Interactions of putative telomere-binding proteins in *Arabidopsis thaliana*: identification of functional TRF2 homolog in plants

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Received 1 September 2004; revised 1 November 2004; accepted 5 November 2004

Available online 23 November 2004

Edited by Horst Feldmann

Abstract Telomere-binding proteins are required for forming the functional structure of chromosome ends and regulating telomerase action. Although a number of candidate proteins have been identified by homology searches to plant genome databases and tested for their affinity to telomeric DNA sequences in vitro, there are minimal data relevant to their telomeric function. To address this problem, we made a collection of cDNAs of putative telomere-binding proteins of *Arabidopsis thaliana* to analyse their protein–protein interactions with the yeast two-hybrid system. Our results show that one myb-like protein, AtTRP1, interacts specifically with AtKu70, the latter protein having a previously described role in plant telomere metabolism. In analogy to the interaction between human Ku70 and TRF2 proteins, our results suggest that AtTRP1 is a likely homolog of TRF2. The AtTRP1 domain responsible for AtKu70 interaction occurs between amino acid sequence positions 80 and 269. The protein AtTRB1, a member of the single myb histone (Smh) family, shows self-interaction and interactions to the Smh family proteins AtTRB2 and AtTRB3. Protein AtTRB1 also interacts with AtPot1, the *Arabidopsis* homolog of oligonucleotide-binding-fold-containing proteins which bind G-rich telomeric DNA. In humans, the TRF1-complex recruits hPot1 to telomeres by protein–protein interactions where it is involved in telomere length regulation. Possibly, AtTRB1 has a similar role in recruiting AtPot1.

Keywords: Plant; Telomere-binding protein; Interaction; Yeast two-hybrid assay; AtKu70; AtPot1

1. Introduction

Proteins associated with telomeres have diverse functions that include chromosome protection and maintenance, and the regulation of telomeric DNA length. In addition to telomerase (a ribonucleoprotein enzyme complex which elongates linear chromosomes by reverse transcription of its endogenous RNA template), a number of proteins participate in telomere homeostasis. These include proteins that bind to the double-stranded part of telomeric DNA, to terminal single-stranded 3′-overhang of the G-rich DNA strand, and to telomeres by protein–protein interactions (see [1] for recent review). These proteins are best characterised in yeast, protozoan and mammalian model organisms. However, a number of putative plant homologs have been found by searching *Arabidopsis* and rice genome databases and their affinities to telomeric DNA sequence tested in vitro.

Double-stranded telomere-repeat DNA-binding proteins share a characteristic Myb domain referred to as a telobox that is responsible for binding to telomeric DNA sequences [2]. Proteins from *Arabidopsis thaliana* in this group are: AtTRP1, AtTBP1, AtTRB1, AtTRB2 and AtTRB3 [3–5]. AtTRP1 and AtTBP1 contain a Myb domain in the C-terminal region similar to human myb-like telomere-binding proteins TRF1 and TRF2 [6–8] and yeast protein Rap1 [9,10]. The proteins AtTRB1, AtTRB2 (syn. AtTBP3) and AtTRB3 (syn. AtTBP2) have a Myb domain at the N-terminus. Although these proteins bind specifically to telomeric repeats in vitro their functions and localisation in vivo have not been determined. Yeast two-hybrid experiments revealed interactions between proteins AtTRB2 and AtTRB3 and between themselves, while no in vitro interactions with the telomerase catalytic subunit were observed [4].

Another candidate protein with specific function(s) at telomere ends could be AtPot1 (Accession No. BT012568). This protein is the *Arabidopsis* homolog of oligonucleotide-binding (OB)-fold-containing proteins that specifically bind G-rich strands of telomeric DNA in *Oxytricha nova* (TEBP), budding yeast (Cdc13), fission yeast (spPot1) and human (hPot1) [11]. Human protein hPot1 binds specifically to the G-rich telomere strand and can act as a telomerase-dependent positive regulator of telomere length [12]. Loayza and de Lange [13] showed that hPot1 can also be recruited to the dsDNA part of telomeres via its interaction with the TRF1 complex. They proposed that this interaction affects the loading of hPot1 on the single-stranded telomeric DNA and transmits information about telomere length to the telomere terminus where telomerase is regulated. The recruitment of hPot1 is mediated by PTOP protein, a component of the TRF1 complex [14].

Besides DNA sequence-specific telomere-binding proteins, there are proteins that are not restricted to telomeres. For example, the heterodimer of two human proteins hKu70 and hKu80, which is known to mediate the non-homologous end joining pathway to repair DNA double-strand breaks, is recruited to telomeres via protein–protein interaction with TRF1. Here, the heterodimer performs the opposite function and prevents end-joining of mammalian telomeres [15]. Song et al. [16] found that hKu70 alone interacts with the protein

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.11.021

TRF2. In *A. thaliana*, two analogous proteins AtKu70 and AtKu80 have been found on the basis of sequence similarity to their human orthologs. Yeast two-hybrid analysis demonstrated that AtKu70 and AtKu80 form a heterodimer and electrophoretic mobility-shift assays revealed that this heterodimer binds to double-stranded telomeric and non-telomeric DNA sequences, but not to single-stranded DNA. The AtKu70/AtKu80 heterodimer possesses single-stranded DNA-dependent ATPase and ATP-dependent DNA helicase activities [17]. Ku [18] regulates the extension of the telomeric G-strand by acting as a negative regulator of telomerase. Deficiency of AtKu70 and AtKu80 results in the lengthening of telomeric G-overhangs [19–21]. However, in plants lacking both AtKu70 and the catalytic subunit of telomerase, AtTERT, the telomeres shorten approximately two or three times faster than in the plants with only inactive AtTERT [18]. One possible role of AtKu70 at telomeres is the maintenance of the telomeric C-rich strand by action on proteins that control telomere elongation [20].

The above examples clearly show that elucidation of protein interaction networks provides keys for understanding telomere functions. In plant cells, understanding lags behind that achieved for human cells due to the lack of function-related data on candidate plant telomere-binding proteins. An understanding in plants is however highly desirable because of some unique features of telomeres in plant model systems. These include, e.g., reversible and precise regulation of plant telomerase during growth and differentiation which results in telomere length stability [22,23], presence of human-type of telomeres in many plant families [24], and plant-specific phenotypes of telomerase and Ku knockout *Arabidopsis* mutants [18–21,25]. We focused in this work on detection and mapping of protein–protein interactions within a subset of known, or candidate, *A. thaliana* telomere-binding proteins AtTRP1, AtTBP1, AtKu70, AtKu80, AtPot1, AtTRB1, AtTRB2 and AtTRB3 using the yeast two-hybrid system.

2. Materials and methods

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

An overview of cloned candidate telomere-binding proteins is given in Table 1.

cDNA sequences of AtTRB2 (AtTBP3) and AtTRB3 (AtTBP2) have been cloned as described previously [4]. cDNA sequences coding for proteins AtTRP1, AtTBP1, AtKu70, AtPot1 and AtTRB1 were obtained using RT-PCR from total RNA isolated from *A. thaliana* seedlings and cloned into vectors pGADT7 and pGBKT7 (Clontech) for yeast two-hybrid system experiments. Sequence-specific primers with restriction sites were used for cloning of individual cDNAs as shown in Table 2 (primers 1–10). AtKu80 was cloned by endonuclease cleavage of AtKu80 in pBluecript II SK+ (between restriction sites *Eco*RI × *Bam*HI) (clone obtained from M.E. Gallego, University of Blaise Pascal, Aubiere, France) and ligation into vectors pGADT7 and pGBKT7.

To localise the interaction domain of AtTRP1, deletion forms of AtTRP1 were generated by PCR and cloned into vector pGBKT7. In total, six fragments were generated using primers and restriction enzyme cleavage: D1 (coding for amino acid (aa) positions 1–335), D2 (aa positions 1–269), D3 (aa positions 1–212), D4 (aa positions 134–335), D1E (aa positions 80–335) and D2E (aa positions 80–269) (for details see Fig. 3 in Section 3).

Prior to two-hybrid system screening, cloned constructs were checked for their correct reading frame and absence of non-sense mutations using the Protein Truncation Test. In vitro transcription

Table 1
Overview of cloned proteins

Protein	Protein group	Characteristic domain	Accession number	Reference
AtTRP1	dsDNA binding proteins	C-terminal Myb domain	CAB50690	[26]
AtTBP1			AAK31590	[3]
AtTRB1		N-terminal Myb domain	AAL73123	[5]
AtTRB2			AAL73441	[4]
AtTRB3			NP_190554	[4]
AtPot1	ssDNA binding proteins	Pot1 domain	BT012568	[11]
AtKu70	other telomere-associated proteins	Ku70/80 consensus	AAG44852	[17,18]
AtKu80			AAG44851	[17,21]

Table 2
Complete list of primers used for cloning

Primer	Cloned sequence	Restriction site	Sequence of primer (5′->3′)
1	AtTRP1	*Bam*HI	TTGGATCCATATGGTGTCGCGTAAGTGTG
2	AtTRP1	*Xho*I	ATCCTCGAGTTAGAGAAGTAACAGACCCTC
3	AtTBP1	*Xho*I	ATTCTCGAGGTATGGTGGTCAAAAGGAAGT
4	AtTBP1	*Xho*I	ATTCTCGAGTTACATGGACGAACCTGC
5	AtKu70	*Bam*HI	TAGGATCCACATGGAATTGGACCCAGATGATG
6	AtKu70	*Xho*I	ACGCTCGAGTTATTTACCAATGTGAGTCAGAATCC
7	AtPot1	*Bgl*II	GAAGATCTTAATGGAGGAGGAGAGAAGAG
8	AtPot1	*Xho*I	CCGCTCGAGACATGAAGCATTCATCCAAG
9	AtTRB1	*Bam*HI	ATGGATCCGAATGGGTGCTCCTAAGCAG
10	AtTRB1	*Xho*I	TGGCTCGAGAGGCACGGATCATCATTTTG
11	D4	*Eco*RI	GCGAATTCTTCAGTCAAGATGTAAAACCG
12	D1	*Bam*HI	TAGGATCCACTCGGTGTCCATCACTC
13	D2	*Bam*HI	TAGGATCCACGGTAATCATCAGTAACGG
14	D3	*Bam*HI	AAGGATCCAATACGAGGTACAGTCCTAG

and in vitro translation were performed by the TNT T7 Coupled Reticulocyte Lysate System (Promega). [^{35}S]Methionine (Amersham Biosciences) was added to the reaction for autoradiographic detection of translation products after separation by 15% discontinuous SDS–PAGE.

Clones producing truncated or no proteins were excluded from further testing, while the others were analysed by DNA sequencing on an ABI PRISM 310 sequencer (Perkin–Elmer).

2.2. Yeast two-hybrid system

Two strains of *Saccharomyces cerevisiae*, PJ69-4a and PJ69-4α, were used [27]. AtTRP1, AtTBP1, AtKu70, AtKu80, AtPot1, AtTRB1, AtTRB2 and AtTRB3 in the yeast vector pGADT7 were expressed in *Saccharomyces cerevisiae* strain PJ69-4a and the ones in vector pGBKT7 in strain PJ69-4α. Both strains, identical except for the mating type, were mixed on Petri dishes with YPD medium (1.1% yeast extract, 2.2% bacteriological peptone, 2% glucose and 2% agar) to fuse the yeast haploid cells of different strains. The diploid cells were printed by velvet stamp into selection medium (0.67% yeast nitrogen base, 2% glucose, 0.12% amino acid mixture-Ade only and 2% agar, pH adjusted by NaOH to 6.8) and were incubated at 30 °C for a few days until colonies had grown.

To assay β-galactosidase (LacZ) reporter gene expression, the colonies grown in the selection medium were reprinted by velvet stamp into selection medium with X-gal (selection medium containing X-gal 80 mg/l and 1× BU salts (10× BU: 0.26 M Na_2HPO_4 and 0.25 M NaH_2PO_4)). The colonies were incubated at 30 °C for 4 days and checked for the development of a blue colour.

2.3. Co-immunoprecipitation

Proteins were synthesised using TNT® Coupled Wheat Germ Extract System (Promega) either as [^{35}S]methionine labelled (AtTRB1 and AtKu70) or c-myc-tagged forms (AtPot1 and AtTRP1). Protein mixtures (AtTRB1 + AtPot1 and AtTRP1 + AtKu70) and negative controls (AtTRB or AtKu70 alone) were incubated at 4 °C for 1 h, immunoprecipitated with 1 μg of rabbit anti c-myc antibody (0.1 mg/ml, ICN Biomedicals) and captured by Seize® Classic (G) Immunoprecipitation Kit (Pierce). Immune complexes were eluted and analysed using SDS–PAGE and autoradiography.

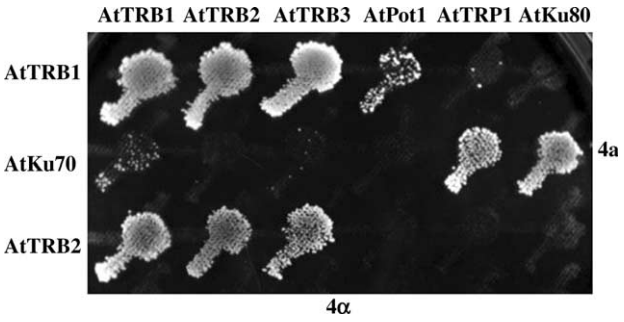

Fig. 1. Two-hybrid assay for interactions among *A. thaliana* putative telomere-binding proteins. 4a (horizontal rows) and 4α (vertical rows) denote different mating types of the yeast strain (PJ69-4a and PJ69-4α, respectively). Previously identified interactions of AtKu70 × AtKu80 and AtTRB2 × AtTRB3 are used as positive controls.

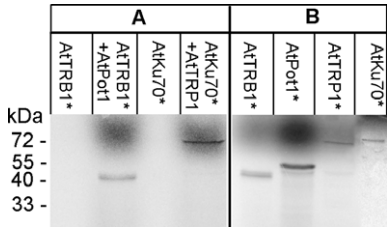

Fig. 2. Co-immunoprecipitation of in vitro translated AtTRB1, AtPot1, AtTRP1 and AtKu70 proteins. Radioactively labelled proteins AtTRB1 and AtKu70 (marked with asterisks) co-immunoprecipitated with c-myc-tagged proteins AtPot1 and AtTRP1, respectively (panel A). No radioactively labelled proteins were immunoprecipitated in the absence of their c-myc-tagged partners. To check the molecular weight of in vitro translation products, all proteins were prepared in labelled form in parallel experiments and their aliquots run on the same gel (panel B) without immunoprecipitation.

3. Results and discussion

The cDNAs of AtTRB1, AtTRB2 (syn. AtTBP3), AtTRB3 (syn. AtTBP2), AtTRP1, Ku70 and AtPot1 from *A. thaliana* were cloned. AtTRP1, AtTRB2 and AtTRB3 are known to bind specifically telomeric repeats TTTAGGG, and AtKu70 is associated with telomere maintenance. A new protein, AtPot1 (Accession No. BT012568), from *A. thaliana* containing a Pot-like motif was found on the basis of amino acid sequence comparison with TEBP alfa (Accession No. P29549) from *Oxytricha nova*, which binds the single-stranded DNA of telomere overhangs. To identify interactions among these candidate proteins, the two-hybrid system in *Saccharomyces cerevisiae* was used. The proteins were cross-tested with each other to assay all combinations in vectors pGADT7 and pGBKT7, so each protein was used as a bait or prey.

The telomere-binding proteins AtTRB2 and AtTRB3 are known to interact together and form homomultimers in yeast experiments [4]. The protein AtTRB1 is similar to proteins AtTRB2 and AtTRB3 at the level of amino-acid sequence and together they belong to the Smh family of proteins [5]. As expected, the two-hybrid assay showed that protein AtTRB1 interacts with proteins AtTRB2 and AtTRB3 and also forms homomultimers (Fig. 1). Additionally, AtTRB1 interacts with AtPot1 (Fig. 1), a result confirmed by co-immunoprecipitation of in vitro translated proteins (Fig. 2). This result corresponds to the interaction of human telomeric ssDNA binding protein hPot1 with the TRF1 protein complex [13,14]. It is not known whether AtPot1 protein binds telomeric ssDNA, but it does contain a sequence homologous to the Pot1-like domain found in the human protein hPot1 [11]. Interestingly, the interaction with AtPot1 is limited to AtTRB1 only, while there is no interaction with the other *Arabidopsis* members of the same family (AtTRB2 and AtTRB3).

Another interaction of potential functional importance was observed between protein AtKu70 and telomere-binding protein AtTRP1 and confirmed by co-immunoprecipitation of in vitro translated proteins (Fig. 2). Similar results were reported previously in human protein Ku70, which interacts with telomere-binding protein TRF2 [16]. The facts that AtTRP1 and TRF2 share both the C-terminal Myb domain enabling specific binding of telomeric DNA and have analogous interaction with Ku proteins suggest that the *Arabidopsis* protein AtTRP1 could be an ortholog of human TRF2. With the exception of AtKu80 and AtTRP1, none of the other proteins tested (including related *Arabidopsis* C-terminal Myb-like protein AtTBP1) showed interaction with AtKu70.

To localise the region of AtTRP1 responsible for the interaction with AtKu70, AtTRP1 deletion mutants were generated and cloned into vector pGBKT7. Based on the interaction of human protein TRF2 with protein Ku70, the interaction domain of AtTRP1 was expected to occur in the N-terminal region. Therefore, we designed deletion mutants that comprise

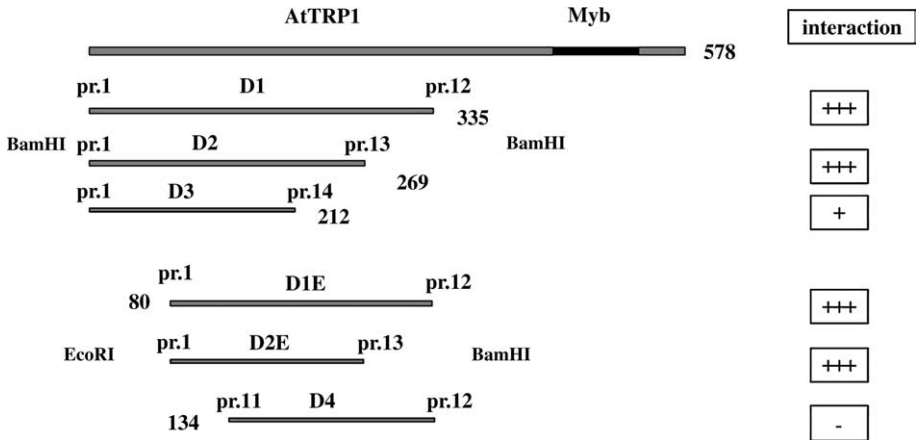

Fig. 3. Generation of AtTRP1 fragments using PCR primers (pr. 1, 11, 12, 13 and 14) and endonuclease cleavage. The interactions of the expression products with protein AtKu70 are shown.

this region and the central part of protein AtTRP1. In particular, six cloned fragments D1 (coding for aa positions 1–335), D2 (aa positions 1–269), D3 (aa positions 1–212), D4 (aa positions 134–335), D1E (aa positions 80–335) and D2E (aa positions 80–269) were fused with GAL4 DNA-binding domain and expressed in yeast as a bait. We observed that the clones bearing fragments D1, D2, D1E and D2E showed interaction with AtKu70 (Fig. 3). The clone D3 showed weak interaction, indicating that its C-end includes only part of the interaction domain. These results suggest that the interaction domain of AtTRP1 responsible for the interaction with AtKu70 is localised between amino acid positions 80 and 269. This position corresponds approximately to the region (aa positions 45–245) of the domain of human TRF2 responsible for interaction with hKu70. The clone D4 did not grow in the selection medium at all, reflecting a complete loss of interaction and the critical importance of amino acid positions 80–134.

The growth rate of clones bearing fragments D1, D2, D1E and D2E was higher than that of the clone bearing the full-length protein AtTRP1. However, the growth rate (promoted by the level of expression of the reporter gene ADE2) is an ambiguous measure of interaction strength as growth rate may also be affected by the size and structure of the two-hybrid complex and by direct interference of the expressed fusion proteins with yeast cell metabolism. Therefore, to evaluate the strength of interactions of truncated AtTRP1 forms more precisely, we used the expression of β-galactosidase (LacZ) reporter gene. This assay also confirmed that the cloned fragment D3 showed significantly weaker expression of LacZ reporter gene than clones D1E, D2, D2E and the full-length protein sequence (Fig. 4).

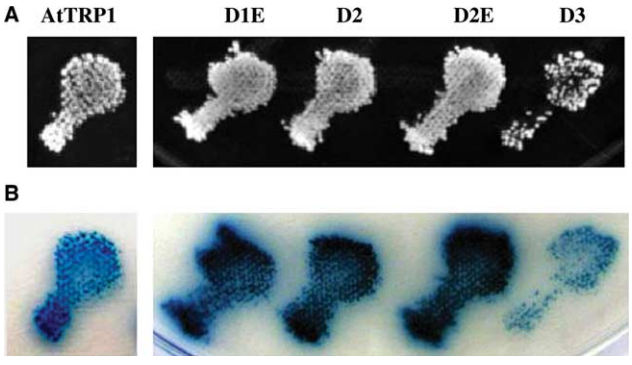

Fig. 4. Evaluation of interactions of full-length and truncated AtTRP1 with AtKu70. Growth of diploid yeasts on selection medium (-Ade) (panel A) and on the same medium containing X-gal (B). Clone nomenclature corresponds to Fig. 3.

During cloning of AtKu70, a mutant clone expressing the full-length, but mutated protein AtKu70 was identified in two-hybrid system. This clone did not interact with AtTRP1 or with AtKu80. Sequencing of this clone revealed two point mutations, first in amino acid position 117 substituting arginine (codon AGG) for lysine (codon AAG) and second in position 319, where glycine (codon GGA) was substituted by arginine (codon AGA). Because of the similar properties of arginine and lysine residues, the first substitution is not expected to disrupt protein interactions. The second mutation is in a highly conserved region found by Tamura [17] and it is likely to be responsible for protein inactivation. It is not known whether this mutation in AtKu70 occurs within the interaction domain required for the interaction with proteins AtKu80 and AtTRP1, or indeed whether the same domain is involved in both cases. However, since proteins AtKu70 and AtKu80, as well as their orthologs in other organisms function as heterodimers, we would expect that the AtKu70 domain responsible for interaction with AtKu80 is permanently blocked and, therefore, unavailable for AtTRP1 binding. The human protein hKu70 has been shown to use different regions for interactions with proteins TRF2 and hKu80 [16]. This suggests that AtKu70 also may use two distinct interaction domains for analogous interactions.

Acknowledgements: We thank Karel Říha for providing us with cloned AtKu70 cDNA, Maria Eugenia Gallego for cloned AtKu80 cDNA and valuable discussion regarding the AtKu proteins, Jana Hradilová for the total cDNA from *Arabidopsis thaliana*, and Tatiana Kubičárová for technical assistance with immunoprecipitation. This work was supported by The Grant Agency of the Czech Republic (project 204/02/0027) and the institutional support (MSM143100008 and Z5004920).

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