoside complex; 1.5% (wt/vol) polyvinylpyrrolidone and 10% glycerol] with a ratio of 4ml/g tissue. After centrifuging in Eppendorf tubes at 14K RPM at $4^oC$ for 15 minutes, the supernatants were transferred to new tubes. For each extract, PEG 800 was added to a final concentration of 10%, mixed thoroughly at 4°C for 30 minutes, and centrifuged again at 14K RPM for 10 minutes. The pellet was resuspended in a quarter of the original volume of buffer W for 30 minutes at 4°C and then centrifuged for 2 minutes. The supernatant was stored at –80°C until used.

**Telomere repeat amplification protocol (TRAP) assay**

Telomerase activity was detected by a modified version of the TRAP protocol (Kim et al. 1994; Fitzgerald et al. 1996). The following primers obtained from the Gene Technology Laboratory at Texas A&M University were used in the TRAP assay: Forward primer (TS21) 5'GACAATCCGTCGAGCAGAGTT3' and Reverse primer 5'CCCTAAACCCTAAACCCTAAA3'. The 48ul reaction mixtures were prepared to contain 50mM Tris acetate (pH 8.3), 50mM potassium glutamate, 0.1% Triton X-100, 1mM spermidine, 1mM DTT, 50uM each dNTP, 5mM $MgCl_2$, 10mM EGTA, 0.5ul ($\alpha$-$^{32}$P)dGTP (3,000 mCi/mM; New England Nuclear), 100ng/ul BSA, 0.5uM T4 gene 32 product, 0.5ul *Taq* polymerase and 1ul (50ng/ul) forward primer (TS21). After the addition of nuclear extracts containing 0.25-1.0ug protein, the telomerase extension reaction was allowed to proceed at 30°C for 45 minutes. Then the reverse primer (50ng) was added, and the reaction mixture was amplified by 30 cycles of PCR at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 90 seconds. After 30 cycles, an additional 5 minute 72°C extension step was run. The reaction was stopped by adding 50ul telomerase stop buffer (10mM Tris-HCl pH 7.5 and 21mM EDTA) and then ethanol precipitated. The samples were resolved on 6% sequencing gels. The gels were dried and exposed on X-ray film or PhosphorImage screen. Extracts from *Arabidopsis* wild-type flowers or califlower were used on each gel as positive controls.

### Identification of individual plant from telomerase positive pools

Once telomerase-positive pools were identified, seeds from the original pools were sown in soil as mentioned above, and leaves from each individual plant were collected and subjected to TRAP assayed to identify individual plants with altered telomerase activity. Seeds from mutant plants were harvested and used for further experiments.

### Plasmid rescue and sequence

Given that a BlueScript plasmid was harbored on the activation tagging vector and flanked by several restriction enzyme sites, the T-DNA and adjacent plant DNA sequences from mutant plants can be recovered by plasmid rescue (Weigel, et al., 2000). The activation tagging vector used for generating *Arabidopsis* activation tagged lines was shown in Figure 1. Because of imprecise transfer of right border sequences there is often no unique sequence at the right end of the T-DNA insertion, which made it

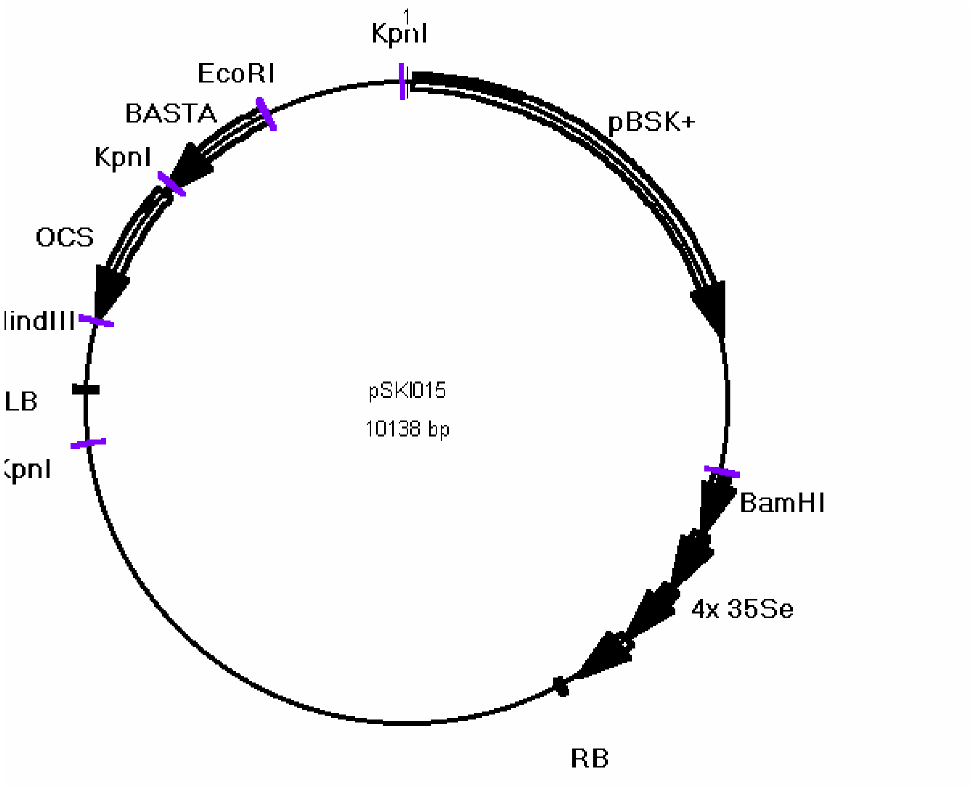

1
KpnI
EcoRI
BASTA
KpnI
OCS
lindIII
LB
‹pnI
pBSK+
pSKI015
10138 bp
BamHI
4x 35Se
RB

**Figure 1.** Vector map of pSKI015. pSKI015 was used for Arabidopsis activation-tagged-line generation.

impossible to sequence into plant DNA from this end of the T-DNA. For this reason, in our experiments, we chose to use restriction enzyme BamHI to rescue the T-DNA and adjacent plant DNA through the left border. Genomic DNA from telomerase-positive individual plants was prepared, and about 1ug of genomic DNA was digested overnight with BamHI in a 100ul reaction. After phenol-chloroform extraction and ethanol precipitation, digested DNA was dissolved in 50ul TE buffer and 10ul were used and allowed to ligate overnight at 16$^{o}$C in a total volume of 100ul. Ligated DNA was precipitated, and about one-fifth was transformed by electroporation into competent *E. coli DH10B* cells. The rescued plasmids were sequenced with either a left border primer (LB) or a T7 primer.

### Mapping of *tac1* and *tac2* mutations

Given that there was no sequence information corresponding to any rescued clones at the time of this experiment, to uncover T-DNA insertion sites, a high density TAMU *Arabidopsis* BAC membrane was purchased from *Arabidopsis* Biological Resource Center. To identify individual BAC clones covering T-DNA insertion sites in both *tac1* and *tac2*, a plant DNA fragment was cut from rescued clones with BamHI and XbaI and used to probe a high density BAC membrane. The membrane was reused after stripping off radioactive probe by boiling 2 min in 1% SDS. Identified BAC clones were matched to contigs generated by the genome sequencing project, thereby giving the chromosomal location of the T-DNA insertion.

## Segregation analysis

Seeds from heterozygous TAC1 plants were sown directly in soil. After overnight cold treatment, they were moved to a growth chamber with continuous light. Leaves from individual plants were collected and then sprayed with a 1:200 dilution of commercially available Finale (AgrEvo, Montvale, NJ), which contains 5.78% (w/v) ammonium glufosinate (also known as Basta). Plants were sprayed twice three days apart. Excised leaves were subjected to TRAP assay and results were compared to BASTA resistance/susceptibility data to see if telomerase activity in leaves was linked to T-DNA insertion.

## Semi-quantitative RT-PCR analysis of *AtTERT* expression in *tac1* and *tac2*

Total RNA was extracted from wild-type, *tac1* and *tac2* leaves and wild-type flowers using the Tri-Reagent (Sigma) following the manufacturer's instruction. RNA concentrations were determined with the aid of a spectrophotometer. In each reverse transcription reaction using the reverse transcriptase-SuperScript II (Invitrogen) and oligo d(T), 2.5ug of total RNA was used. To prevent the effect of RNA secondary structure prior to synthesizing first strand cDNA, total RNA was heated at $65^oC$ for 5 min. RT was performed at $42^oC$ for 1.5 hr and then the enzyme was inactivated by incubation at $75^oC$ for 15 min. To amplify *AtTERT* expression products, 1 ul of first strand cDNA was used in a 20 ul reaction. The primer set used for amplification was as follows: TERT 5 (GCCCTTGATGGATATGTCC) and TERT 48 (CCAACTGCAG CATGTTGTTC). After 3 min denaturation at 94°C, PCR was carried out for 20 cycles under the following conditions: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec followed by a final elongation period of 7 min at 72°C. A 15 ul aliquot of the reaction was resolved on a 1.8% agarose gel and subjected to Southern blot analysis using probes generated from each genomic DNA product. As a quantitation control, RT-PCR was carried out with primers specific for the cystosolic glyceraldehyde-3-phosphate dehydrogenase gene (forward: GACCTTACTGTCAGAC-TCGAG and reverse: CGGTGTATCCAAGGATTCCCT). Signals were quantified on a Fuji PhosphorImager.

## RESULTS

### Screening strategy

To faciliate isolation of genes regulating telomerase activity, a screening strategy was developed for *Arabidopsis thaliana*. Basically, TRAP assays were used to screen activation-tagged lines for mutants that ectopically express telomerase activity. These lines were created by using the T-DNA of *Agrobacterium tumefaciens* to insert a strong, constitutive enhancer at random sites throughout the whole genome. If the enhancer lands near and activates a gene that is required for expression of telomerase, it may result in ectopical telomerase expression. The highly sensitive TRAP assay allows us to detect telomerase activity in nanogram level of plant extracts (Fitzgerald et al., 1996), and thus a rare positive signal can be identified in a background of many negative signals. Practically, we screened activation tagged lines in pools of 10 or 20 by collecting a leaf from each plant, grinding them in bulk and performing the telomerase assay. Once a positive signal was detected, individual plants in rare pools with telomerase activity were then re-sampled to find the mutant plant.

## Isolation of *tac1* and *tac2* mutants

Our previous results showed that leaves from wild-type *Arabidopsis* do not have detectable telomerase activity (Fitzgerald et al., 1999). Through screening of about 2,000 *Arabidopsis* activation-tagged lines by a modified version of TRAP assay, two telomerase activator mutants, *tac1* and *tac2*, were isolated, with strong telomerase activity in their leaves. *tac1* was isolated from original pool CS20857 and *tac2* from pool CS20897. Figure 2 shows telomerase expression in the *tac1* mutant.

The T-DNA insertion and flanking genomic DNA were recovered by plasmid rescue for both *tac1* and *tac2*. Rescued clones were subjected to restriction analysis. By comparing the genomic DNA fragment size released from restriction digestion, two-different-type clones for both *tac1* and *tac2* were identified. Clone B1 and B3 are the

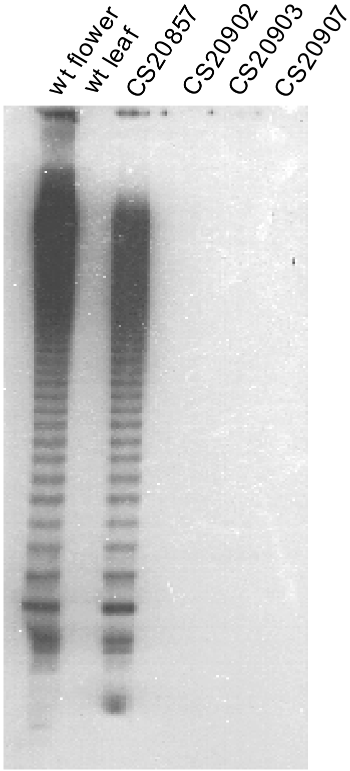

wt flower
wt leaf
CS20857
CS20902
CS20903
CS20907

**Figure 2.** TRAP assay of activation-tagged lines. Nuclei proteins from leaves of activation-tagged lines in pools of 10 or 20 were used for this assay. Wild-type flower and leaf were used as a positive and a negative control respectively. CS numbers represent different pools of activation tagged lines.

representatives of *tac1* and B2 and B6 for *tac2*. DNA sequencing confirmed that B1 and B3 in *tac1* are different and B2 and B6 in *tac2* are different. BLASTing resulted in no information, indicating that all four sequences were in uncharacterized regions at the time of these experiments.

## Chromosome localization of *tac1* and *tac2*

To determine chromosome locations of the T-DNA insertions in both *tac1* and *tac2*, genomic DNA fragments from B1 and B3 of *tac1* and B2 and B6 of *tac2* were isolated and used as probes to hybridize to a high density membrane of the *Arabidopsis* TAMU BAC library. Results are summarized in Table 1. In the case of *tac1*, 8 BAC clones were detected by genomic DNA fragment cut from clone B1. When using B3 fragment as a probe, the same set of BAC clones was isolated. By a combination of Southern blotting and DNA sequencing, we determined that the T-DNA insertion was complex with two left borders (LB) flanking the insertion site in *tac1* mutant. An *Arabidopsis* database search identified that the T-DNA insertion in *tac1* was located on a BAC end mapped to the short arm of chromosome III. Upon completion of *Arabidopsis* genome sequencing, a fine structure of the T-DNA insertion site in *tac1* was confirmed, and this insertion also resulted in the deletion of ~100 nt. Figure 3 shows the chromosome localization and structure of the T-DNA insertion site in the *tac1* mutant.

In the case of TAC2, 11 overlapped BAC clones were identified by genomic DNA fragment from the rescued B6 clone. While when using B2 fragment as probe, 8

**Table 1.** BAC clones identified by rescued genomic DNA in *TAC1* and *TAC2*

| Mutant | Probe | Identified BAC clones | Chr. Location |
|---|---|---|---|
| *TAC1* | B1 Fragment | T1A6, T1J13, T5P16, T25I21, T14B5, T18H19, T15C1, T4J13 | III |
| | B3 Fragment | T1A6, T1J13, T5P16, T25I21, T14B5, T18H19, T15C1, T4J13 | III |
| *TAC2* | B2 Fragment | T10G10, T2K3, T2K2, T3F19, T10C10, T27H2, T19D14, T7N16 | V |
| | B6 Fragment | T13J2, T21F3, T17O4, T17M4, T10C5, T6K12, T2F14, T15G6, T7P7, T15H14, | I |

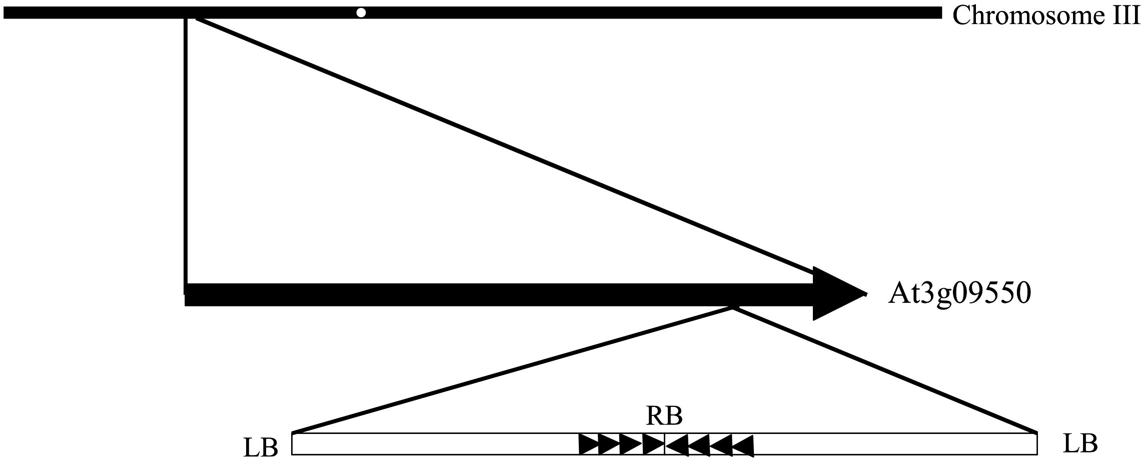

Chromosome III
At3g09550
RB
LB
LB

**Figure 3.** T-DNA insertion structure and its chromosome localization in *tac1* mutant. Two head-to-head orientated T-DNAs were inserted near 3’ end of At3g09550 on Chromosome III.

overlapped BAC clones, which were different from those detected by B6 clone, were detected. This demonstrated that two copies of T-DNA were inserted in TAC2 mutant. Database searching further confirmed one copy of T-DNA was located on chromosome I, and the other was on chromosome V.

### Co-segregation analysis

The original *tac1* mutant plant was hemizygous. In the following generation, 2-week old plants were sprayed with BASTA. Out of 275 individual plants, 199 survived. The ratio of BASTA resistant to BASTA susceptible plants corresponds to a segregation ratio of 3 to 1, confirming the status of a single T-DNA locus in the TAC1 mutant. Furthermore, TRAP assays showed that BASTA resistant individuals were telomerase positive while BASTA susceptible plants were telomerase negative (Figure 4). Taken together, these data suggested that the *tac1* mutation is dominant and segregated as a single locus linked to the BASTA resistance marker. The data shown here also indicated that *tac1* is a gain-of-function mutant, presumably caused by the enhancers harbored on the T-DNA insertion.

### Expression of *AtTERT* in *tac1* and *tac2*

In wild-type *Arabidopsis*, the expression of telomerase activity in various organs is correlated with the level of *AtTERT* transcripts (Fitzgerald et al., 1999). However,

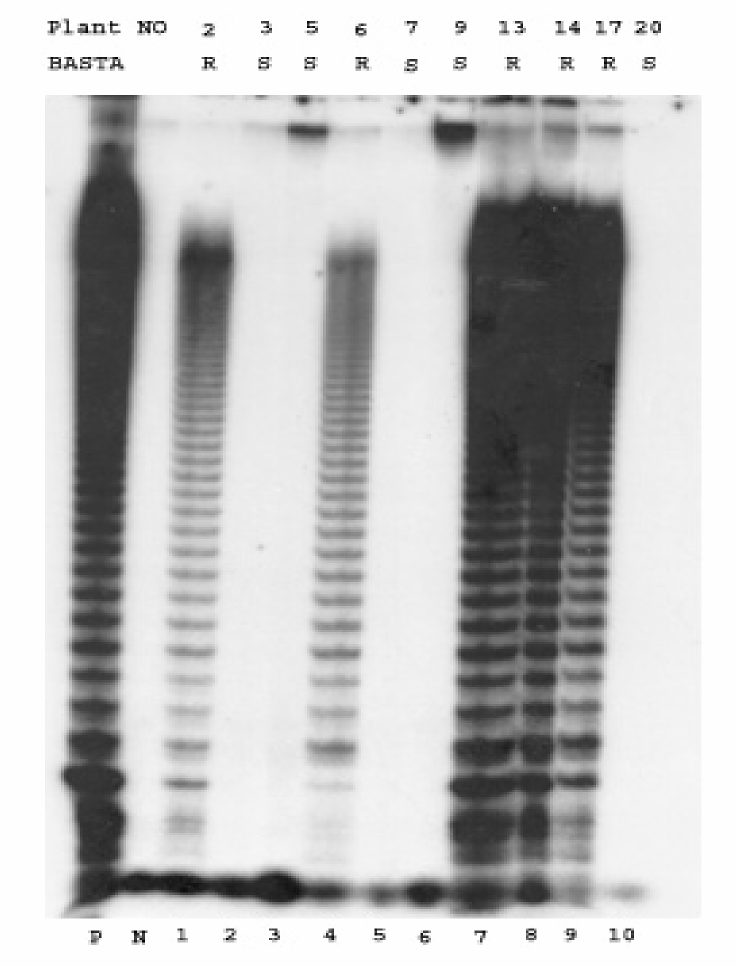

Plant NO 2 3 5 6 7 9 13 14 17 20
BASTA R S S R S S R R R S
P N 1 2 3 4 5 6 7 8 9 10

**Figure 4.** Co-Segregation of telomerase activity and *BASTA* resistance in *tac1*mutant. Individual plants from *tac1* heterzygous population were subject both to TRAP assay and *BASTA* resistance test. R represents resistance; S, for susceptible.

telomerase activity can also be regulated at post-transcriptional, post-translational levels.

To determine at what levels telomerase activity has been regulated in *tac1* and *tac2* mutants, we tested mRNA level expression of the *AtTERT* gene (Figure 5). Steady state levels of mRNA for *AtTERT* were increased in *tac1* over that in wild-type leaves, but not to the level seen in wild-type flowers, indicating that telomerase expression in *tac1* is regulated at the transcriptional level. On the other hand, the expression of *AtTERT* in *tac2* was barely detectable by RT-PCR, similar to that in wild-type leaves, suggesting that telomerase expression may be enhanced by a different mechanism in *tac2*.

## DISCUSSION

### Biochemical screening of mutant sources to identify new genes

In this chapter, we used a modified version of the TRAP assay to screen *Arabidopsis* activation tagged lines for mutants that alter the expression pattern of telomerase. Through screening of 2,000 lines in pools of 10 or 20, we successfully identified two mutants, *tac1* and *tac2*, which can ectopically activate the telomerase in *Arabidopsis* leaves where telomerase is normally undetectable in wild-type.

*Arabidopsis*, as a plant model system, has been intensively studied in many aspects of biology. A simple *Agrobacterium*-mediated transformation technique makes it most powerful in functional genomic studies. Currently, through the efforts of the *Arabidopsis* research community, hundreds of thousands of T-DNA tagged lines, including both

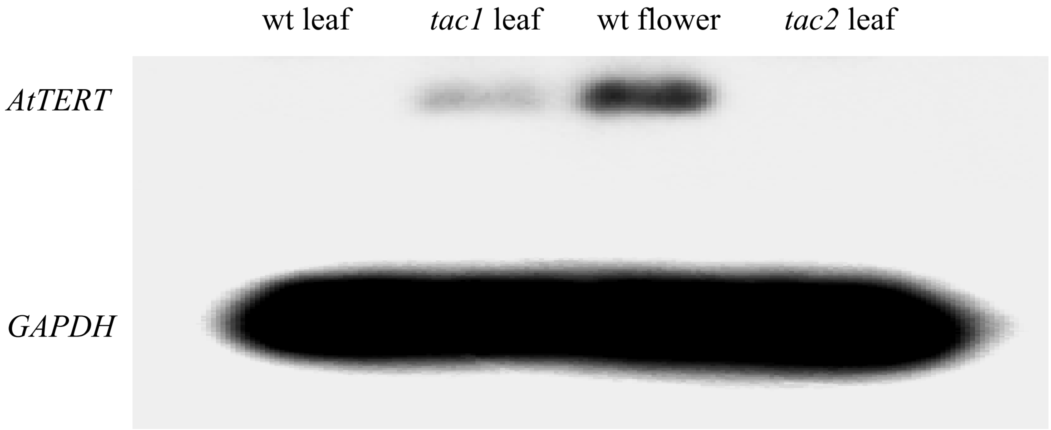

wt leaf
tac1 leaf
wt flower
tac2 leaf
AtTERT
GAPDH

**Figure 5.** Examination of *AtTERT* mRNA expression in *tac1* and *tac2*. Wild-type flower and leaf were used as positive and negative controls. GAPDH as loading control.

loss-of-function T-DNA insertion and gain-of-function T-DNA insertion lines, have been generated and made available to all *Arabidopsis* researchers through the *Arabidopsis* Resource Center, located at Ohio State University. These valuable sources have been intensively used in functional genomic studies. However, most screens on these mutant lines have been based on a visible phenotype (Weigel et al., 2000). Although a PCR based technique has been developed to identify T-DNA knockouts for any genes of interest (Krysan et al., 1999), it can only be used when researchers know what genes they are looking for.

Following the complete sequence of *Arabidopsis* genome, the next goal for the *Arabidopsis* community has been set to dissect functions of each individual gene (Chory et al., 2000). Apparently not all genes show visible phenotypes right after knocking-out and/or over-expressing their function, such as *AtTERT* (Riha et al., 2001), or they may never show a visible phenotype. To aid new gene identification in functional genomic studies, new strategies for screening those valuable stocks need to be developed. Biochemical assay of the mutant stocks may at least in part identify some new components involved in a specific signal transduction pathway or metabolic system. Here through screening of *Arabidopsis* activation tagged lines by a modified version of the TRAP assay, we demonstrated that biochemical screening of these mutant lines to identify new genes is feasible for *Arabidopsis* research and may be applicable to other species, if the mutant sources are available or easy to generate.

## Mechanisms of telomerase regulation in *tac1* and *tac2*

RT-PCR analysis of *AtTERT* mRNA expression in *tac1* and *tac2* mutants indicated that *tac1* regulates telomerase activity by increasing levels of mRNA for *AtTERT*, the catalytic subunit of the telomerase complex, while *tac2* controls telomerase regulation by a different mechanism. A vast amount of research has been focused on the transcriptional regulation of *TERT* genes in other organisms. These studies identified many potential binding sites on the *TERT* promoter region for multiple activators and repressors. The transcription factors well known to up-regulate *hTERT* gene are the oncoprotein *c-Myc* and *Sp1* and the repressors of *hTERT* include *Mad1* and *p53* and many others.

Despite such massive studies, insight into mechanisms that specially control telomerase expression at the transcriptional level is yet unknown. On the other hand, increasing data indicate that regulation of telomerase is a complex and dynamic process involving many steps in the eventual ability of telomerase to locate, access and function on telomeres. Such post-transcriptional control of telomerase activity may play significant roles in regulating the enzyme activity.

Although plants are different from animals both evolutionarily and developmentally, the overall patterns of telomerase expression are similar in these two multicellular kingdoms. Mechanisms uncovered from plants may also hold true in other organisms. In this study, we identified *tac1* as a telomerase activator that somehow increases AtTERT message. In the following chapter, we describe the cloning of the TAC1 gene and the elucidation of its possible mechanism in controlling enzyme activity. Although not described in this study, the characterization of the *tac2* mutant may uncover a novel pathway in controlling telomerase activity.

### ***Arabidopsis* serves as a new model system for telomerase regulation**

Telomerase activity is barely detectable in most normal human somatic tissues, but it is highly expressed in germline cells and reactivated in over 90% of all cancerous tissue examined, covering more than 30 different types of cancer. This nearly ubiquitous expression in human cancers makes telomerase a powerful diagnostic marker and therapeutic target and attracts more attention in studies of telomerase regulation mechanisms in many higher eukaryotic organisms. However, in terms of mutant isolation, none of the mammalian systems are comparable to *Arabidopsis*.

Plants are developmentally different from human; for example, many plant cells are totipotent, they produce new organs throughout their lifespan, and their germ line is specified very late. Because of these differences, it is interesting to investigate the telomerase regulation pathway in plant systems. However, previous results showed that the pattern of telomerase expression in plants is similar to that in human. Telomerase activity is undetectable in most vegetative tissues, but is abundant in reproductive organs, plant tumors, and other rapidly dividing dedifferentiated cells (Shippen and McKnight, 1998; Fitzgerald et al., 1996; Heller, 1996). These similarities in basic telomere biology could indicate that at least some regulatary pathways for telomerase expression may be conserved between higher plants and animals and hence some genes identified in *Arabidopsis* will have functional homologs in other organisms.

As mentioned before, there are powerful genetic tools and nearly saturated mutant stocks available in *Arabidopsis thaliana*. This means that *Arabidopsis* provided a unique opportunity for identifying new genes regulating telomerase activity. In the current study, two telomerase activator mutants were isolated through screening of 2,000 activation tagged lines. Further screening of an additional 6,000 mutant lines in our lab identified at least another two mutants which alter telomerase expression. More than 16,000 activation tagged mutants are available in *Arabidopsis*, and through screening the rest of the mutant lines, we believe that more telomerase activation mutants will be identified. These mutants can serve two major roles. First, they will provide a systematic way to elucidate telomerase regulation pathways in *Arabidopsis* and help us to understand the mechanisms for telomerase regulation in higher plants, and possibly other organisms. Second, at least some of these genes should have functional homologs in humans that can be used to develop novel anticancer treatments.

Given that it is so convenient to identify new genes controlling telomerase regulation in *Arabidopsis*, and studies of these mutants in this system should be straightforward, we propose that *Arabidopsis* can be served as a new model system for studying telomerase regulation.

# CHAPTER III

# SYNERGISTIC ACTION OF TAC1 AND AUXIN ACTIVATES TELOMERASE IN *ARABIDOPSIS THALIANA*

## OVERVIEW

Telomerase, a key enzyme with reverse transcriptase activity, synthesizes and maintains telomeres at the termini of most eukaryotes. Telomerase expression is abundant in the germline cells but is undetectable in most other differentiated tissues of multicellular eukaryotes. Intensive studies of telomerase have focused on human cancerous cells, where over 90% of all cancerous tissues examined have telomerase activity. However, our understanding of mechanisms that specifically control telomerase expression is incomplete. Here we report the cloning and characterization of a telomerase activator gene, *TAC1* in *Arabidopsis*. *TAC1* encodes a small protein with a single zinc finger. Recapitulation experiments confirm that this gene activates telomerase in fully-differentiated leaves. This induction of telomerase activity is uncoupled from the cell cycle and can be diminished by over-expressing iaaL, an enzyme that conjugates free IAA to lysine. Telomere length is unperturbed in the mutant, but other phenotypes, such as altered root development and the ability of cells to grow in culture without exogenous auxin, indicate that TAC1 not only is part of the previously reported link between auxin and telomerase expression, but also potentiates other classic responses to this phytohormone.

## INTRODUCTION

Telomeres are nucleoprotein complex containing non-histone proteins and repetitive, non-coding DNA at the ends of linear chromosomes. In most eukaryotes telomeric DNA consists of tandem arrays of GT-rich repeat sequences. Telomeres serve a variety of purposes, including preserving chromosomal integrity by preventing degradation, end-to-end fusions and rearrangements, and organizing chromosome order during meiosis (for reviews see Blackburn, 1991; Greider, 1991; McKnight et al., 2002).

Telomerase is a ribonucleoprotein complex with reverse transcriptase activity that synthesizes and maintains telomeric DNA in most eukaryotic organisms (Collins and Mtichell, 2002). In the absence of telomerase, the consequence of semiconservative DNA replication results in the successive shortening of telomeres with each cell division, and this shortening eventually causes genome instability, typified by chromosome fusions (Lee et al., 1998; Riha et al., 2001). Critical shortening of one or a few telomeres is sufficient to trigger the onset of cellular senescence in most normal cells and cause genetic instability (Harley et al., 1990; Hemann et al., 2001).

In humans, telomerase is active in the germline and cells with high capacity for proliferation, but it is undetectable in most somatic cells. Forced expression of telomerase activity in telomerase-negative cells by expression of *hTERT*, the gene encoding the catalytic protein component, results in telomere-length stabilization and extension of lifespan to the extent that cells are deemed to have been immortalized (Bodnar et al., 1998). This potential extension of lifespan by expression of *hTERT* has driven much of the recent research into the regulation of telomerase with a special interest on understanding of the transcriptional regulation of TERT genes (Meyerson et al., 1997). These studies identified many potential binding sites in the *hTERT* promoter region for multiple activators and repressors. The transcription factors known to up-regulate *hTERT* include the oncoprotein c-Myc, Sp1, and estrogen and progesterone receptors (Takakura et al., 1999; Kyo et al., 1999; Misiti et al., 2000; Wu et al., 1999; Kyo et al., 2000; Wang et al., 2000). The repressors of *hTERT* include WT1, MZF-2, p53, and Mad1. In addition, the presence of a CpG island and high overall GC content in the *hTERT* regulatory region suggests a possible role of methylation in regulation of hTERT (Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999; Wick et al., 1999).

While a number of molecules have been implicated in the regulation of *hTERT* (Ducrest 2002), insight into mechanisms that specifically control telomerase expression at the transcriptional level is lacking. In addition, increasing data indicate that regulation of telomerase is a complex and dynamic process involving many steps in the eventual ability of telomerase to locate, access and function on telomeres. These post-transcriptional/translational events, such as alternative splicing (Kilian et al., 1997; Ulaner et al., 1998) and phosphorylation (Li et al., 1997; Li et al., 1998; Kang et al., 1999), may play significant roles in controlling enzyme activity (Aisner, et al., 2002).

Despite the evolutionary and developmental divergence between plants and animals, overall patterns of telomerase expression are similar in these two multicellular lineages. In plants, telomerase activity is restricted to reproductive organs, embryos, and immortalized, dedifferentiated cells growing in culture (Fitzgerald et al., 1996; Heller et al., 1996; Kilian et al., 1998). Because plants specify a germline late in their development, telomerase must be induced during the transition from the vegetative phase to the reproductive phase. Mechanisms of telomerase regulation are only beginning to be elucidated in plants, but telomerase activity in *Arabidopsis* correlates with level of *AtTERT* mRNA (Fitzgerald et al., 1999). Although not identified in *Arabidopsis*, there are several alternative spliced forms of TERT mRNA in rice (Kilian et al., 2002). In addition, there is also strong correlation between cell cycle progression and telomerase expression. In synchronized tobacco cells in culture, telomerase activity is restricted to late S-phase. Exposure to auxin-type phytohormones such as indole acetic acid (IAA) induces telomerase activity to higher levels and an earlier appearance during S phase (Tamura et al., 1999). At least part of this cell cycle-dependent, auxin-mediated increase in telomerase activity is regulated by protein phosphorylation (Yang et al., 2002).

As mentioned in the previous chapter, visual screening of activation tagged lines of *Arabidopsis* is a powerful method for identifying novel genes involved in many aspects of plant development (Weigel et al., 2000). Using TRAP assays, we have demonstrated that biochemical screening of such sources would have equal power in functional genomic studies. Through screening of 2,000 *Arabidopsis* activation tagged lines, we identified two *telomerase activatior* (*tac*) mutants that ectopically express telomerase in their leaves. In this chapter, we will describe the cloning and characterization of *TAC1, a* gene encoding a small protein with single zinc finger. Induction of telomerase activity in the *tac1* mutant is uncoupled from the cell cycle, and this induction is due to the synergistic action between TAC1 protein and phytohormone auxin. In addition, TAC1 also potentiates other classic responses to auxin.

## MATERIALS AND METHODS

### Plant materials and growth condition

All activation tagged lines used in this chapter were from the *Arabidopsis* Biological Resource Center; and *tac1-2D* was from Dr. Joe Ecker's SIGNAL collection. The *Arabidopsis* iaaL overexpressing line, and *yucca* mutant line were kindly provided by Dr. Yunde Zhao (UCSD). All materials, including the *tac1* mutant, were grown in soil and cold-treated overnight at 4ºC prior to being placed in a growth chamber on continuous light at 23ºC. Crosses between *tac1* and *iaaL*, and *tac1* and *yucca* were performed to generate double mutants. F1 hybrids were grown in the same condition. Since all three mutants used in crossing experiment are dominant, F1 hybrids were directly used for experiments. However, once following generations were needed, PCR was used to identify double mutants in the segregating population. Leaf samples were harvested and frozen at –80$^{o}$C for later extraction of DNA, RNA and nuclear proteins.

### Plant extract preparation

The collected samples were ground to fine powder in a mortar and pestle under liquid nitrogen, then suspended in buffer W [50mM Tris acetate, pH 7.5; 5mM $MgCl_2$; 100mM potassium glutamate; 20mM EGTA; 1.0mM DTT; 0.1mM PMSF; 0.6mM vanadyl ribonucleoside complex; 1.5% (wt/vol) polyvinylpyrrolidone and 10% glycerol] with a ratio of 4ml/1g tissue. After centrifuging at 14K RPM at $4^oC$ for 15 minutes, the supernatants were transferred to new eppitubes. For each extract, PEG 8000 was added to a final concentration of 10%, mixed thoroughly at $4^oC$ for 30 minutes, and centrifuged at 14K RPM for 10 minutes. The pellet was resuspended in a quarter of the original volume of buffer W for 30 minutes at $4^oC$ and then centrifuged for 2 minutes. The supernatant was stored at $–80^oC$ until use.

### Telomere Repeat Amplification Protocol (TRAP) assay

Telomerase activity was detected by a modified version of the TRAP protocol (Kim et al. 1994; Fitzgerald et al. 1996). The following primers obtained from the Gene Technology Laboratory at Texas A&M University were used in the TRAP assay: Forward primer (TS21) 5’GACAATCCGTCGAGCAGAGTT3’ and Reverse primer 5’CCCTAAACCCTAAACCCTAAA3’. The 48ul reaction mixtures contained 50mM Tris acetate (pH 8.3), 50mM potassium glutamate, 0.1% Triton X-100, 1mM spermidine, 1mM DTT, 50uM each dNTP, 5mM $MgCl_2$, 10mM EGTA, 0.5ul ($\alpha$-$^{32}P$)dGTP (3,000 mCi/mM; New England Nuclear), 100ng/ul BSA, 0.5uM T4 gene 32 product, 0.5ul *Taq* polymerase and 1ul (50ng/ul) forward primer (TS21). After the addition of 0.25-1.0ug of nuclear proteins, the telomerase reaction was allowed to proceed at 30°C for 45 minutes. Then reverse primer (50ng) was added and the reaction mixture was amplified by 30 cycles of PCR at 94°C for 30 second, 65°C for 30 second, and 72°C for 90 second. After 30 cycles, an additional 5 minute 72°C extension step was performed. The reaction was stopped by adding 50ul telomerase stop buffer (10mM Tris-HCl pH 7.5 and 21mM EDTA) and then ethanol precipitated. The samples were resolved on 6% sequencing gels. After gel drying, they were exposed on X-ray film or PhosphorImage screen. As a positive control, *Arabidopsis* wild-type flower or califlower extracts were used on each gel.

### Terminal Restriction Fragment (TRF) analysis

Genomic DNA was extracted, using the protocol described in chapter I, from rosette leaves of *tac1* and wild-type four weeks after germination. One microgram of genomic DNA from either *tac1* or wild-type was digested with Tru9I (Promega) for 2 hrs. The digested DNA was resolved on a 0.8% agarose gel, and subjected to Southern blot analysis. A telomere fragment was cut from the AA44 clone with EcoRI (Fitzgerald et al., 2001), labeled with [$^{32}$P] dGTP and was used as a probe. Radioactive signals were detected by a FUJI PhosphorImager.

## DNA content and cell cycle analysis by flow cytometry

Healthy young leaves from age-matched wild-type and *tac1-1D* plants were manually chopped with a new razor blade to release nuclei into ice-cold buffer as described (Johnston *et al.*, 1999) in a Petri dish (also on ice). The chopped leaf-buffer suspensions were filtered through a 53 μm nylon mesh to recover 1 ml of nuclei suspension which was kept on ice. For all comparisons, propidium iodide was added to samples to a final concentration of 50 *ppm* and the mixture maintained in the dark at $4^{o}$C for 1-2 hrs.

The mean fluorescence of stained nuclei was quantified using a Coulter Elite flow cytometer with a laser tuned at 514 nm and 300 mW. Fluorescence at >615 nm was detected by a photomultiplier screened by a long pass filter. Cell cycle analysis was based upon the ungated propidium fluorescence of no less than 25,000 nuclei (no less than 3,500 from each of 6 independently analyzed leaves). The proportion of cells in S phase between the G1 and the combined G2/4C nuclei was determined using the Multicycle program (Phoenix Flow Systems, Inc., San Diego).

## Total RNA and mRNA extraction and RT-PCR analysis

Total RNA was extracted from wild-type tissues (leaves, stems, flowers, roots and callus) and *tac1-2D*, *tac1-1D iaaL* double mutant leaves using the Tri Reagent (Sigma) following the manufacturer's instruction. The mRNA was isolated from 50ug total RNA using the QIAGEN mRNA isolation kit. mRNA concentrations were determined by absorbance at 260nm. In each reverse transcription reaction using the reverse transcriptase-SuperScript II (Invitrogen) and oligo d(T), 50ng of mRNA was used. To prevent the effect of RNA secondary structure prior to synthesizing first strand cDNA, total RNA was heated at 65$^{o}$C for 5 minutes. RT was performed at 42$^{o}$C for 1.5 hrs and then the enzyme was inactivated by incubation at 75$^{o}$C for 15 minutes. To compare *TAC1* and *AtTERT* expression products, 1 ul of first strand cDNA was used in a 20 ul reaction. The primer set used for *AtTERT* amplification was as following: TERT 5 (GCCCTTGATGGATATGTCC) and TERT 48 (CCAACTGCAGCATGTTGTTC). The primer set for TAC1 was as follow: TAC1_5'(ATGGAAAACATCAAAAACCCTA) and TAC1_3' (CTATGTTGTCTTCTTCTTTAC). In the case of the *tac1-2D* mutant, TAC1_3' was changed to TAC1_M (ATGATCATTAGTATCAAGCTTG). Following 3 minutes denaturation at 94$^{o}$C, PCR was performed at 94$^{o}$C 30'', 60$^{o}$C 30'' and 72$^{o}$C 45'' for 20 cycles with additional 10' 72$^{o}$C extension. PCR products were separated on a 1.8% agarose gel and subjected to Southern Blot analysis. As a quantitation control, RT-PCR was performed with primers specific for the cystosolic glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene (forward: GACCTTACTGTCAGACTCGAG and reverse: CGGTGTATCCAAGGATTCCCT).

**Auxin independent growth of *tac1-1D* callus**

Both *tac1-1D* and wild-type seeds were sterilized with 50% bleach and rinsed with sterilized $H_2O$ for 5 times. Sterilized seeds were planted on MS medium. Prior to 23$^{o}$C dark growing for hypocotyl initiation, seeds were cold treated at 4°C overnight. Callus was initiated by placing excised hypocotyls of 6-day-old plants on solid MS medium supplemented with 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 mg kinetin per liter. After initiation, callus was transferred to the same medium without 2,4-D and maintained there for 4 weeks. Then callus was transferred to fresh MS medium lacking 2,4-D and maintained in the dark at 23°C. After 4 weeks growth, photographs were taken.

### Root investigation

To investigate root growth, seed from wild-type, *tac1-1D*, *iaaL* and *tac1-1D iaaL* double mutant, were sterilized with 50% bleach and rinsed with sterilized $H_2O$ for 5 times. After sterilizing, seeds were plated on solidified B5 medium and pretreated at 4°C overnight. Plants were grown vertically in a growth chamber at 23°C with continuous light. Photos were taken 10 days after germination.

### Isolation of *TAC1* gene

For plasmid rescue, about 1*ug* of genomic DNA from *tac1-1D* plants was digested with *BamHI* in a 100 *ul* reaction. Following phenol/chloroform extraction and ethanol precipitation, digested DNA was allowed to self-ligate overnight at 16°C and then transformed into competent DH10B cells.

BAC clone T14B5, which spanned the T-DNA insertion site of *tac1* was digested with EcoRI and fragments subcloned into pBlueScript. Ninety-six subclones were randomly picked. Dot blots were prepared following the lifting protocol. The prepared dot blots were subjected to Reverse Northern analysis to identify subclones containing the corresponding genes in *tac1-1D*. 2.5 ug of total RNAs from both wild-type and *tac1-1D* were used to generate radiolabeled first strand cDNA by a reverse transcriptase reaction with SuperScript II. Radiolabeled cDNA was probed to dot blots. By comparing the signals from wild-type and *tac1-1D*, subclone B12 was selected as a putative clone containing activated genes. To confirm the result, plasmid DNA from this subclone was then digested with BamHI and restriction fragments were probed with radiolabeled cDNA from wild-type and *tac1-1D*.

### Over-expression of *TAC1*

The *TAC1* coding region was generated by PCR and placed immediately downstream of the CaMV *35S* promoter. This construct was transformed back into wild-type *Arabidopsis*. The transformants were selected on MS medium with 50mg/L kanamycin, and the survivors were transferred to soil and subjected to TRAP assay to measure the telomerase activity in their leaves.

# RESULTS

## *TAC1* gene isolation and confirmation

As mentioned in the previous chapter, the activating T-DNA and flanking plant DNA were recovered from *tac1-1D* by plasmid rescue and DNA sequencing of the flanking region revealed that two head-to-head copies of the activating T-DNA with multimers of the CaMV *35S* enhancer in the center were inserted on chromosome 3. Further analysis indicated that this complex DNA was inserted into the 3’ end of the uncharacterized At3g09550 gene. RT-PCR analysis of leaf RNA from this gene and flanking genes showed that none of them had increased expression compared to wild-type. To isolate activated genes in *tac1-1D*, a reverse-northern approach was used to probe restriction fragments from a BAC clone (T14B5), which spanned the T-DNA insertion site with radiolabeled cDNA from both wild-type and *tac1-1D* plants. As shown in Figure 6, only one fragment displayed increased hybridization to *tac1* RNA relative to wild-type RNA. This fragment carried a gene designated *TELOMERASE ACTIVATOR 1* (At3g09290), which encodes a 19 kD protein with a single zinc-finger domain of the C2H2 type and is not represented in EST or cDNA databases (Figure 7).

*TAC1* was located 78 kilobases away from the activating T-DNA (Figure 7). Multiple copies of enhancers from other pararetroviruses can activate promoters from over 175 kb away (Flajolet et al., 1998), but 78 kb between the CaMV 35S enhancer and the target gene is an unusually long distance compared to other activation-tagged mutants reported

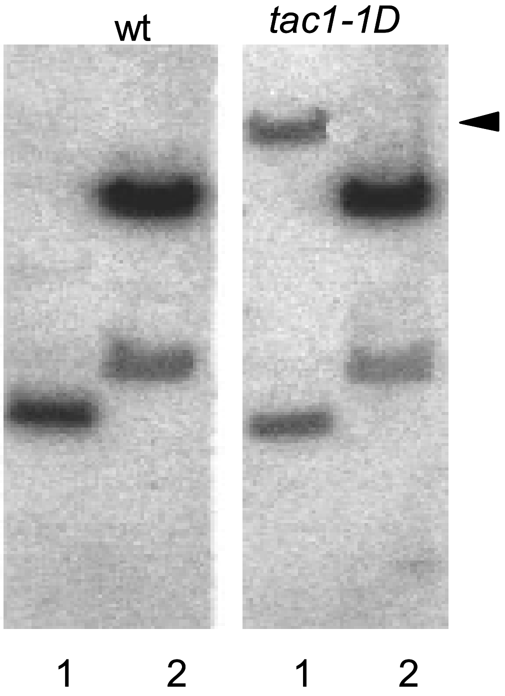
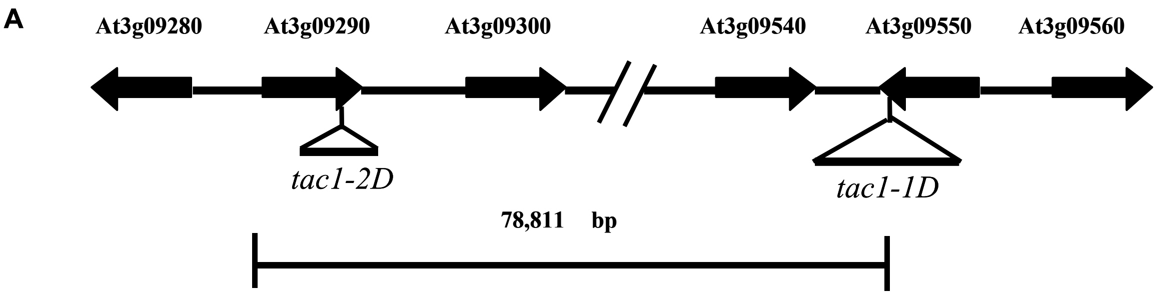


**Figure 6.** Reverse northern analysis. Two subclones of T14B5 were probed with $^{32}$P-labeled cDNA from wt and *tac1*. Arrow head indicated the difference between *tac1* and wt.


A
At3g09280
At3g09290
At3g09300
At3g09540
At3g09550
At3g09560
tac1-2D
tac1-1D
78,811 bp

**B**

MENKNPKNADDSCDSISKNSHQGVDDSLNQSRSY
VCSFCIRGFSNAQALGGHMNIHRRDRAKLRQKLM
EDNKDDVVAESDASEVVSLDLNEQQQQQGEALTC
DDHDQYVDNDISPKQKLEFWVQESKLDTNDHGK
VTEASIDGSSSSHHRDIEVLDLELRLGQSVVKKKTT
▲

**Figure 7.** Fine mapping of *tac1* T-DNA region and TAC1 protein sequence. A. *tac1* is ~80 kb away from T-DNA insertion and *tac1-2D* is created by a T-DNA inserted at 98bp upstream of *TAC1* stop codon. B. TAC1 amino acid sequence. Zinc-finger motif is underlined and arrow head indicates the insertion site for *tac1-2D*.

from this population (Weigel et al., 2000; Zhao et al., 2001; Li et al., 2002). In fact, most of the genes identified in this population of activation-tagged lines are within 4 kb of the T-DNA insertion site (Weigel et al., 2000). To determine whether the T-DNA insertion in the *tac1-1D* plant led to a rearrangement that would place the enhancers closer to the TAC1 gene, we analyzed a 20 kb region on the *TAC1* site toward the T-DNA insertion site and another 20 kb region on the T-DNA site toward the *TAC1* gene in both wild-type and *tac1-1D* plants with multiple PCR reactions that spanned 4 to to 5 kb at a time. No differences were detected between wild-type and *tac1-1D* (Figure 8). We did not examine the central region between the T-DNA and *TAC1*, but even in the unlikely event that this DNA had been deleted, the enhancers would still be acting over a minimum of 40 kilobases.

To confirm that over-expression of *TAC1* was responsible for inducing telomerase activity, the *TAC1* coding region was placed immediately downstream of the CaMV *35S* promoter and was transformed into wild-type *Arabidopsis*. This simpler T-DNA, where *TAC1* is directly activated, resulted in telomerase expression in the leaves (Figure 9). Additional confirmation of the regulatory nature of *TAC1* was unexpectedly provided by another T-DNA insertion line designated *tac1-2D*. This line was generated at the Salk Institute Genomic Analysis Laboratory as part of a large project to disrupt expression of *Arabidopsis* genes. The T-DNA in the SALK_013353 line lies in the 3' portion of the *TAC1* gene (Figure 7). We expected this would eliminate *TAC1* function and hence telomerase expression. TRAP assays of the leaves, however, showed abundant expression of telomerase in this line (Figure 10). RT-PCR showed high levels of *TAC1*

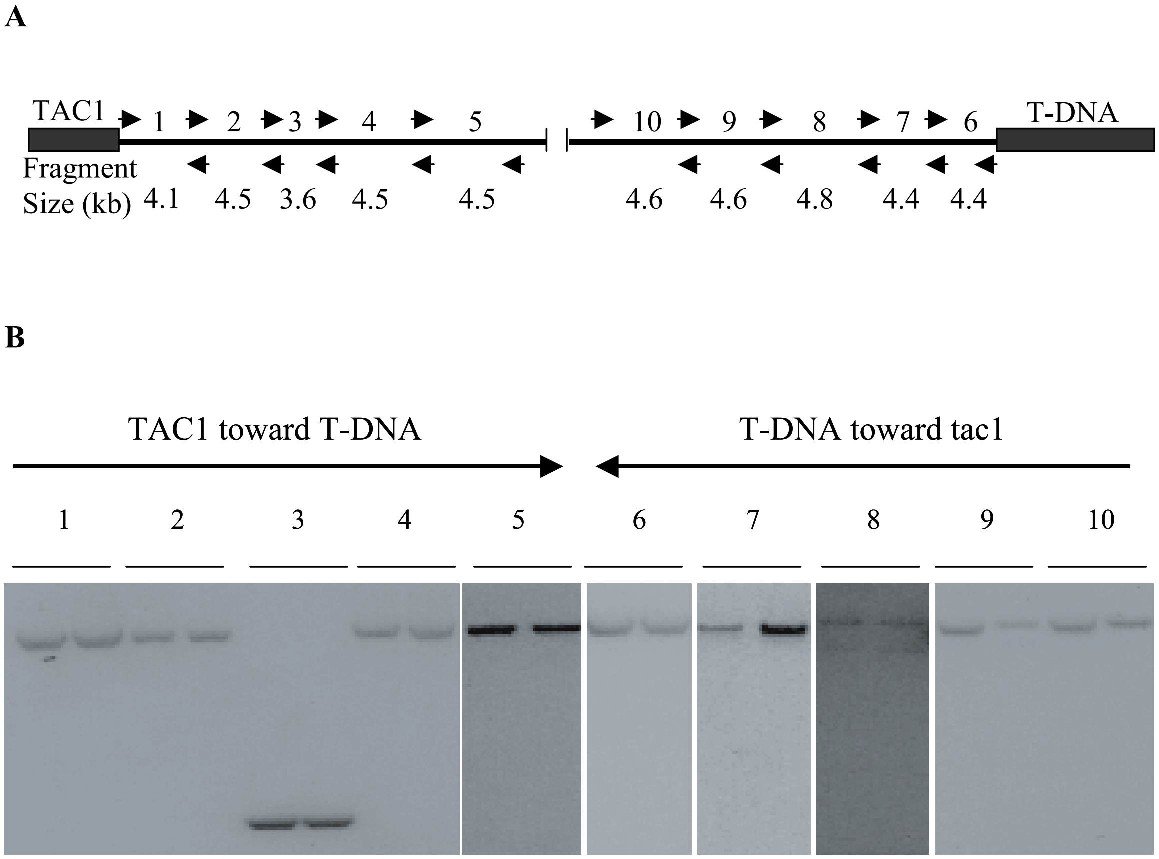

A
TAC1
1
2
3
4
5
10
9
8
7
6
T-DNA
Fragment
Size (kb)
4.1
4.5
3.6
4.5
4.5
4.6
4.6
4.8
4.4
4.4
B
TAC1 toward T-DNA
T-DNA toward tac1
1
2
3
4
5
6
7
8
9
10

**Figure 8.** PCR analysis of *TAC1* T-DNA region. No deletion or rearrangement occurred near T-DNA in *tac1-1D* genome. A. Genomic map of *TAC1* T-DNA region with primer pairs and fragment sizes indicated. B. PCR analysis of *TAC1* T-DNA region. Genomic DNA from both wild type and *tac1-1D* were subjected to PCR analysis. 1 to 10 represent different fragments as indicated in A. For each primer pair, left fragment is wild type and right is *tac1-1D*. Based on database, fragment 3 should be 3.6 kb, however, PCR shows only 1.0 kb in both wild type and *tac1-1D*.

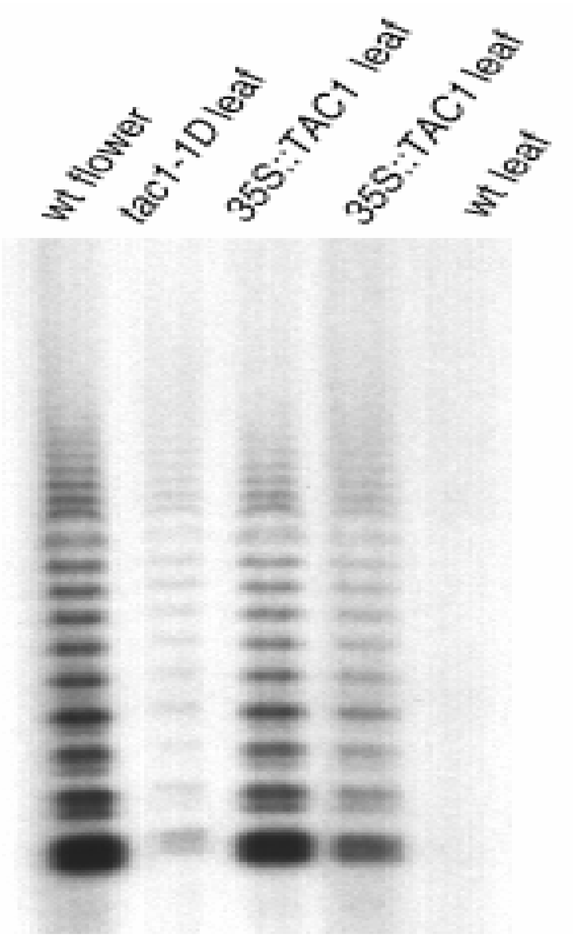

wt flower
tac1-1D leaf
35S::TAC1 leaf
35S::TAC1 leaf
wt leaf

**Figure 9.** TRAP analysis of constitutive expressed TAC1 lines. Crude proteins from two individual *35S::TAC1* transgenic plants, together with other controls were extracted and subjected to TRAP assay.

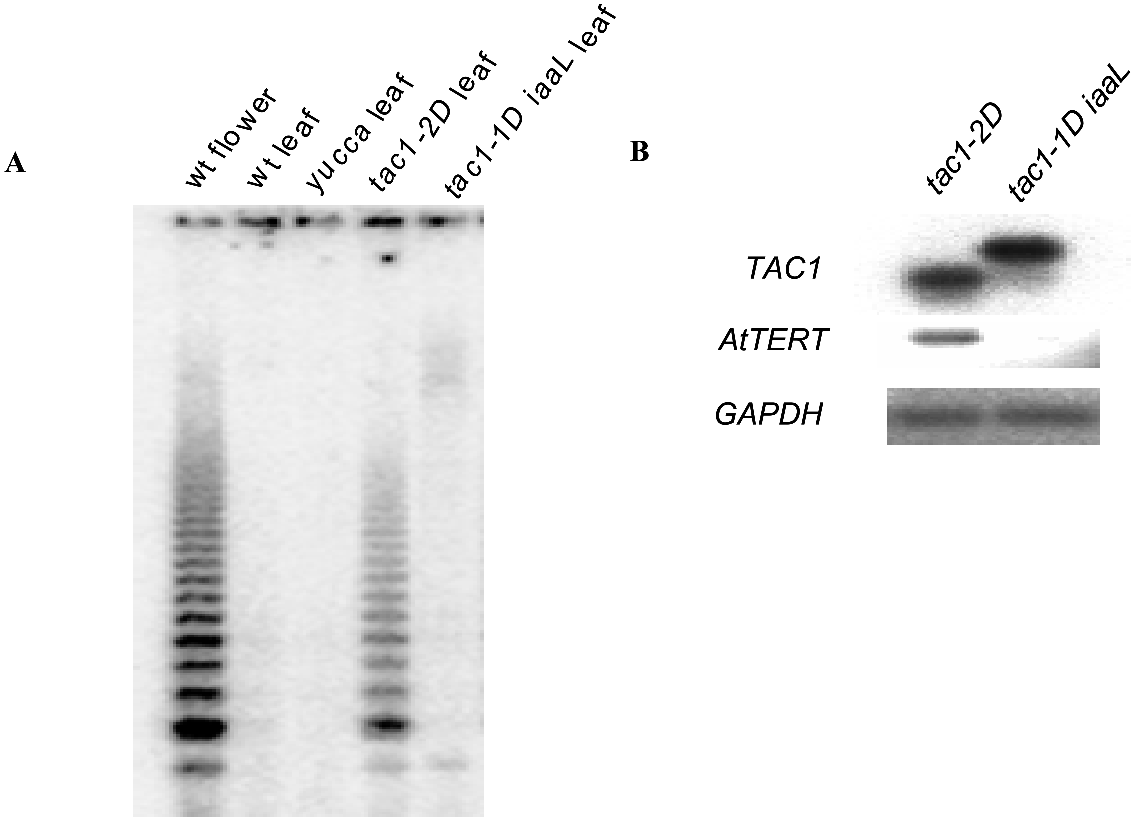
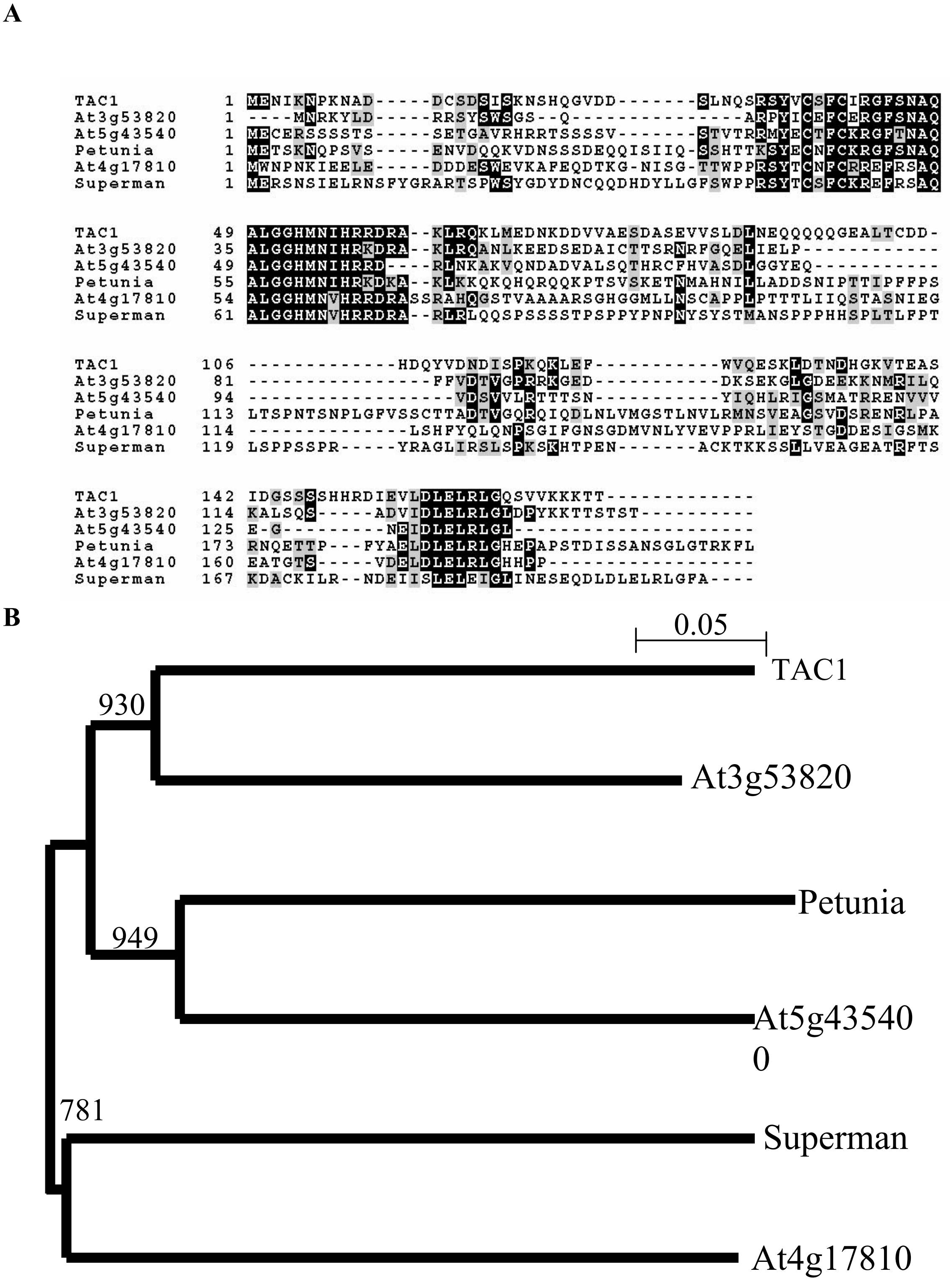

A
wt flower
wt leaf
yucca leaf
tac1-2D leaf
tac1-1D iaaL leaf
B
tac1-2D
tac1-1D iaaL
TAC1
AtTERT
GAPDH

**Figure 10.** TAC1 induces telomerase activity through synergistic action with auxin. A. TRAP assay of *tac1* related mutants: *yucca* is an arabidopsis mutant with 50% more IAA than wild-type; *tac1-2D* is another dominant mutant of *TAC1* gene; *iaaL* is an transgenic line where free IAA can be conjugated to lysine. B. RT-PCR analysis of *tac1-2D* and *tac1-1D iaaL* double mutants. In *tac1-2D*, the size of *TAC1* messenger is smaller than normal size because of different set of primers were used.

mRNA in *tac1-2D* leaves relative to wild-type leaves and *AtTERT* was expressed in *tac1-2D* leaves (Figure 10). The T-DNA in this line carries a full CaMV *35S* promoter whose enhancer evidently activates the *TAC1* promoter. Because the TAC1 protein produced by *tac1-2D* is missing the last 33 amino acids, we conclude that these residues in the carboxy-terminal domain are not required for telomerase activation.

## Evolutionary divergence of $C_2H_2$-type single zinc-finger proteins

*TAC1* encodes a putative $C_2H_2$-type single zinc-finger protein. $C_2H_2$-type single zinc-finger proteins are conserved among flowering plants but are not found outside this lineage. In the *Arabidopsis* genome, at least 30 genes encode putative $C_2H_2$-type single zinc-finger proteins including *SUPERMAN* (*SUP*), a well-characterized single zinc-finger protein that functions as a transcription factor. Phylogenetic analysis indicated that genes in this family are very divergent. The five closest genes including *SUP* and *TAC1* and a gene from petunia were chosen to build up a phylogenetic tree and aligned for their amino acids sequences. Shown in Figure 11, the most closest related gene to *TAC1* is At3g53820. The product of this gene shares only about 32% amino acids identity with TAC1 protein. The known gene *SUP* shares only 26% identity with TAC1 protein.



```
A

TAC1         1 MENIKNPKNAD-----DCSDSISKNSHQGVDD-------SLNQSRSYVCSFCIRGFSNAQ
At3g53820    1 ----MNRKYLD-----RRSYSWSGS--Q---------------ARPYICEFCERGFSNAQ
At5g43540    1 MECERSSSSTS-----SETGAVRHRRTSSSSV-------STVTRRMYECTFCKRGFTNAQ
Petunia      1 METSKNQPSVS-----ENVDQQKVDNSSSDEQQISIIQ-SSHTTKSYECNFCKRGFSNAQ
At4g17810    1 MWNPNKIEELE-----DDDESWEVKAFEQDTKG-NISG-TTWPPRSYTCNFCRREFRSAQ
Superman     1 MERSNSIELRNSFYGRARTSPWSYGDYDNCQQDHDYLLGFSWPPRSYTCSFCKREFRSAQ

TAC1        49 ALGGHMNIHRRDRA--KLRQKLMEDNKDDVVAESDASEVVSLDLNEQQQQQGEALTCDD-
At3g53820   35 ALGGHMNIHRKDRA--KLRQANLKEEDSEDAICTTSRNRFGQELIELP------------
At5g43540   49 ALGGHMNIHRRD----RLNKAKVQNDADVALSQTHRCFHVASDLGGYEQ-----------
Petunia     55 ALGGHMNIHRKDKA--KLKKQKQHQRQQKPTSVSKETNMAHNILLADDSNIPTTIPFFPS
At4g17810   54 ALGGHMNVHRRDRASSRAHQGSTVAAAARSGHGGMLLNSCAPPLPTTTLIIQSTASNIEG
Superman    61 ALGGHMNVHRRDRA--RLRLQQSPSSSSTPSPPYPNPNYSYSTMANSPPPHHSPLTLFPT

TAC1       106 -------------HDQYVDNDISPKQKLEF-----------WVQESKLDTNDHGKVTEAS
At3g53820   81 ----------------FFVDTVGPRRKGED-----------DKSEKGLGDEEKKNMRILQ
At5g43540   94 ------------------VDSVVLRTTTSN-----------YIQHLRIGSMATRRENVVV
Petunia    113 LTSPNTSNPLGFVSSCTTADTVGQRQIQDLNLVMGSTLNVLRMNSVEAGSVDSRENRLPA
At4g17810  114 --------------LSHFYQLQNPSGIFGNSGDMVNLYVEVPPRLIEYSTGDDESIGSMK
Superman   119 LSPPSSPR-----YRAGLIRSLSPKSKHTPEN-------ACKTKKSSLLVEAGEATRFTS

TAC1       142 IDGSSSSHHRDIEVLDLELRLGQSVVKKKTT-------------
At3g53820  114 KALSQS-----ADVIDLELRLGLDPYKKTTSTST----------
At5g43540  125 E-G---------NEIDLELRLGL---------------------
Petunia    173 RNQETTP---FYAELDLELRLGHEPAPSTDISSANSGLGTRKFL
At4g17810  160 EATGTS-----VDELDLELRLGHHPP------------------
Superman   167 KDACKILR--NDEIISLELEIGLINESEQDLDLELRLGFA----
```

**Figure 11.** Phylogenetic study of $C_2H_2$-type single zinc-finger proteins. A. Amino acid alignment, and B. Phylogenetic tree of five most closest $C_2H_2$-type single zinc-finger proteins.

### Telomerase activation in *tac1-1D* is uncoupled from the cell cycle

Because telomerase expression is closely related with an active cell cycle in plants (Tamura et al., 1999; Yang et al., 2002), it was possible that TAC1 acted by stimulating cell division in mature organs. The normal appearance of the leaves was consistent with the typical pattern and low rate of cell division in mature leaves (Talbert et al., 1995). To determine whether telomerase activation in *tac1-1D* was correlated with cell cycle, both mutant and wild-type leaf cells were subject to flow cytometry analysis. As shown in Figure 12, no difference in the number of cells in S phase, where telomerase is activated (Tamura et al., 1999), was detected between *tac1-1D* and wild-type plants. Determination of cells in G2 was complicated by ploidy levels ranging from 2C to 16C, but again there was no difference between wild-type and *tac1-1D* leaves in the proportion of cells at each level. This result also ruled out increased endoreduplication in *tac1-1D*, which would allow the cells to cycle through S phase without an intervening cytokinesis. This analysis clearly demonstrated that telomerase activation in *tac1-1D* mature leaves was uncoupled from progression through cell cycles.

### *TAC1* mRNA expression correlates with *AtTERT* expression

Steady state mRNA expression of *TAC1* in various wild-type organs was investigated by RT-PCR analysis. *TAC1* mRNA was isolated from wild-type leaves, flowers, stems, roots and callus and subjected to RT-PCR analysis. As shown in Figure 13, *TAC1* is

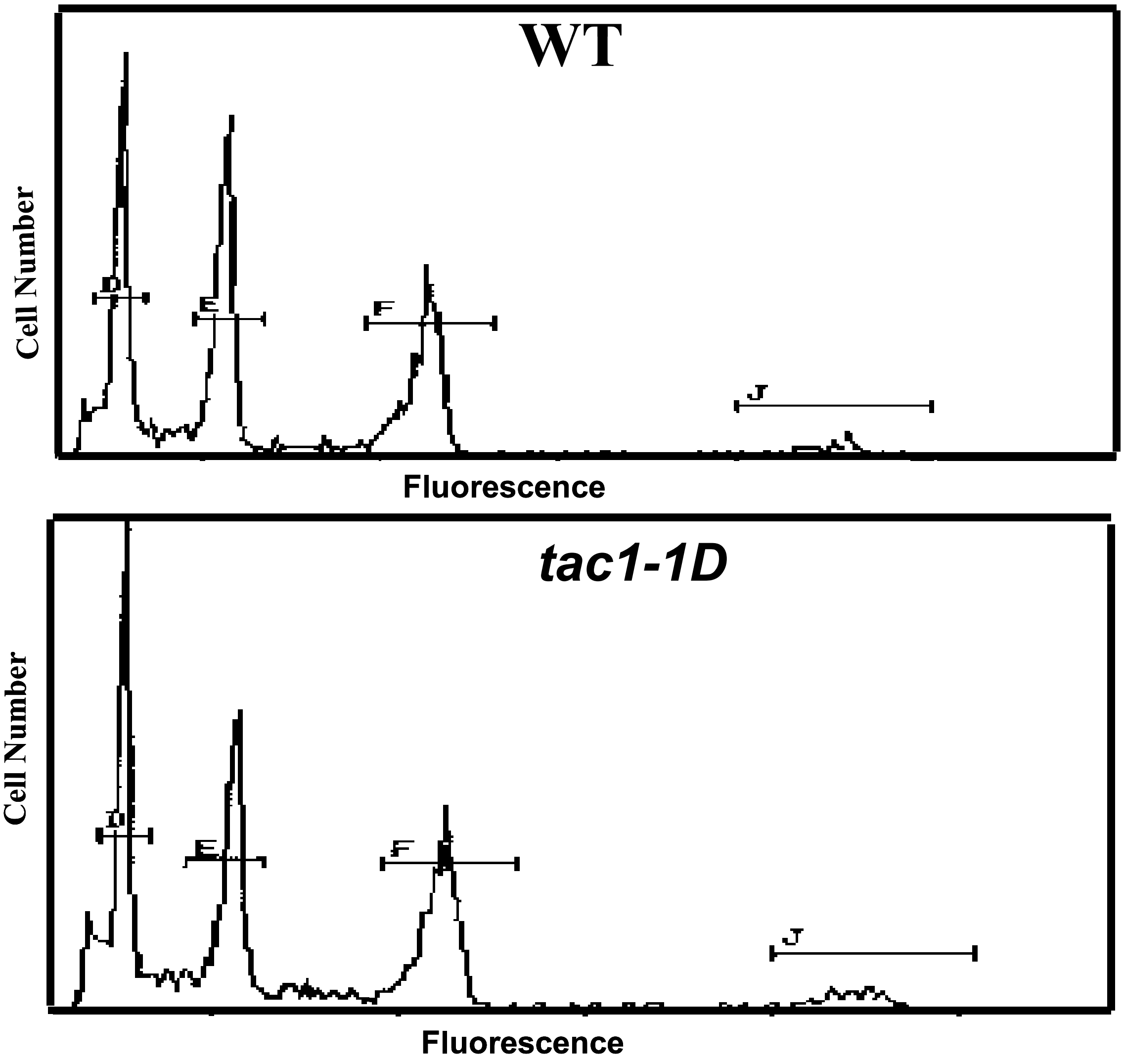


**Figure 12.** Flow cytometry analysis of wild type and *tac1-1D* leaves.

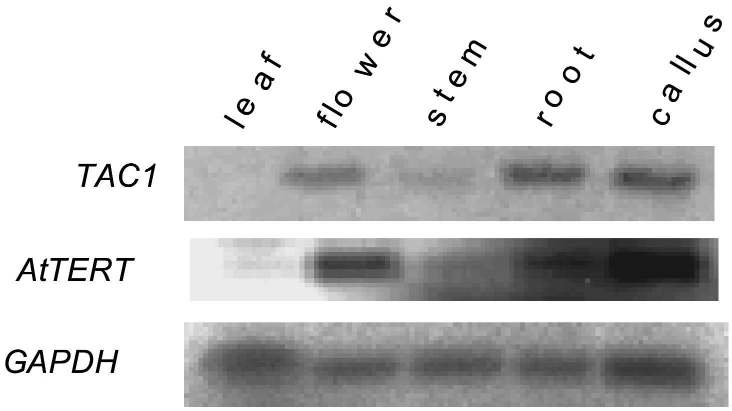

leaf
flower
stem
root
callus
TAC1
AtTERT
GAPDH

**Figure 13.** Expression profile of *TAC1* and *AtTERT*. mRNA from leaf, flower, stem, root and callus were tested for their steady-state level expression of *TAC1* and *AtTERT* by semiquantative RT-PCR analysis. GAPDH was used as loading control.

expressed in flower, root, stem and dedifferentiated callus, but not in vegetative tissue such as leaves, an expression pattern, that strongly correlates with that of *AtTERT* expression.

### *TAC1* induces telomerase activity by synergistic action with auxin

Because exposure to exogenous auxin also can induce telomerase expression (Tamura et al., 1999; Yang et al., 2002), we asked whether auxin metabolism was altered in the *tac1-1D* mutant. First, we examined the formation and sustained growth of *tac1-1D* callus in response to phytohormones. Callus from both *tac1-1D* and the *35S::TAC1* transgenic lines, but not from wild-type plants, could be grown in the absence of exogenous auxin (Figure 14), suggesting a connection between this hormone and induction of telomerase by TAC1. To further explore the role of auxin in telomerase activation, we crossed *tac1-1D* with a line constitutively expressing *iaaL*, a bacterial enzyme that decreases the concentration of free IAA by conjugating it to lysine (Romano et al., 1991). TRAP assays of leaves of the *tac1-1D iaaL* double mutant were negative (Figure 10), suggesting that TAC1 activates telomerase either by increasing the concentration of auxin or by increasing sensitivity to the wild-type concentration of auxin. Telomerase activity was present in the flowers of the *tac1-1D iaaL* double mutant (not shown) implying that telomerase induction in reproductive organs may require lower levels of auxin or the existence of additional mechanisms for controlling this enzyme.

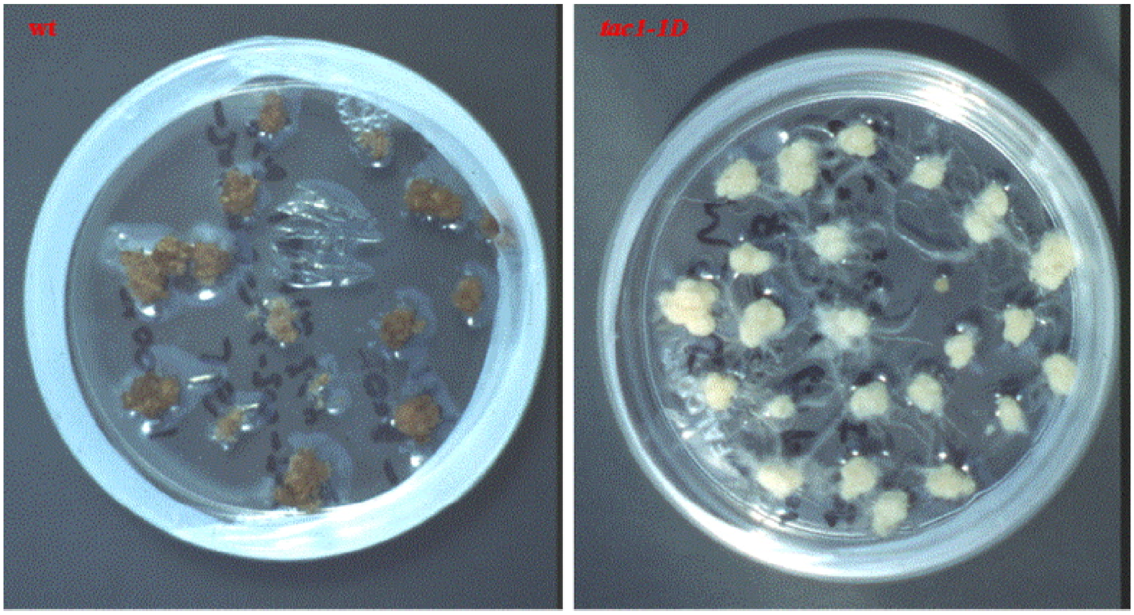


**Figure 14.** Auxin-independent callus growth of *tac1-1D* mutant. Both wild-type and tac1-1D calli were grown on MS medium with only 0.05mg/L Kinetin but no 2,4-D. Photographs were taken after 4 weeks growing.

To determine whether increased levels of endogenous auxin were sufficient induce telomerase expression, we performed TRAP assays on leaves of the *yucca* mutant, which contains 50% more IAA than wild-type (Zhao et al., 2001). As shown in Figure 10, there was no detectable telomerase activity indicating that increased auxin alone is not sufficient for telomerase induction. Therefore, we conclude that over-expression of TAC1 appears to potentiate telomerase's response to normal levels of auxin. To further confirm this synergistic action of *TAC1* with auxin, we performed a cross between *tac1-1D* and *yucca* plants. Consistent with our prediction, *tac1-1D* exacerbated the high auxin phenotype of the *yucca* mutant, the growth of *tac1 yucca* double mutant was much weaker than either of parental lines (Figure 15).

### Over-expression of *TAC1* does not affect plant growth and telomere length

Although TAC1 activates telomerase activity in leaves where there is no detectable telomerase in wild-type, there is no phenotypic difference in the shoots between mutant and wild-type plants. Furthermore, telomere length was not significantly altered in the mutant relative to wild-type (Figure 16), consistent with studies in yeast and mammals indicating that telomerase access to telomeres is regulated by other factors (LaBranche et al., 1998; Griffith et al., 1999; Chardra et al., 2001). The slight difference apparent between wild-type and *tac1* in Figure 16 falls within the natural variation among plants within the population. This telomere length variation was also observed in ecotype Columbia population (Eugene Shakirov, personal communication).

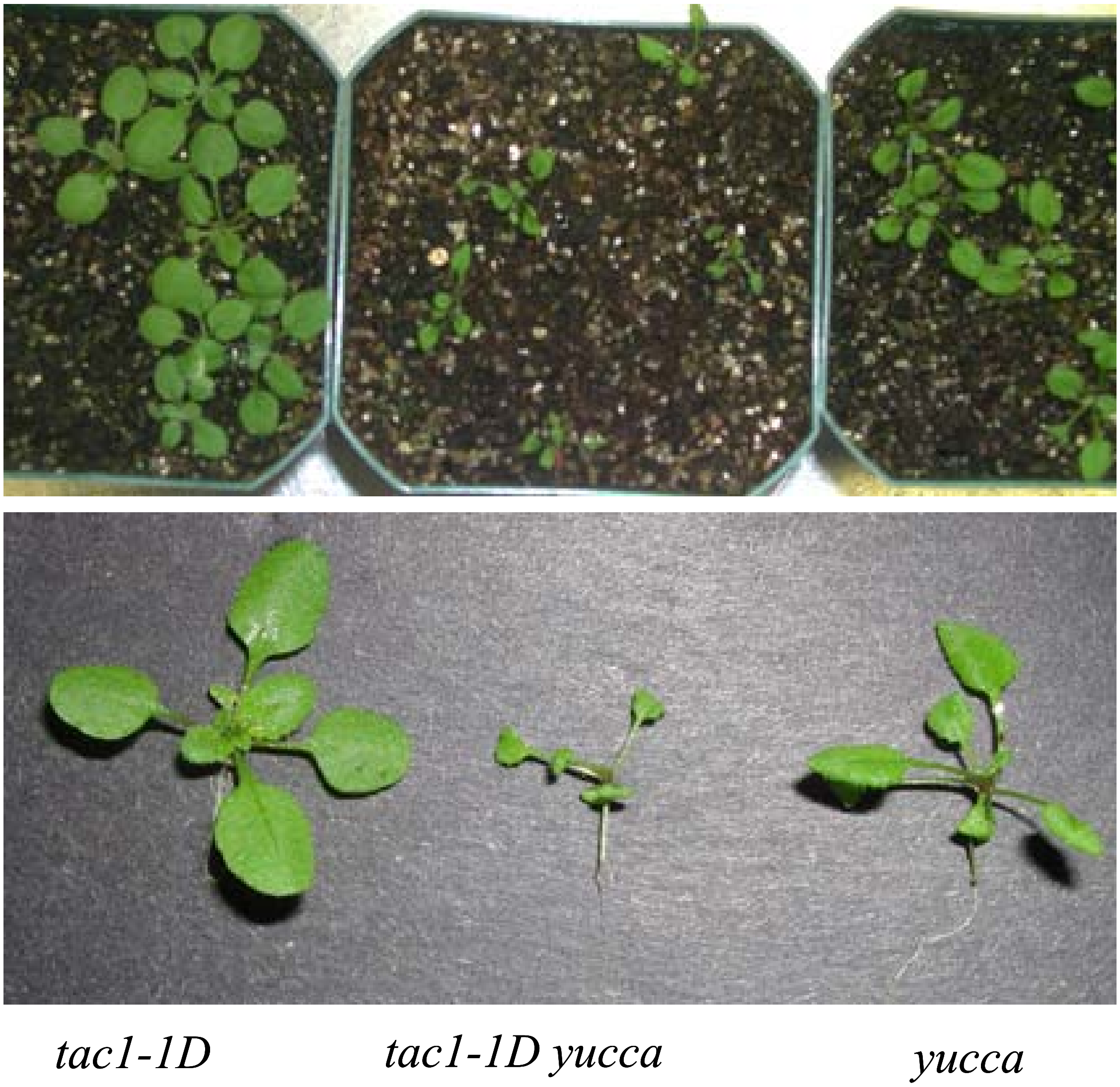


**Figure 15.** Phenotype of *tac1 yucca* double mutant. Upper penal showed a population of *tac1(*left), *tac1yucca* double mutant (middle) and *yucca* (right); lower penal showed individual plants. Photos were taken after two weeks growing at 23 degree under continuous light condition.

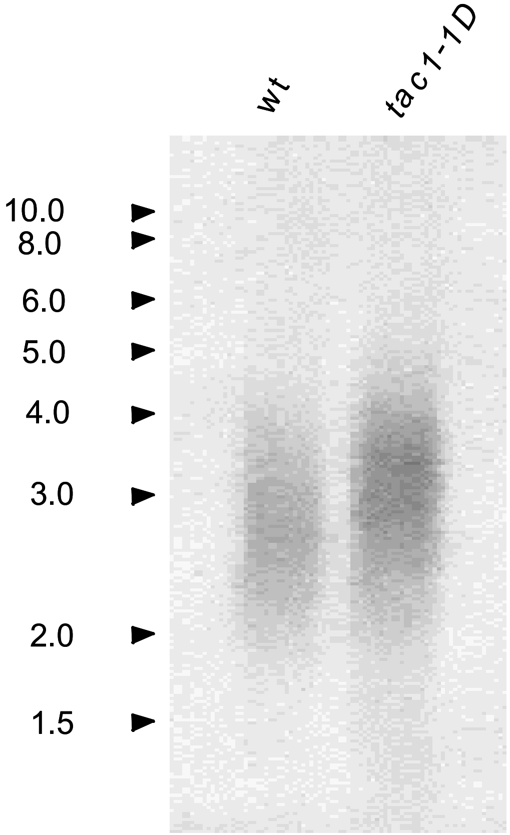


**Figure 16.** Terminal restriction fragment analysis of *tac1-1D*. One microgram genomic DNA from wt and *tac1-1D* were digested with *Tru9I* and separated on 0.8% agarose gel. Blot was probed by telomeric DNA. Expression of telomerase in tac1-1D does not significantly increase length of telomeres.

### Over-expression of *TAC1* rescues the short-root phenotype of *iaaL* plants

Although *iaaL* eliminates the ability of *TAC1* to activate telomerase, over-expression of *TAC1* is still able to influence at least one process in the double mutant. In *iaaL* plants grown on B5 medium, the roots are much shorter than wild-type, as might be expected for a mutant with low levels of free auxin. Roots of the *tac1-1D iaaL* double mutant are partially restored to normal length (Figure 17) indicating that root growth, like activation of telomerase, is also potentiated by *TAC1*. However, these two responses appear to require different levels of auxin as demonstrated by the differential effect of *iaaL*. Over-expression of *TAC1* alone also showed an auxin-related phenotype in its root. Compared to *yucca*, which has more and longer root hairs, the *tac1-1D* mutant also has more and longer root hairs relative to wild-type plants (Figure 18).

## DISCUSSION

In this chapter, we identified and characterized *TELOMERASE ACTIVATOR 1*, the first gene shown to regulate telomerase expression in plants. By recapitulation, its function in activating telomerase in *Arabidopsis* leaves was confirmed. Further studies demonstrated that telomerase is activated by a synergy between TAC1 and auxin. This synergistic action of TAC1 can also partially restore the root length in a *tac1 iaaL* background.

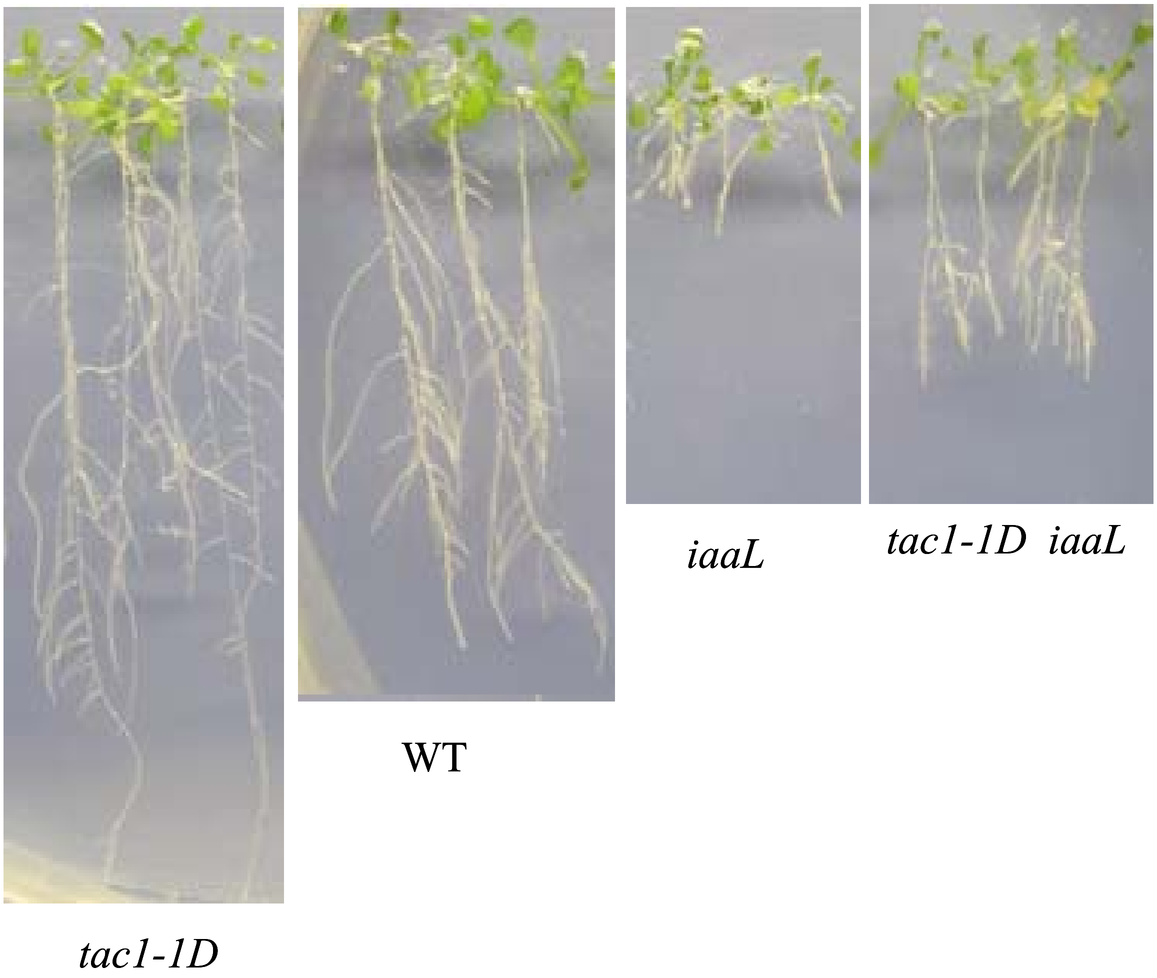

*tac1-1D*
WT
*iaaL*
*tac1-1D iaaL*

**Figure 17.** *tac1-1D* partially rescued root phenotype of *iaaL* mutant. All lines were grown on B5 medium vertically in the same plate. Photo was taken after 9 days growing.

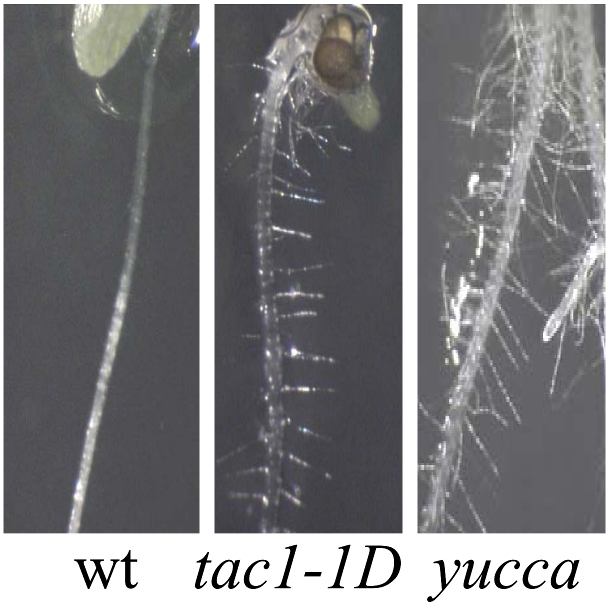


**Figure 18.** Root hair formation of *tac1-1D* mutant. Seeds were germinated and grown on MS medium vertically. Photos were taken after 6 days growing. Only top of roots were captured in the photos.

### ***TAC1* encodes a single zinc-finger protein**

T*AC1*, which regulates telomerase expression, encodes a 19 kD protein with a single zinc finger domain of the $C_2H_2$ type. This zinc finger protein could activate telomerase expression in several different ways. One possibility is that TAC1 serves as a transcription factor and directly binds to the *AtTERT* promoter. However, we failed to detect any sequence-specific interaction between recombinant TAC1 protein and the *AtTERT* promoter in either a yeast one-hybrid system or electrophoretic mobility shift assays. Nevertheless, this mechanism could not be ruled out completely, since the single zinc finger in TAC1 would contact only 4 nucleotides (Wuttke et al., 1997) and may require additional specificity, provided perhaps by another protein. A second possibility is that TAC1 still serves as a transcription factor, but it activates another gene whose expression can activate telomerase expression. If this is the case, yeast one-hybrid screening could be used to identify the specific regulatory element to which the TAC1 protein binds. Another possible mechanism that TAC1 could use to activate telomerase expression is that it may interact with other proteins to form a complex and this complex can regulate telomerase expression directly or indirectly. In this case, the yeast two-hybrid system could be used to identify its interacting proteins. On the other hand, further screening of T-DNA tagged lines may also result in identifying genes directly regulated by or interacting with TAC1. Further study of the *TAC1* gene may help us understand the telomerase regulatory pathway.

## **Enhancers can act over long distance in *Arabidopsis***

To our surprise*, TAC1* was 78 kb away from the activating CaMV *35S* enhancers. One possible explanation is that large-scale deletions or rearrangements occurred around the T-DNA insertion site, which would place enhancers closer to *TAC1*. However, PCR analysis of the TAC1 genomic region in wild-type and *tac1-1D* ruled out this possibility, although one primer pair amplifies a region smaller than that predicted from the published genome in both wild-type and *tac1-1D*. Multiple copies of enhancers from other pararetroviruses can activate promoters from over 175 kb away (Flajolet et al., 1998). However, this unusually long distance between target gene and enhancer in plants is not reported previously. In fact, most of the genes identified in this population of activation-tagged lines are adjacent to the T-DNA insertion site ranging from 380 bp to 3.6 kb (Weigel et al., 2000). Studies in other eukaryotic organisms suggest that the specialized DNA sequence elements called insulators, which flank some genes, may function as a positional enhancer blocker to prevent enhancer-mediated activation of the promoter (Geyer and Corces, 1992; Dorsett, 1993). The long distance action ofan enhancer could be simply explained by the loop domain model (Gerasimova and Corces, 1998; Gerasimova et al., 2000). Based on this model, if there is no insulator between the enhancer and the target gene, looping will drive the enhancer and target gene closer. Our data suggested that, when researchers cannot find activated genes nearby the T-DNA insertion in activation-tagged mutants, they should consider investigating longer distance to identify the corresponding genes.

## Induction of telomerase in *TAC1* mutant is uncoupled from the cell cycle

Flow cytometry analysis of DNA content indicated that neither the percentage of cells in S-phase nor the fraction of leaf cells at each ploidy level from 2C to 16C was significantly different between wild-type and *tac1-1D* mutant. This result demonstrated that TAC1 induces telomerase activity in fully-differentiated leaves without activating cell division. There exist contradictory results in the literature about the cell cycle regulation of telomerase activity. Some studies have shown that telomerase activity is regulated at each stage of the cell cycle (Zhu, et al., 1996), while others have found that telomerase activity does not change significantly at the different stages of the cell cycle (Holt et al., 1996; Mantell & Greider, 1994). In human cells, the extracted telomerase activity in FACS sorted cells is detected at approximately equal amounts at each stage of the cell cycle (Holt et al., 1997) indicating that telomerase activity is not cell cycle regulated. On the other hand, Zhu et al. (1996) discovered an increase in telomerase activity during S phase in the cells synchronized using chemical compounds such as aphidicolin or hydroxyurea, and a sharp decrease during mitosis. However, later study by Holt et al. (1997) found that some chemicals used by Zhu et al. (1996) to arrest cells at different stages of the cell cycle have toxic effects on cells that decrease the levels of telomerase activity. Although Holt et al. (1997) demonstrated that telomerase activity is not cell cycle regulated, when the cells exited the cell cycle, however, telomerase activity was decreased to undetectable level. In plants, the relationship between cell cycle progression and telomerase activity has been studied only in tobacco synchronized cells (Tamura et al., 1999). In synchronized tobacco B2 cells, telomerase activity is restricted to late S phase. When treated with auxin-type hormone indole acetic acid (IAA), telomerase activity is induced to a higher level and detected earlier during S phase. Tamura's results indicated that telomerase activity in plants, at least in tobacco B2 cells, is cell-cycle dependent and auxin mediated. As in human cells, telomerase activity remains undetectable in wild-type organs where no cell division occurred, such as leaves (Fitzigerald et al., 1996).

Although it is unlikely that telomerase is necessary in organs where cell division has stopped, as telomeres do not continuously shorten in the absence of cell division, our finding that induction of telomerase is uncoupled with cell cycle may suggest that differentiated cells could have alternative means of regulating the expression and activity of telomerase to compensate for the lack of cellular proliferation. Further understanding the mechanism by which telomerase induction is uncoupled from the cell cycle in *tac1* mutants may lead to discoverr of a new signal transduction pathway through which telomerase is regulated.

### TAC1 synergistically acts with auxin to induce telomerase activity in *Arabidopsis* differentiated organs

Although we did not demonstrate a direct biochemical connection between TAC1 protein and phytohormone auxin, our genetic results demonstrate that induction of telomerase in *tac1* is due to synergistic action between TAC1 and auxin. Three pieces of evidence support this conclusion. (1) Callus from *tac1* mutant, but not from wild-type, could be grown in the absence of exogenous auxin (Figure 13), suggesting a connection between auxin and induction of telomerase activity by TAC1. (2) Crossing *tac1-1D* with a line constitutively expressing *iaaL*, which conjugates free indole acetic acid (IAA) to lysine, eliminated telomerase activity in the *tac1-1D iaaL* double mutant (Figure 10A), although TAC1 was still expressed in their leaves (Figure 10B). This indicated that over-expression of TAC1 alone cannot induce telomerase activity. (3) There is no detectable telomerase activity (Figure 10A) in the *yucca* mutant, which contains 50% more IAA than wild-type (Zhao et al., 2001), suggesting that increasing the endogenous auxin level alone is not sufficient to induce telomerase activity. Based on this, we conclude that induction of telomerase activity by over-expression of TAC1 is due to a potentiation of the response to normal levels of auxin and not an increase in auxin concentration.

Although it is well known that the phytohormone auxin induces telomerase activity in both synchronized cell lines (Tamura et al., 1999) and immortalized, dedifferentiated callus tissue, no genetic link was established. Our study identified TAC1 as a potentiator of auxin response in activation of telomerase, and provides the first direct genetic link between auxin and telomerase expression.

Regarding the roles of phytohormones, conventional studies demonstrated that cytokinin controls cell division, while auxin is in charge of cell elongation. Although the relationship between cell division and telomerase activity is well established, we do not fully understand why auxin, rather than cytokinin, regulates telomerase expression (Tamura et al., 1999). Our results that TAC1 induces telomerase in an auxin-mediated manner without activating the cell cycle may explain this paradox. The results shown here, together with that of Tamura et al. (1999) suggest that auxin regulates telomerase expression in both cell cycle-dependent and independent pathways.

### TAC1 potentiates other responses of auxin

In this study, we also observed that the *tac1-1D* mutant has other classic auxin responses, such as increased root growth and root hair formation, during root development. In addition, *tac1-1D* can partially rescue the short-root phenotype of *iaaL* over-expression lines and exacerbates the *yucca* phenotype. All these demonstrate that TAC1 not only potentiates telomerase activation, but also at least a few other auxin responses. However, potentiation of telomerase activation and root growth appear to require different levels of auxin, as demonstrated by the differential effect of *iaaL* on these two aspects of the *tac1-1D* phenotype.

Our data suggest that auxin perception, rather than biosynthesis, is disturbed by over-expression of TAC1. First, *tac1-1D*, *tac1-2D* and *35S::TAC1* shoots have a normal appearance, whereas *yucca* mutants have a distinct shoot phenotype, including reduced apical dominance and epinastic leaves with longer petioles (Zhao et al., 2001). Second, the level of IAA that is responsible for the *yucca* phenotype is not sufficient to induce telomerase expression. If TAC1 acted simply by increasing the IAA concentration, it would have to produce levels higher than *yucca*, and the mutants would therefore have a pronounced phenotype. The high-auxin phenotype is exacerbated in the *yucca tac1-1D* double mutant, consistent with the idea that TAC1 acts synergistically with auxin. When TAC1 is overexpressed, the normal, wild-type concentration of auxin is sufficient to induce expression of telomerase in fully differentiated, non-cycling cells, and hence uncouple telomerase activation from cell cycle progression. Further characterization of this and additional telomerase-activated mutants should help to understand the complex signal pathways that regulate this essential enzyme.

# CHAPTER IV

# OVER-EXPRESSION OF TAC1 CAUSES DROUGHT TOLERANCE IN *ARABIDOPSIS THALIANA*

## OVERVIEW

DNA microarrays were used to analyze the expression profile of the *tac1-1D* mutant which was isolated originally as a telomerase activator mutant. TAC1 acts synergistically with auxin to induce telomerase activity in *Arabidopsis* fully differentiated leaves without activating cell cycle. However, microarray data revealed that several drought-induced genes were up-regulated 5~10 fold in the *tac1-1D* mutant. RT-PCR analysis further confirmed this up-regulation for 4 of these genes. Investigation of root growth also indicated that *tac1-1D* root is ~20 % longer relative to wild-type. Because of these molecular and morphological phenotypes, *tac1-1D*, *tac1-2D* and 3 individual *35S::TAC1* transgenic *Arabidopsis* lines were then subjected to drought tolerance experiments and demonstrated that over-expression of TAC1 was able to confer drought tolerance in all these lines. Salt tolerance was also examined for the *tac1-1D* mutant, but there was no difference between tac1-1D and wild-type. In addition, preliminary results showed that treatment with a low concentration of IAA can induce drought tolerance in wild-type *Arabidopsis*. Possible applications of *TAC1* in improving crop tolerance to drought in agriculture are discussed.

# INTRODUCTION

Plants are continuously exposed to biotic and abiotic stresses that endanger their survival. However, they have a remarkable ability to cope with these highly variable environmental stresses. Nevertheless, these stresses represent the primary cause of crop loss worldwide (Boyer, 1982), reducing average yields for most major crops by more than 50% (Bray et al., 2000). Among abiotic factors, water stress is one of the most severe, mainly caused by drought, high salinity and cold. To respond to these environmental challenges, plants have developed various systems to alter their gene expression profiles, which eventually leads to various adaptive responses at the cell and whole-plant levels (Bray, 1993; Zhu et al., 1997; Thomashow, 1998; Hasegawa et al., 2000).

Many genes have been demonstrated to respond to drought stress in various plant species. Functions for many induced proteins have been predicted from sequence homology with known functional proteins. Genes induced under stress conditions are thought to function in protecting cells from water deficit by the production of several different gene products: ater channel proteins involved in altering cellular water potential (Chrispeels et al., 1994; Yamada et al., 1995; Jones & Mullet, 1995; Bohnert et al., 1995; Bartels & Nalson, 1994); the enzymes required for the biosynthesis of various osmoprotectants such as sugars and proline (Delauney & Verma, 1993; Kishor et al., 1995; Yoshiba et al., 1995); lipid desaturase for membrane modification (Thomashow, 1994; Bohnert et al., 1995; Bartels & Nalson, 1994); protective proteins such as LEA proteins, osmotin and mRNA-binding proteins (Goday et al., 1994; Kiyosue et al., 1994a); thiol proteases (Williama et al., 1994); the detoxification proteins such as soluble epoxide hydrolase, ascorbate peroxidase (Kiyosue et al., 1994b; Mittler & Zilinskas, 1994); and protein kinases, phospholipase C, and transcription factors, which are involved in further regulation of signal transduction and gene expression (Hiyayama et al., 1995; Urao et al., 1994; Holappa & Walker-Simmons, 1995; Urao et al., 1993; Kusano et al., 1995). Among these, some stress-inducible genes have been over-expressed in transgenic plants, producing a stress-tolerant phenotype of the plant, confirming that the gene products function in stress tolerance.

At least three independent signal pathways function under drought conditions: two are ABA (abscisic acid)-dependent and one is ABA-independent (Iwassaki et al., 1995). In ABA-dependent signal pathways, drought-induced genes can also be induced by exogenous ABA treatment. These genes contain potential ABA-responsive elements (ABREs) in their promoter regions (Finkelstein & Lynch, 2000). The ABREs function as *cis*-acting DNA elements, involved in ABA-regulated gene expression. For the ABA-independent signal pathway, genes are induced by drought in both wild-type and absisaic acid biosynthesis (*aba*) or absisaic acid insensitive (*abi*) mutants, suggesting that these genes do not require ABA for their expression under drought conditions. Genes induced by the ABA-independent pathway under drought condition always contain a dehydration responsive element (DRE), MYCRS or MYBRS elements (Zhu et al., 2002). Although not fully understood, crosstalk between ABA-dependent and ABA-independent pathways could exist and together would drive plants responsiveness to drought condition (Zhu et al., 2002).

Three steps are required for plants to respond to such stresses: osmotic stress recognition, signal transduction, and production of physiological responses. Knowledge of the first step in plants is lacking, and is mostly available from other species such as bacteria and yeast. The second and third steps have been relatively well studied in plants.

The first step is largely controlled by the osmosensor, which recognizes changes in osmotic pressure. In *E. coli* and yeast, such signals are detected by the osmosensors EnvZ and SLN1, respectively (Maeda et al., 1994; Mizuno, 1998). A similar protein, AtHK1, has been found in *Arabidopsis* (Urao et al., 1999). However, its function in plants is yet not understood. This class of genes has been identified as transmembrane two-component histidine kinases. Some of these sensor proteins form homodimers, their conformation easily changing upon mechanical stimuli to the membrane (Yaku and Mizuno, 1997). Such conformational alteration is believed to relay the signal into the cell interior (Posas et al., 1996; Lohrmann and Harter, 2002).

Once the signal is perceived by the sensor, the second step involves signal transduction. In plants, Ca2+-coupled phosphoprotein cascades are involved (Martin & Busconi, 2000). Ca2+-dependent protein kinases (CDPKs) are serine/threonine protein kinases with a C-terminal calmodulin-like domain that can directly bind Ca2+. These kinases, together with the SOS3 family of Ca2+ sensors, play a major role in transducing stress signals through protein phosphorylation cascades. In addition to Ca2+-regulated protein kinase pathways, plants also use other phosphoprotein modules for abiotic stress signaling (Kiegerl et al., 2000; Yang et al., 2001; Zhang and Klessig, 2001).

The final step is that plants respond to the abiotic stress signal by either activating or suppressing downstream stress-responsive genes. A number of genes have been identified and characterized as osmotic-stress regulated (Bohnert et al., 1995). Examples are those encoding the late embryogenesis abundant proteins (LEA), which are known to respond to and reduce the effects of osmotic stress (Thomashow, 1998). Many other genes encoding proteins involved in osmolite biosynthesis, transporters, and regulatory functions have also been isolated (Zhu et al., 1997).

In our study of telomerase regulation in *Arabidopsis*, we initially identified TAC1 as a telomerase activating protein, that acts synergistically with auxin to induce telomerase activity in *Arabidopsis* fully differentiated leaves without activating cell cycle. Here, we described the characterization of another function of TAC1 and show that over-expression of TAC1 can induce drought tolerance in both *Arabidopsis* and tobacco. Also, we showed that a low concentration of exogenous IAA (1 nm) can result in wild-type *Arabidopsis* drought tolerance, confirming our previous observation that TAC1 acts in conjunction with auxin.

## MATERIALS AND METHODS

### Microarray analysis

*tac1-1D* and its parental line Col-7 were obtained from the *Arabidopsis* Biological Resources Center. Both lines were grown in the same pot and cold–treated overnight at 4°C prior to be placed in a growth chamber with continuous light at 23°C. Water was routinely supplied. After two weeks of growth, leaves from both wt and *tac1-1D* were collected and subjected to total RNA isolation. Total RNA was extracted from both wt and *tac1-1D* leaves using Tri-Reagent (Sigma) following the manufacturer's instruction. Labeling of the RNA, hybridization to Affymetrix microarrays, and analysis of the data were performed according to protocols and software from Affymetrix.

### Root investigation

Seeds from wt and *tac1-1D* were sterilized with 50% bleach and rinsed with sterilized $H_2O$ for 5 times. Sterilized seeds were plated on solidified $B_5$ medium (Gamborg et al., 1968) and pretreated at 4°C overnight. Plants were grown vertically in a growth chamber at 23°C with continuous light. Photos were taken 10 days after germination and root length was measured at the same time.

## Drought stress treatment

*tac1-1D* and its parental line were obtained from ABRC, *tac1-2D* from Dr. Joe Ecker's SIGnAL collection and transgenic plants over-expressing TAC1 cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter were derived from this study (See chapter III). All lines were grown in soil after overnight cold treatment at $4^oC$. Plants were continuously growing at $23^oC$ with continuous light for 2 weeks with sufficient water supply. After that, plants were subjected to drought stress treatment for 10 days or until plants completely wilted. Water was re-supplied after drought stress treatment. Photos were taken 4 days after re-watering or right after apparent differences were observed.

## RT-PCR analysis

To confirm microarray data, RT-PCR was used to check five drought-induced genes. A different set of materials was grown and leaves were collected for total RNA isolation. RT-PCR analysis was following manufacturer's instruction (Invitrogen). The primer sets for each drought-induced gene were as following: Di21_5' (CTCCGGTGCCGTTAAATCTC) and Di21-3' (CAAAAGCAACTCTCTGAGCTC) for Di21; RD21a_5' (GCAAACGACGAATCTGCTCTC) and RD21a_3' (CGATGGAGCTTTTATCGGTAG) for RD21a; RD21b_5' (GTGGAAGGCTTAAACAAGATTG) and RD21b_3' (AGGCAACCGAAACTTTATCCG) for RD21b; Dr4_5' (GCCACCATATCCATCACTACC) and

Dr4_3’ (CAGTAGAAAGGGAACATCCTC) for Dr4 and Xero_5’ (ATTCTCACCAG AATCAAACCG) and Xero_3’ (TAGTGATGACCACCGGGAAG) for Xero-2. Following 4 min denaturation at 94$^{o}$C, PCR was performed at 94$^{o}$C 30 sec, 60$^{o}$C for 30 sec and 72$^{o}$C 45 sec for 30 cycles with an additional 10 min extension at 72$^{o}$C. PCR products were separated on a 1.8% agarose gel. As a quantitation control, RT-PCR was performed for the cystosolic glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene with primer set forward (GACCTTACTGTCAGACTCGAG) and reverse (CGGTGTA TCCAAGGATTCCCT).

**IAA treatment**

Seeds from wild-type Columbia were grown in soil and subjected to overnight cold treatment at 4$^{o}$C. Plants were continuously growing at 23$^{o}$C with continuous light for 2 weeks with sufficient water supply. Then stop watering for a week. After that, plants in same tray were divided into two parts. Half was sprayed with 50ml 1nM IAA/day for 7 days. The other half was sprayed with same amount of water every time as a mock control. The experiment was duplicated. Photographs were taken after 7-day IAA treatment.

# RESULTS

## Microarray analysis reveals new function of TAC1 over-expression

The *tac1-1D* mutant has no morphological differences in the shoot relative to wild-type ecotype Columbia. However, it can act synergistically with auxin to induce telomerase activity in *Arabidopsis* fully-differentiated leaves without altering cell cycle. In addition, TAC1 can partially rescue the *iaaL* root phenotype in a *tac1-1D iaaL* double mutant. To further determine whether additional molecular differences existed, total RNA from leaves of *tac1-1D* mutant and parental plants was extracted and used in a DNA microarray experiment using Affymetrix chips containing 8,200 *Arabidopsis* genes. After hybridization and normalization of data, expression levels of most of genes are similar in *tac1-1D* and the parental plants (Figure 19). However, ~5% of the genes were identified with changes in mRNA levels greater than 2-fold. Table 2 listed top 25 genes which are up-regulated in *tac1-1D* relative to wild-type. Most of the genes up-regulated in *tac1-1D* were stress related genes. Five of these, listed in Table 3, are well-known, drought-induced genes. Their expression levels were 3-10 fold greater than in wild-type. Because we did not have a duplicate design for this experiment, our first interpretation was that many stress-related genes were up-regulated due to slight differences in watering the plants.

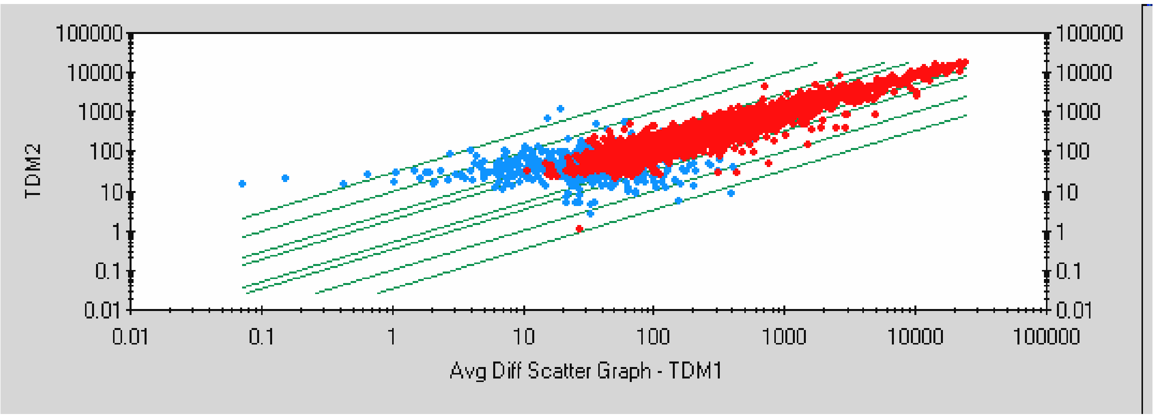


**Figure 19.** Expression of 8,200 genes in wild type vs. *tac1-1D* leaves. Red dots represent genes with same expression levels in *tac1-1D* and wild-type; blue dots represent genes with more than two fold difference between *tac1-1D* and wild-type.

**Table 2.** Top 25 genes up-regulated in *tac1* in microarray analysis

| Affy Number | Gene Name | Function | Fold Increased |
|---|---|---|---|
| 15189_s_at | PDF1 | Protodermal factor | 52.6 |
| 15562_at | F20B18.120 | Putative peroxidase | 24.0 |
| 17012_at | Thi2.1 | Thionin | 16.8 |
| 13680_at | L04637 | Lipoxygenase | 13.8 |
| 18607_at | U78721 | Unknown | 13.5 |
| 19839_at | AC005727 | Unknown | 10.8 |
| 16048_at | Dr4 | Drought induced | 10.7 |
| 19622_g_at | PRXR4 | Peroxidase | 9.2 |
| 14547_at | AC005275 | Putative homolog of transport inhibitor response | 8.9 |
| 12746_i_at | DR21A | Drought-induced cysteine proteinase | 8.1 |
| 12748_f_at | DR21A | Drought-induced cysteine proteinase | 7.1 |
| 13261_at | U37697 | Glutathione reductase | 6.7 |
| 16968_at | AL021691 | Glucosyltransferase-like protein | 6.3 |
| 20585_at | AC006072 | Unknown | 6.2 |
| 15101_s_at | AF071788 | Phosphoenolpyruvate carboxylase | 6.1 |
| 16131_s_at | PAP2 | Phytchrome-associated protein | 6.0 |
| 12727_f_at | AtMYB76 | Transcription factor | 6.0 |
| 12332_s_at | ChiB | Basic endochitinase | 5.8 |
| 17322_at | AL049655 | Aquaporin/MIP-like protein | 5.7 |
| 20604_at | AL049608 | Unknown | 5.6 |
| 14657_s_at | AJ002585 | Thionin | 5.5 |
| 18587_s_at | AC007166 | Unknown | 5.5 |
| 17273_at | AC004697 | Unknown | 5.5 |
| 19471_at | AL021811 | S-receptor kinase-like protein | 5.5 |
| 20201_at | AL078470 | Glycine-rich protein | 5.3 |

**Table 3.** Drought-induced genes up-regulated in *tac1-1D*

| Affy Number | Gene Name | Function | Fold induced |
|---|---|---|---|
| 16048_at | *Dr4* | Drought-induced | 10.7 |
| 12746_i_at | *RD21A* | Drought-induced | 8.7 |
| 12748_f_at | *RD21B* | Drought-induced | 7.1 |
| 19186_s_at | *Xero 2* | Drought-induced | 3.6 |
| 18231_at | *Di21* | Drought-induced | 2.9 |

### ***tac1-1D* root is 20% longer than wild-type Columbia**

To investigate the root phenotype of *tac1-1D*, both *tac1-1D* and wild-type Columbia ecotype were plated on solid B5 medium and grown vertically. After 10 days, root lengths were measured and compared to that of wild-type Columbia. As shown in Figure 20A, *tac1-1D* mutants have longer roots. Quantitative measure of the root length indicated that the *tac1-1D* root is 20% longer than wild-type Columbia, as is evident from the histograms shown in Figure 20B. The fact that *tac1-1D* mutants have longer roots than wild-type Columbia prompted us to re-evaluate data from the microarray experiment.

### **RT-PCR analysis confirms the up-regulation of drought induced genes in *tac1-1D***

To determine whether the transcript changes for drought-induced genes identified in the *tac1-1D* mutant microarray analysis were reproducible, total RNA was extracted from different set of *tac1-1D* mutants and parental plants grown in the same conditions and examined by RT-PCR analysis. As shown in Figure 21, all of the genes examined exhibited increased mRNA abundance relative to wild-type Columbia. However fold changes were difference in some cases. This result demonstrated that, at least for drought-induced genes, microarray data were reliable and had biological reproducibility.

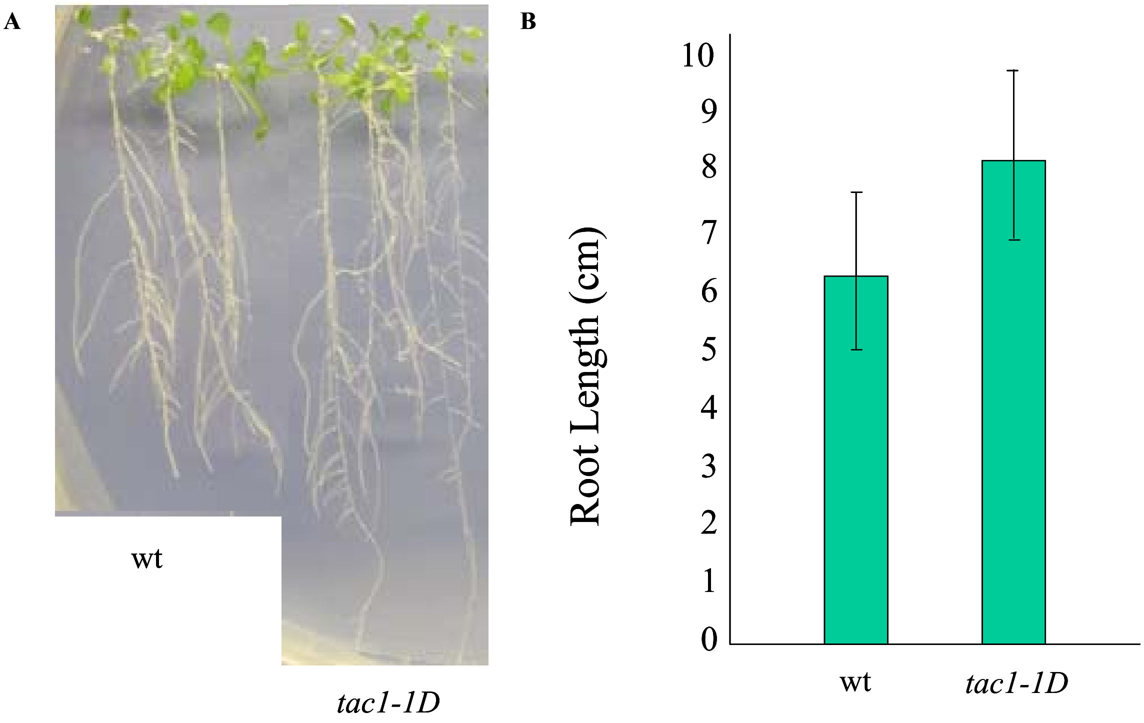


**Figure 20.** Comparison of root length between wild type and *tac1-1D*. A. Root length of *tac1-1D* is longer than wild-type. B. Quantitative measurement of root length for wild-type and *tac1-1D*. Plants were grown on B5 medium vertically. Photo was taken after 9 days growing.

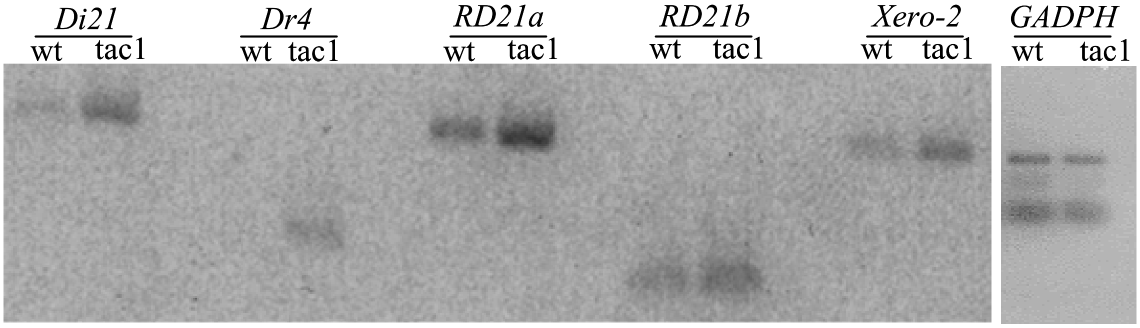

Di21
wt tac1
Dr4
wt tac1
RD21a
wt tac1
RD21b
wt tac1
Xero-2
wt tac1
GADPH
wt tac1

**Figure 21.** Semiquantative RT-PCR analysis of drought-induced genes. GADPH serves as a loading control.

### ***tac1-1D*** **mutant has drought tolerance phenotype**

Our initial experiment focused on the *tac1-1D* mutant. Both *tac1-1D* and wild-type Columbia were grown in the same pot. After two-weeks of growth in sufficient water conditions, water supplies were stopped and the plants were not watered for at least 10 days, or until the plants were completely wilted. The plants were then re-watered and drought tolerance was checked after 4-days additional growth. Shown in Figure 22, *tac1-1D* plants survived this treatment while wild-type Columbia plants died, confirming that *tac1-1D* is more drought tolerant than wild-type Columbia.

### ***tac1-1D*** **mutant is not salt tolerant**

To determine whether TAC1 over-expression can induce multiple stress tolerances, we evaluated the *tac1-1D* mutant for salt tolerance. When 150mM and 300mM NaCl were applied, there was no difference between wild-type Columbia and *tac1-1D* (not shown) indicating that over-expression of TAC1 cannot confer salt tolerance.

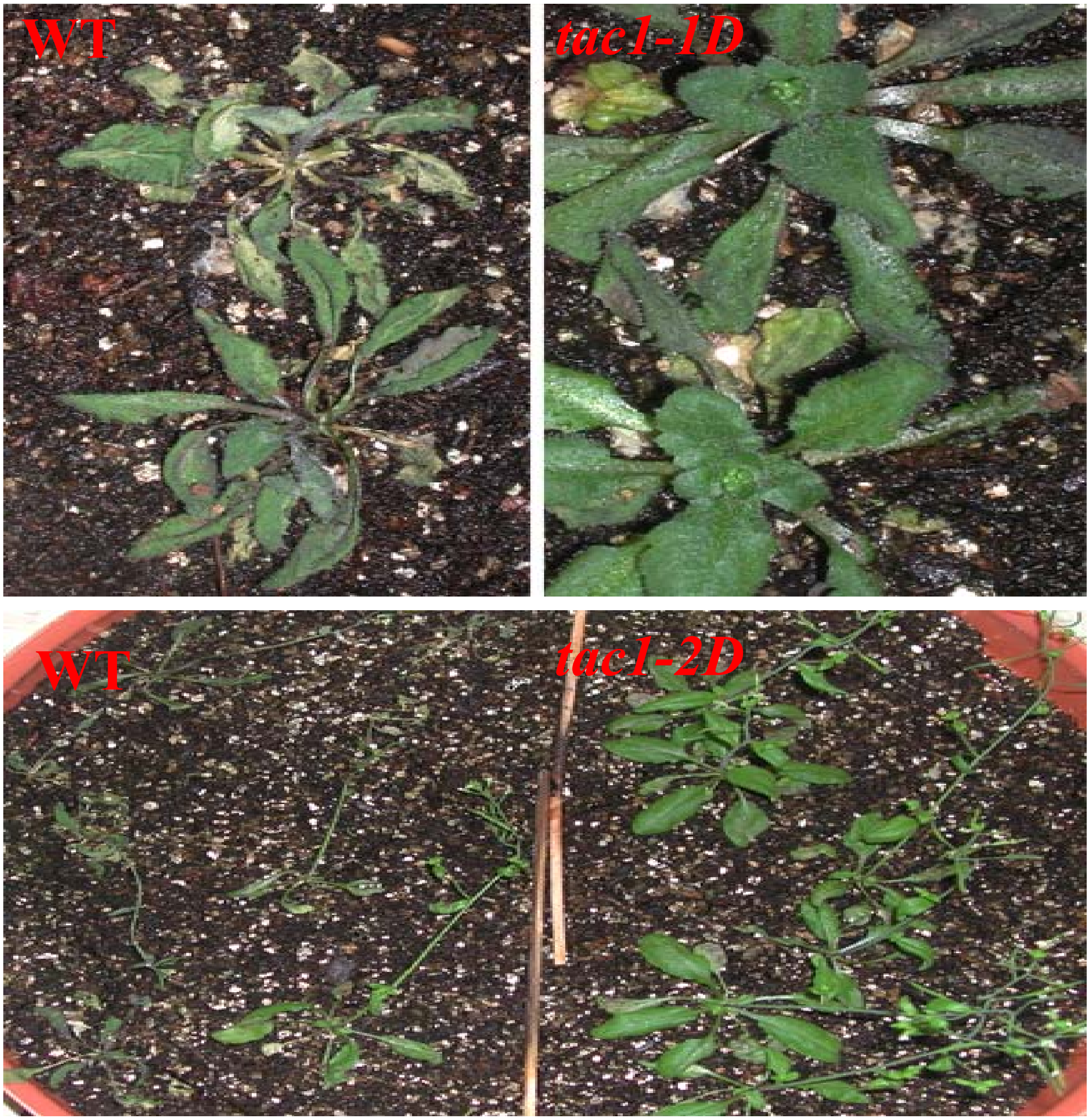


**Figure 22.** Drought phenotype of *tac1-1D* and *tac1-2D*. Upper panel: comparison of wt (left) and *tac1-1D* (right). Lower panel: comparison of wt (left) and *tac1-2D* (right). All plants were subjected to 10 days non-water treatment. Photos were taken 4 days after re-watering.

## Over-expression of TAC1 is responsible for drought tolerance

As mentioned in chapter III, the T-DNA insertion site in *tac1-1D* is 78 kb away from the activated *TAC1* gene. This raises a possibility that drought tolerance in *tac1-1D* might be due to the activation of some other genes near the *35S* enhancer in this mutant. To rule out this possibility, we examined *tac1-2D*, another independent *TAC1* mutant, and three independent transgenic lines expressing TAC1 from the strong constitutive CaMV *35S* promoter for their drought-tolerance phenotype. The results are shown in Figure 22 and Figure 23. Compared with wild-type Columbia, *tac1-2D* and all transgenic lines showed a drought tolerance phenotype. These results confirmed the involvement of TAC1 in drought tolerance. Furthermore, all three individual *35S::TAC1* transgenic lines have much stronger drought tolerance phenotype. They can survive a longer time during the period of lacking water. When wild-type Columbia plants were completely wilted, plants from all three transgenic lines were still normal and growing regularly (Figure 23). These results clearly demonstrated that over-expression of TAC1 is responsible for the drought tolerance phenotype.

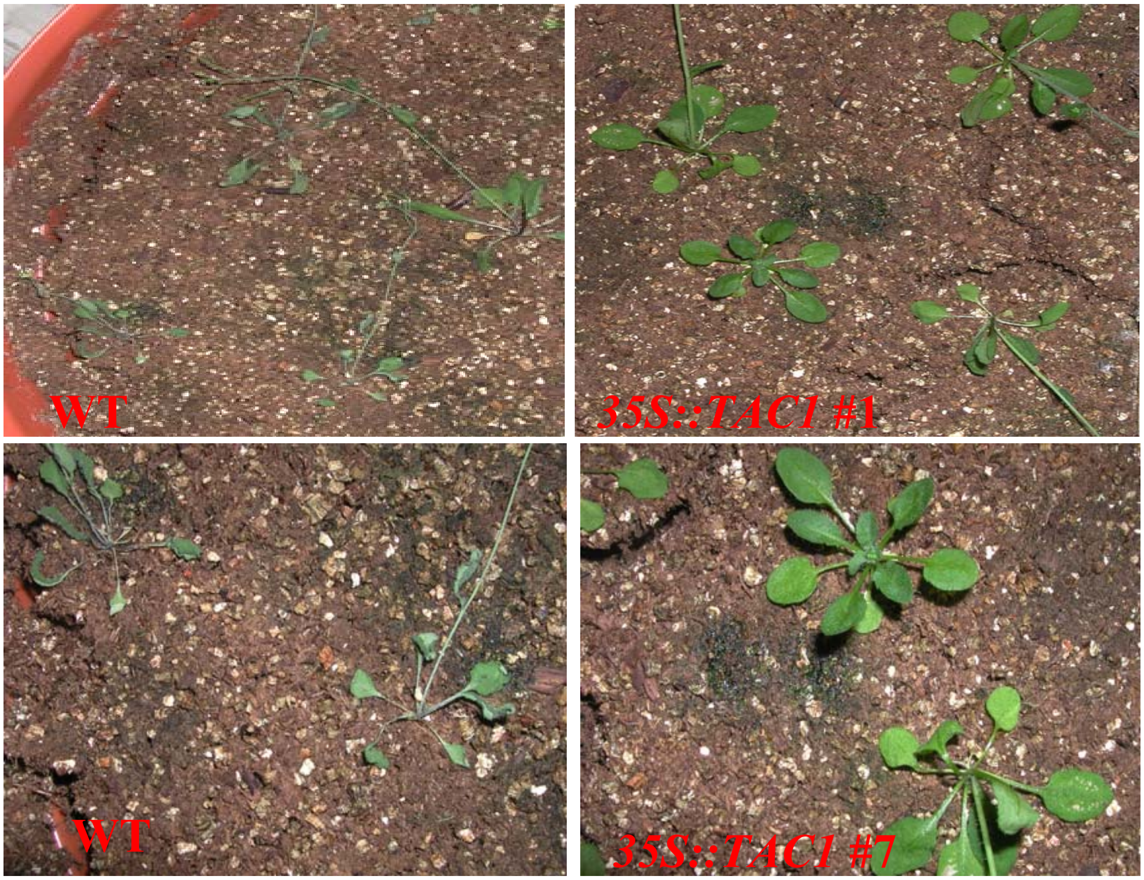


**Figure 23.** Drought phenotype of constitutively expressed TAC1 lines. Upper panel: comparison of wt and *35S::TAC1* line #1; lower panel: comparison of wt and *35S::TAC1* line #7. Photos were taken after15-day none-watering growth.

### Low concentration of IAA induce drought tolerance in wild-type Columbia

To dissect possible mechanisms by which TAC1 induces drought tolerance, we checked the effect of exogenous IAA on drought tolerance. As reported in the literature, auxin and ABA act antagonistically in induction of drought tolerance, where ABA can induce drought tolerance, but an extra amount of auxin will make plants sensitive to drought condition. On the other hand, some drought-induced genes can also be induced by auxin treatment (Kiyosue et al., 1994; Bianchi et al., 2002). Since high concentrations of IAA will cause the opposite effect for drought tolerance, we chose a low IAA concentration (1 nM) to treat the wild-type Columbia. As a mock control, the same amount of water was sprayed for the control panel. Shown in Figure 24, 1 nM IAA treatment significantly increased drought tolerance of wild-type Columbia plants. Because over-expression of TAC1 potentiates responses to normal levels of auxin as inferred in previous chapter, the result shown here might partially explain how *TAC1* confers drought tolerance.

## DISCUSSION

In this chapter, we described a new role of TAC1 over-expression in drought tolerance. Initially TAC1 was described as a telomerase activating protein. It can induce telomerase activity in fully-differentiated *Arabidopsis* leaves through synergistic action with auxin. However, micrroarray analysis indicated that many stress related genes are up-regulated.

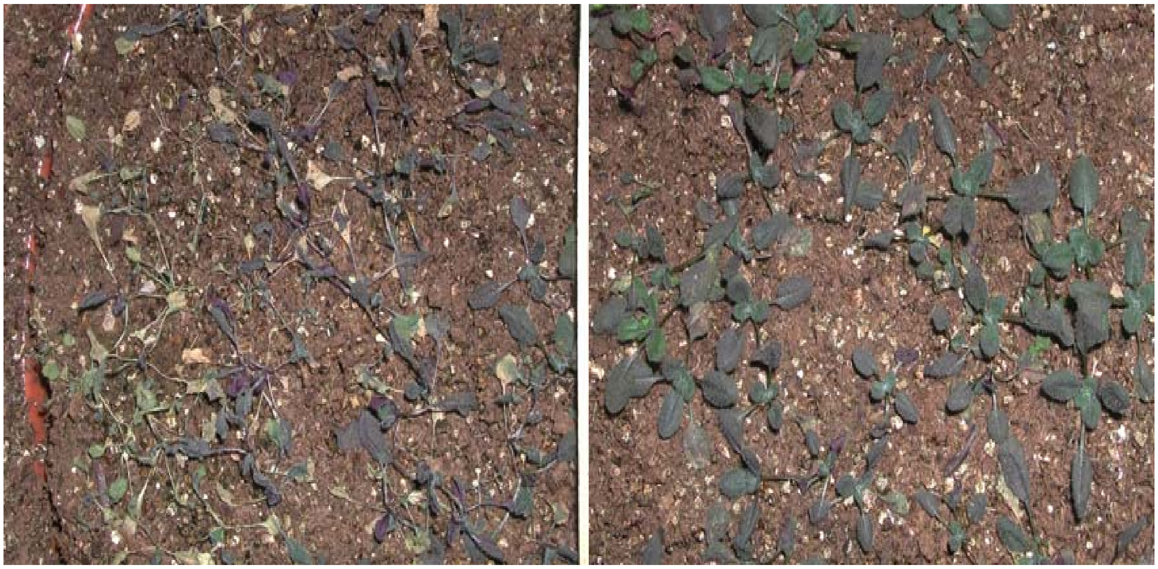


**Figure 24.** Effect of low concentration IAA on drought tolerance. Two-week old wild-type plants were drought treated for 7 days, then were subjected to 1 nM IAA treatment for a week. As a mock control, equal amount of water were sprayed to control panel. Photo was taken at 14th day after stopping watering.

Among these, 5 genes are drought induced. Further experiments demonstrated that TAC1 over-expression could induce drought tolerance, but not salt tolerance.

### Over-expression of TAC1 confers drought tolerance but not salt tolerance

To determine if TAC1 over-expression really could induce drought tolerance, *tac1-1D*, *tac1-2D* and three independent *35S::TAC1* transgenic lines were analyzed for their stress responses. In all cases, these transgenic plants showed a tolerance to osmotic stress, as clearly seen by plant recovery after re-watering from severe wilting caused by drought condition, or for *35S::TAC1* transgenic lines, the difference could be revealed even before re-watering. In the same experiments, control plants, ecotype Columbia, did not survive. Another notable finding was that plants over-expressing TAC1 were susceptible to salt stress, showing a similar sensitive response as the wild-type control. This could be explained by the possibility that transgenic plants produced some compatible solutes which confer tolerance to osmotic stress, but not to sodium ion toxicity. Although we do not know the mechanism by which TAC1 over-expression confers drought tolerance, it might act as a regulatory protein and be involved in the signaling pathway to activate osmotic stress responsive genes.

### Is TAC1 drought tolerance due to its synergistic action with auxin?

Since over-expression of TAC1 appears to potentiate some, but not all, of the plant's responses to auxin (for example, *tac1-1D* mutants have longer roots and more root hairs than wild-type Columbia), it is reasonable to ask if TAC1's drought tolerance phenotype is due to its synergistic action with auxin. Although a vast amount of research has been done on the adaptive physiological and molecular responses of plants to osmotic stress, knowledge about the molecular mechanism that operates in the signal transduction pathway of cellular response to osmotic stress is quite limited (Bray, 1993; Skriver and Mundy, 1990; Hong et al., 1997; Zhu, 2001). Studies of the effect of auxin on drought tolerance are even fewer. Conventional thought is that auxin and ABA act antagonistically on plant responses to environmental stresses, particularly drought stress (Swarup et al., 2002). Based on this idea, exogenous ABA can induce drought tolerance, while exogenous auxin can make plants sensitive to drought conditions. The major role of ABA in drought tolerance is best illustrated by mutant plants that cannot produce ABA, such as *Arabidopsis aba1*, *aba2*, and *aba3*. Without water stress, these mutants grow and develop relatively normally (Koornneef et al., 1998), but under drought stress they wilt readily and die if the stress continues. This function of ABA is partially through guard cell regulation where ABA can make the stomata close and hence do not lose too much water under drought stress. Regarding the effect of auxin on drought stress, the main knowledge is that auxin can make stomata open and hence lose water rapidly. Kovtun et al. (2000) provides evidence of crosstalk between $H_2O_2$ stress and auxin signaling, where constitutively active ANP genes (*Arabidopsis* MAPKKKs that initiate an $H_2O_2$ stress activated MAPK cascade) effectively suppressed the GH3 promoter induction by auxin. Also, Sadiqov et al. (2002) demonstrated the involvement of IAA and Ca2+ during stress-induced proline accumulation.

Limited information indicated that the polypeptide products of the AXR1 and AXR2 genes could play a role both in the auxin and drought response pathways. However, this auxin-dependent drought stress response in *Arabidopsis* is disrupted when a mutation occurs in the AXR1 gene (Leymarie et al., 1996). Here we show that over-expression of TAC1, a new gene, which can potentiate some responses to auxin, conferred drought tolerance. Furthermore, treatment with 1 nM IAA significantly increases wild-type Columbia ecotype's drought tolerance. However, whether or not TAC1's drought tolerance phenotype is due to its synergistic action with auxin is not yet known. This question could be answered by examining the drought tolerance phenotype of *tac1 iaaL* double mutants. If this is true, TAC1 may provide a link between auxin and plant drought stress response. However, in our preliminary experiment, 1 nM IAA induced drought tolerance in wild-type plants. This data contradicts to the idea that synergistic action of TAC1 with auxin causes drought tolerance and could simply indicate that a low level of IAA is required for drought tolerance. Further experiments should be designed to involve a series of IAA concentrations from low to high to examine the effects of auxin on plant drought tolerance responses.

## TAC1 over-expression provides a new way to manipulate crop drought tolerance

Drought tolerance is one of the most important traits for crops because world arable lands are continuously being injured from desiccation. Molecular genetic approaches have been used previously to improve plant tolerance to stresses through alteration of osmolytes, osmoprotectants, membrane fatty acids, channels, and transcription factors (Jaglo-Ottosen et al, 1998; Liu et al., 1998; Bohnert and Sheveleva, 1998). However, in some cases increased levels of osmoprotectants provide drought tolerance only in the lab, but not in the field. Over-expression of transcription factors, such as DREB1, which activates drought responsive genes, provides a good tolerance to drought. However, it is always coupled with a dwarf phenotype, which hinders its use in crop improvement. Over-expression of *TAC1* appears to increase drought tolerance without affecting other aspects of shoot growth and development, at least in *Arabidopsis*. In addition, increased root length in TAC1 may help plants acquiring water and other nutrients more effectively. Further manipulation of TAC1 through transgenic technology may provide a novel strategy for drought tolerance improvement in agriculturally important plants such as cotton and maize.

# CHAPTER V

# CONCLUSIONS AND PERSPECTIVES

Telomerase, a ribonucleoprotein complex, is the key enzyme that synthesizes telomeric DNA in most eukaryotic organisms. In mammals, telomerase expression is abundant in the germline cells but is undetectable in most other differentiated organs. Intensive studies of telomerase have focused on human cancerous cells where over 90% of all cancerous tissues examined have telomerase activity. In wild type *Arabidopsis*, telomerase expression is abundant in reproductive organs and dedifferentiated tissues such as flowers, siliques and callus but barely detectable in vegetative tissues (both rosette and cauline leaves).

In this study, a biochemical screen strategy was developed for isolation of telomerase activating mutants in *Arabidopsis thaliana.* Through screening of *Arabidopsis* activation-tagged lines by a PCR-based TRAP assay, two *tac* (for Telomerase ACtivator) mutants were isolated. Plasmid rescue indicated that *tac1* mapped to the left arm of chromosome 3, while the *tac2* mutant harbored two independent T-DNAs: one on chromosome 1, the other on chromosome 5. Segregation analysis demonstrated that telomerase expression in *tac1* was dominant and linked with the T-DNA insertion. RT-PCR analysis of *AtTERT* expression revealed that different mechanisms are involved in alternating telomerase activity in *tac1* and *tac2*.

*TAC1* gene was cloned and characterized. *TAC1* encodes a zinc finger protein with a single finger structure. Recapitulation experiments demonstrated that this gene activated telomerase in fully differentiated *Arabidopsis* leaves. This induction of telomerase activity was uncoupled from the cell cycle and was diminished by over-expressing iaaL, an enzyme that conjugates free IAA to lysine. Telomere length was unperturbed in the mutant, but other phenotypes, such as altered root development and the ability of cells to grow in culture without exogenous auxin, indicated that TAC1 not only is part of the previously reported link between auxin and telomerase expression, but also potentiates other classic responses to this phytohormone.

Because of a strong relationship between telomerase activity and human cancers, studies of telomerase regulation have been given a lot attention in humans. Although a number of molecules have been implicated in the regulation of *hTERT*, insight into mechanisms that specifically control telomerase expression is yet unclear. Plants are developmentally different from humans. However, the overall pattern of telomerase expression in plants is similar to that in humans. These similarities in basic telomere biology indicate that at least some of telomerase regulation pathways are conserved between plants and humans and this promotes us using *Arabidopsis* as another experimental system to study the mechanisms of telomerase regulation. Hundreds of thousands of available T-DNA tagged lines in *Arabidopsis* make it powerful and unique comparing to mammalian systems. Through current study, a biochemical screening strategy for *tac* mutant isolation was developed and this strategy was demonstrated a powerful through screening first 2,000 *Arabidopsis* activation tagged lines. Because more than 16,000 such lines are available in *Arabidopsis*, complete screening of these lines will result in identifying more *tac* mutants. Cloning and characterization of these identified mutants will provide us a systemic way to elucidate telomerase regulation pathways in *Arabidopsis*, and help us to understand the whole picture of telomerase regulation in plant kingdom, and possibly other organisms. On the other hand, some of these genes should have functional homologs in humans that can be used to develop novel anticancer treatments.

*TAC1* gene encodes a single zinc finger protein and it can act synergistically with auxin to induce telomerase activity in fully differentiated tissues without activating cell cycle. However, how *TAC1* functions in regulating telomerase is yet not clear. In *Arabidopsis*, there are about 30 $C_2H_2$-type single zinc finger proteins. Most of these proteins are not elucidated for their functions. The only known gene, which was well characterized, is *superman* (*SUP*), a transcription factor involved in flower development. The identity between *TAC1* and *superman* is about 26%. It is of interesting to further determine if TAC1 functions also as transcription factor or through protein-protein interaction in regulating telomerase activity. Because no sequence specific interaction between TAC1 recombinant protein and *AtTERT* promoter was detected in current study, next step should focus on a yeast one-hybrid screening strategy to identify the specific regulatory element where TAC1 protein can bind to. If TAC1 acts through protein-protein interaction, then yeast two-hybrid system should be used for isolation of its interacting proteins.

In this study, we provide evidence that *TAC1* may be a linker between telomerase regulation pathway and auxin regulation pathway. However, how does *TAC1* exactly work in these pathways is not known. Through screening libraries by either yeast one-hybrid or two-hybrid systems as mentioned above, the biochemical evidence could be identified to tight these two regulatory pathway together.

Flow cytometry analysis of DNA content demonstrated that cell cycle does not activated in *tac1* leaves comparing to wild type leaves indicating that TAC1 induce telomerase activity in fully differentiated leaves without activating cell division. To further test this cell cycle-independent telomerase regulation in *tac1* mutant, a *tac1* cell line should be established and synchronized to each different stage of cell cycle. These synchronized *tac1* cells should be tested for their telomerase activity. If TAC1 can activate telomerase expression in cell cycle-independent manner, telomerase activity should be detected in all different synchronized cells.

DNA microarrays were used to analyze the expression profile of the *tac1* mutant. Although *tac1-1D* was isolated originally as a telomerase activator mutant, microarray data revealed that several drought-induced genes were up-regulated 3~10 fold in the *tac1-1D* mutant relative to wild type. RT-PCR analysis further confirmed this up-regulation for five of these genes. Investigation of root growth also indicated that *tac1-1D* roots were ~20% longer relative to wild type. *tac1-1D*, *tac1-2D* and 3 individual *35S::TAC1* transgenic *Arabidopsis* lines were then subjected to drought tolerance experiments and demonstrated that over-expression of TAC1 was able to confer drought tolerance in all these lines. Salt tolerance was also examined for the *tac1-1D* mutant, but there was no difference between *tac1-1D* and wild type. In addition, our preliminary result showed that treatment with a low concentration of IAA could induce drought tolerance in wild type *Arabidopsis*. Although plants with constitutive expression of telomerase have no practical utility, the ability of TAC1 to confer drought tolerance could have significant agricultural applications.

Although not tested in this study, it is of interesting to know that if this drought tolerance phenotype in *tac1* is related to its constitutive expression of telomerase. Loss of function of *TERT* in yeast affected many genes' expression genome-wide including stress induced genes. However, no report describes the effects of over-expression of telomerase on genes' expression genome-wide in any organisms. To test relationship between telomerase activity and drought tolerance in *tac1*, we should generate a double mutant between *tac1* and *AtTERT*$^{-/-}$ line and set this double mutant in drought tolerance experiments. If drought tolerance phenotype is independent of telomerase expression, then the double mutant should also confer drought tolerance, otherwise should not.

Because drought tolerance is grouped into at least two classes: ABA dependent and ABA independent. It is also interested to know that if *tac1* drought tolerance is ABA dependent or independent. To do this, a double mutant between *tac1* and *aba1* or *aba2* or *aba3* needs to be generated to block the production of ABA in *tac1* background. Then testing drought tolerance status for the double mutant.

Conventional thought is that auxin and ABA act antagonistically on plant responses to drought stress (Swarup et al, 2002), however, auxin concentration is relatively high in this consideration. No any report describes the effect of low concentration of auxin on drought tolerance. Our preliminary result shows that treatment with 1 nM IAA significantly increases wild type Columbia ecotype's drought tolerance indicating that low level of IAA may be required for drought tolerance. Because over-expression of

TAC1 potentiates responses to normal levels of auxin as inferred in our experiments, this result might partially explain how *TAC1* confers drought tolerance. However, further experiments should be designed to involve a series of IAA concentrations from low to high to examine the effects of auxin on plant drought tolerance responses.

Drought tolerance is most important trait for crops growing in dry-land areas. The fact that over-expression of TAC1 increases drought tolerance without affecting other aspects of shoot growth and development may provide a way in manipulating drought tolerance in agriculturally important crops. To best understand the mechanism of drought tolerance in *tac1* mutant, we should further characterize *tac1* mutant at molecular level, in the meantime, we should over-express TAC1 in other important crops such as cotton and maize through genetic engineering.

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