Characterization of two *Arabidopsis thaliana* myb-like proteins showing affinity to telomeric DNA sequence

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Abstract: Telomere-binding proteins participate in forming a functional nucleoprotein structure at chromosome ends. Using a genomic approach, two *Arabidopsis thaliana* genes coding for candidate Myb-like telomere binding proteins were cloned and expressed in *E. coli*. Both proteins, termed AtTBP2 (accession Nos. T46051 (protein database) and GI:638639 (nucleotide database); 295 amino acids, 32 kDa, pI 9.53) and AtTBP3 (BAB08466, GI:9757879; 299 amino acids, 33 kDa, pI 9.88), contain a single Myb-like DNA-binding domain at the N-terminus, and a histone H1/H5-like DNA-binding domain in the middle of the protein sequence. Both proteins are expressed in various *A. thaliana* tissues. Using the two-hybrid system interaction between the proteins AtTBP2 and AtTBP3 and self interactions of each of the proteins were detected. Gel-retardation assays revealed that each of the two proteins is able to bind the G-rich strand and double-stranded DNA of plant telomeric sequence with an affinity proportional to a number of telomeric repeats. Substrates bearing a non-telomeric DNA sequence positioned between two telomeric repeats were bound with an efficiency depending on the length of interrupting sequence. The ability to bind variant telomere sequences decreased with sequence divergence from the *A. thaliana* telomeric DNA. None of the proteins alone or their mixture affects telomerase activity in vitro. Correspondingly, no interaction was observed between any of two proteins and the *Arabidopsis* telomerase reverse transcriptase catalytic subunit TERT (accession No. AF172097) using two-hybrid assay.

Key words: plant telomere-binding protein, two-hybrid assay, protein expression, telomerase.

Résumé : Les protéines qui se lient au télomère contribuent à former une structure nucléoprotéique fonctionnelle aux extrémités des chromosomes. À l'aide d'une approche génomique, deux gènes de l'*Arabidopsis thaliana*, codant pour des protéines de type Myb se liant putativement aux télomères, ont été clonés et exprimés chez *E. coli*. Les deux protéines, appelées AtTBP2 (acc. No. T46051 (banque de protéines), GI:638639 (banque de séquences nucléotidiques) ; 295 aa, 32 kDa, pI 9,53) et AtTBP3 (BAB08466, GI:9757879 ; 299 aa, 33 kDa, pI 9,88), contiennent un unique domaine de liaison à l'ADN de type Myb à l'extrémité N-terminale et un domaine de liaison à l'ADN de type histone H1/H5 au milieu de la protéine. Les deux protéines sont exprimées dans divers tissus chez l'*A. thaliana*. À l'aide de la technique double hybride, des interactions entre les protéines AtTBP2 et AtTBP3 ainsi que des interactions avec elles-mêmes ont été détectées. Des analyses de retard sur gel ont révélé que chacune des deux protéines est capable de se lier au brin riche en G ainsi qu'à l'ADN bicaténaire des séquences télomériques avec une affinité qui est proportionnelle au nombre de répétitions télomériques. L'interaction avec des substrats composés d'ADN non-télomérique situé entre des répétitions télomériques dépendait de la taille de cette séquence discordante. L'aptitude à se lier à des variants télomériques diminuait avec un accroissement de la divergence par rapport à la séquence de l'ADN télomérique chez l'*A. thaliana*. Aucune des deux protéines, seule ou en mélange, n'affectait l'activité de la télomérase in vitro. Pareillement, aucune interaction n'a été observée entre ces protéines et la sous-unité catalytique de l'activité transcriptase inverse (TERT; acc. No. AF172097) de la télomérase au moyen d'un essai double hybride.

Mots clés : protéine de liaison aux télomères chez les plantes, essai double hybride, expression protéique, télomérase.

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Introduction

Eukaryotic telomeres function as supramolecular nucleoprotein complexes of telomeric DNA, general chromatin proteins, and telomere-specific proteins. Besides telomerase, a ribonucleoprotein enzyme complex responsible for the resynthesis of incompletely replicated telomeres ends, many other specific proteins or their complexes have a role in telomere metabolism, e.g., to organise telomeres into protective capping structures, quadruplexes, or t-loops; to regulate access of telomerase to its substrate, the G-strand overhang; or to mediate anchoring of telomeres to the nuclear envelope. Another class of proteins also exists that does not associate with telomeric DNA directly, but interacts with DNA-binding proteins and modulates their affinity for telomeric DNA.

In yeast, protozoan, and mammalian cells, a number of proteins have been found that interact either with the single-stranded G overhang (e.g., α/β heterodimeric protein from *Oxytricha* (Gottschling and Zakian 1986; Price and Cech 1987), Cdc13p in *S. cerevisiae* (Nugent et al. 1996), and Pot1 in *S. pombe* and human (Baumann and Cech 2001)) or with double-stranded telomeric DNA (Rap1p in *S. cerevisiae* (Berman et al. 1986; Buchman et al. 1988), Taz1p in *S. pombe* (Cooper et al. 1997), and TRF1 and TRF2 in human and mouse (Bilaud et al. 1997; Broccoli et al. 1997; Chong et al. 1995)). All proteins known to bind to double-stranded telomeric DNA share a telomeric DNA-binding motif similar to the c-Myb family of transcriptional activators (Bilaud et al. 1996). The c-Myb proteins typically consist of three tandem repeats of the Myb DNA-binding motif (R1, R2, and R3) and at least two are required for sequence-specific binding (Tanikawa et al. 1993). But TRF1, TRF2, and Taz1p contain a single Myb-like domain at their C termini and predominantly bind to DNA as homodimers, whereas Rap1p possesses two Myb-like subdomains linked by a flexible region (Konig et al. 1996).

The importance of telomere-binding proteins for telomere architecture and telomerase regulation (see (Shore 1997) for review) has lead to the identification and partial characterization of several telomere-binding proteins in plants. The first such protein identified was found in *Chlamydomonas reinhardtii* specifically binding to the G-strand overhang (Petracek et al. 1994). The 34-kDa protein is called G-strand binding protein (GBP) and it binds two or more single-strand TTTTAGGG telomeric repeats.

Among higher plants, the first telomere-binding protein to be isolated was in *Arabidopsis thaliana* (Zentgraf 1995). This 67-kDa protein (ATBP1) binds to both single- and double-stranded $(TTTAGGG)_4$ oligonucleotides in vitro. Both kinds of DNA–protein complexes were salt resistant and insensitive to RNase. An additional protein of merely 22 kDa (ATBP2) was associated via protein–protein interaction with ATBP1 to form a higher-order complex exclusively during the onset of leaf senescence (Zentgraf et al. 2000). Single-strand telomere binding proteins have been identified in gel retardation assays of rice nuclear extracts (rice G-rich telomere binding proteins, RGBP) (Kim et al. 1998) and three types of complexes of RGBP with two or more single-stranded (ss) TTTAGGG repeats were detected, which showed sequence specificity, high-salt resistance, and no affinity to double-stranded telomeric DNA. Similar nucleoprotein complexes have also been described in nuclear extracts of mung bean (Lee et al. 2000). Recently, nucleoprotein complexes with G-rich telomeric strand were identified in nuclear extracts of telomerase-negative tissues of *Nicotiana tabacum* and *Silene latifolia*. They were specific to $(TTTAGGG)_n$ sequence and resistant to high salt concentrations, and caused species-non-specific inhibition of plant telomerase owing to a reduced accessibility of the G overhang (Fulneckova and Fajkus 2000).

The possibility of applying a genomic approach to search for plant Myb-like telomere-binding proteins lead to the characterization of double stranded (ds) telomeric DNA-binding proteins from rice (RTBP1) and *Arabidopsis* (AtTBP1) (Hwang et al. 2001; Yu et al. 2000). Both proteins have a similar molecular mass (70 kDa) and contain a single Myb-like domain at the C-terminus related to that of TRF1, TRF2, Taz1p and Tbf1p. The Myb-like domain of both proteins is capable of sequence-specific binding to ds oligonucleotides comprising at least a dimer of the TTTAGGG sequence. Recently, a similar myb-like telomere-binding protein, NgTRF1 (75 kDa), which is selectively expressed in G1 phase, has been described in tobacco (Yang et al. 2003).

Here we report on the characterization of two new Myb-like telomere-binding proteins (AtTBP2 and AtTBP3) expressed in various *Arabidopsis thaliana* tissues. In contrast to AtTBP1, RTBP1, and NgTRF1, these proteins contain their Myb-like domain near the N-terminus and an H1/H5-like DNA-binding domain near the centre of their amino acid sequences. In addition to being able to bind ds telomeric sequences, they can also bind to the G-rich ss DNA, although with lower affinity compared with ds telomeric sequences. Both proteins show strong self and mutual interactions in yeast two-hybrid assays and no detectable interactions with the telomerase catalytic subunit. Correspondingly, they do not affect telomerase activity in vitro, but rather they could participate in regulation of telomere metabolism indirectly in analogy to the other Myb-like telomere-binding proteins.

Materials and methods

Cloning of AtTBP2 and AtTBP3 cDNA

A BLAST search (http://www.ncbi.nlm.nih.gov/) was applied to search for the presence of *Arabidopsis thaliana* homologs containing a Myb-like telobox domain e.g., in PcMYB1, hTRF1, hTRF2, and TBF1 (*S. cerevisie*) (Bilaud et al. 1996; Broccoli et al. 1997; Feldbrugge et al. 1997). Two cDNA clones (see Results) without characterized functions were chosen. Using reverse transcription mediated PCR (RT-PCR) these two predicted sequences were obtained using total RNA isolated from three-week-old seedlings using an RNeasy Plant minikit (Qiagen, Valencia, Calif.). Sequence-specific primers with restriction sites for *Eco*RI at the 5′ and *Bam*HI at the 3′ end were used for AtTBP2 (forward primer, 5′-GGAATTCATGGGAGCTCCAAAGCTGAAG-3′; reverse primer, 5′-CGGGATCCTTACCGAGTTTGGCTATGCAT-3′) and AtTBP3 (forward primer, 5′-GGAATTCATGGGTGCACCAAAGCAGAAG -3′; and reverse primer, 5′-CGGGATCCTCACCAAGGATGATTACGGA-3′).

Table 1. Overview of oligodeoxynucleotides used in electrophoresis mobility shift assays (EMSA).

Name	Stand	No. of residues	Sequence (5′→3′)*	Notes
AtTR-2	-C	14	[**CCCTAAA**]$_2$	*Arabidopsis thaliana* telomeric sequence, C-strand
	-G	14	[**TTTAGGG**]$_2$	*A. thaliana* telomeric sequence, G-strand
AtTR-3	-C	21	[**CCCTAAA**]$_3$	*A. thaliana* telomeric sequence, C-strand
	-G	21	[**TTTAGGG**]$_3$	*A. thaliana* telomeric sequence, G-strand
AtTR-4	-C	28	[**CTAAACC**]$_4$	*A. thaliana* telomeric sequence, C-strand
	-G	28	[**GGTTTAG**]$_4$	*A. thaliana* telomeric sequence, G-strand
AtTR-6	-C	42	[**CCCTAAA**]$_6$	*A. thaliana* telomeric sequence, C-strand
	-G	42	[**TTTAGGG**]$_6$	*A. thaliana* telomeric sequence, G-strand
HuTR-4	-C	28	[CTAACC]$_4$CTAA	*Homo sapiens* telomeric sequence, C-strand
	-G	28	[TTAGGG]$_4$TTAG	*H. sapiens* telomeric sequence, G-strand
ChTR-4	-C	31	[CTAAAACC]$_3$CTAAAAC	*Chlamydomonas reinhardtii* telomeric sequence, C-strand
	-G	31	[GTTTTAGG]$_3$GTTTTAG	*Ch. reinhardtii* telomeric sequence, G-strand
BoTR-4	-C	29	[CTAAC]$_5$CTAA	*Bombyx mori* telomeric sequence, C-strand
	-G	29	[TTAGG]$_5$TTAG	*B. mori* telomeric sequence, G-strand
AsTR-4	-C	28	[CTAAGC]$_4$CTAA	*Ascaris lumbricoides* telomeric sequence, C-strand
	-G	28	[TTAGGC]$_4$TTAG	*A. lumbricoides* telomeric sequence, G-strand
PPG-AtTR-4	-G	28	[*GG*TTTA*G*]$_4$	Analogue of the AtTR-4-G modified by substitution of guanines by 7-deaza-8-aza-G (pyrazolo[3,4-d]pyrimidine) (for ds-form combined with AtTR-4-C)
AtTR-2-10	-C	24	**AAATCCC**CGGTACTGGT**AAATCCC**	Nontelomeric ODN harboured by telomeric C-strand sequence at both ends
	-G	24	**GGGATTT**ACCAGTACCG**GGGATTT**	Nontelomeric ODN harboured by telomeric G-strand sequence at both ends
AtTR-2-6	-C	20	**CCCTAAA**CATGGC**CCCTAAA**	Nontelomeric ODN harboured by telomeric C-strand sequence at both ends
	-G	20	**TTTAGGG**GCCATG**TTTAGGG**	Nontelomeric ODN harboured by telomeric G-strand sequence at both ends
Non-TR-4	-C	24	TCATGGCTGGTCATGGCTGGTACT	Nontelomeric ODN analogue of TR10-C
	-G	24	AGTACCAGCCATGACCAGCCATGA	Nontelomeric ODN analogue of TR10-G (complementary to TR10-24C)
I	-G	17	GCCATGACCA**TTTAGGG**	Forming a blunt ending ds-ODN
	-C	17	3′-CGGTACTGGT**AAATCCC**-5′	
II	-G	24	GCCATGACCA**TTTAGGGTTTAGGG**	Forming a G overhang adjacent to telomeric sequence in ds-ODN
	-C	17	3′-CGGTACTGGT**AAATCCC**-5′	
III	-G	17	GCCATGACCA**TTTAGGG**	Forming G overhang adjacent to nontelomeric sequence in ds-ODN
	-C	10	3′-TGGTCATGGC-5′	

Note: *Arabidopsis thaliana* telomeric sequence is shown in bold letters.
*Unless otherwise indicated.

Expression of AtTPB2 and AtTBP3 in *Escherichia coli*

PCR products were digested by *Eco*RI and *Bam*HI restricton enzymes and ligated using T4 ligase (Gibco BRL) to pET30a+ (Novagen) and transformed into *E.coli* BL21(DE3)pLysS. Clones expressing His-tag-fused proteins were grown on Luria–Bertani (LB) medium with kanamycin (1 μg/mL) and chloramphenicol (0.5 μg/mL) to prepare proteins for testing telomeric DNA-binding activity. Expressed proteins were purified on Ni-NTA Superflow matrices (Qiagen), their concentration determined by Bradford assays (Bradford 1976), and stored in aliquots at –70 °C.

To prove successful purification, the proteins were blotted (10 μg per lane) from a 10% denaturing acrylamide gel to Hybond-C extra nitrocellulose membrane (Amersham, U.S.A.) and detected by specific monoclonal mouse anti-polyhistidine (Sigma, St. Louis, Mo.) and by anti-mouse IgG peroxidase conjugate antibody (Sigma), followed by ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, N.J.).

Electrophoresis mobility shift assay (EMSA)

DNA probes and competitors used are described in Table 1. To reduce non-specific DNA–protein binding, 10 μg of purified AtTBP2 or 3 were preincubated with 10 μg of a nonspecific competitor DNA (either poly(dG–dC)·poly(dG–dC) (Amersham), or 2.5, 50, or 250 pmol of a specific competitor (oligodeoxynucleotides of non-telomeric or competitor telomeric sequence in single-stranded or double-stranded form, as indicated in Results)) for 20 min in EMSA buffer (10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithio-

Fig. 1. Structure of the proteins AtTBP2 and AtTBP3. The localization of the Myb-like and histone H1/5-like domains is shown.

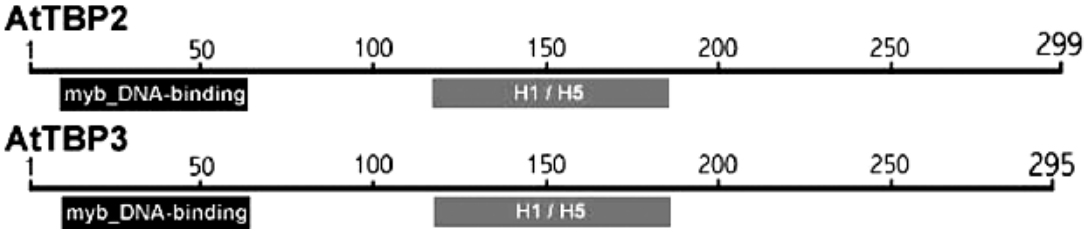


Fig. 2. Expression of AtTBP2 and AtTBP3 in various *A. thaliana* tissues tested by RT-PCR. RNA isolated from root (1), stem (2), leaf (3), flower (4) and seedling (5) was used as template.

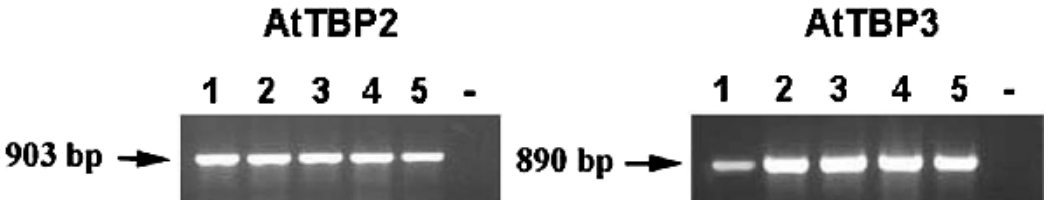
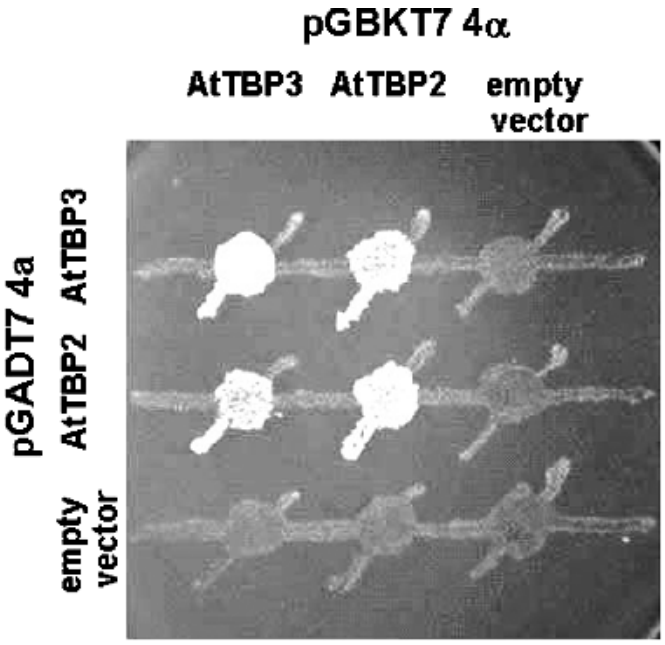


Fig. 3. Detection of interactions between AtTBP2 and AtTBP3 and of self-interaction of each of the proteins by two-hybrid assay.



treitol, 50 mM NaCl, and 5% w/v glycerol). Probes were end-labelled using [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs, Beverly, Mass.) (Sambrook et al. 1989). A probe (2.5 pmol) was added to the reaction mixture on ice and incubated for 45 min before the mixture was loaded onto a 7.5% w/v non-denaturing polyacrylamide gel (AA:BIS = 47:1, 0.5× TBE, 1.5 mm thick). Electrophoresis was at 15 °C for 3 h at 10 V/cm. Binding activity was quantified using phosphorimager STORM860 and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif) as the decrease of the free probe signal.

Two-hybrid system

Two strains of *Saccharomyces cerevisiae*, PJ69-4a and PJ69-4α, were used (James et al. 1996). AtTBP2 and AtTBP3 in the yeast vector pGADT7 (Clontech, Palo Alto, Calif.) were expressed in PJ69-4a and those in vector pGBKT7 (Clontech) were expressed in PJ69-4α. Both strains, identical except for the mating type, were mixed on Petri dishes to fuse yeast haploid cells of different strains. The diploid cells were printed by velvet stamp onto selection medium (0.67% w/v yeast nitrogen base, 2% w/v glucose, 0.12% w/v amino acid mixture (-Leu, -Trp, -Ade), and 2% agar 20 g, pH adjusted by NaOH to 6.8) and were incubated at 30 °C for a few days until colonies had grown.

Detection of AtTBP2 and AtTBP3 expression in plant tissues by RT-PCR

RNA was isolated from 100 mg of various tissue types of *Arabidopsis thaliana* 'Columbia': root, stem, leaf, and flower, as well as 6-day-old seedlings, using TRIZOL® reagent (Invitrogen, Carlsbad, Calif.) and the manufacturer's instructions. Five micrograms of each RNA sample was used per reaction volume and cDNA was synthesized by SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen) using oligo $(dT)_{12–18}$ priming. cDNA of all samples was then amplified by *Taq* polymerase using the primers for AtTBP2 and AtTBP3 described above.

Detection of telomerase activity

Extracts for telomerase assays were prepared from *A. thaliana* seedlings as described by (Fitzgerald et al. 1996) with minor modifications (Fajkus et al. 1998). The amount of protein in extracts was determined by a standard assay (Bradford 1976). The reaction mixture composed of 45 µL telomerase assay buffer (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 $MgCl_2$, 10 mM EGTA, 100 µg bovine serum albumin (BSA)/mL), 10 pmol of primer CaMV35S, proteins AtTBP2 or AtTBP3 (concentration) 5, 50 or 500 ng, and telomerase extract from *Arabidopsis* (50 or 500 ng of protein) was incubated at 26 °C for 45 min. The extension products were amplified by Dynazyme II polymerase (Finnzymes) using 10 pmol of the reverse primer TELPR as described previously (Fajkus et al.

1998). The products were separated on a 12.5% w/v polyacrylamide gel stained with Syber Green (Amersham) and scanned on a phosphorimager STORM860 (Molecular Dynamics).

Results

AtTBP2 and AtTBP3: structural domains and expression pattern

Searching *Arabidopsis thaliana* databases for putative genes coding for telobox-containing proteins resulted in two candidate protein sequences, T46051 and BAB08466 (nucleotide database accession Nos. GI:638639 and GI:9757879, respectively). The translated proteins of those sequences, termed AtTBP2 (295 amino acids, 32 kDa, pI 9.53) and AtTBP3 (299 amino acids, 33 kDa, pI 9.88), contain a single Myb-like DNA-binding domain at the N-terminus, and a histone H1/H5-like DNA-binding domain in the middle of the protein sequence (Fig. 1). In recently updated database records, the AtTBP2 and AtTBP3 proteins reported here correspond to records of AtTRB3 (GenBank accession NO. ATU83838) and AtTRB2 (accession No. ATU83837), respectively. cDNAs of both proteins were cloned as described above.

To find out if these proteins are expressed in *Arabidopsis* tissues, RT-PCR was applied to total RNA from root, stem, leaf, flower, and seedlings (see Materials and methods section). The results showed ubiquitous expression of both proteins in the tissues examined (Fig. 2).

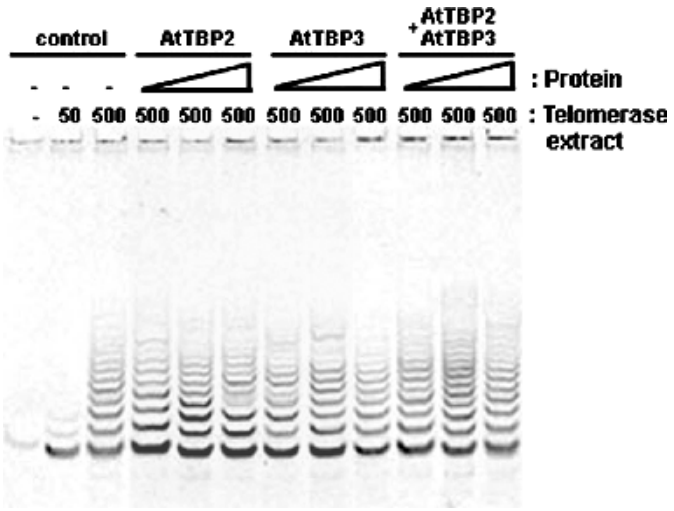
Protein–protein interactions of AtTBP2 and AtTBP3

Using the two-hybrid assays, both proteins were tested for self and mutual interactions, and also for interactions with five overlapping subcloned fragments of the cloned catalytic subunit of telomerase (Fitzgerald et al. 1999; Tamura et al. 1999) spanning the whole AtTERT cDNA sequence. While no interactions with telomerase protein were detected, both AtTBP2 and AtTBP3 showed strong mutual and self interactions (Fig. 3). Neither of the AtTBP2 and AtTBP3, nor their mixture, had any effect on TRAP assays (Fig. 4).

DNA-binding specificity

The DNA binding preference for oligodeoxynucleotide (ODN) substrates (see Table 1) was tested using EMSA as described above. The types of ODNs were chosen to make possible the assessment of the effect of the sequence, length, and structure (ss- or ds-, G-4, and G overhang, respectively) of the DNA substrate on protein binding. The results obtained (see Tables 2, 3 and 4) can be summarized as follows: both AtTBP2 and AtTBP3 preferentially bind the G-rich strand and dsDNA of *A. thaliana* telomeric sequence, the latter being a better substrate for binding by both proteins (see Figs. 5 and 6 for comparison). The C-rich telomeric strand is not preferentially bound (Table 2). Although both proteins show strong mutual interaction in two-hybrid assays, a mixture (in equimolar ratio) did not significantly change binding efficiencies compared with the single protein alone. Binding of both proteins to telomeric dsODNs is highly specific, because even a 100-fold abundance of non-telomeric sequence cannot displace their binding to tetrameres of the telomeric sequence (AtTR-4-ds) (Table 4*a* and Fig. 5).

Fig. 4. Telomerase activity assay (TRAP) in the presence of AtTBP2 and AtTBP3 proteins. The triangle shows increasing concentration (5, 50, and 500 ng) of either of the proteins or of their 1:1 mixture. The amount of telomerase extract (ng total protein) is indicated above each lane.



dsODN bearing two repeats of the G-rich strand and one repeat of the C-rich strand, thus forming a G overhang of 7 nt (ODN II in Table 4), is bound with a similar affinity as ODNs bearing two telomeric repeats on both strands (AtTR-2-ds). A blunt-ended ODN with just a single telomeric repeat on both strands (ODN I) does not show preferential binding, as well as ODN with the G overhang formed by one telomeric repeat and no other telomeric repeats on either strand (ODN III) (Table 4*b*).

The binding affinity to ds and ssODNs of the plant telomeric sequence is roughly proportional to the number of telomeric repeats (Table 4*c*). A minimum of two double-stranded repeats is required for preferential binding of either of the proteins; two single-stranded repeats are sufficient for binding of AtTBP2, but not AtTBP3 (Table 3).

The interaction of AtTBP2 or AtTBP3 with a plant telomeric sequence dimer interrupted by a 10-nt intervening sequence (spacing telomere motifs in dsODN by approximately one helix turn) is similar to the uninterrupted telomeric dimer in ss and ds forms. Two telomeric repeats of the G strand are apparently too short to be bound by AtTBP3, though they are sufficient for AtTBP2 binding whether uninterrupted or interrupted by the 10-nt spacer. AtTBP2 also binds more strongly than AtTBP3 to the ds form of the uninterrupted dimer (AtTR-2-ds) and the dimer interrupted by the 10-nt spacer (Table 3). In contrast, a 6-nt-long spacer sequence that positions the corresponding sites in the telomere repeat approximately at the opposite sides of the DNA helix completely abolishes binding of either protein to both ss and dsODNs (AtTR-2-6-G and AtTR-2-6-ds, respectively). Addition of nonspecific DNA, poly(dG–dC)·poly(dG–dC), is able to efficiently compete with interaction of both AtTR-2-10-G and AtTR-2-10-ds with either of the proteins (data not shown).

Table 2. Electrophoretic mobility shift assay of proteins AtTBP2 and AtTBP3 (10 μg).

	AtTBP2	AtTBP3	AtTBP2+AtTBP3
AtTR-6-C*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●
AtTR-6-G*	❍❍◒●●	❍❍◒●●	❍❍◒●●
AtTR-6-ds*	●●●●●	●●●●●	●●●●●

Fraction of retarded oligo*	
❍❍❍❍❍	(free oligo*)
❍❍❍❍●	<20%
❍❍❍●●	20-40%
❍❍◒●●	40-60%
❍●●●●	60-80%
●●●●●	>80%

Note: Asterisks indicate end-labelled oligonucleotide whose retardation was evaluated as indicated on the right. Both proteins preferentially bind the G-rich strand and dsDNA, but not the C-rich strand of a hexamer of the telomeric repeat AtTR-6 (2.5 pmol) in competition with the nonspecific competitor poly (dG-dC)·poly(dG-dC).

Table 3. Affinity of AtTBP2 and AtTBP3 for two telomeric repeats (G-strand, ds) arranged in tandem or interrupted by 10 or 6 nt of spacer sequence.

	AtTBP2	AtTBP3
TR-2-G*	❍❍❍●●	❍❍❍❍❍
TR-2-10-G*	❍❍❍●●	❍❍❍❍❍
TR-2-6-G*	❍❍❍❍❍	❍❍❍❍❍
TR-2-ds*	❍●●●●	❍❍❍●●
TR-2-10-ds*	❍●●●●	❍❍❍●●
TR-2-6-ds*	❍❍❍❍❍	❍❍❍❍❍

Note: The fraction of retarded ODN was evaluated as described in Table 2. EMSA revealed that a 10 nt interruption does not affect affinity of AtTBP2 and AtTBP3 compared to tandemly arranged repeats, whereas a 6 nt interruption completely abolishes binding of both poteins.

A modified G-rich telomeric ODN (PPG-AtTR-4-ds), in which all G positions are substituted with 7-deaza-8-aza-G (pyrazolo[3,4-d]pyrimidine, PPG), shows reduced AtTBP3 binding even in the absence of competitor DNA, whereas the interaction with AtTBP2 is similar compared with the unmodified tetramer telomeric sequence (AtTR-4). In both proteins, a 100-fold excess of nontelomeric sequence is able to displace the protein from its binding to PPG-AtTR-4-ds in contrast to AtTR-4 (Table 4*d*). Correspondingly, PPG-AtTR-4 is unable to displace AtTBP2 or AtTBP3 bound to the shorter (trimeric) AtTR-3-ds sequence, whereas unmodified AtTR-4 is able to do so efficiently when in 20-fold excess (Table 4*c*).

When using ODNs of variant telomeric sequence to compete for binding of the *A. thaliana* telomeric sequence by the AtTBP2 or AtTBP3 protein, the efficiency of competition followed the degree of homology to the *A. thaliana* telomeric sequence $(TTTAGGG)_n$ (Table 4*e*). For example, the weakest competitor from those tested is the sequence at *Ascaris* telomeres (AsTR-4-ds, $(TTAGGC)_4$) with the AtTBP2 protein, whereas the *Chlamydomonas* sequence (ChTR-4-ds, $(TTTTAGGG)_4$) competed relatively well. The protein AtTBP3 shows less specific binding than AtTBP2 as indicated by complete displacement of AtTBP3 from the AtTR-4-ds by a hundred-fold excess of the human sequence (HuTR-4, $(TTAGGG)_4$). Partial displacement of AtTBP3 from AtTR-4-ds can also be accomplished by other sequences, including AsTR-4-ds.

Discussion

Our results show that both proteins characterized in this study are ubiquitously expressed in various *Arabidopsis thaliana* tissues (Fig. 2), indicating a physiological function. In analogy to myb-like telomere binding proteins from other organisms (TRF1, TRF2, Taz1p, Tbf1p, and RTBP1), and to the previously characterized AtTBP1 from *Arabidopsis thaliana* (Hwang et al. 2001), the AtTBP2 and AtTBP3 proteins contain a myb-like telobox domain. However, in contrast to these proteins, AtTBP2 and AtTBP3 have the myb-like domain near their N- rather than their C-terminus. Besides this domain, they have a centrally positioned H1/H5-like DNA-binding domain (Fig. 1). This domain may be responsible for at least one of two kinds of non-specific sequence binding observed in our experiments: first, the low-affinity binding of nontelomeric dsDNA substrates that could be fully inhibited by the addition of telomeric dsDNA substrate, and second, the binding of a non-telomeric ssDNA substrate that is promoted by binding of a telomeric substrate, especially the G strand (see Table 4*a*). In the absence of the protein, no interaction between telomeric and non-telomeric ODNs was observed. The cooperative mode of binding is especially notable with AtTBP3, which binds labelled nontelomeric ssDNA weakly, whereas the addition of unlabelled AtTR-4-G enhances binding, suggesting that the two ODNs do not compete for the same binding site (Fig. 6). The proteins that are able to form dimers by protein–protein interaction (yeast two-hybrid data and in analogy to TRF1 and TRF2) (Fairall et al. 2001), can probably further be grouped into high-molecular-weight (HMW) complexes bound via protein–DNA–protein interactions. Possibly, the telomeric G-strand ODN forms the bridge. Although the details of this mode of binding and its eventual biological significance are to be determined, this result demonstrates the advantage of the approach chosen in this work, using complete protein for in vitro studies rather than the common use of only the myb-like domain.

Another difference between AtTBP2 and AtTBP3 and previously characterized telomere-binding proteins (e.g., TRF1 and TRF2, Broccoli et al. 1997) is their ability to interact mutually, as shown by yeast two-hybrid assay (Fig. 3). Neither AtTBP2, nor AtTBP3, nor their mixtures displayed interaction with the catalytic subunit of *Arabidopsis* telomerase, AtTERT, consistent with the absence of an observed effect (either positive, or negative) on telomerase activity in vitro (Fig. 4). Experimental data were essential to demonstrate the telomere-binding character of AtTBP2 and

Table 4. Competition assay for AtTBP2 and AtTBP3 (10 µg) binding to radiolabeled oligo* (2.5 pmol) with 2.5 pmol, 50 pmol or 250 pmol of unalabeled specific competitor ODNs of non-telomeric sequence or competitor telomeric sequence in single-stranded or double-stranded form (see Table 1).

	AtTBP2					AtTBP3				
		No competitor DNA	Oligo* : Competitor DNA				No competitor DNA	Oligo* : Competitor DNA		
	Oligo*		1 :1	1 : 20	1 : 100	Competitor DNA	Oligo*		1 :1	1 : 20	1 : 100	Competitor DNA
a	AtTR-4-G*	❍❍☯●●	❍❍☯●●	❍❍❍●●	❍❍❍❍❍	non-TR-G	**AtTR-4-G***	❍●●●●	❍●●●●	❍❍☯●●	❍❍❍●●	**non-TR-G**
	non-TR-G*	❍❍☯●●	❍❍❍●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-4-G	**non-TR-G***	❍❍❍❍●	❍❍❍●●	❍❍❍●●	❍❍❍❍●	**AtTR-4-G**
	AtTR-4-ds*	❍●●●●	●●●●●	●●●●●	●●●●●	**non-TR-ds**	AtTR-4-ds*	●●●●●	❍●●●●	❍●●●●	❍❍☯●●	non-TR-ds
	non-TR-ds*	❍❍☯●●	❍❍❍●●	❍❍❍❍❍	❍❍❍❍❍	**AtTR-4-ds**	non-TR-ds*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍❍	❍❍❍❍❍	AtTR-4-ds
b	I*	❍❍❍●●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	non-TR-ds	I*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	non-TR-ds
	non-TR-ds*	❍❍☯●●	❍❍☯●●	❍❍☯●●	❍❍❍❍●	I	non-TR-ds*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍❍	❍❍❍❍❍	I
	II*	❍●●●●	❍❍☯●●	❍❍☯●●	❍❍❍❍●	non-TR-ds	II*	❍❍☯●●	❍❍☯●●	❍❍☯●●	❍❍☯●●	non-TR-ds
	non-TR-ds*	❍❍☯●●	❍❍☯●●	❍❍❍●●	❍❍❍❍●	II	non-TR-ds*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍❍	❍❍❍❍❍	II
	III*	❍❍❍❍●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	non-TR-ds	III*	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	non-TR-ds
	non-TR-ds*	❍❍☯●●	❍❍☯●●	❍❍☯●●	❍❍☯●●	III	non-TR-ds*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	III
c	AtTR-3-G*	❍❍❍●●	❍❍❍●●	❍❍❍●●	❍❍❍●●	AtTR-2-G	AtTR-3-G*	❍❍☯●●	❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-2-G
		❍❍❍●●	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	AtTR-3-G		❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	AtTR-3-G
		❍❍❍●●	❍❍❍●●	❍❍❍●●	❍❍❍❍❍	AtTR-4-G		❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	AtTR-4-G
		❍❍❍●●	❍❍❍●●	❍❍❍❍●	❍❍❍❍❍	PPG-AtTR-4-G		❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	PPG-AtTR-4-G
		❍❍❍●●	❍❍❍●●	❍❍❍●●	❍❍❍❍❍	AtTR-6-G		❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	❍❍❍❍❍	AtTR-6-G
	AtTR-3-ds*	❍●●●●	❍●●●●	❍●●●●	❍●●●●	AtTR-2-ds	AtTR-3-ds*	❍●●●●	❍●●●●	❍●●●●	❍●●●●	AtTR-2-ds
		❍●●●●	❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-3-ds		❍●●●●	❍❍☯●●	❍❍❍●●	❍❍❍●●	AtTR-3-ds
		❍●●●●	❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-4-ds		❍●●●●	❍❍☯●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-4-ds
		❍●●●●	❍●●●●	❍❍☯●●	❍❍❍●●	PPG-AtTR-4-ds		❍●●●●	❍❍☯●●	❍❍❍●●	❍❍❍●●	PPG-AtTR-4-ds
		❍●●●●	❍❍☯●●	❍❍❍●●	❍❍❍❍❍	AtTR-6-ds		❍●●●●	❍❍❍●●	❍❍❍❍❍	❍❍❍❍❍	AtTR-6-ds
d	PPG-AtTR-4-G*	❍❍☯●●	❍❍☯●●	❍❍❍●●	❍❍❍❍❍	non-TR-G	PPG-AtTR-4-G*	❍❍❍●●	❍❍❍●●	❍❍❍❍❍	❍❍❍❍❍	non-TR-G
	non-TR-G*	❍❍☯●●	❍❍☯●●	❍●●●●	❍❍❍●●	PPG-AtTR-4-G	non-TR-G*	❍❍❍❍●	❍❍❍●●	❍❍☯●●	❍❍❍●●	PPG-AtTR-4-G
	PPG-AtTR-4-ds*	❍●●●●	❍●●●●	❍●●●●	❍❍❍❍❍	non-TR-ds	PPG-AtTR-4-ds*	❍❍☯●●	❍❍❍❍●	❍❍❍❍❍	❍❍❍❍❍	non-TR-ds
	non-TR-ds*	❍❍☯●●	❍❍☯●●	❍❍❍❍●	❍❍❍❍❍	PPG-AtTR-4-ds	non-TR-ds*	❍❍❍❍●	❍❍❍❍●	❍❍❍❍●	❍❍❍❍❍	PPG-AtTR-4-ds
e	AtTR-4-ds*	❍●●●●	❍●●●●	❍●●●●	❍❍❍●●	HuTR-4-ds	AtTR-4-ds*	●●●●●	❍●●●●	❍❍❍❍❍	❍❍❍❍❍	HuTR-4-ds
		❍●●●●	❍●●●●	❍❍❍●●	❍❍❍❍●	ChTR-4-ds		●●●●●	❍●●●●	❍❍❍●●	❍❍❍●●	ChTR-4-ds
		❍●●●●	❍●●●●	❍●●●●	❍❍❍●●	BoTR-4-ds		●●●●●	❍●●●●	❍❍☯●●	❍❍❍❍●	BoTR-4-ds
		❍●●●●	❍●●●●	❍●●●●	❍●●●●	AsTR-4-ds		●●●●●	❍●●●●	❍❍☯●●	❍❍❍●●	AsTR-4-ds

Note: The evaluation mode is illustrated in Fig. 5.

Fig. 5. An example of an EMSA result showing specific binding of AtTBP2 to ds-telomeric sequence (*a*). While a 100 fold abundance of non-telomeric dsODN is not able to displace the protein from its binding to labeled telomeric dsODN (left), telomeric dsODN is able to fully displace labeled non-telomeric dsODN already at a 20-fold excess (right). The evaluation is illustrated below (*b*) (corresponding to Table 4*a*), highlighted by bold letters.

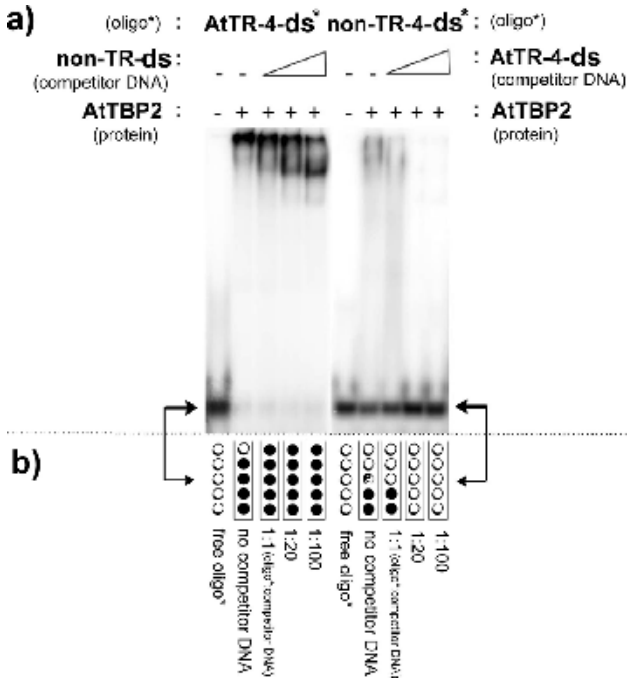
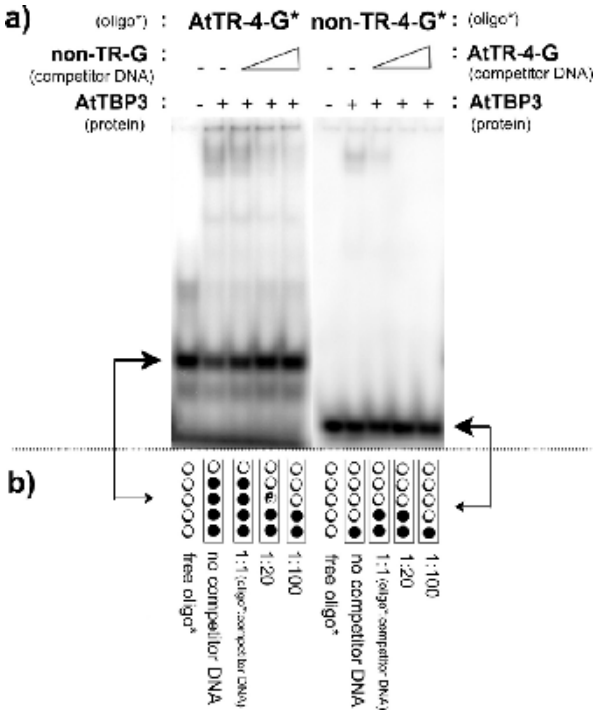


Fig. 6. Binding of AtTBP3 to G-strand-telomeric sequence (*a*). Non-telomeric ss-ODN is not able to displace the protein from its binding to labeled telomeric G-strand ODN (AtTR-4-G) completely (left). Labeled non-telomeric ssODN binds weakly in the absence of telomeric competitor, but the addition of unlabelled AtTR-4-G enhance binding at lower concentrations (right). The evaluation is illustrated below (*b*) (corresponding to Table 4*a*), highlighted by bold letters.



AtTBP3, particularly since a large family of R2R3 Myb-like transcription factors exists in *Arabidopsis thaliana* (Kranz et al. 1998). A certain flexibility in binding related telomeric DNA sequences (human, *Chlamydomonas*) was observed, which may be important to maintain protein function at variant sequences produced by plant telomerases (Fitzgerald et al. 2001). Similarly, the putative rice telomere binding protein RTBP1 can also bind the human telomere repeat sequence in vitro (Yu et al. 2000). The flexibility of the protein may be important for telomere function in those plants where the *Arabidopsis*-like telomere sequence has been substituted for human (Weiss and Scherthan 2002) or other variants of the minisatellite repeat (Sýkorová et al. 2003*a*, 2003*b*).

Since our proteins display affinity to both ds and G-strand telomeric ODNs, the possibility of G4-specific-binding was also considered. However, under the conditions of our experiments, G-4 formation was not observed. Nevertheless, a modified ODN (PPG-AtTR-4), in which formation of G-4 DNA is abolished, displayed significantly lower binding affinity to both proteins. This is probably a consequence of the displaced nitrogen in the pyrazol ring (N7→N8), a position critical for binding of TRF1 to human telomeric substrates, as it contacts R425 and K421 amino acid residues of the TRF1 protein.

We conclude that besides merely enriching the family of Myb-like telomere-binding proteins, our data further show their capability to form not only homodimers, but also heterodimers, a feature that is absent from the analogous mammalian proteins TRF1 and TRF2. On the other hand, in analogy to TRF proteins neither AtTBP2 nor AtTBP3 alone, nor their mixture, inhibits telomerase activity in vitro, which corresponds to the absence of any interactions with the catalytic subunit of telomerase, AtTERT, in the yeast two-hybrid assay.

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