TELOMERASE ACTIVATOR1 Induces Telomerase Activity and Potentiates Responses to Auxin in Arabidopsis

Shuxin Ren, a J. Spencer Johnston, b Dorothy E. Shippen, c and Thomas D. McKnight a,1

a Department of Biology, Texas A&M University, College Station, Texas 77843-3258
b Department of Entomology, Texas A&M University, College Station, Texas 77843-2475
c Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-2128

Telomerase activity is highly regulated, abundant in rapidly dividing cells and reproductive organs, but undetectable in most other differentiated tissues. Little is known about mechanisms that regulate telomerase. Here, we used a biochemical assay to screen activation-tagged lines of Arabidopsis thaliana for mutants that ectopically express this enzyme in their leaves. In one such mutant, a previously uncharacterized zinc-finger protein we designate TELOMERASE ACTIVATOR1 (TAC1) is overexpressed and induces telomerase in fully differentiated leaves without stimulating progression through the cell cycle. Reducing endogenous concentrations of auxin in the mutant blocks the ability of TAC1 to induce telomerase. This result, along with other phenotypes of the mutant, such as the ability of cells to grow in culture with exogenous auxin and increased sensitivity of primary root growth to exogenous auxin, indicates 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

Telomerase is a specialized reverse transcriptase responsible for synthesizing and maintaining telomeric DNA (reviewed in Collins and Mitchell, 2002; Cech, 2004). In most angiosperms, this DNA consists of tandem repeats of the heptanucleotide TTAGGG, although some monocot telomeres comprise the related hexanucleotide TTAGGG (Adams et al., 2001; Weiss-Schneeweiss et al., 2004). Tracts of telomeric DNA extend for several kilobases and loop back to form higher order structures called t-loops (Cesare et al., 2003). Specialized nonhistone proteins that bind telomeric DNA have been well characterized in yeast (Saccharomyces cerevisiae) and humans. In plants, many putative telomeric DNA binding proteins have been described (reviewed in McKnight et al., 2002), but it is not yet clear which, if any, of these proteins associate with telomeres in vivo.

In Arabidopsis thaliana, telomeres range in size from 2 to 9 kb, varying slightly among the ecotypes (Shakirov and Shippen, 2004). As for other eukaryotes, proteins that interact, directly or indirectly, with telomerase regulate telomere length. For example, the Ku70/80 heterodimer is a negative regulator of telomere length (Bundock et al., 2002; Riha et al., 2002; Gallego et al., 2003) and may act by limiting access of telomerase to the ends of chromosomes. Mutations affecting the Rad50/Mre11/Xrs2 DNA repair complex are also reported to affect telomere length (Gallego and White, 2001; Bundock et al., 2002). In the absence of telomerase, the telomeric DNA tract shortens because of the inability of DNA-dependent DNA polymerases to fully synthesize the 5’ end of the lagging strand. In Arabidopsis, the absence of telomerase results in a loss of 250 to 500 bp per plant generation (Riha et al., 2001). Initially, the effects of telomere shortening are minimal, but eventually telomeres lose their ability to protect chromosomes from end-to-end fusions, and the genome suffers multiple rounds of chromosome breakage and fusion (Riha et al., 2001; Siroky et al., 2003). After surviving six or seven generations without telomerase, the mutants experience increasingly severe developmental defects in subsequent generations, culminating in severely stunted and sterile plants. No telomerase-deficient plants survive past the tenth generation (Riha et al., 2001).

In plants, as in humans, telomerase expression is largely restricted to reproductive organs, embryos, and rapidly dividing cells such as immortalized, dedifferentiated cells growing in culture (Fitzgerald et al., 1996; Heller et al., 1996; Kilian et al., 1998). Human telomerase is undetectable in most somatic cells but is reactivated in a large majority of cancerous tumors, conferring unlimited potential for proliferation (Kim et al., 1994; Shay and Bacchetti, 1997). A major point of control of telomerase activity in humans is transcription of the telomerase reverse transcriptase gene hTERT (Meyerson et al., 1997; Nakamura et al., 1997). Many molecules have been implicated in the regulation of hTERT expression, either by repressing or activating transcription (reviewed in Ducrest et al., 2002), and pathways that regulate telomerase in humans are rapidly being elucidated. The hTERT promoter contains binding sites for many transcription factors, including estrogen receptors and c-Myc. Exposure to estrogen results in increased transcription of hTERT by the direct binding of the estrogen receptor to estrogen-responsive elements in the promoter and indirectly by activating transcription of c-Myc (Kyo et al., 1999). Posttranscriptional events,
including estrogen-mediated phosphorylation of hTERT (Kimura et al., 2004) and sequestration within the nucleolus (Wong et al., 2002), are also involved in regulating telomerase activity in humans.

Mechanisms of telomerase regulation are only beginning to be elucidated in plants, but enzyme activity correlates with TERT mRNA levels (Fitzgerald et al., 1999). In rice (Oryza sativa), TERT transcripts are subject to alternative splicing (Heller-Uszynska et al., 2002), possibly providing additional opportunities for regulation. There is also a strong correlation between cell cycle progression and telomerase expression. In synchronized tobacco (Nicotiana tabacum) cells in culture, telomerase activity is restricted to late S phase. Exposure to auxin-type phytohormones, such as indole acetic acid (IAA), induces higher levels of telomerase activity and an earlier appearance of that activity during S phase (Tamura et al., 1999). This cell cycle–dependent, auxin-mediated increase in telomerase activity is regulated by protein phosphorylation and can be blocked by protein kinase inhibitors (Yang et al., 2002).

In an effort to identify genes that regulate telomerase expression in Arabidopsis, we used a biochemical assay to screen activation-tagged lines (Weigel et al., 2000) for ectopic expression of telomerase in their leaves. We recovered three telomerase activation mutants and designated the first one telomerase activator1-1 Dominant (tac1-1D). The gene activated in this line (TAC1) encodes a small, zinc-finger protein related to SUPERMAN, a well-characterized transcription factor involved in floral organ development (Bowman et al., 1992; Isernia et al., 2003). Reducing endogenous concentrations of auxin in the mutant blocks the ability of TAC1 to induce telomerase. Primary root growth of mutant seedlings is more sensitive to inhibition by exogenous auxin, whereas a tac1-null mutant is more resistant. Additionally, growth of tac1-1D callus is auxin independent. These results suggest that TAC1 is part of a pathway linking auxin signaling and telomerase expression.

RESULTS

Identification and Characterization of tac Mutants

To identify genes that regulate telomerase expression, we screened 7976 activation-tagged lines using the telomerase repeat amplification protocol (TRAP; Kim et al., 1994) as modified by Fitzgerald et al. (1996). Because telomerase activity is normally repressed and undetectable in leaves and because TRAP is exquisitely sensitive, we initially screened the plants in pools of 10 or 20. Leaves from three pools of plants exhibited telomerase activity (Figure 1A). Individual plants within the pools were screened to identify tac mutants. Here, we describe one tac1 mutant and the gene activated in this line.

TRAP assays revealed telomerase expression in leaf extracts from the activation-tagged pool CS20857 (Figure 1A). Ectopic telomerase expression was dominant and genetically linked to the herbicide resistance trait conferred by the T-DNA. We designated this line tac1-1D. RT-PCR revealed that the steady state level of AtTERT mRNA was elevated in tac1-1D above that in wild-type leaves, although not to the level in wild-type flowers (Figure 1B). Because telomerase is a ribonucleoprotein complex, this result indicates either that other essential components of the holoenzyme are coordinate induced in tac1-1D or that TERT is the limiting component in plants, as it is in humans (Meyerson et al., 1997; Nakamura et al., 1997). Although increased telomerase expression might be expected to result in telomere elongation, telomere length in tac1-1D plants was well within the range seen in wild-type plants (Figure 1C). The fact that telomere length is unperturbed by constitutive telomerase activity is consistent with studies indicating that access to the ends of chromosomes by telomerase is regulated by telomere-associated proteins (reviewed in Vega et al., 2003).

Identification of TAC1

In most previously characterized activation-tagged lines, the activated gene responsible for the altered phenotype is very near
or adjacent to the site where the 35S enhancers are inserted (Weigel et al., 2000; Zhao et al., 2001; Dinneny et al., 2004). To determine the site of insertion in tac1-1D, we recovered the activating T-DNA and flanking plant DNA from the mutant by plasmid rescue. DNA sequencing revealed that two head-to-head copies of the T-DNA, with multimers of the Cauliflower mosaic virus (CaMV) 35S enhancer in the center, were inserted on chromosome III into the 3' end of the uncharacterized locus At3g090550 (Figure 2A). This was the only T-DNA insertion detected by DNA gel blotting (data not shown). RT-PCR analysis of genes near the T-DNA insertion site indicated that none of them had increased expression compared with the wild type (data not shown).

To identify the gene (or genes) activated in the tac1-1D mutant, we used radiolabeled cDNAs from wild-type and tac1-1D leaves to probe restriction fragments from the bacterial artificial chromosome T14B5, which spans the T-DNA insertion site. Only one fragment displayed increased hybridization to cDNA from tac1-1D leaves relative to the wild type (Figure 2B). This fragment was 78 kb away from the activating T-DNA (Figure 2A) and carried the gene we designated TAC1 (GenBank accession number NM_111763; locus At3g09290). TAC1 encodes a 19-kD protein with a single C2H2 zinc-finger domain and a Leu-rich region near the C terminus identified as an ethylene response factor–associated amphiphilic transcriptional repression (EAR) domain (Ohta et al., 2001; Hiratsu et al., 2002) (Figure 2C). The Arabidopsis genome encodes 29 proteins with the same overall structure as TAC1 (Dinkins et al., 2002), including SUPERMAN (Bowman et al., 1992), RABBIT EARS (Takeda et al., 2004), and JAGGED (Dinneny et al., 2004; Ohno et al., 2004), but there is little similarity in amino acid sequence outside the short zinc-finger and EAR domains. RT-PCR analysis of TAC1 mRNA in the wild-type plants revealed low or undetectable levels of the transcript in all organs examined (Figure 2D). Expression was lowest in leaves and higher in flowers, roots, and callus (Figure 2D), consistent with levels of AtTERT mRNA and telomerase activity in these settings.

**Figure 2.** Identification of the TAC1 Gene.
(A) Map of TAC1 genomic region (not to scale). Locus numbers are indicated above arrows representing open reading frames. Independent T-DNA insertions in tac1-1D, tac1-2D, and tac1-3 plants are shown below the open reading frames. The TAC1 locus is indicated by a black arrow. The TAC1 start codon is 78,811 nucleotides from the end of the activating T-DNA in tac1-1D.
(B) DNA gel blot identifying TAC1. EcoRI fragments from the T14B5 BAC were subcloned into pBluescript KS +, digested with BamHI, blotted, and hybridized to radiolabeled cDNA from the wild type (left panel) and tac1-1D leaves (right panel). One subclone (in lane 1) revealed that a single BamHI fragment (containing the At3g09290 locus) hybridized to cDNA from the activation-tagged mutant but not the wild type. The subclone in lane 2 (and many others not shown) had identical hybridization patterns when probed with cDNA from wild-type and tac1-1D leaves.
(C) Predicted amino acid sequence of TAC1. The single C2H2 zinc-finger motif and the EAR domain (near the C terminus) are underlined. The arrowhead indicates the site of the T-DNA insertion in the tac1-2D allele.
(D) RT-PCR analysis of TAC1 and AtTERT mRNA in the wild-type plants. A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used to indicate the amount of template (bottom panel).
(E) RT-PCR analysis of TAC1 expression in tac1 mutants. A GAPDH gene was used to indicate the amount of template and to ensure the absence of contaminating genomic DNA (bottom panel).
139 amino acids. Plants expressing this TAC1-139 PCR to create a truncated version of telomerase induction. To test this hypothesis directly, we used codon 139, displacing the region encoding the EAR transcrip
tivate the cleotides upstream of the designated SALK_013353, contains a T-DNA inserted 100 nu-

tive enhancer. Apparently, this single SALK lines contains a complete vector (Baulcombe et al., 1986) used to disrupt genes in the line had increased T-DNA confirmed that the T-DNA was not spliced out of the polyadenylation site from the bulk of the coding region. RT-PCR confirmed that the T-DNA was not spliced out of the TAC1: T-DNA message (data not shown). We expected this line to lack TAC1 mRNA and possibly telomerase activity. Instead, this tac1-2D line had increased TAC1 mRNA (Figures 2E and 3C) and copious telomerase activity in its leaves (Figure 3B), in accordance with the high level of TERT mRNA (Figure 3C). The pROK2 vector (Baulcombe et al., 1986) used to disrupt genes in the SALK lines contains a complete 3SS promoter, including the enhancer. Apparently, this single 3SS enhancer is sufficient to activate the TAC1 promoter. The T-DNA in tac1-2D occurs after codon 139, displacing the region encoding the EAR transcriptional repres sor domain (Figure 2C). Telomerase activity in tac1-2D suggested that the EAR domain may not be required for telomerase induction. To test this hypothesis directly, we used PCR to create a truncated version of TAC1 encoding only the first 139 amino acids. Plants expressing this TAC11-139 construct from the 3SS promoter have telomerase activity in their leaves (Figure 3C), confirming that the EAR domain is not required to induce telomerase. Although tac1-2D was not the null mutant we expected, it was valuable as a second, independent activation-tagged line to further confirm that increased TAC1 expression results in telomerase activity in mature, differenti ated organs.

We obtained a TAC1-null mutant that does not express detectable amounts of TAC1 transcript (Figure 2E) from the GABI-Kat collection of T-DNA insertions (Rosso et al., 2003) and designated this null mutant tac1-3 (GABI_368A07). The pattern of telomerase expression in tac1-3 is similar to the wild-type pattern (data not shown). The persistence of telomerase activity in tac1-3 may result from a redundant function supplied by one of the other 28 members of the SUPERMAN-like family or a completely redundant pathway for telomerase induction. As described later, this null mutant has an altered response to auxin.

**Characterization of Additional tac1 Lines**

Further confirmation of the regulatory nature of TAC1 was unexpectedly provided through a T-DNA insertion line generated by the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003) that we refer to as tac1-2D (Figure 2A). This line, originally designated SALK_013353, contains a T-DNA inserted 100 nucleotides upstream of the TAC1 stop codon, which separates the last 33 codons, the presumed 3’ untranslated region, and the polyadenylation site from the bulk of the coding region. RT-PCR confirmed that the T-DNA was not spliced out of the TAC1: T-DNA message (data not shown). We expected this line to lack TAC1 mRNA and possibly telomerase activity. Instead, this tac1-2D line had increased TAC1 mRNA (Figures 2E and 3C) and copious telomerase activity in its leaves (Figure 3B), in accordance with the high level of TERT mRNA (Figure 3C). The pROK2 vector (Baulcombe et al., 1986) used to disrupt genes in the SALK lines contains a complete 3SS promoter, including the enhancer. Apparently, this single 3SS enhancer is sufficient to activate the TAC1 promoter. The T-DNA in tac1-2D occurs after codon 139, displacing the region encoding the EAR transcriptional repres sor domain (Figure 2C). Telomerase activity in tac1-2D suggested that the EAR domain may not be required for telomerase induction. To test this hypothesis directly, we used PCR to create a truncated version of TAC1 encoding only the first 139 amino acids. Plants expressing this TAC11-139 construct from the 3SS promoter have telomerase activity in their leaves (Figure 3C), confirming that the EAR domain is not required to induce telomerase. Although tac1-2D was not the null mutant we expected, it was valuable as a second, independent activation-tagged line to further confirm that increased TAC1 expression results in telomerase activity in mature, differenti ated organs.

We obtained a TAC1-null mutant that does not express detectable amounts of TAC1 transcript (Figure 2E) from the GABI-Kat collection of T-DNA insertions (Rosso et al., 2003) and designated this null mutant tac1-3 (GABI_368A07). The pattern of telomerase expression in tac1-3 is similar to the wild-type pattern (data not shown). The persistence of telomerase activity in tac1-3 may result from a redundant function supplied by one of the other 28 members of the SUPERMAN-like family or a completely redundant pathway for telomerase induction. As described later, this null mutant has an altered response to auxin.

**Overexpression of TAC1 Increases Telomerase Activity without Activating the Cell Cycle**

How might overexpression of TAC1 activate telomerase? Al-though its zinc-finger motif and similarity to SUPERMAN sug-
gested that TAC1 is a transcription factor, we failed to detect a sequence-specific interaction between the recombinant pro-
tein and the AtTERT promoter by either yeast one-hybrid or electrophoretic mobility shift assays with recombinant protein (data not shown).

Because telomerase expression is closely correlated with an active cell cycle in plants (Tamura et al., 1999; Yang et al., 2002), we investigated the possibility that TAC1 acts by stimulating cell division in mature organs. Arabidopsis plants with increased rates of cell division have larger cotyledons, curled leaves, smaller stature at maturity, and other developmental abnormalities (Dewitte et al., 2003; Yu et al., 2003). However, the normal appearance of shoots in both tac1-1D and tac1-2D was consist-ent with the typical pattern and rate of cell division in the mature, wild-type leaves, arguing that telomerase activity was uncoupled from cell cycle progression. To further investigate cell cycle regulation in tac1-1D, we analyzed DNA content in leaf cells by flow cytometry (Table 1). There was no difference in the percentage of cells in S phase between the wild type (19.5% ± 1.6%) and tac1-1D (17.7% ± 1.7%). Continued progression of leaf cells through the cell cycle leads to a decrease in cells in G1 (with nuclei at the 2C ploidy level) and to a very pronounced decrease in endoreduplication (Dewitte et al., 2003). However, the fraction of cells at each ploidy level from 2C to 16C was nearly identical in the wild-type and tac1-1D leaves (Table 1). The similar numbers of cells in S phase and at each ploidy level in both mutant and wild-type leaves confirm that overexpression of TAC1-activated telomerase occurs in mature leaves without increasing the percentage of cycling cells.

**TAC1 Acts Synergistically with Auxin to Activate Telomerase**

Exposure to exogenous auxin also can induce telomerase ex-
pression (Tamura et al., 1999; Yang et al., 2002), prompting us to ask whether auxin metabolism was altered in the tac1-1D mutant. We examined the formation and sustained growth of tac1-1D callus in response to phytohormones. Callus from both tac1-1D plants and the 3SS:TAC1 transgenic lines, but not from the wild-type plants, could be grown in the absence of exoge-
nous auxin (Figure 4A; data not shown) suggesting a connection between this hormone and induction of telomerase by TAC1. We also examined the ability of auxin to induce telomerase in a mutant defective in auxin signaling. The double mutant axr1-3 axr4-2 is defective in many auxin responses and is more resistant to exogenous auxin than either parental mutant (Hobbie and Estelle, 1995). Nevertheless, the synthetic auxin 2,4-D is able to induce telomerase in the leaves of this double mutant (Figure 3D).
Figure 3. Effect of TAC1 Overexpression in Different Genetic Backgrounds.

(A) Recapitulation of tac1 phenotype by direct overexpression of TAC1 from the CaMV 35S promoter. TRAP assays were performed on tac1-1D leaves and leaves from two independent 35S:TAC1 transformants. Extracts from wild-type flowers and leaves were used as positive and negative controls, respectively.

(B) Telomerase activity in other mutant backgrounds. TRAP assays indicate that activation of telomerase in leaves requires both elevated levels of TAC1 and the wild-type concentrations of auxin. Increased auxin concentrations in the yucca mutant are not sufficient to induce telomerase expression. Decreased auxin levels in the tac1-1D iaaL double mutant eliminate telomerase expression in leaves but not flowers. Activation of TAC1 in the tac1-2D mutant also induces telomerase activity.

(C) Telomerase activation by a truncated version of TAC1. TRAP assays of leaves from three independently transformed transgenic plants expressing the first 139 amino acids of TAC1. This truncated version of TAC1 lacks the EAR transcriptional repressor domain and corresponds to the TAC1 protein predicted to be expressed in the tac1-2D mutant.

(D) Telomerase induction in an auxin-signaling mutant. TRAP assays of leaf tissue from the wild type and the axr1-3 axr4-2 double mutant with and without treatment with exogenous 2,4-D. Extracts from wild-type flower and leaf are positive and negative controls, respectively.

(E) Expression of TAC1 and AtTERT mRNA in tac1-1D iaaL and tac12D mutants. RT-PCR analysis indicates that expression of AtTERT in leaves is eliminated by iaaL and correlates with loss of telomerase activity in the double mutant. The T-DNA insertion in tac1-2D truncates the TAC1 mRNA, resulting in the deletion of the last 33 amino acids, including the repressor domain, but this is still sufficient to activate expression of AtTERT.
Table 1. DNA Content Analysis of Wild-Type and tac1-1D Leaves

<table>
<thead>
<tr>
<th>Ploidy or Phase</th>
<th>Wild Type</th>
<th>tac1-1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C</td>
<td>24.3 ± 4.1</td>
<td>26.0 ± 1.7</td>
</tr>
<tr>
<td>4C</td>
<td>28.2 ± 8.0</td>
<td>32.8 ± 3.7</td>
</tr>
<tr>
<td>8C</td>
<td>17.2 ± 6.0</td>
<td>11.9 ± 5.8</td>
</tr>
<tr>
<td>16C</td>
<td>3.6 ± 1.5</td>
<td>1.9 ± 1.2</td>
</tr>
<tr>
<td>S phase</td>
<td>19.5 ± 1.6</td>
<td>17.7 ± 1.7</td>
</tr>
</tbody>
</table>

*Numbers are the average percentage of nuclei at each ploidy level or phase from five size-matched leaves (±SD).

To further explore the role of auxin in telomerase induction, we crossed tac1-1D plants with a line constitutively expressing iaaL, a bacterial enzyme that decreases the concentration of free IAA by conjugating it to Lys (Romano et al., 1991). Telomerase assays of leaves in the tac1-1D iaaL double mutant were negative (Figure 3B) and correlated with a decrease in mRNA for AtTERT (Figure 3E). Hence, TAC1 may activate telomerase expression either by increasing the concentration of auxin or by increasing sensitivity to the wild-type concentration of auxin. To determine whether an increased level of endogenous auxin was sufficient to induce telomerase, we performed telomerase assays on leaves of the yucca mutant, which contains 50% more IAA than the wild type (Zhao et al., 2001). There was no detectable telomerase activity in yucca leaves (Figure 3B). Together, the results from telomerase assays in yucca and the tac1-1D iaaL double mutant indicate that TAC1 acts synergistically with auxin to activate telomerase in mature leaves. Although auxin clearly plays a role in inducing telomerase in vegetative organs, whether it plays any role in the developmental induction of the enzyme during the transition to flowering is less certain because flowers of the low-iaaL mutant have telomerase activity (Figure 3B).

To determine whether other aspects of auxin metabolism were altered in tac1-1D, we examined expression of the DR5:GUS reporter gene, which carries multiple copies of an auxin-responsive element in the synthetic DR5 promoter (Ulmasov et al., 1997). Quantitative β-glucuronidase (GUS) assays revealed a small, but statistically significant, increase in expression in the tac1-1D background more than the very low level of expression in a wild-type background (Figure 5A). The effect of the tac1-1D background on GUS expression disappeared when promoter was induced with exogenous auxin. We also used RNA gel blots to compare the expression of the auxin-inducible genes IAA2, IAA4, IAA8, and IAA12 (Abel et al., 1995) in tac1-1D and wild-type backgrounds but found little difference (Figure 5B; data not shown). These results suggest that TAC1 appears to interact with auxin to activate telomerase, but overexpression of the protein does not lead to a large-scale alteration of normal responses to auxin.

Although we did not directly measure IAA concentrations in our mutants, two lines of reasoning lead us to postulate that induction of telomerase activity by overexpression of TAC1 is because of a potentiation of the response to normal levels of auxin rather than an increase in auxin concentration. First, tac1-1D plants have a normal appearance, whereas shoots of the yucca mutant, with a twofold increase in IAA concentration, have a distinctive phenotype, including reduced apical dominance and epinastic leaves with longer petioles (Figure 4B). Furthermore, wild-type and tac1-1D plants have identical ratios of shoot mass to root mass (7.2:1) for 3-week-old seedlings grown on MS medium and primary root length (Figure 4C) and nearly identical numbers of lateral roots (averages of 6.4 for the wild type and 6.6 for tac1-1D for the roots measured in Figure 4C).

The level of IAA responsible for the yucca phenotype is not sufficient to induce telomerase expression, implying that if...
IAA2 appears to be induced by IAA. The same RNA preparation was used for all blots.

We also examined expression of similar in wild-type and tac1-1D did not detect a signal in any tissue (data not shown). For 1 h. In untreated leaves, message levels for of wild-type and (B) Quantitative assay of GUS expression driven by the synthetic DR5 promoter. Seven-day-old seedlings, doubly homozygous for tac1-1D and DR5:GUS, were treated with various concentrations of IAA for 12 h. Data represent the averages of four separate treatments, each using four seedlings. In the absence of exogenous auxin, there is a slight but statistically significant (double asterisks, $a = 0.05$), but no significant difference at higher concentrations.

There is also a slight difference at 10 nM IAA (single asterisk, $a = 0.05$), but no significant difference at higher concentrations.

(B) N analysis of auxin-inducible genes. RNA was extracted from leaves of wild-type and tac1-1D plants with and without exposure to 20 μM IAA for 1 h. In untreated leaves, message levels for IAA2, IAA4, and IAA8 are similar in wild-type and tac1-1D leaves. Under these conditions, only IAA2 appears to be induced by IAA. The same RNA preparation was used for all blots. GAPDH message was probed to indicate relative amounts of RNA in the lanes. We also examined expression of IAA12 but did not detect a signal in any tissue (data not shown).

overexpression of TAC1 activated telomerase simply by increasing the IAA concentration, it would have to produce levels higher than that of the yucca plants. The tac1 mutants should therefore have a pronounced phenotype. Second, the high-auxin phenotype is exacerbated in a yucca tac1-1D double mutant, resulting in a smaller plant with a more distorted leaf blade to petiole ratio (Figure 4B) and even lower fertility (data not shown) than the yucca parent. These observations reinforce the idea that overexpression of TAC1 potentiates at least some of the many responses to auxin, including activation of telomerase.

TAC1 Potentiates Root Sensitivity to Exogenous Auxin

One classic response to exogenous auxin is inhibition of primary root elongation (Estelle and Somerville, 1987). To determine whether this response was also affected by overexpression of TAC1, we grew seedlings on medium containing various concentrations of 2,4-D and measured root length. As shown in Figure 6, roots of the overexpressing tac1-1D line are more sensitive to intermediate concentrations of 2,4-D than the wild type. The role of TAC1 in modulating responses to auxin is further supported by the tac1-3 null line, whose roots are less sensitive to inhibition, even at the highest concentration tested (Figure 6).

Additionally, although the ability of TAC1 to activate telomerase was eliminated by iaaL in the tac1-1D iaaL double mutants, overexpression of TAC1 partially rescued the short root phenotype associated with the low auxin concentration in iaaL plants (Figure 4C). The results presented here indicate that TAC1 is part of the previously described pathway linking auxin signaling and telomerase expression in plants (Tamura et al., 1999; Yang et al., 2002) and are consistent with a model where TAC1 potentiates some of the myriad responses to auxin.

DISCUSSION

Despite the evolutionary and developmental divergence between Arabidopsis and humans, overall patterns of telomerase expression are similar in these two multicellular organisms. The highly flexible pattern of plant development might have needed a broader pattern of telomerase expression, but this is not the case (Fitzgerald et al., 1996; Heller et al., 1996; Kilian et al., 1998; Riha et al., 1998). Suppression of telomerase activity in somatic tissues may provide a selective advantage for mammals because constitutive expression of TERT leads to an increased incidence of cancer in transgenic mice (Artandi et al., 2002; Canela et al., 2004). Because plants, including our tac1 mutants, do not form tumors (except in response to specialized bacterial, viral, and insect pathogens), it is not clear whether suppression of telomerase in vegetative tissues provides any selective advantage. It is possible that telomerase was initially suppressed stochastically and, in the absence of any negative consequences, has remained suppressed in most tissues. Of course, the plants must retain a mechanism to activate this essential enzyme when needed.

In this study, we exploited some of the transgenic tools available for Arabidopsis to identify genes that control expression of telomerase. Using the PCR-based TRAP assay, we screened ~8000 activation-tagged lines of Arabidopsis and recovered three mutants that expressed telomerase in their leaves (Figure 1A). Activation-tagged populations of Arabidopsis have been examined for mutants many times before, but usually only by visual inspection for developmental abnormalities. The recovery of our tac1-1D mutant indicates that biochemical analysis of this mutant population also can be successful, particularly where a high signal-to-noise ratio permits screening in large pools.

Overexpression of TAC1, a SUPERMAN-Like Zinc-Finger Protein, Induces Telomerase Activity

TAC1, the gene responsible for activating telomerase in our mutant, encodes a member of the plant-specific
SUPERMAN-like family of transcription factors. The 29 SUPERMAN-like proteins encoded by the Arabidopsis genome contain a single C2H2 zinc-finger domain and a Leu-rich domain near the C terminus (Dinkins et al., 2002). Because a zinc-finger domain can bind only three nucleotides, most zinc-finger transcription factors characterized from other organisms use multiple zinc fingers to increase the specificity of interactions with DNA (Pavletich and Pabo, 1991). SUPERMAN, and presumably other members of this single-zinc-finger family, use basic amino acids on either side of the canonical-finger domain to stabilize DNA binding (Dathan et al., 2002). The only other conserved feature of this family, the C-terminal Leu-rich domain, is an active transcriptional repressor (Ohta et al., 2001; Hiratsu et al., 2003) and is essential for normal function of SUPERMAN (Hiratsu et al., 2002). In TAC1, this domain is not required for the activation of telomerase because it has been removed in tac1-2D and the TAC1-139 construct. Complete elimination of TAC1 mRNA in the tac1-3 null mutant has no apparent effect on telomerase expression, implying that TAC1 may activate telomerase in wild-type plants under conditions we did not examine. Alternatively, another protein, possibly one of the other members of the SUPERMAN-like family, provides a redundant function.

**TAC1 Is Activated over a Long Distance**

In theory, activation tagging depends on the ability of multiple copies of the strong, constitutive CaMV 35S enhancer carried by the T-DNA to override endogenous transcriptional control of genes in the vicinity of the insertion. Somewhat surprisingly, genes near the T-DNA insertion in tac1-1D appeared to be immune to the effects of the 35S enhancer. Instead, the enhancers activated a gene ~80 kb away in this line. DNA gel blots of genomic DNA from the mutant and the wild-type plants showed no deletions or rearrangements that would have moved TAC1 closer to the enhancers in the mutant. Multiple copies of enhancers from other pararetroviruses can activate promoters from more than 175 kb away (Flajolet et al., 1998), and there are many examples of long distance activation of promoters by distant enhancers in a variety of other species, from Drosophila (Merli et al., 1996; Calhoun and Levine, 2003) to humans (Nobrega et al., 2003). Nevertheless, most reports of activation tagging in Arabidopsis indicate that the activated genes are near or adjacent to the enhancers (Weigel et al., 2000). The discrepancy between our results and those previously described for this population of tagged lines raises the possibility that distal activation is more prevalent than previously recognized because the relatively simple cases of proximal activation are easier to discern and report. Intriguingly, none of the genes between TAC1 and the T-DNA were activated in tac1-1D, implying that only a small subset of promoter sequences or chromatin conformations are susceptible to activation by the 35S enhancer.

**A Second Activation-Tagged Allele from an Unexpected Source**

Our tac1-2D line, from the SALK collection (Alonso et al., 2003), harbors a T-DNA insertion in the 3′ end of the coding region that removes the last 33 codons in the coding region including the EAR repressor domain. We initially investigated this line as a potential tac1-null mutant but found that it expresses telomerase in leaves similar to the tac1-1D mutant. Presumably, the single enhancer in the 35S promoter in the pROK2 T-DNA (Baulcombe et al., 1986) activates transcription from the TAC1 promoter, and the truncated TAC1 protein that results from this allele is capable of activating telomerase without the repressor domain. The position of the T-DNA in the 3′ end of the coding region eliminates the possibility that transcription of TAC1 is the result from read-through directly from the 35S promoter or the

---

**Figure 6. Auxin Sensitivity of tac1 Mutants.**

Elongation of primary roots from the overexpressing line tac1-1D are slightly more sensitive to inhibition by auxin than wild-type roots. Roots from the tac1-3 T-DNA insertion line are more resistant to auxin than wild-type roots. Asterisks indicate significant difference ($\alpha = 0.05$) from the wild type at each concentration. Double asterisks indicate significance at $\alpha = 0.01$. Error bars indicate SD.
ARFs are usually associated with Aux/IAA repressor proteins. Only four different ARF–Aux/IAA pairs are diagrammed, but the Arabidopsis genome encodes 23 ARFs and 29 Aux/IAA proteins. As indicated in the top panel, when auxin levels increase, the Aux/IAA proteins are targeted for ubiquitin-mediated degradation, thereby releasing the ARFs to modulate gene expression and trigger the whole range of auxin responses. TAC1, as indicated in the bottom panel, may act by disrupting one, or a very few, of the ARF–Aux/IAA pairs, leading to a limited set of auxin responses, including telomerase induction. Most of the ARFs remain bound to their repressors. It is also possible that TAC1 partially destabilizes other ARF–Aux/IAA pairs, allowing them to fully dissociate at lower concentrations of auxin than they normally do. This feature could explain the potentiating effect of TAC1. Low levels of auxin, as seen in the iaaL background, block the effects of TAC1.

Figure 7. Model for TAC1 Action.

Constitutive Expression of Telomerase Has Little Effect on Development

The higher levels of mRNA for \textit{AtTERT} in leaves of mutants that overexpress TAC1 are likely to be the proximal cause for the increase in telomerase activity in \textit{tac1-1D} and \textit{tac1-2D}. In mammals, overexpression of TERT alone is sufficient to confer telomerase activity on normally telomerase-deficient cells (Bodnar et al., 1998). This constitutive expression of telomerase confers apparent immortality to some cell types (Bodnar et al., 1998) but can lead to increased incidence of cancer in transgenic mice (Artandi et al., 2002; Canela et al., 2004). There are no obvious defects in Arabidopsis plants that constitutively express telomerase. Root length, shoot to root mass ratios, and the frequency of lateral root initiation was similar between \textit{tac1-1D} and wild-type plants. Even telomere length is unaltered from the wild type (Figure 1C). A similar situation is seen in telomerase-positive immortalized human cells (Counter et al., 1992; Kim et al., 1994), where telomeres are not perceptibly extended. Telomere lengths remain stable under these conditions because telomerase access is regulated by a suite of telomere binding proteins (reviewed in Vega et al., 2003).

Activation of Telomerase by TAC1 Is Uncoupled from Cell Cycle Progression

Previous reports have linked telomerase expression in plants to progression through the cell cycle, specifically indicating that telomerase is active during late S phase (Tamura et al., 1999; Yang et al., 2002). Overexpression of TAC1 appears to uncouple activation of telomerase from the cell cycle because the number of cells in S phase is not increased in the activation-tagged line, and the shoots do not show developmental defects described for plants with continuous cell division (Dewitte et al., 2003; Yu et al., 2003). Some of these defects may be avoided if cells progress through S phase without subsequent division, but ploidy levels are unchanged in \textit{tac1-1D}, indicating that endoreduplication is unaffected. Thus, TAC1 has the ability to induce telomerase expression in fully differentiated, mature tissues, without triggering dedifferentiation or activating the cell cycle.

TAC1 Potentiates Some Responses to Auxin

Telomerase activity appears earlier in S phase and obtains higher levels in cells treated with exogenous auxin (Tamura et al., 1999; Yang et al., 2002). Several results presented here imply that TAC1 is part of this auxin–telomerase link and may act by altering at least part of the auxin-signaling pathway. First, callus from plants overexpressing TAC1 grows vigorously in the absence of exogenous auxin. Second, induction of telomerase by increased TAC1 expression can be blocked by reducing the auxin concentration in a \textit{tac1-1D iaaL} double mutant. Third, TAC1 expression affects inhibition of primary root elongation by exogenous auxin. TAC1-overexpressing plants are more sensitive to auxin, whereas TAC1-null plants are less sensitive. We did not quantitate auxin levels in our mutants; however, the normal appearance of \textit{tac1-1D} plants, especially in contrast with high-auxin phenotype in \textit{yucca} mutants (Zhao et al., 2001), suggests that auxin perception, rather than biosynthesis, is disturbed by overexpressing TAC1. The effects of TAC1 appear to be limited to a subset of auxin responses because the expression of several auxin-inducible genes is unaffected in a \textit{tac1-1D} background.

The exact mechanism by which TAC1 potentiates a small subset of responses to auxin is not yet known, but we favor a model where a branch of the auxin-signaling pathway leading to telomerase expression becomes activated, whereas most other aspects of auxin signaling remain inactive. Auxin responses are mediated by auxin responsive factors (ARFs), transcription factors that bind to auxin responsive elements upstream of target genes. In the presence of wild-type concentrations of auxin, the ARFs are associated with inhibitory Aux/IAA proteins. When auxin concentration increases, Aux/IAA proteins are ubiquitinated and targeted for destruction by proteasomes,
thereby releasing the ARFs from inhibition (reviewed in Leyser, 2002). The Arabidopsis genome encodes 23 ARFs and 29 Aux/IAA proteins (Remington et al., 2004). TAC1 may act by disrupting interactions between a limited number of ARFs with their repressors (Figure 7). Twenty-seven of the 29 Aux/IAA proteins contain an EAR domain (Tiwari et al., 2004), as does TAC1. It is possible that high levels of TAC1 allow it to compete for proteins that bind to the EAR domain on Aux/IAA proteins, although our tac1-2D mutant and 3SS: TAC1 transgenic plants indicate that telomerase induction does not require the EAR domain of TAC1. In addition to fully releasing an ARF to activate telomerase expression, TAC1 may also act to partially destabilize other ARF–Aux/IAA pairs. This partial destabilization would not by itself activate auxin responses, but it may potentiate the responses and allow them to occur at lower auxin concentrations than they would in the absence of TAC1.

In summary, we find that overexpression of TAC1, in the presence of wild-type levels of auxin, is sufficient to induce expression of telomerase in fully differentiated, noncycling cells and to uncouple telomerase activation from cell cycle progression. Neither TAC1 nor increased endogenous auxin concentrations alone are sufficient for this induction, suggesting that TAC1 is involved in a branch of the auxin-signaling pathway that regulates telomerase. Characterization of additional telomerase-activated mutants should help to clarify the pathways that control expression of this essential enzyme in plants.

METHODS

Plant Material

Activation-tagged lines (Weigel et al., 2000) and the axr1-3 axr4-2 mutant were purchased from the ABRC (Columbus, OH). Our tac1-2D line, also obtained from the ABRC, was generated as part of the Salk T-DNA insertion collection and originally designated SALK_013353 (Alonso et al., 2003). The TAC1-null line, tac1-3, was purchased from the GABI-Kat collection (Rosso et al., 2003), where it was designated GABI_368A07. Seeds for the yucca mutant (Zhao et al., 2001) and the iaaL transgenic plant (Romano et al., 1991) were generously provided by Yunde Zhao. DRS:GUS (Umasov et al., 1997) seeds were kindly supplied by Thomas Guilfoyle. GUS activity was measured as described by Jefferson et al. (1987). Statistical significance of the differences was evaluated by t tests.

Mutant Screen

Activation-tagged plants were screen in pools of 10, 12, or 20 independently transformed lines. Populations of 50 to 100 plants from each pool were grown in soil in a growth chamber with a 20-h-light (22°C), 4-h-dark (18°C) cycle. Leaves from all plants in the pool were harvested in bulk before flowering. Modified TRAP assays were performed as previously described (Fitzgerald et al., 1999). Leaves from pool CS20857 contained telomerase activity, and assays were repeated on individual plants to find the tac1-1D mutant.

Identification of TAC1

The T-DNA and flanking plant DNA were recovered by plasmid rescue, as described by Weigel et al. (2000). DNA sequencing of the recovered plasmid indicated that the T-DNA had inserted into the portion of the genome contained on the BAC T14B5, which we obtained from the ABRC. BAC DNA was digested with EcoRI and subcloned into pBlue-script KS+ (Stratagene, La Jolla, CA). Individual EcoRI subclones were prepared, cleaved with BarnHI, electrophoresed on 1% agarose gels, and blotted onto Hybond N+ membranes (Amer sham, Piscataway, NJ).

Total RNA was prepared from wild-type and tac1-1D leaves with TRIzol (Sigma Chemical, St. Louis, MO) according to the manufacturer’s protocol. Five micrograms of total RNA was radiolabeled by reverse transcription with Superscript II (Invitrogen, Carlsbad, CA) in the presence of 50 μCi [α-32P]dGTP (Perkin-Elmer, Wellesley, MA). Radiolabeled first-strand cDNA was added to the membrane and allowed to hybridize overnight. The filter was washed in successive baths of 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 1× SSC, and 0.5× SSC, all containing 0.1% SDS for 15 min, each at 65°C. The filter was dried and analyzed on a Fuji phosphor imager (Tokyo, Japan). One BarnHI fragment hybridized to cDNA from tac1-1D but not to cDNA from the wild type. Sequencing of this fragment indicated that it came from the At3g09290 locus that we designated TAC1.

RT-PCR

RT-PCR reactions were performed in 25 μl with one step Superscript II RT-PCR kit from Invitrogen. Reverse transcription was initiated with an oligo(dT)19 primer and proceeded at 42°C for 45 min. The reaction was stopped by incubation at 75°C for 15 min. PCR was performed with gene-specific primers using 1 μl of the RT mixture as template under the following conditions: 94°C, 3 min, followed by 20 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 45 s) and a final elongation period of 10 min at 72°C. ActIN cDNA was amplified with Tst 5 and Tst 48 primers, and GAPDH cDNA was amplified with forward and reverse primers as previously described (Fitzgerald et al., 1999). TAC1 cDNA was amplified with forward (5\'ATGGAAAAATGAATGGAAGAA3\') and reverse (5\'-ATCGGAGCAAAAGGAATTTGAGA-3\') primers. Products were resolved by electrophoresis on 1.5% agarose gels and detected by DNA gel blotting.

RNA Gel Blot Analysis

Leaves from 3-week-old tac1-1D and wild-type plants were grown in MS medium and treated with 20 μM IAA for 1 h with shaking at 100 rpm. Total RNA was isolated from Tri reagent treated and untreated leaves. Fifteen micrograms of total RNA was separated on a 1.2% agarose gel and transferred to a Hy bond N+ membrane. Blots were probed with 32P-labeled PCR products from IAA2, IAA4, IAA8, and IAA12 genes (Abel et al., 1995). As loading control, the membrane was stripped and re-probed with 32P-labeled GAPDH gene.

Construction of Transgenic Plants

Both full-length and truncated versions (first 139 codons only) of the TAC1 coding region were amplified by PCR, sequenced, and cloned into pBluescript II (Jefferson et al., 1987) between the BamHI and SacI sites replacing the GUS coding region of the original vector. Binary vectors were transformed into Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983) and used to transform Arabidopsis thaliana by the floral dip method (Clough and Bent, 1998).

Callus and Root Growth

Seeds from tac1-1D and wild-type plants were sterilized in 20% household bleach for 20 min, rinsed five times with sterile water, and germinated on MS medium. Hypocotyls were excised from 6-d-old plants and transferred to MS medium supplemented with 2 mg of 2,4-D and 0.05 mg of kinetin per liter. After initiation, callus was transferred to the
same medium without 2,4-D and maintained for 4 weeks. Then, callus was transferred to fresh MS medium lacking 2,4-D and maintained in the dark at 23°C. Photographs were taken after an additional 4 weeks of auxin-independent growth.

Seeds were analyzed for response to auxin on MS medium containing various concentrations of 2,4-D from 10 pM to 100 nM. Primary root length was measured after 6 d of growth. Statistical significance of the differences was evaluated by t tests.

Flow Cytometric Analysis of DNA Content and Cell Cycle

Healthy, young leaves from age-matched wild-type and tac1-1D plants were manually chopped using a razor blade to release nuclei into ice-cold buffer as described (Johnston et al., 1999). The chopped leaves were filtered through a 53-μm nylon filter to recover 1 mL, which was kept on ice. Propidium iodide was added to samples to a final concentration of 50 ppm and the mixture maintained in the dark at 4°C for 1 to 2 h. The mean fluorescence of stained nuclei was quantified using a Coulter Elite flow cytometer (Beckman Coulter, Fullerton, CA) with a laser tuned at 514 nm and 300 mW. Fluorescence at >615 nm was detected by a photo-multiplier screened by a long-pass filter. Cell cycle analysis was based upon the unagated propidium fluorescence of no fewer than 25,000 nuclei (no fewer than 3500 from each of six independently analyzed leaves). The proportion of cells in S phase between the G1 and the combined G2/M phases for each sample was calculated using the Multicycle program (Phoenix Flow Systems, San Diego, CA).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number NM_111763.

ACKNOWLEDGMENTS

We thank Yunde Zhao for yucca and iaaL seeds, the ABRC for activation-tagged seeds and seeds for the axt1-3 axt4-2 mutant, the Salk Institute Genomic Analysis Laboratory for tac1-2D (SALK_013353), the GABI-Kat group for tac1-3 (GABI_388A07), and C.K. Becker for help with analysis flow cytometry data. This work was funded by Geron Corporation, the National Science Foundation (MCB 0244159 to T.D.M. and MCB 0233987 to D.E.S.), and the Texas Advanced Technology Program.

Received June 9, 2004; accepted August 17, 2004.

REFERENCES


Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2002). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J. 34, 733–739.


TELOMERASE ACTIVATOR1 Induces Telomerase Activity and Potentiates Responses to Auxin in Arabidopsis
Shuxin Ren, J. Spencer Johnston, Dorothy E. Shippen and Thomas D. McKnight

Plant Cell 2004;16;2910-2922; originally published online October 14, 2004;
DOI 10.1105/tpc.104.025072

This information is current as of July 9, 2017