

# Inhibition of plant telomerase by telomere-binding proteins from nuclei of telomerase-negative tissues

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**Abstract** The activity of telomerase in plant cells is precisely regulated in response to changes in cell division rate. To explore this regulatory mechanism, the effect on telomerase activity of protein extracts from nuclei of telomerase-negative tissues was examined. An inhibition of telomerase activity was found which was species-non-specific. This inhibition was due to proteins which form salt-stable, sequence-specific complexes with the G-rich telomeric strand and reduce its accessibility, as shown by gel retardation and by terminal transferase (TdT) extension of G-rich telomeric and non-telomeric (substrate) primers. A 40 kDa polypeptide was detected by SDS-PAGE after cross-linking the complex formed by extracts from tobacco leaf nuclei. Such proteins may be involved in regulation of telomerase activity in plants.

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**Key words:** Plant telomerase regulation; Telomere-binding protein; Telomerase inhibition

## 1. Introduction

Telomeres, nucleoprotein structures at the ends of linear eukaryotic chromosomes, are essential for maintaining the integrity of the genome by protecting chromosomes against end-to-end fusion, recombination and exonucleolytic degradation. As the functional components of chromosomes, telomeres allow complete replication of the chromosome ends [1,2] and represent a biological clock that determines lifespan [3]. Telomeres seem to be associated with the nuclear matrix and might play a role in nuclear architecture [4,5]. In most eukaryotes, telomeric DNA consists of a tandemly repeated short G-rich motif on the strand oriented from centromere to telomere, e.g. TTAGGG in humans [6] or TTTAGGG in most higher plants studied [7].

Telomeres are synthesised and maintained by the action of telomerase, a ribonucleoprotein complex with reverse transcriptase activity. Their maintenance on human chromosomes strongly correlates with oncogenic potential and the capacity for cellular proliferation [8]. As telomerase activity is repressed in almost all differentiated human cells their replicative capacity is limited by gradual telomere shortening. In

contrast, plant cells possess the ability to reversibly regulate their telomerase activity according to the cell division rate which results in a stable telomere length and a virtually unlimited lifespan [9,10].

In all organisms examined to date, the telomeric G-rich strand, oriented in the 5' to 3' direction towards the end of the chromosome, terminates in a single-stranded 3' overhang [11,12], whose length varies from 16 nucleotides in *Oxytricha* to 50–100 nucleotides in mouse and human telomeres. The biological relevance of this overhang was demonstrated by studies showing that its disruption in mutants is connected with a loss of telomere function [13–15]. The G-rich DNA strand is capable of forming G-DNA, a four-stranded DNA structure under physiological conditions in vitro. Folding of telomere DNA into G-DNA inhibits elongation by telomerase in vitro, suggesting a possible inhibitory effect of G-DNA on telomere elongation in vivo [16].

The telomeric DNA is complexed with specific non-nucleosomal telomere-binding proteins (TBPs) in addition to nucleosomes. Two classes of TBPs have been identified: those that bind the double-stranded (ds) region of telomeric DNA and those that bind the single-stranded (ss) overhangs. The first class (dsDNA-TBP) includes, e.g. Rap1p from *Saccharomyces cerevisiae* [17,18], and two related mammalian proteins, TRF1 and TRF2 [19–21]. All these dsDNA-TBPs are able to affect telomere length [22]. Lack of TRF2 results in loss of the 3' overhang, end-to-end chromosome fusions and activation of p53, which mediates cell cycle arrest and apoptosis [14,23]. The recent discovery of t-loops suggests a possible mechanism by which TRF2 may affect the single-stranded overhang: TRF2 can remodel linear telomeric DNA into large duplex loops in vitro, whose size is proportional to the telomere length. Binding of TRF1 and SSB protein suggested that the t-loops are formed by invasion of the 3' overhang into the duplex telomeric repeat array [24]. The t-loop model for telomere structure proposes a solution to the protection and maintenance of telomeres in mammals.

The other class of telomere-binding proteins interacts with the single-stranded 3' overhang, forming very salt-stable complexes which may act as a molecular chaperone for G-quartet formation [25,26]. The best-characterised protein of this class is the  $\alpha/\beta$  heterodimeric protein from *Oxytricha* [27,28] whose  $\beta$  subunit, in contrast to the  $\alpha/\beta$  heterodimer, facilitates the formation of G-DNA [25,29]. This protein protects the telomeric overhang within a protein core and renders the 3' end inaccessible to telomerase, thereby participating in regulation of telomere length [30].

Although plant telomere sequences have been available for

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a number of years, telomere-binding proteins have not been extensively studied in plant systems. The first and so far only protein showing affinity to the dsDNA of plant telomeric sequence detected in *Arabidopsis thaliana* has an affinity for the telomeric dsDNA as well as for the G-rich ssDNA which is much higher than for C-rich ssDNA [31]. DNA–protein complexes of three types were identified by gel retardation assays using rice nuclear extracts, produced by binding proteins of at least three different proteins termed rice G-rich telomere-binding proteins (RGBPs) which, however, have not been further characterised. In contrast to the protein from *A. thaliana*, RGBPs show binding only to the G-rich DNA strand and no affinity to double-stranded telomere repeats or single-stranded C-rich sequences [32].

The nucleoprotein structures formed by these proteins may explain the remarkable stability of plant telomere lengths during ontogenesis [9]. Our knowledge of nucleoprotein complexes of plant telomeres is very poor and the effect of telomere-binding proteins on the synthesis of telomeric repeats by telomerase has not been analysed in plants so far.

Here we present results showing that plant cell nuclei contain telomere DNA-binding proteins which can inhibit telomerase activity by altering the accessibility of telomeric DNA, and thus may participate in telomere length regulation.

## 2. Materials and methods

### 2.1. Preparation of nuclear protein extracts containing TBPs

Cell nuclei were prepared according to Espinas and Carballo [33] from 10 g of fresh leaves of young *Silene latifolia* or *Nicotiana tabacum* plants. The nuclei were stirred in a small volume of buffer C (20 mM HEPES, 40 mM KCl, 5 mM MgCl<sub>2</sub>, 0.2 mM EDTA) containing 2 M NaCl, 0.5 mM PMSF and 1 mM DTT for 60 min on ice. The crude extract was clarified by centrifugation at 25 000 × g for 20 min at 4°C; the supernatant was extensively dialysed against buffer C containing 20% glycerol, 0.1 mM DTT and 0.01 mM PMSF and then was quickly frozen in liquid nitrogen and stored at –70°C.

### 2.2. Preparation of plant cell extracts containing telomerase

Cell extract from *S. latifolia* seedlings was prepared as described previously by Fitzgerald et al. [34]. Approximately 1 g of material was ground in liquid nitrogen, suspended in 4 ml of buffer W (50 mM Tris–acetate pH 7.5, 5 mM MgCl<sub>2</sub>, 100 mM potassium glutamate, 20 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 0.6 mM vanadyl ribonucleoside complex (NEB), 1.5% (w/v) polyvinylpyrrolidone, 10% glycerol) and centrifuged at 16 000 × g for 15 min at 4°C. The supernatant was completed with PEG 8000 (Sigma) to a final concentration of 10%, stirred for 30 min at 4°C, and centrifuged at 20 000 × g for 5 min at 4°C. The pellet was resuspended in 1 ml of buffer W for 30 min on ice and centrifuged at 20 000 × g for 2 min at 4°C. The supernatant was stored at –70°C until use. The protein concentration in nuclear and cell extracts was determined by the Bradford procedure [35].

### 2.3. Oligonucleotides

Synthetic deoxyoligonucleotides were purified on 20% polyacrylamide gels. The oligonucleotide PtelG [TTAGGG]<sub>6</sub> was used as ssDNA probe for gel retardation assays. The oligonucleotide CaMV35S (5′-CGTCTCAAAGCAAGT GGATT-3′) was used as telomerase substrate [10] in the telomere repeat amplification protocol (TRAP). The C-telomeric primer TP (5′-CCGAATTCAACCTAAACCCTAAACCCTA ACCCC-3′) [36] was used in the PCR step of TRAP. The amplification of the control fragment ROMAN2 was done with TS(21) (5′-GACAATCCGTCGAGCAGAGTT-3′) [34]. For gel retardation assay, gel-purified oligonucleotides were 5′-end labelled using γ-[<sup>32</sup>P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs) following standard protocol, and were purified with a QIAquick nucleotide removal kit (Qiagen).

### 2.4. Detection of telomerase activity

A version of TRAP modified for plant telomerase was used [10,34]. Telomerase assay buffer contained 50 mM Tris–acetate pH 8.3, 50 mM potassium glutamate, 0.1% Triton X-100, 1 mM spermidine, 1 mM DTT, 50 μM of each dNTP, 5 mM MgCl<sub>2</sub>, 10 mM EGTA, 100 μg/ml bovine serum albumin (BSA). The telomerase substrate primer CaMV35S was denatured for 3 min at 95°C and cooled on ice prior to addition to reactions. The reaction mixtures, composed of telomerase assay buffer (48 μl), 1 pmol of primer CaMV35S and nuclear extracts containing TBPs (0.5–25 μg of protein), were combined on ice. After addition of telomerase-containing extract (50 ng of protein), samples were incubated at 26°C for 45 min in a thermocycler. After the elongation step of TRAP, reaction mixtures (including those without nuclear protein extracts) were purified using a QIAquick nucleotide removal kit (Qiagen) to avoid possible interference of protein extracts with DNA polymerase in the subsequent PCR step. The extension products were amplified by 25 cycles of hot-start PCR at 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s, using 10 pmol of CaMV35S primer, 10 pmol of TP primer and 2 U Dynazyme II DNA polymerase (Finnzymes) in each reaction. The products were separated on a 12.5% polyacrylamide gel which was stained with Vistra Green (Amersham) and scanned on a STORM PhosphoFluorImager (Molecular Dynamics). Analogous methodology was used when BSA or cytochrome *c* was used instead of nuclear extracts. To detect possible inhibition of DNA polymerase in the PCR step, 1 amol of control template ROMAN2 [10] was used in a parallel reaction. Formation of protein–DNA complexes in telomerase assay buffer was confirmed by gel mobility shift assays.

### 2.5. Gel mobility shift assays

In order to reduce non-specific DNA–protein binding interactions, nuclear protein extract was pre-incubated with an equal amount of salmon sperm DNA in DNA-binding buffer (10 mM Tris–HCl pH 8, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM DTT, unless otherwise indicated) for 20 min on ice. End-labelled DNA probe (1 pmol) was then added to reaction mixtures and incubated with the nuclear protein extract for 20 min on ice. The DNA–protein complexes were resolved by electrophoresis on 6% polyacrylamide gel (acrylamide:bisacrylamide 47:1) in 0.5 × TBE at 10 V/cm for 2.5 h. Gels were dried and subjected to autoradiography. For UV cross-linking in gels (see Section 2.6), the drying step was omitted.

### 2.6. UV cross-linking in gel

After the gel mobility shift assay, the gel was placed on ice and irradiated for 45 min from a distance of 10 cm by a UV lamp (312 nm), the positions of the free and shifted probe were visualised by autoradiography, and bands corresponding to both free and complexed probe were excised. The gel slices were boiled for 5 min in 1.5 ml of a modified SDS–PAGE sample buffer (1% SDS, 3 mM DTT, 125 mM Tris–HCl pH 6.8 [37]) and placed in the stacking gel of a 12% SDS–PAGE gel [38]. Covalently bound DNA–protein complexes were separated from free probe and the gel was dried and autoradiographed.

### 2.7. Terminal transferase (TdT) assay

Prior to the assay, formation of DNA–protein complexes in TdT buffer was tested by a modified gel mobility shift assay. The nuclear protein extract was incubated with 1 pmol of end-labelled DNA oligonucleotide in 1 × TdT buffer (New England Biolabs) for 30 min on ice, and then loaded onto 6% polyacrylamide gel (see Section 2.5). Salmon sperm DNA (25 μg) was used as non-specific competitor. In order to investigate the effect of protein–DNA complexes on the efficiency of TdT extension, the nuclear protein extract was incubated with 10 pmol of substrate oligonucleotide in 1 × TdT buffer for 30 min on ice. 10 units of TdT (New England Biolabs) and 0.4 μl α-[<sup>32</sup>P]dATP (3000 Ci/mmol, 10 mCi/ml; Amersham) were added to a final volume of 40 μl, and the reaction mixture was placed at 37°C for 1 h. Elongation products were separated from proteins and non-incorporated nucleotides by QIAquick nucleotide removal kit (Qiagen), and resolved on 8% sequencing gel, which was then dried and autoradiographed. Non-specific DNA–protein binding interactions was reduced by pre-incubation of the nuclear extract with supercoiled DNA (250 μg) prior to addition to reaction.

### 3. Results

#### 3.1. Detection of complexes between the G-rich telomeric strand and proteins in nuclear extracts

We assayed for the ability of TBPs in nuclear extracts to form complexes with the telomeric G-rich strand by gel mobility shift assays. Three sequence-specific complexes were detected between end-labelled G-rich telomeric strand (PltelG) and proteins from *S. latifolia* leaf nuclei, whereas a single tobacco complex was formed. The intensities of the shifted bands seen as complexes that migrated more slowly than the free probe, increased with increasing amounts of nuclear extract (Fig. 1). To evaluate the salt sensitivity of TBP binding, nuclear extracts were incubated with end-labelled PltelG in the presence of increasing concentrations of NaCl in DNA-binding buffer. These experiments showed the resistance of the complexes to high salt concentrations (0.6–1.0 M NaCl) (Fig. 2A).

#### 3.2. UV cross-linking of tobacco TBP to G-rich strand DNA

To estimate the apparent molecular mass of the protein in the single tobacco complex formed with tobacco nuclear extracts (Fig. 1), UV cross-linking of the complex was performed in the gel. After brief autoradiography, the cross-linked complex was excised from the mobility shift gel, denatured, and subjected to SDS-PAGE, when proteins covalently linked to DNA usually migrate with the same mobility as the protein alone [39]. This assay showed a prominent single polypeptide band with an apparent molecular mass of 40 kDa. As controls, gel slices from the same region of a lane without TBPs, and from the region corresponding to the free probe showed no 40 kDa band (Fig. 2B).

#### 3.3. TBPs from telomerase-negative tissues inhibit telomerase activity

To investigate whether the formation of these complexes affects telomerase activity, we examined telomerase activity

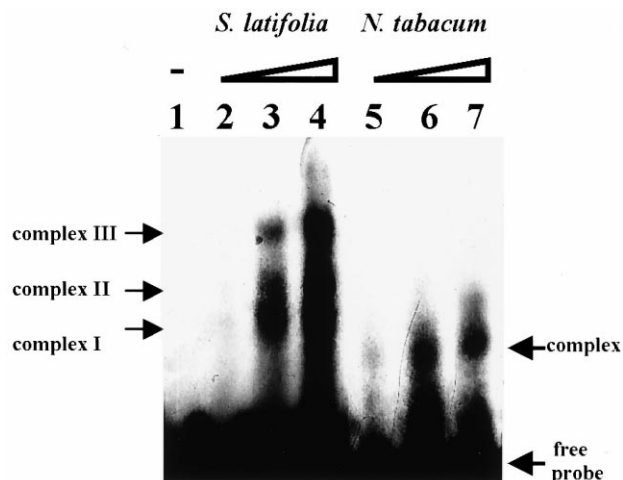


Fig. 1. Detection of G-rich telomeric strand binding activities in nuclear extracts from *S. latifolia* and *N. tabacum* leaves. Gel mobility shift assays in DNA-binding buffer were performed with 1 pmol of  $^{32}$ P-end-labelled PltelG as a probe and increasing amounts of nuclear extracts from *S. latifolia* (lanes 2–4) and *N. tabacum* (lanes 5–7). Both sets of lanes show reactions with nuclear extracts at protein concentrations of 1, 5, 10  $\mu$ g per assay. Lane 1 contained probe alone. Salmon sperm DNA (25  $\mu$ g) was used in all reactions as non-specific competitor.

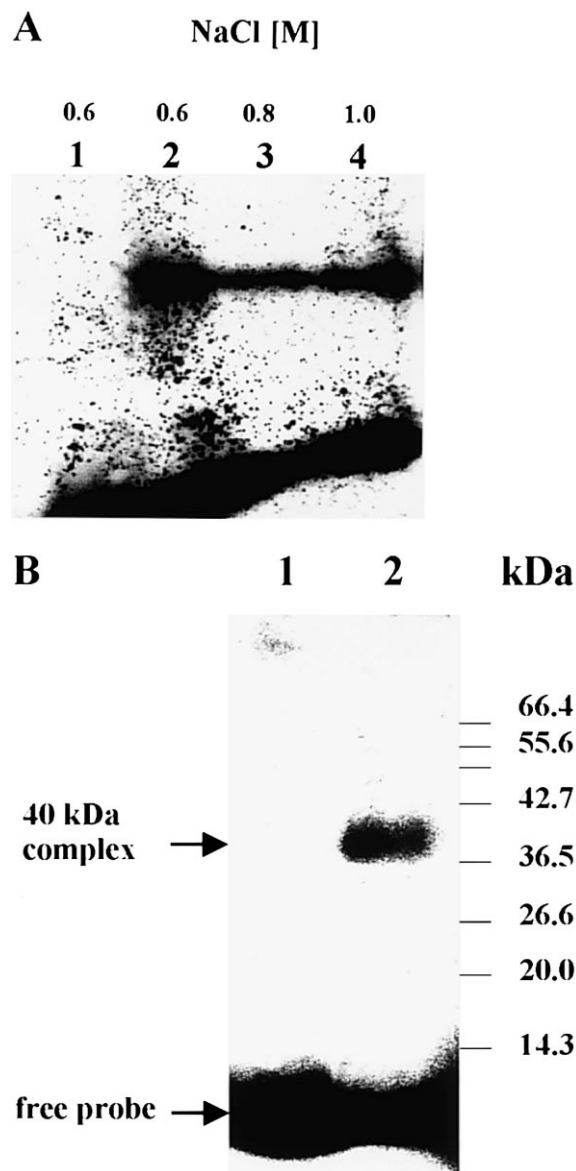


Fig. 2. Characterisation of the complex formed by tobacco TBP with PltelG. A: To evaluate the salt sensitivity of the complex, nuclear extracts were incubated with end-labelled PltelG in the presence of 0.6, 0.8, 1.0 M NaCl (lanes 2–4) in DNA-binding buffer. Lane 1 contained free probe alone in the presence of 0.6 M NaCl in DNA-binding buffer. Salmon sperm DNA (25  $\mu$ g) was used as non-specific competitor. B: To estimate the apparent molecular mass of the single tobacco complex, UV cross-linked complex was excised from the mobility shift gel and subjected to SDS-PAGE (lane 2). Lane 1, control gel slice from the region corresponding to free probe was also analysed. Protein size markers are indicated on the right in kDa.

in extracts from *S. latifolia* seedlings upon addition of nuclear extracts from telomerase-negative tissues. Telomerase added multiple repeats to the non-telomeric substrate CaMV35S primer in the absence of these extracts but this activity was reduced in the presence of nuclear protein extracts from either *S. latifolia* or *N. tabacum* leaf nuclei; increasing amounts of nuclear extract resulted in a corresponding decrease in telomerase activity (Fig. 3). This inhibition occurred during the elongation step, because proteins were eliminated after this step (see Section 2.4) and controls with the template RO-

MAN2 showed no inhibition of the PCR step even at the highest concentration of TBPs. To test whether this inhibition of telomerase activity is specific for TBPs in nuclear extracts from leaves, analogous TRAP assays were performed with 25  $\mu$ g of either BSA or the more basic protein cytochrome *c*. These proteins showed no effect on telomerase activity (Fig. 3), although cytochrome *c* binds non-specifically to single- and double-stranded DNA [30]. These results show that the observed modulation of telomerase activity is specifically exerted by telomere-binding proteins, and further that these proteins are not species-specific.

### 3.4. TBPs are able to displace telomerase from its substrate

The formation of complexes between TBPs and the G-rich telomeric strand under the conditions of TRAP assays was verified by gel mobility shift assays using either the non-telomeric CaMV35S primer or the telomeric G-rich primer PtelG as probes. No complex was detected with the non-telomeric primer. The G-rich telomeric strand (PtelG) formed a significant fraction of complexes upon addition of 25  $\mu$ g of nuclear protein extracts, whereas in the presence of up to 10  $\mu$ g of nuclear proteins either only a trace amount or no complex was found (Fig. 4). This result suggests that telomere-binding proteins forming sequence-specific complexes with the G-rich strand had a lower binding affinity for PtelG under the conditions of the telomerase assay than in DNA-binding buffer. Consistent with the results of mobility shift assays, a significant reduction of telomerase activity occurred in TRAP assays only when sequence-specific complexes were formed between the G-rich strand and TBPs. Considering the results of TRAP

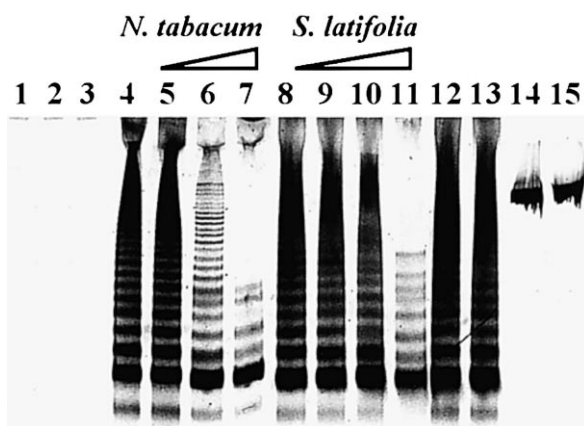


Fig. 3. Inhibition of telomerase by TBPs from leaf nuclei. A telomerase activity assay (see Section 2.4) was performed without (lanes 1–3, 14, 15) or with telomerase extract from *S. latifolia* seedlings (lanes 4–13). Lane 1 shows reaction without telomerase and nuclear extracts. Telomerase activity in the presence of nuclear extracts from *N. tabacum* leaves, 25  $\mu$ g (lane 2) or from *S. latifolia* leaves, 25  $\mu$ g (lane 3) was tested. The nuclear extracts either from *N. tabacum* (lanes 5–7) or *S. latifolia* leaves (lanes 9–11) were added during the extension phase of TRAP. Before the PCR step of reaction products (lanes 1–15) were purified using a QIAquick PCR purification kit. The following concentrations of nuclear proteins were used: 0  $\mu$ g (lanes 4, 8), 0.5  $\mu$ g (lanes 5, 9); 10.0  $\mu$ g (lane 6, 10); 25  $\mu$ g (lanes 7, 11). The addition of either 25  $\mu$ g of BSA (lane 12) or 25  $\mu$ g of cytochrome *c* (lane 13) instead of nuclear extract did not affect telomerase activity. The absence of inhibition of the PCR step was confirmed by amplification of the control template ROMAN2 in a parallel reaction in the presence of 25  $\mu$ g of nuclear proteins from *N. tabacum* leaves (lane 14) or 25  $\mu$ g of nuclear proteins from *S. latifolia* leaves (lane 15).

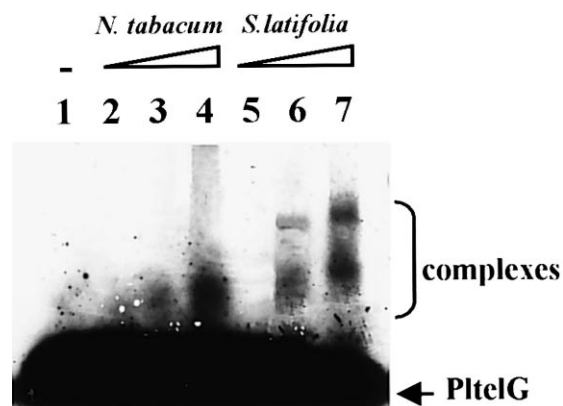


Fig. 4. Formation of nucleoprotein complexes with PtelG under the conditions of TRAP assays. The gel mobility shift assay was performed with 1 pmol  $^{32}$ P-end-labelled telomeric PtelG primer as probe that was incubated with nuclear proteins from *N. tabacum* leaves (lanes 2–4) or from *S. latifolia* (lanes 5–7). The following concentrations of nuclear proteins were used: 0  $\mu$ g (lane 1), 1  $\mu$ g (lanes 2, 5); 10  $\mu$ g (lane 3, 6); 25  $\mu$ g (lanes 4, 7). Salmon sperm DNA (25  $\mu$ g) was used as non-specific competitor.

assays using the non-telomeric primer together with the sequence specificity of the G-strand-binding proteins, we conclude that telomerase inhibition by the TBPs occurs only after initial addition of several telomeric repeats by telomerase, and thus that they are capable of displacing telomerase from its substrate.

### 3.5. TBPs inhibit primer extension by TdT

To further analyse the mechanism of the inhibition of telomerase activity by TBPs, their effect on terminal transferase extension of G-rich telomeric and non-telomeric primers was tested. Unlabelled primers were incubated with nuclear proteins extracted from leaves to form complexes followed by the addition of TdT and  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ . Nucleotide addition by TdT was monitored by denaturing gel electrophoresis (Fig. 5). An arrest of DNA extension was observed in the early stage of the TdT reaction, being more pronounced in the case of the telomeric primer. When a sub-saturating concentration of TBPs was used, the extension of excess primer molecules was not affected. Gel mobility shift assays confirmed formation of sequence-specific complexes only between G-rich strand and TBPs from *S. latifolia* and *N. tabacum* leaf nuclei under the conditions of the TdT reaction (Fig. 6). In these complexes, the access of TdT to the substrate G-strand is restricted. Since the formation of complexes modulates TdT extension as well as telomerase activity, it is evident that these proteins act by binding to the substrate rather than by a specific protein–protein interaction. This conclusion is further supported by the fact that inhibition of telomerase activity is not species-specific. Thus, we propose that the G-strand-binding proteins detected here regulate telomerase activity in vitro by controlling the accessibility of the telomeric DNA substrate.

## 4. Discussion

Telomerase activity in plant cells is precisely regulated in response to changes in division rate, resulting in developmentally stable telomere lengths in contrast to mammalian so-

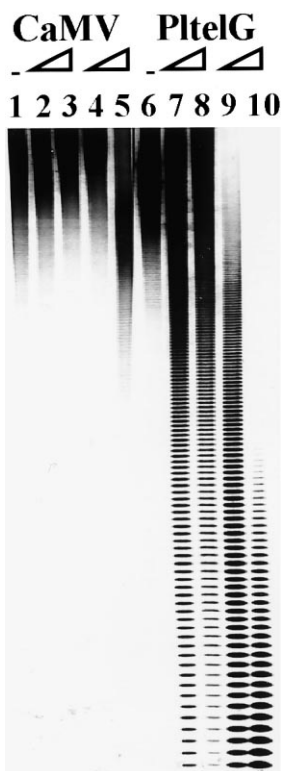


Fig. 5. TBPs affect the accessibility of TdT to non-telomeric (CaMV35S, lanes 1–5) and telomeric (PtelG, lanes 6–10) sequence primers. Equal concentrations of both primers were used throughout. In the absence of TBPs (lanes 1 and 6) as well as in the pre-incubation of non-telomeric primer CaMV35S with 1  $\mu$ g (lanes 2, 4) and 10  $\mu$ g (lanes 3, 5) of nuclear proteins from *S. latifolia* (lanes 2, 3) or from *N. tabacum* (lanes 4, 5) leaves, predominantly long extension products are produced by TdT. Pre-incubation of primers with 1  $\mu$ g (lanes 7, 9) and 10  $\mu$ g (lanes 8, 10) of proteins from *S. latifolia* (lanes 7, 8) or from *N. tabacum* (lanes 9, 10) leaf nuclei resulted in frequent abortion of extension at the early stage of reaction in PtelG (lanes 7–10). Formation of long extension products, apparently arising from excess primer, was not affected. Supercoiled DNA (250  $\mu$ g) was used as non-specific competitor.

matic cells. This stability has been observed in tomato, in *S. latifolia* and *N. tabacum* [9,10,40]. In human as well as in plant cells, telomerase activity is usually correlated with the expression of its catalytic subunit [41] and even telomerase-negative human cells express the RNA component of telomerase [42]. Tissue-specific, long-term regulation of telomerase activity in plant cells may also be achieved by regulated expression of the catalytic subunit, but further mechanisms appear to be necessary for the precise control of the extent and timing of telomerase action. At this level, modulation of the interaction of telomerase with telomeric DNA may be involved and could be mediated by proteins which specifically bind to telomeric DNA as well as by the conformation of telomeric DNA. Here we show that nuclear extracts from telomerase-negative tissues have an inhibitory effect on the activity of telomerase *in vitro*; in the presence of increasing amounts of nuclear extracts from leaf cells, telomerase activity in extracts from *S. latifolia* seedlings was decreased. Our data suggest that this modulation of telomerase activity is due to specific telomere-binding proteins, since we found sequence-specific complexes of proteins with the G-rich telomere strand after incubation with nuclear extracts from leaf cells. In the

case of tobacco extracts the apparent molecular mass of the polypeptide in the complex was 40 kDa, and the relatively high salt resistance of its DNA binding resembles that of the telomere end-binding proteins found in *Oxytricha*, *Euplotes*, and *Xenopus* (e.g. 2 M NaCl) [43–45]. The telomeres of *Pisum sativum* and *Vicia faba* are associated with the nuclear matrix [5] and it is plausible that plant telomere-binding proteins might be involved in nuclear architecture. Three complexes of TBPs with the telomeric G-strand were found here in *S. latifolia*, a ‘short telomere plant’, whereas a single complex was detected in tobacco, a ‘long telomere plant’, possibly reflecting differences in telomerase regulation between these species which we have previously observed [9,10,40]. Nuclear extracts from leaf cells of both species inhibited the ability of telomerase from *S. latifolia* seedlings to elongate the CaMV35S primer. The species-non-specific inhibition of primer extension by both telomerase and TdT indicates that the TBPs identified here act by binding to the DNA substrate rather than by a specific protein–protein interaction. Based on work with unicellular organisms, the telomeric nucleoprotein complex could sequester the ends of chromosomes by a capping function on the single-stranded 3’ overhang by stabilising G-quartet formation at the 3’ overhang of the G-rich strand [25–27,45–47]. The proteins may also participate in other aspects of telomere function. In *Oxytricha*, they may contribute to telomere length regulation by repressing telomerase [30] and in *S. cerevisiae*, the single-stranded telomeric DNA-binding protein Cdc13p is required to serve as a positive regulator of telomerase by mediating its access [48]. The recent discovery of t-loops suggests that in mammalian cells, telomere protection can be mediated by sequestering the G strand 3’ overhang inside a double-stranded DNA rather than by tenacious protein binding in mammalian cells [24], and both *S. latifolia* and *N. tabacum* have telomeres long enough (2.5–4.5 kb and 20–170 kb, respectively) to form t-loops but the actual conformation of their telomeres is unknown. In contrast, *Oxytricha* telomeres consist of just 20 bp of double-stranded telomeric repeats, far too short to form t-loops, and in species with such short telomeres the t-loop structure may be replaced by end-binding proteins which protect the ends and regulate telomere length.

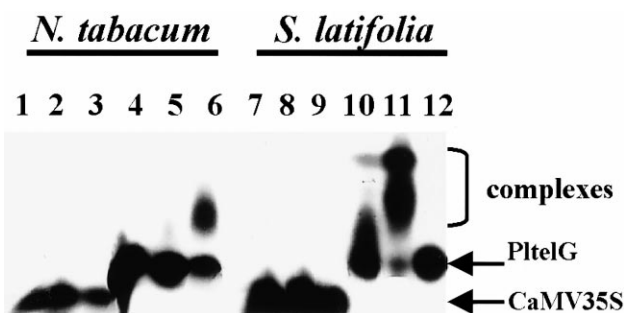


Fig. 6. Formation of sequence-specific nucleoprotein complexes in reaction conditions for TdT. The gel mobility shift assay was performed with 1 pmol  $^{32}$ P-end-labelled non-telomeric CaMV35S primer (lanes 1–3, 7–9) or telomeric PtelG primer (lanes 4–6, 10–12) as probe that was incubated with no nuclear extract (lanes 1, 4, 7, 12), with 1  $\mu$ g of nuclear proteins from *N. tabacum* leaves (lanes 2, 5) or from *S. latifolia* (lanes 8, 10), or with 10  $\mu$ g of nuclear proteins either from *N. tabacum* leaves (lanes 3, 6) or from *S. latifolia* leaves (lanes 9, 11). Salmon sperm DNA (25  $\mu$ g) was used as non-specific competitor.

In summary, the results presented here show that nuclei from telomerase-negative plant tissues contain factors which repress telomerase activity *in vitro*, and that TBPs which form sequence-specific complexes with the G-rich telomeric strand are candidates for this function.

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