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Mapping of interaction domains of putative telomere-binding proteins AtTRB1 and AtPOT1b from *Arabidopsis thaliana*

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Abstract **We previously searched for interactions between plant telomere-binding proteins and found that AtTRB1, from the single-myb-histone (Smh) family, interacts with the Arabidopsis POT1-like-protein, AtPOT1b, involved in telomere capping. Here we identify domains responsible for that interaction. We also map domains in AtTRB1 responsible for interactions with other Smh-family-members. Our results show that the N-terminal OB-fold-domain of AtPOT1b mediates the interaction with AtTRB1. This domain is characteristic for POT1- proteins and is involved with binding the G-rich-strand of telomeric DNA. AtPOT1b also interacts with AtTRB2 and AtTRB3. The central histone-globular-domain of AtTRB1 is involved with binding to AtTRB2 and 3, as well as to AtPOT1b. AtTRB1-heterodimers with other Smh-family-members are more stable than AtTRB1-homodimers. Our results reveal interaction networks of plant telomeres.**

Structured summary:
MINT-6440051:
AtTRB1 (uniprotkb:Q8VWK4) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *two-hybrid* (MI:0018)
MINT-6440068:
AtTRB2 (uniprotkb:Q8VX38) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *two-hybrid* (MI:0018)
MINT-6440083:
AtTRB3 (uniprotkb:Q9M2X3) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *two-hybrid* (MI:0018)
MINT-6440099:
AtPOT1b (uniprotkb:Q6Q835) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *two-hybrid* (MI:0018)
MINT-6440119:
AtPOT1b (uniprotkb:Q6Q835) *physically interacts* (MI:0218) with *AtTRB2* (uniprotkb:Q8VX38) by *two-hybrid* (MI:0018)
MINT-6440138:
AtPOT1b (uniprotkb:Q6Q835) *physically interacts* (MI:0218) with *AtTRB3* (uniprotkb:Q9M2X3) by *two-hybrid* (MI:0018)
MINT-6440216:
AtPOT1b (uniprotkb:Q6Q835) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *coimmunoprecipitation* (MI:0019)
MINT-6440157:
AtTRB2 (uniprotkb:Q8VX38) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *coimmunoprecipitation* (MI:0019)
MINT-6440177:
AtTRB3 (uniprotkb:Q9M2X3) *physically interacts* (MI:0218) with *AtTRB1* (uniprotkb:Q8VWK4) by *coimmunoprecipitation* (MI:0019)

Keywords: Plant; Telomere; Protein–protein interaction; AtTRB1; AtTRB2; AtTRB3; AtPOT1b

1. Introduction

Telomere proteins play a role in the protection and maintenance of chromosome ends. In human cells, the minimal functional set of proteins participating in telomere protection is collectively called "shelterin" [1]. Shelterin consists of three proteins (TRF1, TRF2 and POT1) that directly recognize telomeric DNA and are interconnected by at least three other proteins (TIN2, TPP1 and Rap1), forming a telomere-specific protective cap. Similar complexes are also likely to exist in plants and these are particularly attractive to study due to the telomerase-competent status (i.e., reversible telomerase activity regulation) of plant somatic cells [2,3]. A number of putative plant telomeric proteins have been found by homology searches of DNA and protein sequence databases and tested for their affinity to telomeric DNA sequences in vitro (reviewed in [4]). There is however very little data relevant to their telomeric function. Of the putative "plant shelterin" components, functional data relevant to telomere homeostasis is available for two *Arabidopsis thaliana* POT1-like proteins, AtPOT1a and AtPOT1b. These proteins contain the oligonucleotide-binding (OB) fold domain which binds to the G-rich strand of telomeric DNA but their overall sequence similarity is low (49%). The functions of AtPOT1a and AtPOT1b proteins are different: AtPOT1a functions mainly in telomerase regulation, while AtPOT1b contributes to chromosome end-protection and genome stability [5–9].

Recently, another *Arabidopsis* protein, AtTBP1, has been shown to be involved in telomere length regulation [10]. This

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protein binds double-stranded telomeric DNA in vitro via a characteristic Myb-like domain, referred to as a telobox, located at its C-terminus [11,12]. To identify other components of ''plant shelterin'', we analyzed a number of putative *A. thaliana* telomere proteins for their mutual interactions. We previously found that AtTRP1, the *Arabidopsis* myb-like protein bearing a C-terminal telobox, interacts with AtKu70 [5], which itself plays a role in plant telomere homeostasis [13,14]. Furthermore AtTRP1 may be a functional homolog of mammalian TRF2 [5]. In addition, an *Arabidopsis* POT1-like protein, AtPOT1b, interacts with AtTRB1, a protein from the single myb histone (Smh) family [5]. The Smh family is characterised by a unique triple motif structure containing a N-terminal myb-like domain, a central GH1/GH5 histone globular domain and a C-terminal coiled-coil domain [15]. Proteins of this family in *Arabidopsis* show specific binding to telomeric DNA and can form homo- and heteromeric protein–protein complexes [16].

The abundance of candidate telomere proteins in plants, arising from numerous paralogs of telomere-binding protein and plant-specific proteins, coupled with an apparent absence of some constitutive animals and fungi shelterin components, makes imperative analyses of interactions between the candidate plant telomere proteins. Using a combination of the yeast two-hybrid system (Y2H) and co-immunoprecipitation (CoIP), we characterise here the protein domains involved in interactions between AtTRB1 and AtPOT1b, as well as domains engaged in the formation of homomeric and heteromeric complexes of AtTRB proteins.

2. Materials and methods

2.1. Cloning of full length proteins and their deletion variants for two-hybrid assay

An overview of cloned candidate telomeric DNA-binding proteins is given in Table 1. cDNA sequences of AtTRB1, AtTRB2, AtTRB3 and AtPOT1b have been cloned as described previously [16]. To localize the interaction domains the deletion forms of AtTRB1 and AtPOT1b were generated by PCR and cloned into the vector pGBKT7 or pGADT7, respectively. Sequence-specific primers with restriction sites were used for cloning individual cDNAs as shown in Table 2.

To localize the interaction domain(s) in AtTRB1, cDNA fragments were cloned in pGADT7 and denominated according to primers used (for example, the fragment F1R1 was generated using TRB1 F1 as forward and TRB1 R1 as reverse primers – see Figs. 1A and 2B). Similarly, AtPOT1b fragments were generated to localize the region of AtPOT1b responsible for interaction with AtTRB1 (see Fig. 2C).

Prior to two-hybrid screening, cloned constructs were checked for the correct reading frame and absence of mutations by DNA sequencing on an ABI PRISM 310 sequencer (Perkin–Elmer).

2.2. Yeast two-hybrid (Y2H) system

Two strains of *Saccharomyces cerevisiae*, PJ69-4a and PJ69-4α were used [17]. Protein AtPOT1b, its deletion variants and AtTRB2, AtTRB3 were expressed from the yeast vector pGBKT7 in strain PJ69-4α, and AtTRB1 and its fragments from vector pGADT7 in strain PJ69-4a. This division enabled proper combining of the proteins and their deletion variants in interaction assays. 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 yeast haploid cells of different strains, and incubated at 30 °C for 8–10 h. The diploid cells were printed by velvet stamp onto control -Leu,-Trp selective plates (0.67% yeast nitrogen base, 2% glucose, 0.12% amino acid mixture without Leu and Trp, 2% agar, pH adjusted by NaOH to 6.8) and then onto -Ade selective plates to test the interaction (0.67% yeast nitrogen base, 2% glucose, 0.12% amino acid mixture without Ade, 2% agar, pH adjusted by NaOH to 6.8) and were incubated at 30 °C for a few days until colonies had grown. Alternatively, PJ69-4a cells were cotransformed with both pGBKT7 and pGADT7 plasmids and grown on -Leu,-Trp plates. Colonies were inoculated into YPD liquid medium and incubated at 30 °C overnight. Ten-times diluted aliquots were dropped onto both -Ade and -His plates. For a semi-quantitative test, 5 μl aliquots were dropped onto selective -His plates containing increasing concentrations of 3-aminotriazol (3-AT). As the 3-AT inhibits His3 activity, the ability of yeast cells to grow on higher concentrations correlates with the higher binding affinity of the hybrid proteins.

To verify our results we also used the yeast strain MaV203, where the His3-reporter gene is under a less tightly controlled promoter (Invitrogen). The drop test was executed in the similar way as with the PJ69 strain.

Table 2
Complete list of primers used for cloning

Primer	Restriction site	Sequence of primer (5′ → 3′)
POT1b F	BamHI	ATGGATCCTAATGGAGGAGGAGAGAAGAG
POT1b F1	BamHI	ATGGATCCTAAAGATTGTGCTGATTAACC
POT1b F2	BamHI	TAGGATCCACTTCTTATCGAATCTGAGAG
POT1b F3	BamHI	TTGGATCCTTAAGTCAGAAAGGCTTC
POT1b R	XhoI	ATTCTCGAGTCATGAAGCATTGATCCAAG
POT1b R1	XhoI	TTACTCGAGCCCTTCATCAGCATATAGAG
POT1b R2	XhoI	TTACTCGAGCCTGTGATTTCAGAATGTG
POT1b R3	XhoI	TTACTCGAGGGTTGAAGACAGTGAATG
POT1b R4	XhoI	TTACTCGAGATCTTCAAACTTGTACGTG
POT1b R5	XhoI	CTTCTCGAGGGTTAATCAGCACAATCTTTA
TRB1 F	BamHI	ATGGATCCGAATGGGTGCTCCTAAGCAG
TRB1 F1	BamHI	CGGGATCCAAGATGCGACCTCTGGACTCC
TRB1 F2	BamHI	GAGGATCCAAGGTCTGGGGGTGTTTGGA
TRB1 F01	BamHI	CGGGATCCTAGTCATGGCAAATGGCTGG
TRB1 R	XhoI	TGGCTCGAGAGGCACGGATCATCATTTTG
TRB1 R1	BamHI	TCGGATCCTCCAAACACCCCCAGACC
TRB1 R2	BamHI	GAGGATCCGGAGTCCAGAGGTCGCATC
TRB1 R12	BamHI	CAGGATCCGCGTTTGAAGTCTGGTGGAG

2.3. In vitro translation and co-immunoprecipitation

Proteins were co-expressed from the same constructs as were used in Y2H system with an hemagglutinin tag (pGADT) or a myc-tag (pGBKT) by use of the TNT Quick Coupled Transcription/Translation System (Promega) in 15–25 μl of each reaction according to the manufacturer's instruction. For Myc pull-down experiments, 15–25 μl of in vitro-expressed proteins in total volume of 100 μl of HEPES buffer (25 mM HEPES, 150 mM KCl, 50 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, 2 mM PMSF, 2 μg/μl leupeptine, 1 μg/μl pepstatine) were mixed with 1 μg anti-Myc-tag polyclonal antibody (Abcam) and incubated overnight at 4 °C (Input fraction). 10 μl of Protein G magnetic particles (Dynabeads, Invitrogen-Dynal) were then added, and the mixture was incubated for 1 h/4 °C (Un-

Table 1
Overview of cloned proteins

Protein	Protein group	Characteristic domain	GenBank accession number	Reference
AtTRB1	dsDNA binding	N-terminal Myb domain	AAL73123	[16]
AtTRB2	Proteins		AAL73441	[17]
AtTRB3			NP_190554	
AtPOT1b	ssDNA binding proteins	Pot1 domain	NP_196249	[5,9,23]