

## Functional characterization of domains in AtTRB1, a putative telomere-binding protein in *Arabidopsis thaliana*

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### ABSTRACT

Telomeres are nucleoprotein structures ensuring the stability of eukaryotic chromosome ends. Two protein families, TRFL (*TFL*-Like) and SMH (Single-Myb-Histone), containing a specific *telobox* motif in their Myb domain, have been identified as potential candidates involved in a functional nucleoprotein structure analogous to human “shelterin” at plant telomeres. We analyze the DNA-protein interaction of the full-length and truncated variants of AtTRB1, a SMH-family member with a typical structure: N-terminal Myb domain, central H1/5 domain and C-terminal coiled-coil. We show that preferential interaction of AtTRB1 with double-stranded telomeric DNA is mediated by the Myb domain, while the H1/5 domain is involved in non-specific DNA-protein interaction and in the multimerization of AtTRB1.

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### 1. Introduction

Telomeres are nucleoprotein structures which form the ends of linear chromosomes, ensuring their stability and complete replication. The telomeric DNA is composed of minisatellite tandem repeats, for example (TTAGGG)<sub>n</sub> in the majority of plants or (TTAGGG)<sub>n</sub> in vertebrates, which form a terminal 3'-single-stranded G-rich overhang (Moyzis et al., 1988; Richards and Ausubel, 1988). Telomeric DNA is complexed with histones and non-histone proteins which mediate the flexibility of telomere structure (Griffith et al., 1999), regulate its accessibility for other proteins including telomerase, prevent recombination and serve as recognition marks of intact chromosomal termini to distinguish them from double-strand breaks (Bertuch and Lundblad, 2006).

Proteins bind telomere either by directly interacting with the double-stranded (ds) or single-stranded (ss) telomeric DNA or through protein-protein interactions with the DNA-binding proteins. In humans, the constitutive complex of six telomere-binding proteins has been termed “shelterin”, encompassing the dsDNA-binding proteins TRF1 and TRF2, the ssDNA-binding protein POT1 and the proteins TIN2, TPP1 and hRap1 which are bound to the

complex via protein-protein interactions (de Lange, 2005). Telomeric dsDNA-binding proteins show the presence of a single highly conserved motif, the three-helical Myb domain in which the third helix is defined by the presence of a conserved amino acid sequence termed a “*telobox*” (Bilaud et al., 1996), which is responsible for the recognition of telomeric dsDNA.

Numerous proteins have been suggested to participate in the plant analog of human shelterin. Based on the presence of a *telobox* within their Myb domain, a group of TBP-like (Telomere-Binding-Protein-like) proteins has been found *in silico* in *Arabidopsis thaliana* (Yanhui et al., 2006). The plant Myb domain of the TBP-like proteins can be placed either at the C-terminus of the protein, as in TRFL (TRF-like) proteins (Karamysheva et al., 2004), or at the N-terminus, as in SMH (Single-Myb-Histone) proteins (Marian et al., 2003). In TRFL proteins, the Myb domain can either be extended by a fourth helix (TRFLI family) or the three-helical structure of the Myb domain is maintained (TRFLII) (Hwang and Cho, 2007; Hwang et al., 2005; Karamysheva et al., 2004; Sue et al., 2006).

SMH proteins are plant-specific and have been reported to preferentially bind plant telomeric dsDNA. Apart from the N-terminally localized three-helical Myb-domain, they contain a histone-like H1/5 domain in the central part of the protein and a coiled-coil domain at the C-terminus (Marian et al., 2003; Schruppfova et al., 2004). The *A. thaliana* SMH family of genes encodes five proteins (AtTRB1-5), two of which (AtTRB2 and AtTRB3) have been characterized previously and are capable to bind preferentially to plant

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telomeric dsDNA. Considering their unique structure, it has been suggested that binding of these proteins to dsDNA is mediated and affected not only by the Myb domain but possibly also by the H1/5 domain, whose role in DNA binding has however not been elucidated (Schrumpfova et al., 2004). AtTRB1 (At1g49950), a member of the SMH family, interacts with the telomere-associated protein AtPOT1b, one of the POT1-like proteins in *A. thaliana* (Kuchar and Fajkus, 2004), which in turn participates in telomere capping and maintenance of genome stability (Shakirov et al., 2005).

In this study, we analyze the mode of binding of AtTRB1 to DNA. We show that the high-affinity sequence-specific interaction between telomeric dsDNA and AtTRB1 is mediated predominantly by the Myb domain, although other parts of the protein also contribute to the interaction. Furthermore, we show that AtTRB1 is capable of forming multimers and that this interaction is mediated mainly by the H1/5 domain. We suggest that AtTRB1 shows the characteristics of a telomere-binding protein and may be a part of the protein complex safeguarding plant telomeres.

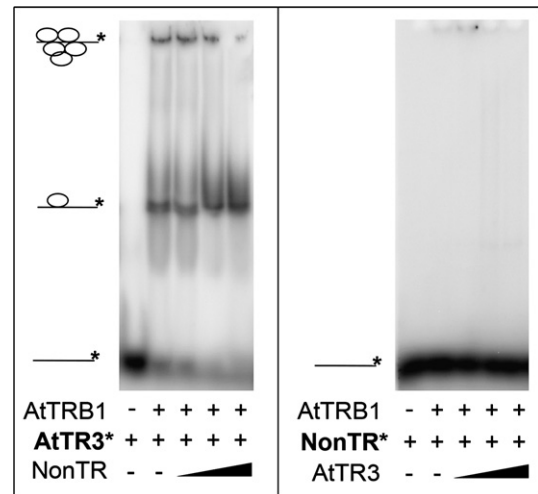
## 2. Results and discussion

### 2.1. AtTRB1 preferentially binds telomeric dsDNA

Full-length AtTRB1 (Fig. 1) was subjected to EMSA (Electrophoretic Mobility Shift Assay) using ss (AtTR3-G, AtTR3-C) or ds (AtTR3) trimers of a plant telomeric sequence and a non-telomeric oligonucleotide (NonTR-C, NonTR-G, NonTR). The telomeric oligonucleotide was used as the labeled probe while the non-telomeric oligonucleotide served as competitor DNA and vice versa. The results show that AtTRB1 is indeed a telomeric dsDNA-binding protein, similarly to proteins AtTRB2 and AtTRB3 which we have described previously (Schrumpfova et al., 2004), and shows no or very low-affinity to non-telomeric dsDNA (Fig. 2) or ssDNA of either telomeric or non-telomeric sequence (data not shown).

### 2.2. The Myb domain mediates the specific interaction between AtTRB1 and telomeric dsDNA

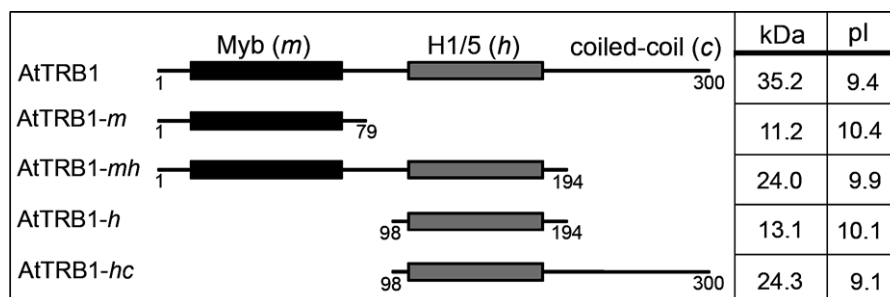
In order to determine which domain of AtTRB1 is primarily responsible for binding telomeric dsDNA, truncated variants of AtTRB1 (Myb domain only – *m*; Myb + H1/5 domains – *mh*; H1/5 domain only – *h*; and H1/5 + coiled-coil domains – *hc*) were used in EMSA (Fig. 1). All truncated variants of AtTRB1 showed a certain affinity towards telomeric dsDNA (Fig. 3); the level and character of the binding differed in each case. The Myb domain showed the strongest interaction with telomeric dsDNA of all the protein fragments tested; telomeric DNA was not released from the DNA-protein complex even upon addition of 100-fold excess of non-telomeric competitor (Fig. 3a). Two telomeric repetitions were adequate for the binding of Myb domain (data not shown). Unlike in TRFLI proteins, the Myb domain of SMH proteins does not contain



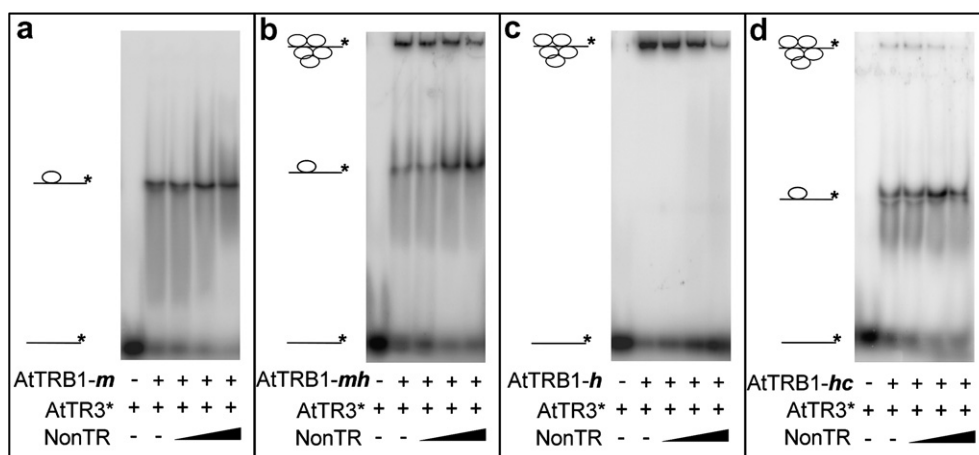
**Fig. 2.** EMSA of full-length AtTRB1 binding a radioactively-labeled ds telomeric (AtTR3\*) or non-telomeric (NonTR\*) oligonucleotide with unlabeled NonTR or AtTR3 respectively as competitor DNA. The concentration of unlabeled competitor increases from 1-, 20- to 100-fold the concentration of the labeled probe (as depicted by the triangle). DNA probe/protein concentration is 1/100.

the C-extension domain which has been proposed to condition the ability of the proteins to bind to telomeric DNA (Hwang and Cho, 2007; Karamysheva et al., 2004). However, recent solution of the structure of the C-extension-containing Myb domain of AtTRP1 has shown that the contact between DNA and AtTRP1 is mediated merely by amino acid residues of helix 1 (H1) and H3 and the loop connecting H3 and H4, while H4 itself stabilizes the Myb domain into a tetrahedral structure (Sue et al., 2006). The level of conservation of the amino acid sequence within the C-extension region thus may not be critical for binding of telomeric DNA, as the domain-stabilizing function may be provided by a different amino acid sequence.

The fragment *mh* bound telomeric dsDNA in a similar mode as fragment *m*, but formation of a high molecular weight complex, which did not migrate into the gel, was observed. This complex was partially disassembled by the addition of a 100-fold excess of non-telomeric competitor, which however did not release the protein from the complex with telomeric DNA (Fig. 3b). The H1/5 domain of AtTRB1 formed a high molecular weight complex with the telomeric DNA which was released from the complex by addition of non-telomeric competitor (Fig. 3c). Similarly, the fragment *hc* retarded the telomeric DNA probe but was partially loosened from the complex by a 100-fold excess of non-telomeric competitor (Fig. 3d). None of the fragments of AtTRB1 displayed affinity towards ssDNA or non-telomeric dsDNA with the exception of the H1/5 domain, which bound non-telomeric dsDNA in a similar manner as the telomeric dsDNA (data not shown). In this case, how-



**Fig. 1.** Cloned regions of AtTRB1 schematic representation of the full-length AtTRB1 and its truncated variants. Numbers in diagrams denote amino acid residues. pI denotes isoelectric point as determined using pI/MW calculator at ExPASy Proteomics Server.



**Fig. 3.** EMSA of fragments of AtTRB1 binding a radioactively-labeled ds telomeric (AtTR3\*) oligonucleotide with unlabeled non-telomeric oligonucleotide (NonTR) as competitor DNA. The concentration of unlabeled competitor increases from 1-, 20- to 100-fold the concentration of the labeled probe (as depicted by the triangle). DNA probe/protein concentration is 1/100.

ever, telomeric dsDNA did not compete for the interaction as efficiently, and the non-telomeric probe was not released from the complex. This shows that the H1/5 domain interacts non-specifically with any DNA without preference for either telomeric or non-telomeric sequence (Ellen and van Holde, 2004) which, together with the high pI of the AtTRB1 fragments, suggests that electrostatic interactions take part in the interaction of the fragments of AtTRB1 with telomeric dsDNA. This may interfere with determining the sequence specificity of AtTRB1 binding to telomeric DNA. The specificity of the interaction is nevertheless demonstrated by the fact that the H1/5 domain alone as well as the H1/5 + coiled-coil domains are released from the DNA-protein complex in excess of non-telomeric DNA, while the Myb and Myb + H1/5 domains form stable complexes with telomeric DNA even when competed by non-telomeric DNA.

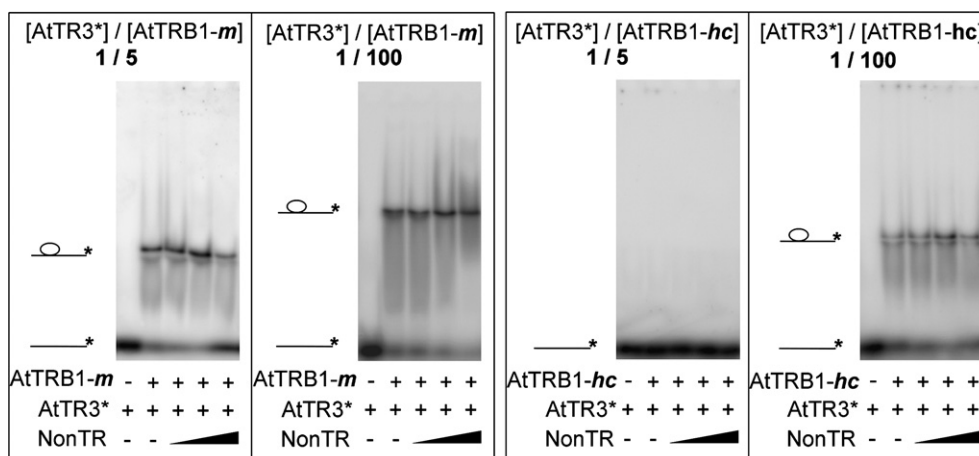
To determine the relative contribution of the Myb domain to the telomeric DNA binding affinity of AtTRB1, the amount of protein required for the formation of a DNA-protein complex which could be clearly and reproducibly detected in the gel was compared between fragments *m* and *hc*. The Myb domain formed a clear retarded band at a DNA/protein ratio 1/5, whereas fragment *hc* formed retarded bands only from a DNA/protein ratio above 1/50 (see Fig. 4).

Taken together, these data suggest that the Myb domain is the main region of the protein responsible for and promoting the binding of AtTRB1 to telomeric dsDNA, but also other regions have the potential to contribute to this interaction.

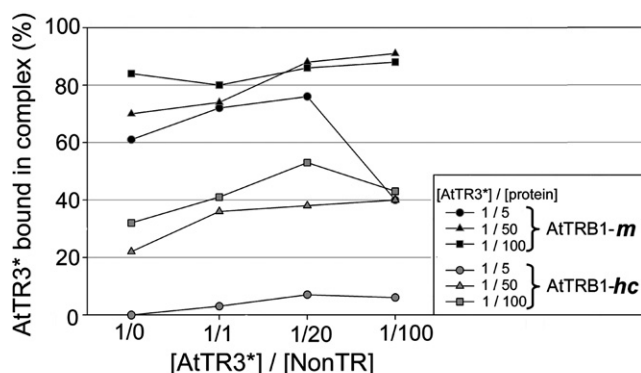
### 2.3. The Myb domain of AtTRB1 binds plant and human telomeric DNA with a similar affinity

To test the specificity of the Myb domain of AtTRB1 in recognition of related telomeric sequences, the binding to plant telomeric dsDNA (AtTR3) and human telomeric dsDNA (HuTR3) was compared when competed by the non-telomeric dsDNA (NonTR), AtTR3 or HuTR3. While the protein was not released from the plant telomeric probe by the non-telomeric DNA, the complex was equally dissociated by an excess of either human or plant telomeric DNA (Fig. 5). Similarly, the fragment covering the Myb domain together with the H1/5 domain (fragment *mh*) did not bind plant telomeric DNA stronger than human telomeric DNA (data not shown).

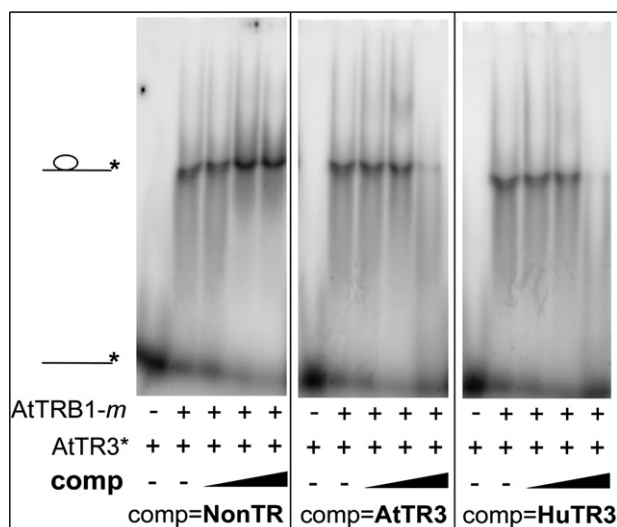
The non-selective binding of the Myb domain to either plant (TTTAGGG) or human (TTAGGG) telomeric sequence appears to be a general feature of the *A. thaliana* SMH proteins. Although the Myb domain has not been studied separately before, the bind-



**Fig. 4a.** Comparison of the binding affinity of the Myb domain (fragment AtTRB1-m) and the H1/5 + coiled-coil domains (fragment AtTRB1-hc) to a radioactively-labeled ds telomeric (AtTR3\*) oligonucleotide with unlabeled non-telomeric oligonucleotide (NonTR) as competitor. The concentration of the protein exceeds the concentration of the labeled probe 5-fold or 100-fold.



**Fig. 4b.** Percentage of labeled telomeric probe (AtTR3\*) bound in a DNA-protein complex by the Myb domain (AtTRB1-m) or the H1/5 + coiled-coil domains (AtTRB1-hc) with increasing concentration of unlabelled non-telomeric competitor (NonTR).



**Fig. 5.** Affinity of the Myb domain (AtTRB1-m) to labeled plant telomeric probe (AtTR3\*) in the presence of increasing amounts of unlabeled non-telomeric (NonTR), plant telomeric (AtTR) or human telomeric (HuTR) competitor. DNA probe/protein concentration is 1/100.

ing of two other full-length SMH proteins, AtTRB2 and AtTRB3, to plant telomeric oligonucleotide has been efficiently competed by human telomeric sequence as well (Schrumpfova et al., 2004). Although the proteins are not able to discriminate between related telomeric sequences of *A. thaliana* and human their stronger binding of telomeric than non-telomeric DNA suggests the following possibilities: (i) the core sequence recognized by the Myb domain without the C-extension is centered so that there is no difference between plant and human sequences (e.g. TTAGGGTT). Myb domains with a C-extension are however centered around the TTT sequence (Chen et al., 2001; Sue et al., 2006; Yang et al., 2003; Yu et al., 2000); (ii) At TRB proteins do not distinguish small changes in the sequence, as there has never been competition of so highly similar sequences throughout the evolution of these proteins in *A. thaliana*. In addition, the ability of AtTRB1 to bind typical plant and human telomeric motifs with a similar affinity could be important for an easier adaptation to a change in telomere sequence from a plant to a human-like motif, which has occurred during the evolution of plants of the order Asparagales (Sykora et al., 2003). This also corresponds with the finding that proteins binding ss telomeric DNA in plants with human-type telomeres show affin-

ity to both the ancestral and present telomeric DNA (Rotkova et al., 2004; Rotková et al., 2007).

#### 2.4. AtTRB1 forms multimers under the conditions of PFO-PAGE (Perfluoro-octanoic acid PolyAcrylamide Gel Electrophoresis)

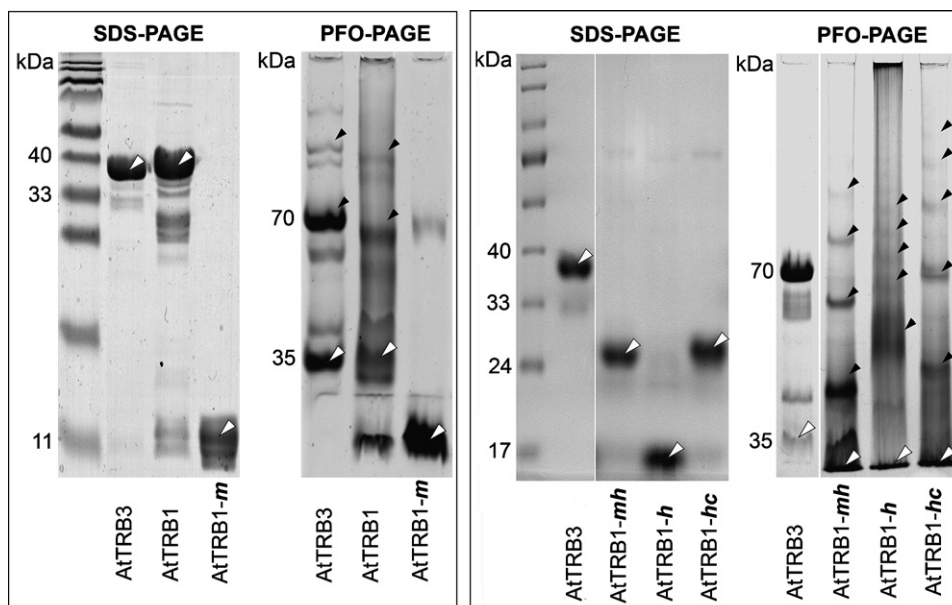
In order to investigate the ability of the AtTRB1 fragments to form self-dimers or multimers, PAGE with a weak detergent, perfluoro-octanoic acid (PFO), was used. This method can be used for detection and molecular mass determination of protein complexes since (in contrast to SDS-PAGE), PFO-PAGE preserves high-affinity protein-protein interactions (Ramjeesingh et al., 1999). The results confirm the strong tendency of the H1/5 domain to multimerize and the same holds true for all the fragments of AtTRB1 which contain the H1/5 domain. Dimerization has been proved to increase the efficiency of the binding of telomere-associated proteins to telomeric DNA (Bianchi et al., 1997, 1999; Fairall et al., 2001) and may also enhance the selectivity for binding longer tracts of telomeric sequence (Karamysheva et al., 2004). Candidate plant telomeric proteins containing the Myb domain on their C-terminus have also been shown to interact with the telomeric DNA as dimers. AtTRP1, a member of the TRFLI family (Karamysheva et al., 2004), has been shown to multimerize via its central domain and even the isolated Myb domain with C-extension has been shown to multimerize through the C-terminal residues (Sue et al., 2006). Myb domain of the rice RTBP also interacts with plant telomeric DNA in the form of a homodimer (Yu et al., 2000). A capability for multimerization therefore seems to characterize telomere dsDNA-binding proteins. In the case of AtTRB1, the N-terminal Myb domain itself does not form higher molecular weight complexes (Fig. 6), therefore the length of the DNA recognition sequence should be determined only by the number of DNA-protein interactions within one molecule of protein. This is in agreement with our finding that two telomeric repetitions are sufficient for the binding of the Myb domain. Similarly, Sue et al. have shown that the monomeric Myb domain of AtTRP1<sup>464–560</sup> interacts with 13 bp of DNA containing a single repeat of *A. thaliana* telomeric sequence (Sue et al., 2006).

### 3. Concluding remarks

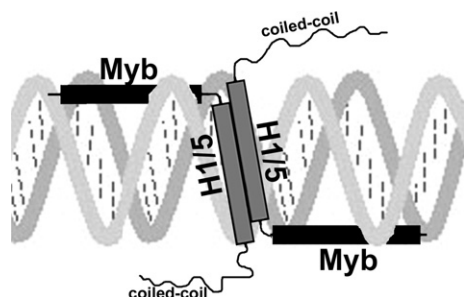
AtTRB1 is a member of the plant-specific SMH family of *A. thaliana* proteins which are characterized by the presence of a *telobox*-containing Myb domain located at their N-terminus, a histone-like H1/5 domain in the centre and a coiled-coil domain at the C-terminus (Marian et al., 2003). Because the presence of a *telobox* is common to telomere dsDNA-binding proteins (Bilaud et al., 1996), the SMH proteins are potential candidates to cap and regulate the plant telomeres.

We show that AtTRB1 is indeed a telomeric dsDNA-binding protein, similarly as proteins AtTRB2 and AtTRB3 which we have described previously (Schrumpfova et al., 2004). The Myb domain of AtTRB1 preferentially binds telomeric over non-telomeric DNA. Two telomeric repeats are sufficient for the Myb domain to bind DNA. Homomultimer formation through the H1/5 domain has been shown by PFO-PAGE. This suggests a possible role for the H1/5 domain of AtTRB proteins in promoting either their self- or mutual interactions or their interaction with other partners. Compared to other dsDNA-binding proteins, the triple-domain AtTRB proteins have a potential for a remarkable multiplicity of modes of binding to telomeres: the Myb-domain primarily ensures direct sequence-specific binding of AtTRB1 to telomeric DNA, the H1/5 domain may enhance this binding by protein dimerization, while extensive protein aggregation is counteracted by competitive loading of the H1/5 domain to non-specific DNA (Fig. 7). Thus, paradoxically, DNA-





**Fig. 6.** Comparison of SDS-PAGE and PFO-PAGE of AtTRB1 and its fragments. White arrowheads point to monomers, black to homomultimers; the AtTRB3 monomer was used as an approximate marker of MW 35 kDa. Left panel: AtTRB1 and AtTRB1-*m* (15% SDS-PAGE gel, AA:BIS 37:1; 9% PFO-PAGE gel, AA:BIS 37:1); right panel: AtTRB1 – *mh*, *h*, *hc* (12.5% SDS-PAGE gel, AA:BIS 37:1; 8% PFO-PAGE gel, AA:BIS 37:1).



**Fig. 7.** Schematic view of binding of AtTRB1 to telomeric DNA. The Myb-domain primarily ensures direct sequence-specific binding of AtTRB1 to telomeric DNA, while the H1/5 domain may enhance this binding by protein dimerization and sequence-non-specific binding to DNA.

sequence-non-specific binding of the H1/5 domain promotes specific interaction of the Myb-domain with telomeric DNA, as illustrated in Figs. 2 and 4b. Moreover, the H1/5 domain of AtTRB proteins was recently shown to interact with the OB-fold domain of the AtPOT1b telomeric protein (Prochazkova Schruppfova et al., 2008) suggesting yet another mode of AtTRB recruitment to telomeres. Interestingly, our results do not show any important role of the coiled-coil domain of AtTRB1 in the interactions studied, although this domain was originally suggested as responsible for protein-protein interactions (Marian et al., 2003). However, we cannot exclude that the coiled-coil domain participates in dimerization upon binding of the AtTRB1 to DNA, when the H1/5 domain becomes engaged in DNA-protein interaction, or in interaction with AtPOT1b. Our data thus reveal at least partial outlines of a plant analogy of the mammalian shelterin complex (de Lange, 2005).

We conclude that AtTRB1 is a telomeric-dsDNA-binding protein which is able to form multimers. The specific binding to telomeric DNA is primarily conducted by the Myb domain, while the multimerization and sequence-non-specific interactions with DNA are mediated by the H1/5 domain.

## 4. Experimental

### 4.1. Cloning, expression and purification of AtTRB1 and its fragments

The cDNA sequence of AtTRB1 (locus At1g49950) was obtained by RT-PCR from total RNA as described previously (Kuchar and Fajkus, 2004). In order to analyze the binding of AtTRB1 to telomeric DNA and to examine the contribution of its three individual major domains in the binding, AtTRB1 and its truncated variants (Fig. 1) were cloned into pET15b (Novagen) between the NdeI and BamHI sites using the following sets of primers:

AtTRB1 (full-length): A (5'-AGTATTCATATGGGTGCTCCTAAGCA-GA-3') + B (5'-CGGGATCCTCAGGCACGGATCATCATT-3'); AtTRB1-*m*: A + C (5'-GAGGATCCCTAAAGGGAGAACCTCCTTTT-3'); AtTRB1-*mh*: A + D (5'-TTGGATCCTCATCTCCTGTGGGAAGACAAGG-3'); AtTRB1-*h*: E (5'-AGTGTTTCATATGGAATGTGGATGCGACCT-3') + D, AtTRB1-*hc*: E + B.

Proteins were expressed by autoinduction in ZYM-5052 (Studier, 2005) in *E. coli* C41(DE3) (Miroux and Walker, 1996) grown for 3 h at 37 °C followed by 18 h at 20 °C. Expression of proteins of the correct molecular weight was confirmed by immunodetection. His-tagged proteins were purified by affinity FPLC (Fast Protein Liquid Chromatography) (Pharmacia ÄKTA purifier system) on BD TALON™ Superflow Resin (BD Biosciences) in 50 mM Na-phosphate buffer with 300 mM NaCl. A step gradient from 10 mM to 80 mM imidazole was used for elution. Protein concentration was estimated according to Bradford (1976).

### 4.2. Electrophoresis mobility shift assay (EMSA)

Oligonucleotides used in EMSA are described in Table 1. Telomeric oligonucleotides containing different numbers of telomere repeat units were adjusted to 28 nt by supplementing their telomeric part with a non-telomeric sequence (NonTR). Ds oligonucleotides were formed by annealing of complementary ss oligonucleotides. Oligonucleotides used as probes were end-labeled using [ $\gamma$ -<sup>32</sup>P]ATP (MP Biomedicals) and polynucleotide kinase (NEB). 2.5–250 pmol of proteins (as indicated in Results)

**Table 1**

Oligonucleotides used in EMSA. The plant telomeric sequence is shown in bold

Name	Length (nt)	Sequence 5'-3'
AtTR2-G	28	<b>GGTTTAGGGTTTAG</b> AGTACCAGCCATGA
AtTR2-C	28	TCATGGCTGGTACTCTAAACCTAAACC
AtTR3-G	28	<b>GGTTTAGGGTTTAGGGTTTAG</b> AGTACCA
AtTR3-C	28	TGGTACTCTAAACCTAAACCTAAACC
AtTR4-G	28	<b>GGTTTAGGGTTTAGGGTTTAG</b>
AtTR4-C	28	<b>CTAAACCTAAACCTAAACCTAAACC</b>
HuTR4-G	24	GGTTAGGGTTAGGGTTAGGGTTAG
HuTR4-C	24	CTAACCTAAACCTAAACCTAAACC
NonTR-G	28	CATCATGGCTGGTATGGCTGGTACTAG
NonTR-C	28	CTAGTACCAGCCATGACCAAGCCATGATG

were preincubated with 2.5, 50 or 250 pmol of competitor DNA for 10 min in 50 mM Na-phosphate buffer pH 8 + 200 mM NaCl; the labeled probe (2.5 pmol) was added, and the reaction was further incubated for 10 min at 25 °C. Total reaction volume was 20 µl. The reaction mixture was loaded onto an 8% w/v non-denaturing polyacrylamide gel (AA:BIS = 37:1, 0.25 × Tris-borate-EDTA (TBE) buffer). Electrophoresis was performed at 4 °C in 0.25 × TBE for 4 h at 120 V. Results were visualized on a STORM840 phosphorimager and evaluated using ImageQuant software (Molecular Dynamics). Protein-DNA interaction was quantified from the signal of unbound DNA probe and expressed as the percentage of labeled probe bound in a protein-DNA complex.

#### 4.3. Polyacrylamide gel electrophoresis with perfluoro-octanoic acid (PFO-PAGE)

PFO-PAGE was performed according to Ramjeesingh et al. (1999) with a slight modification in the content of PFO in the running buffer (0.2% w/v compared to 0.5%). No DTT was used in the loading buffer. Ten micrograms of protein were used per lane. Electrophoresis in 8% or 9% gel (AA:BIS = 37:1) was carried out at 80 V for 3 h. Identical samples were simultaneously separated by SDS-PAGE to compare the denatured state of the proteins.

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