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Arabidopsis POT1 associates with the telomerase RNP and is required for telomere maintenance

Yulia V Surovtseva[1], Eugene V Shakirov[1], Laurent Vespa, Nathan Osbun, Xiangyu Song and Dorothy E Shippen*

Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA

POT1 **is a single-copy gene in yeast and humans that encodes a single-strand telomere binding protein required for chromosome end protection and telomere length regulation. In contrast, *Arabidopsis* harbors multiple, divergent *POT*-like genes that bear signature N-terminal OB-fold motifs, but otherwise share limited sequence similarity. Here, we report that plants null for *AtPOT1* show no telomere deprotection phenotype, but rather exhibit progressive loss of telomeric DNA. Genetic analysis indicates that *AtPOT1* acts in the same pathway as telomerase. *In vitro* levels of telomerase activity in *pot1* mutants are significantly reduced and are more variable than wild-type. Consistent with this observation, AtPOT1 physically associates with active telomerase particles. Although low levels of AtPOT1 can be detected at telomeres in unsynchronized cells and in cells arrested in G2, AtPOT1 binding is significantly enhanced during S-phase, when telomerase is thought to act at telomeres. Our findings indicate that AtPOT1 is a novel accessory factor for telomerase required for positive telomere length regulation, and they underscore the coordinate and extraordinarily rapid evolution of telomere proteins and the telomerase enzyme.**

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Introduction

Telomeres stabilize eukaryotic genomes by facilitating the complete replication of the chromosome terminus, and sequestering the ends from recognition by DNA damage checkpoint machinery that would otherwise lead to inappropriate engagement of recombination and DNA repair activities. Telomeres typically consist of simple G-rich repeats that terminate in a single-strand 3′ extension, termed the G-overhang. The G-overhang serves as the substrate for the telomerase ribonucleoprotein (RNP) reverse transcriptase, which replenishes telomeric DNA. How telomerase engages the telomere is unknown, but its activity is modulated in *cis* by cell cycle-regulated interactions with resident telomeric DNA binding proteins (reviewed in Collins, 2006).

G-strand binding proteins play a crucial role in regulating telomerase access to telomeres, and in controlling other telomere-associated activities. The best characterized of these is Cdc13p from budding yeast (Nugent *et al*, 1996). Typical of this class of proteins, Cdc13p binds telomeric DNA via an oligonucleotide–oligosaccharide binding fold (OB-fold) (Mitton-Fry *et al*, 2002). Cdc13p is a multifunctional protein (reviewed in Lustig, 2001). It dynamically interacts with other constituents of the chromosome terminus and contributes to positive and negative regulation of telomerase, coupling of leading and lagging strand DNA synthesis, and protection of the C-rich strand of the chromosome terminus against nucleolytic attack.

In *Schizosaccharomyces pombe* and in higher eukaryotes, the presumed ortholog of Cdc13p is POT1 (Protection Of Telomeres) (Baumann and Cech, 2001). Although most organisms harbor only a single *POT1* gene, ciliates, mouse and *Arabidopsis* possess at least two of these (Wang *et al*, 1992; Shakirov *et al*, 2005; Hockemeyer *et al*, 2006; Jacob *et al*, 2006; Wu *et al*, 2006). POT1 binds telomeric DNA *in vitro* (Baumann and Cech, 2001), but its attachment to the chromosome terminus *in vivo* is mediated primarily through protein interactions in the Shelterin complex (Loayza and de Lange, 2003; de Lange, 2005). Recent studies indicate that POT1 function is conveyed through its association with TPP1, another OB-fold containing protein (Houghtaling *et al*, 2004; Liu *et al*, 2004; Ye *et al*, 2004; Wang *et al*, 2007; Xin *et al*, 2007).

The co-crystal structure of human POT1 bound to its DNA substrate indicates that the 3′ terminal residues of the DNA are sequestered within the protein binding pocket, implying that hPOT1 functions to protect against nucleases and limit accessibility to telomerase (Lei *et al*, 2004). Consistent with this prediction, hPOT1 negatively regulates telomerase activity *in vitro*; this inhibition requires the DNA binding activity of hPOT1 (Kelleher *et al*, 2005; Lei *et al*, 2005). *In vitro* studies suggest that hPOT1 could also promote telomerase action at the chromosome terminus. Disruption of G-quartet structures by hPOT1 facilitates elongation by telomerase *in vitro* (Zaug *et al*, 2005). Moreover, hPOT1 stimulates unwinding of telomeric DNA by WRN and BLM helicases (Opresko *et al*, 2005), and depending on the location of POT1 binding site, hPOT1 can improve telomerase activity and processivity *in vitro* (Lei *et al*, 2005).

Genetic analysis of POT1 in fission yeast and vertebrates reveals a complex role for this protein in telomere length control. Human cells with reduced levels of POT1 display telomere elongation (Veldman *et al*, 2004; Ye *et al*, 2004; Yang *et al*, 2005) as do mice conditionally null for POT1a (Wu *et al*, 2006). Similarly, reduction of telomere-bound POT1 in *S. pombe* results in dramatic telomere elongation (Bunch *et al*,

*Corresponding author. Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, TX 77843-2128, USA. Tel.: +1 979 862 2342; Fax: +1 979 845 9274;
E-mail: dshippen@tamu.edu
[1]These authors contributed equally to this work

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2005). In contrast, overexpression studies implicate *S. pombe* and human POT1 in the positive regulation of telomere length (Colgin *et al*, 2003; Armbruster *et al*, 2004; Liu *et al*, 2004; Bunch *et al*, 2005). Thus, like Cdc13p, POT1 may contribute to both positive and negative regulation of telomere length.

POT1 is also necessary for chromosome end protection. *S. pombe pot1*$^{-}$ mutants suffer immediate and catastrophic loss of telomeric repeats, erosion of subtelomeric DNA, and chromosome mis-segregation (Baumann and Cech, 2001). Depletion of vertebrate POT1 leads to a DNA damage response at telomeres (Hockemeyer *et al*, 2005; Churikov *et al*, 2006), and in chicken cells results in a rapid G2 cell cycle arrest (Churikov *et al*, 2006). Other studies on POT1-depleted mammalian cells reveal genome instability, senescence and apoptosis (Veldman *et al*, 2004; Yang *et al*, 2005). The mouse POT1a and POT1b genes appear to be partially redundant for chromosome end protection. Although POT1b mutants are viable (Hockemeyer *et al*, 2006), conditional knockout of POT1a results in embryonic lethality (Hockemeyer *et al*, 2006; Wu *et al*, 2006). Single POT1a or double POT1a POT1b mutants exhibit a strong telomere DNA damage response, low levels of telomere fusions and endoreduplication, along with proliferative arrest and senescence (Hockemeyer *et al*, 2006). A second study implicated POT1a and POT1b in repression of non-homologous end joining and homologous recombination at telomeres (He *et al*, 2006; Wu *et al*, 2006).

Arabidopsis encodes two POT-like proteins, AtPOT1 and AtPOT2 (Shakirov *et al*, 2005), and possibly a third, AtPOT3 (Surovtseva *et al*, in preparation). In contrast to the mouse POT1a and POT1b proteins, which share 72% similarity (Hockemeyer *et al*, 2006), the plant POT proteins are more divergent and display only 49% overall sequence similarity. AtPOT2 is implicated in chromosome end protection, as overexpression of the N-terminal portion of the protein leads to severe growth and developmental defects, telomere shortening, and a high incidence of anaphase bridges and chromosome mis-segregation. AtPOT1, by contrast, contributes to telomere length regulation. Overexpression of a C-terminal fragment of AtPOT1 lacking the OB-fold motifs results in modest telomere shortening, but plants are wild type in appearance and show no signs of genome instability (Shakirov *et al*, 2005).

In this study, we examined the fate of *Arabidopsis* mutants null for *AtPOT1*. We found no evidence that AtPOT1 contributes to chromosome end protection or genome stability. Instead, AtPOT1 is required for positive regulation of telomere length: *pot1* mutants display progressive telomere shortening at the same rate as telomerase-null plants. Notably, *in vitro* telomerase activity levels are reduced in *pot1* mutants, but not abolished. Finally, we show that AtPOT1 physically associates with the telomerase RNP, and is enriched at telomeres during S-phase. Thus, AtPOT1 appears to be a novel telomerase accessory factor that promotes its activity *in vitro* and *in vivo*.

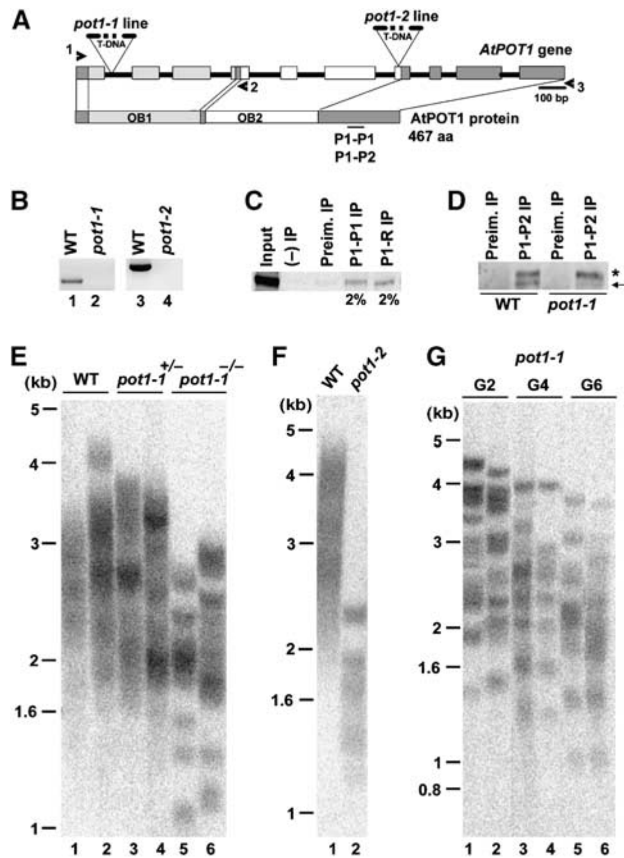

Figure 1 Telomere phenotypes in AtPOT1-deficient *Arabidopsis*. (**A**) Genomic map and coding region of the *AtPOT1* locus. Rectangles are exons; black lines represent introns. The position of T-DNA insertions in the *pot1-1* and *pot1-2* alleles are shown. OB1 and OB2 indicate two predicted OB-folds in the AtPOT1 protein. The position of the peptide used to raise P1-P1 and P1-P2 antibodies is indicated. Both peptides were raised against a similar AtPOT1 region, but P1-P2 peptide is slightly longer (see Materials and Methods). (**B**) RT–PCR analysis of the *AtPOT1* gene expression in *pot1-1* and *pot1-2* mutants. Primer pairs 1–2 and 1–3 (shown as arrowheads in panel A) were used to analyze gene expression. (**C**) IP of recombinant ^{35}S-labeled AtPOT1 protein. IP efficiencies for P1-P1 and P1-R antibodies are indicated. (**D**) Detection of endogenous AtPOT1 protein in wild-type and *pot1-1* callus. AtPOT1 was immunoprecipitated and detected by Western blot analysis using P1-P2 antibody. The arrow indicates the 55 kDa endogenous AtPOT1 protein immunoprecipitated from wild-type callus. The asterisk indicates a nonspecific cross-reacting protein. (**E**) TRF analysis of DNA from six siblings segregating from a heterozygous *pot1-1* parent. (**F**) TRF analysis of a *pot1-2* mutant. (**G**) Multi-generational TRF analysis of *pot1-1*. DNA samples from two individual *pot1-1* plants from the second (G2), fourth (G4), and sixth (G6) generation of self-pollination were analyzed. Blots shown in panels E, F and G were hybridized with a radiolabeled telomeric DNA probe. Molecular weight markers are indicated. Plants analyzed in panel G are from the WS ecotype, plants in panel E are from a Columbia-WS cross, and plants in panel F are from Columbia. Telomeres in wild-type WS plants are typically longer than those in Columbia (Shakirov and Shippen, 2004).

Results

Plants null for AtPOT1 do not exhibit genome instability

To identify an *Arabidopsis* line null for *AtPOT1*, we screened T-DNA collections from the University of Wisconsin *Arabidopsis* Knock-out Facility. Analysis of the ALPHA population uncovered a mutant with an insertion in the first intron of *AtPOT1* (Figure 1A and Supplementary Figure 1). This line was designated *pot1-1*. In the Weigel collection, we found a second *AtPOT1* allele (*pot1-2*), bearing a T-DNA in the seventh exon (Figure 1A and Supplementary Figure 1). To determine if these insertions disrupt *AtPOT1* gene expression, RT–PCR was performed using primers flanking the insertion

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sites (Figure 1B and Supplementary Figure 1). No PCR products were generated in reactions with cDNA from the mutant plants (Figure 1B, lanes 2 and 4), confirming that expression of the full-length *AtPOT1* mRNA was abolished in *pot1-1* and *pot1-2* mutants.

To monitor AtPOT1 protein, antibodies were raised against two peptides corresponding to a segment in the C-terminus of AtPOT1 protein (P1-P1 and P1-P2) (Figure 1A), and against a full-length recombinant AtPOT1 protein (P1-R). All three antibodies detected recombinant AtPOT1 by Western blotting (data not shown), and each immunoprecipitated the recombinant protein with ~2% immunoprecipitation (IP) efficiency (Figure 1C; data not shown). Importantly, P1-P1 and P1-P2 detected a 55 kDa protein that corresponds to endogenous AtPOT1 protein in extracts from wild-type seedlings and callus, but not from *pot1-1* mutants (Figure 1D; data not shown). We conclude that *pot1-1* and likely *pot1-2* (see below) are null for *AtPOT1*.

In striking contrast to yeast and vertebrate cells deficient in POT1, *Arabidopsis pot1* mutants appeared morphologically indistinguishable from wild-type and showed no decrease in fertility or perturbation in growth and development for the six generations they were propagated. Furthermore, chromosome ends were refractory to nuclease attack and non-homologous end joining in the absence of AtPOT1. No anaphase bridges were observed in first (G1) or second (G2) generations of *pot1-1* mutants (Supplementary Table 1; data not shown). The more sensitive telomere fusion PCR assay (Heacock *et al*, 2004) also failed to detect an increased frequency in chromosome end joining reactions in *pot1-1* (data not shown). Thus, AtPOT1 is dispensable for chromosome end protection in *Arabidopsis*.

AtPOT1 is required for telomere length maintenance in vivo

To examine telomere length in *pot1* mutants, terminal restriction fragment (TRF) analysis was performed on plants segregated from self-pollination of a heterozygous *pot1-1* parent. As expected, telomeres in wild-type siblings appeared as a homogeneous smear of products ranging from 1.6 to 4.5 kb (Figure 1E, lanes 1 and 2). As for *AtTERT* (Fitzgerald *et al*, 1999), *AtPOT1* is not haploinsufficient for telomere maintenance in *Arabidopsis*; plants heterozygous for the T-DNA insertion exhibited a wild-type telomere profile (Figure 1E, lanes 3 and 4).

Strikingly, telomere tracts in *pot1-1* were much shorter than in wild-type and showed a more discrete banding pattern (Figure 1E, lanes 5 and 6). To determine whether disruption of the *AtPOT1* gene was responsible for the telomere phenotypes, TRF analysis was performed on *pot1-2* mutants. Telomeres were significantly shorter in *pot1-2* than in wild-type, or even *pot1-1* (Figure 1F, lane 2). Since the *pot1-2* mutant was homozygous when we identified it, we suspect that this line had already been propagated at the *Arabidopsis* stock center for several generations in the absence of AtPOT1 prior to our analysis, leading to more substantial loss of telomeric DNA than in *pot1-1*. Complementation experiments provided further verification that AtPOT1 depletion caused telomere shortening. Plants heterozygous for *pot1-1* were transformed with the wild-type *AtPOT1* coding region under control of the constitutive CaMV *35S* promoter. In plants expressing the *35S*::*AtPOT1* transgene telomeres, particularly the shortest ones in the population, were returned to the wild-type length (Supplementary Figure 2A, lanes 2 and 3). A second complementation experiment performed with *pot1 ku70* double mutants confirmed that AtPOT1 is required for telomere maintenance (Supplementary Figure 2B; see below).

Telomeres in pot1 mutants shorten at the same rate as in tert mutants

We followed the fate of telomeres in *pot1* mutants for several plant generations and found that telomere length in *pot1-1* decreased progressively with each generation (Figure 1G). The ever-shorter-telomere phenotype and sharp TRF banding profile were strikingly similar to the phenotype associated with *tert* mutants, which lose 200–500 bp of telomeric DNA per plant generation (Fitzgerald *et al*, 1999; Riha *et al*, 2001).

To determine if the rate of telomere shortening in *pot1-1* was the same as in *tert*, we used a parent-progeny analysis to measure the rate of bulk telomere loss. DNA extracted from first generation (G1) parents (P) homozygous for the *pot1-1* or *pot1-2* allele, and their progeny (G2) was subjected to TRF analysis. For *pot1-1*, bulk telomeres declined by approximately 200–500 bp, while for *pot1-2*, a loss of approximately 200 bp was observed (Figure 2A and B). To obtain a more accurate estimate of the telomere shortening rate, individual telomeres were examined using subtelomeric TRF analysis (Shakirov and Shippen, 2004). Using probes specific for the South (right) arm of chromosome two (2R), or the North (left) arm of chromosome one (1L) (Figure 2C), only a single discrete band was detected in the parent and its progeny, possibly reflecting the coordinate regulation of telomere length on homologous chromosomes throughout plant development (Shakirov and Shippen, 2004). For both telomeres, the length decreased by approximately 200–300 bp relative to the parent (Figure 2C).

To further examine the rate of telomere shortening in *pot1* mutants, we performed primer extension telomere repeat amplification (PETRA) (Heacock *et al*, 2004). In this assay, telomeres are amplified in a PCR reaction using primers directed at the G-overhang and a unique subtelomeric sequence. PETRA showed a decline of approximately 200 bp on the 2R and 5R telomeres in both *pot1-1* and *tert* mutants (Figure 2D and E; data not shown). The same degree of shortening occurred in *pot1-2* (data not shown). We conclude that disruption of *AtPOT1* leads to a progressive loss of telomeric DNA that proceeds at the same rate as in *tert* mutants.

Telomeres become critically shortened in G6 *tert* mutants, giving rise to end-to-end fusions and genome instability (Riha *et al*, 2001). Because our *pot1-1* mutants were derived from the WS ecotype, which naturally has longer telomeres than the Columbia ecotype (Shakirov and Shippen, 2004), from which the *tert* mutant was obtained, it is not surprising that G6 *pot1-1* mutants do not yet show signs of genome instability. Assuming a telomere shortening rate of 200 bp/plant generation, we expect two or three additional generations are required for some *pot1-1* telomeres to become critically shortened (Heacock *et al*, 2004).

AtPOT1 and AtTERT act in the same genetic pathway

To determine whether *AtPOT1* and *AtTERT* act in the same genetic pathway, plants heterozygous for *pot1-1* were crossed

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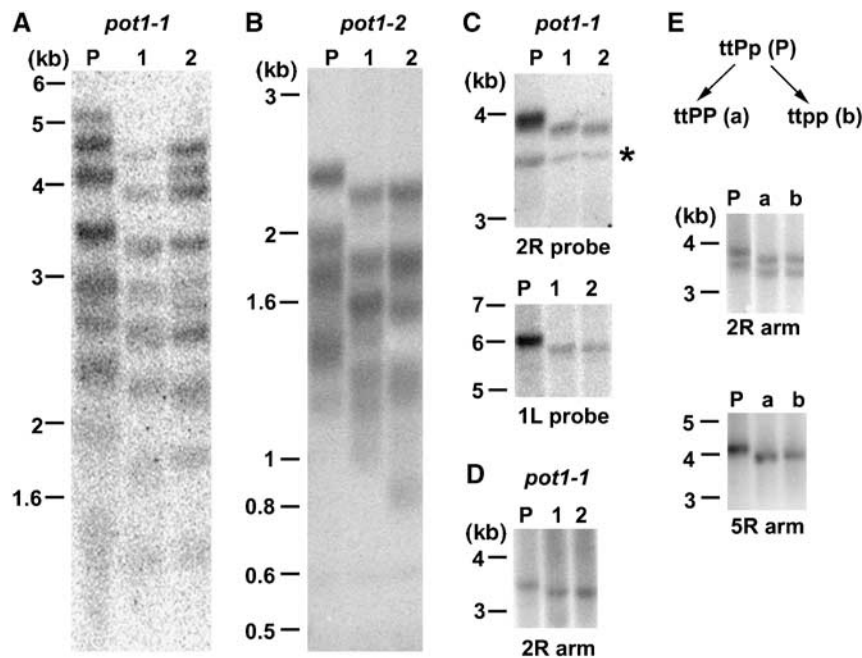

Figure 2 Parent–progeny analysis reveals the same rate of telomere shortening in *pot1-1*, *pot1-2*, and *tert* mutants. (**A, B**) TRF analysis of bulk telomeric DNA from *pot1-1* and *pot1-2* parents (P) and two progeny (1 and 2) using a telomeric probe. (**C**) Subtelomeric TRF analysis of DNA from a *pot1-1* parent and two progeny. DNA blots were hybridized with a probe corresponding to unique subtelomeric regions on 2R and 1L chromosome arms. The asterisk indicates a cross-hybridizing band. (**D**) PETRA analysis of the 2R telomere in a *pot1-1* parent and two progeny. (**E**) PETRA analysis of the 2R and 5R telomeres in a parent homozygous for *tert* and heterozygous for *pot1-1* (ttPp), and its *tert* (ttPP) (a) and *pot1-1 tert* (ttpp) (b) progeny. The two PETRA bands detected in the 2R reaction may represent different size telomeres on homologous chromosomes or two populations of cells (Shakirov and Shippen, 2004). A telomeric probe was used to detect PETRA products.

to plants heterozygous for *tert*. Double heterozygous mutants from F1 were allowed to self-pollinate to generate F2 progeny. As shown in Figure 3A, telomeres of the same length and sharp banding profile were found in *pot1-1 tert*, as in their *tert* and *pot1-1* siblings. PETRA and TRF parent–progeny analysis confirmed that telomeres in *pot1-1 tert* mutants shortened at the same rate as in either single mutant (Figures 2E and 3B).

If AtPOT1 is required for telomerase function *in vivo*, its contribution should be especially obvious in a genetic background, where telomerase generates ultra-long telomere tracts. KU is a negative regulator of telomere length in *Arabidopsis*, and telomeres in mutants deficient in KU70 or KU80 undergo telomerase-dependent expansion to more than twice the normal length in a single generation (Riha *et al*, 2002; Gallego *et al*, 2003) (Figure 3C, lanes 3 and 4). In contrast, *ku70 tert* double mutants display accelerated telomere shortening and a precocious onset of genome stability (Riha and Shippen, 2003). To further investigate the role of AtPOT1, we generated *pot1 ku70* mutants. In contrast to their *ku70* siblings, *pot1 ku70* mutants failed to elongate their telomeres (Figure 3C, lanes 7 and 8), and exhibited a heterogeneous profile of TRF products similar to that seen in *ku70 tert* (Supplementary Figure 3B, lane 3). Notably, telomeres in *pot1 ku70* mutants were significantly elongated when an exogenous copy of *AtPOT1* was introduced (Supplementary Figure 2B), confirming that AtPOT1 is required for telomere elongation in the absence of KU.

We also found that degree of telomere shortening was the same in *pot1 ku70* and *ku70 tert* double mutants as in triple *pot1 ku70 tert* mutants. Parent–progeny analysis confirmed that plants from all three genotypes lost approximately the

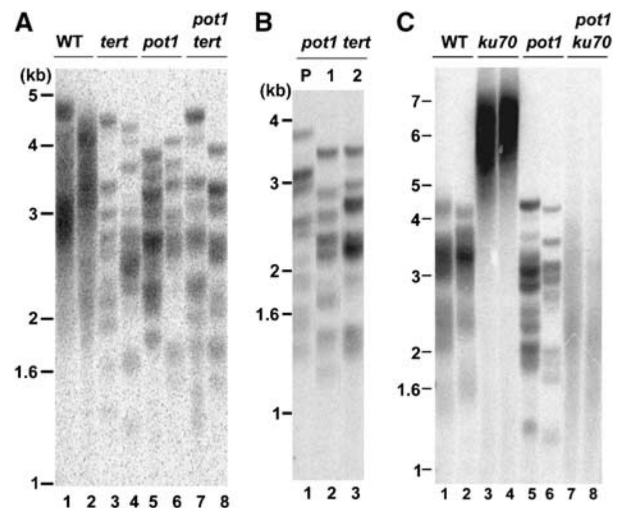

Figure 3 AtPOT1 functions in the telomerase pathway. (**A**) TRF analysis of *pot1-1 tert* mutants. Results for eight progeny (two for each genotype) that were segregated from a parent heterozygous for *pot1-1* and *tert* are shown. (**B**) TRF analysis of telomeres in *pot1-1 tert* parent (P) and its two progeny (1 and 2) are shown. (**C**) TRF analysis of *pot1-1 ku70* mutants. Results for progeny segregated from a parent heterozygous for *pot1-1* and *ku70* are shown. Two different progeny were analyzed for each genotype. The blot was hybridized with a telomeric DNA probe.

same amount of telomeric DNA from G1 (Supplementary Figure 3B, lanes 3, 4, 6) to G2 (Supplementary Figure 3C). All three mutants reached the terminal phenotype in G3. The incidence of anaphase bridges in *pot1 ku70, tert ku70* and

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pot1 ku70 tert mutants in G3 were the same (Supplementary Table 1). These genetic data reinforce the notion that AtPOT1, like AtTERT, does not contribute to chromosome end protection. We conclude that *AtPOT1* acts in the same genetic pathway as telomerase and is specialized for telomere length maintenance *in vivo*.

AtPOT1 is a component of the telomerase RNP required for maximal activity in vitro

We considered the possibility that AtPOT1 is required for telomerase enzyme activity. TRAP assays were performed in parallel with extracts from wild-type and *pot1* seedlings. As shown in Figure 4A (lanes 1 and 3–5), robust telomerase activity was reproducibly detected in extracts from wild type plants and from suspension culture. In contrast, telomerase levels were reduced and more variable in both *pot1-1* and *pot1-2* mutants (Figure 4A, lanes 6–12). Extract mixing experiments indicated that the reduction in enzyme activity was not due to the presence of a soluble PCR inhibitor (data not shown). Interestingly, although the *in vitro* levels of telomerase activity varied among *pot1* mutants, the progressive telomere shortening phenotype observed *in vivo* was extremely consistent among the dozens of *pot1-1* and *pot1-2* mutant plants we examined.

Most TRAP reactions carried out with *pot1* mutants showed reduced, but detectable levels of enzyme activity (Figure 4A, lanes 6–8 and 10–12). Titration of such samples suggested that telomerase activity was decreased by approxi-

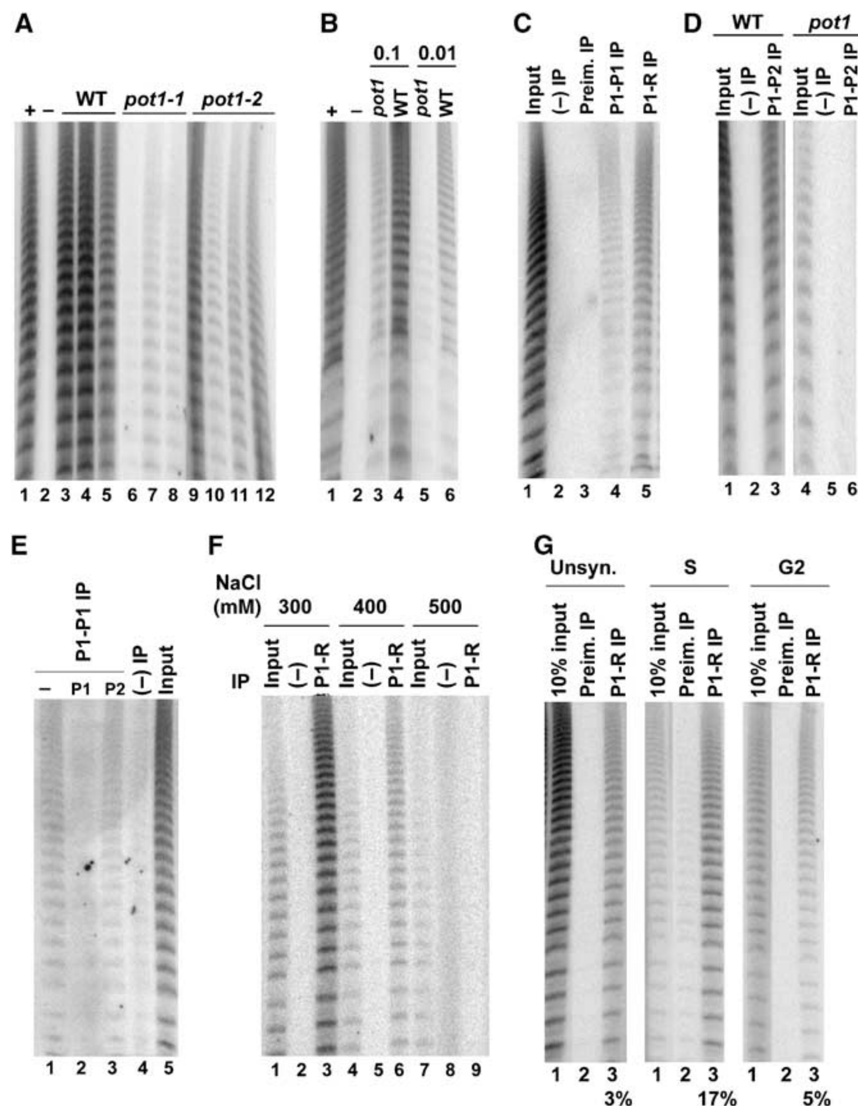

Figure 4 AtPOT1 interacts with the telomerase RNP. (**A**) TRAP assay results for wild-type (WT), *pot1-1*, and *pot1-2* flowers. Results for extracts prepared from 10 different plants are shown in lanes 3–12. (**B**) TRAP assay results for wild-type and *pot1-2* mutant flowers. $10\times$ and $100\times$ dilutions of protein extracts were used for TRAP as indicated. In panels A and B, extract prepared from *Arabidopsis* suspension culture (lane 1) served as the positive control (+). (**C**) TRAP assay following AtPOT1 IP from suspension culture using P1-P1 or P1-R antibodies. (**D**) TRAP assays with P1-P2 immunoprecipitates from wild-type and 4-day-old *pot1-2* seedlings extracts. (**E**) TRAP assay results for P1-P1 antibody immunoprecipitates. IP from suspension culture extract was performed with no addition of peptide (–), $100\times$ excess of P1-P1 peptide (P1), or $100\times$ excess of a nonspecific AtPOT2 peptide (P2). (**F**) TRAP assays with eluates from P1-R IP in the presence of NaCl. NaCl concentrations are indicated. (**G**) TRAP assays with P1-R immunoprecipitates from unsynchronized suspension culture (unsyn.) or S-phase and G2-phase synchronized cells. The relative amount of active telomerase precipitated in the reaction is indicated. In all panels, IP with no antibody added ((–) IP), or with preimmune serum (Preim. IP), was used as a negative control.

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mately 10-fold (Figure 4B, compare lanes 3 and 6). A more detailed understanding of AtPOT1's role in *Arabidopsis* telomerase biochemistry will require the development of a conventional (non-PCR based) primer extension assay to monitor the catalytic properties of the enzyme, a goal that has thus far proven elusive. Nevertheless, our current data allow us to conclude that AtPOT1 promotes telomerase action *in vitro*, but is not absolutely essential for its biochemical activity.

Next we looked for a physical interaction between AtPOT1 and the telomerase RNP. We could not detect a direct association between recombinant AtPOT1 and AtTERT by co-IP or by yeast two-hybrid assay (Y Surovtseva, M Jasti, and D Shippen, unpublished data). However, since AtTERT is the only *Arabidopsis* telomerase subunit isolated so far, AtPOT1 could contact another component of the RNP. To test this idea, IPs were performed with POT1 antibody on extracts from wild-type seedlings and asynchronous *Arabidopsis* cell culture (Menges and Murray, 2002). We confirmed that telomeres in this cell line fall within the wild-type range (Supplementary Figure 4A and B), and chromosome ends are protected against end joining reactions (Supplementary Figure 4C). Moreover, a 55 kDa band corresponding to endogenous AtPOT1 was observed by Western blotting following IP of cell culture extracts (Supplementary Figure 4D).

TRAP assays conducted on immunoprecipitates in the absence of antibody or with preimmune serum did not generate PCR products (Figure 4C, lanes 2 and 3; Figure 4D, lanes 2 and 5). However, telomerase activity could be immunoprecipitated from both seedlings and cell culture using all three of the POT1 antibodies (Figure 4C, lanes 4 and 5; Figure 4D, lane 3). The specificity of the AtPOT1 interaction with telomerase was demonstrated in three ways. First, IP of extracts from *pot1* mutant seedlings failed to precipitate telomerase activity (Figure 4D, lane 6). Second, addition of a 100-fold excess of the P1-P1 peptide to cell culture extracts during the IP with the P1-P1 antibody dramatically decreased the TRAP signal, while a nonspecific AtPOT2 peptide of a similar length failed to compete (Figure 4E, lanes 2 and 3). Third, the AtPOT1 interaction with telomerase was stable in high salt; the association persisted in up to 400 mM NaCl (Figure 4F, lane 6). Since telomerase activity is strongly inhibited in salt concentrations greater than 450 mM (Figure 4F, lane 7), the AtPOT1 interaction may be even more robust.

To investigate whether AtPOT1 association with telomerase is cell cycle regulated, IP was performed on synchronized cell extracts (Menges and Murray, 2002). The level of telomerase activity is approximately the same in unsynchronized cultured cells as in cells arrested in S or G2 (Supplementary Figure 4E). While POT1 antibodies immunoprecipitated telomerase activity at all of the time points examined (Figure 4G, lane 3 in all panels), in three separate experiments TRAP products precipitated from S-phase cell extracts were significantly increased relative to unsynchronized cells (average = 4.5-fold). Hence, AtPOT1 shows a dynamic interaction with the telomerase RNP.

AtPOT1 dynamically associates with telomeres in vivo

Mammalian and fission yeast POT1 bind telomeric DNA *in vitro* (Baumann and Cech, 2001; Wu *et al*, 2006). To investigate AtPOT1 interactions with telomeric DNA, gel-shift experiments were performed. AtPOT1 is extremely insoluble when expressed in *E. coli*, but soluble full-length AtPOT1 or the N-terminal domain containing the OB-folds can be obtained from rabbit reticulocyte lysate (data not shown). Under the same conditions that the OB-fold containing N-terminus of mouse POT1a binds its cognate telomere sequence (Wu *et al*, 2006), AtPOT1 failed to bind *Arabidopsis* telomeric DNA (Supplementary Figure 5). Thus, under these *in vitro* assay conditions, AtPOT1 does not interact with telomeric DNA in the same manner as its mammalian counterpart.

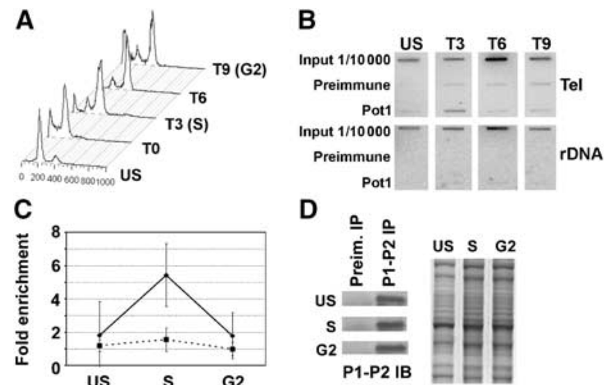

Figure 5 AtPOT1 is associated with telomeric chromatin in S-phase. (**A**) FACS analysis of *Arabidopsis* suspension culture cells synchronized with aphidicolin. Data are shown for unsynchronized (US) and synchronized cells at 0, 3, 6, and 9 h after release from aphidicolin arrest. (**B**) Example of ChIP analysis on synchronized suspension culture extracts using P1-P2 antibody or preimmune serum. Immunoprecipitated DNA was monitored on slot blot using a radiolabeled telomeric or rDNA probe. (**C**) Quantitation of AtPOT1 association with telomeric DNA. The average of results from four independent experiments is shown. The solid black line indicates the ratio of the telomeric DNA signal obtained with the POT1 antibody relative to the preimmune sera control. As a negative control, the rDNA signal obtained with the POT1 antibody relative to the preimmune sera (gray dashed line) is shown. (**D**) Western blot analysis of AtPOT1 protein. Extracts from synchronized cells were precipitated with P1-P2 antibody, followed by P1-P2 Western blot analysis. Commassie-stained inputs (right) are shown as loading controls.

Human POT1 binds telomeres throughout the cell cycle, showing a transient decrease in binding late in G2 (Verdun *et al*, 2005). To investigate AtPOT1 interaction with telomeres *in vivo*, chromatin IP (ChIP) was employed. Since we failed to detect AtPOT1 binding to telomeres in chromatin preparations from plant cell extracts, ChIP assays were performed on suspension culture cells. Slight (1.8-fold) enrichment of AtPOT1 at telomeres was observed in unsynchronized cells relative to the preimmune sera control (Figure 5B and C). Therefore, we asked whether AtPOT1 localization at telomeres was regulated during the cell cycle. Our synchronization protocol did not allow us to examine cells blocked in G1, however more than 75% of the unsynchronized cells are in this phase of the cell cycle (Figure 5A). Using aphidicolin, we could enrich for cells in S-phase and in G2. In four separate experiments, AtPOT1 interaction with telomeres significantly increased in S-phase cells; the enhancement ranged from 3.4 to 7.1-fold (average = 5.4-fold) over the preimmune sera control (Figure 5B and C). As an additional control, we monitored the ratio of the rDNA signal immunoprecipitated by the POT1 antibody relative to the preimmune control. As

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expected, no significant enrichment in S-phase was observed (Figure 5B and C). The AtPOT1 association with telomeres decreased dramatically as cells transitioned into G2 (down to 1.8-fold enrichment), indicating that AtPOT1 association with telomeres in S-phase does not reflect an increased number of binding sites after telomere replication. Furthermore, AtPOT1 protein levels were unchanged during the cell cycle (Figure 5D), arguing that its interaction with telomeres is dynamic and peaks in S-phase.

Discussion

Cdc13p and POT1 from yeast and vertebrates are multifunctional gatekeepers at the chromosome terminus, performing the crucial functions of distinguishing the ends from double-strand breaks, protecting against inappropriate recombination and nucleolytic attack, and controlling telomerase activity (de Lange, 2005; Baumann, 2006). Here, we demonstrate that AtPOT1 exhibits distinctly different interactions with telomeres. Unlike human POT1, disruption of the *AtPOT1* gene is not lethal and *Arabidopsis pot1* mutants display no evidence of chromosome end deprotection. Even in a *ku70 tert* background, where *Arabidopsis* telomeres are severely compromised (Riha and Shippen, 2003), the loss of AtPOT1 does not exacerbate telomere erosion or increase the frequency of chromosome end joining. While it is conceivable that AtPOT1 acts redundantly with another component of the telomere complex to protect the terminus, the dynamic interaction of AtPOT1 with telomeres is inconsistent with a primary role in this pathway.

Further distinguishing AtPOT1 from the previously described POT1 proteins is the ever-shorter-telomere phenotype displayed by *Arabidopsis* null mutants. Depletion of POT1 in mammals leads to telomere elongation (Veldman *et al*, 2004; Ye *et al*, 2004; Yang *et al*, 2005; Wu *et al*, 2006), implying a role in the negative regulation of telomere length. Although it is possible that the mammalian POT1 contribution to positive telomere length regulation is obscured by the other more severe phenotypes associated with POT1 depletion (Churikov *et al*, 2006; Hockemeyer *et al*, 2006; Wu *et al*, 2006), our data argue that the AtPOT1 protein has evolved a pivotal and highly specialized role in promoting telomerase action at telomeres by working in the context of the telomerase RNP.

Four lines of genetic and biochemical evidence strongly implicate AtPOT1 in the telomerase pathway. First, telomeres shorten at the same rate in *tert* and *pot1* mutants, and depletion of telomere tracts is not accelerated in plants with a deficiency in both genes. Second, AtPOT1 is required for the telomerase-dependent elongation of telomeres in *ku70* mutants. Third, *in vitro* telomerase activity levels are significantly reduced in *pot1* mutants. Fourth, enzymatically active telomerase is specifically immunoprecipitated with AtPOT1 antibodies.

How AtPOT1 interacts with the telomerase RNP to facilitate telomere maintenance is unknown. Transient transfection experiments in tobacco with GFP-tagged AtPOT1 and AtTERT show that the two proteins co-localize in the nucleolus (N Kato, E Lam, E Shakirov, and D Shippen, unpublished data), where telomerase RNP biogenesis occurs in both yeast and mammals (Etheridge *et al*, 2002; Teixeira *et al*, 2002). Intriguingly, we found that AtPOT1 association with enzymatically active telomerase is regulated in the cell cycle, increasing by an average of ∼4.5-fold in S-phase relative to unsynchronized cells (predominantly G1) and cells arrested in G2. Thus, AtPOT1 may stabilize an enzymatically active form of the RNP. In support of this model, telomerase activity levels are more variable in the absence of AtPOT1. Although *Arabidopsis* shows no haploinsufficiency with respect to *AtTERT* (Fitzgerald *et al*, 1999) or *AtPOT1* (this study), the more compromised telomerase enzyme found in *pot1*-null mutants may be unable to solve the end replication problem.

It is also possible that AtPOT1 functions to promote telomerase action on its telomeric DNA substrate. Notably, the variability of *in vitro* telomerase activity levels in *pot1* mutants is incongruent with the highly consistent ever-shorter-telomere phenotype displayed by these plants. By virtue of its two OB-folds, AtPOT1 is predicted to directly bind telomeric DNA. However, under conditions where *S. pombe* and mammalian POT1 associate with telomeric DNA *in vitro* (Baumann and Cech, 2001; Wu *et al*, 2006), AtPOT1 showed no binding. While AtPOT1 may simply need different biochemical reaction conditions to associate with telomeric DNA, another more interesting possibility is that AtPOT1 requires a binding partner. Recent studies reveal that the mammalian POT1 binding partner, TPP1, which cannot bind telomeric DNA on its own (Wang *et al*, 2007; Xin *et al*, 2007), not only greatly enhances the affinity of POT1 for telomeric DNA *in vitro*, but also stimulates telomerase activity and processivity (Wang *et al*, 2007). Moreover, like AtPOT1, TPP1 can assume a canonical OB-fold (Wang *et al*, 2007; Xin *et al*, 2007) and physically interacts with the telomerase RNP (Xin *et al*, 2007). Mice deficient in TPP1 show profound developmental defects and animals that survive to adulthood are infertile (Keegan *et al*, 2005). Thus, TPP1 contrasts with *Arabidopsis* POT1 in that it appears to possess additional functions besides stimulating telomerase activity (Xin *et al*, 2007). Altogether our observations underscore the extraordinarily rapid evolution of the telomeric complex, and indicate that OB-fold bearing proteins, such as AtPOT1, are co-evolving with the telomerase RNP.

Materials and methods

Mutant lines

The *pot1-1* allele was identified in ALPHA population of T-DNA insertion lines at the University of Wisconsin *Arabidopsis* Knockout Facility. The collection was screened using primers 5′-TTTGTACTGGCCTCTCCAAGGTTCACCAT-3′ and 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′, according to the protocol available at http://www.biotech.wisc.edu/Arabidopsis/Index2.asp. The *pot1-2* line was identified by screening a pooled genomic DNA collection (ABRC #CD10-A) from the Weigel T-DNA lines using primers 5′-CGGGATCCCACCCAGAAGATACTAAGATG-3′ and 5′-TTGACCATCATACTCATTGCTG-3′. *tert* and *ku70* mutants and plant growth conditions are as described (Riha *et al*, 2001, 2002). All crosses were made between plants heterozygous for the desired mutations. Double- and triple-heterozygous F1 plants were identified by PCR genotyping and then self-propagated to F2 to obtain single-, double-, and triple-homozygous mutants and their wild-type siblings. F2 plants (G1) were self-propagated for several generations. Independent lines from at least two F2 plants were established and analyzed for each genotype. Complementation was performed as described in Supplementary Figure 2. Wild-type and G3 *pot1-1* were used to establish callus. Callus initiation and maintenance were performed as described (Watson *et al*, 2005), with slight modifications. Seeds were germinated on 0.5× Murashige and Scoog (MS) medium plates supplemented with 3% sucrose and 2.8 g/l phytagel. Roots were harvested at 3 weeks, finely chopped,

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and placed on 1× MS medium plates supplemented with 2 mg/l 2,4-D, 0.05 mg/l kinetin, 3% sucrose, and 2.8 g/l phytagel (CIM). Callus was grown on CIM at 25°C in the dark and transferred to fresh medium every 4 weeks.

RT–PCR analysis, telomere analysis, TRAP assays, and cytogenetics

Total RNA was extracted from plant tissue using Tri Reagent solution (Sigma). Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen), as described (Shakirov *et al*, 2005). To evaluate expression of the regions flanking the T-DNA insertion in the *pot1-1* allele, primer 1 (5′-GGATCCATGGCGAAGAAGAGAGAGAGAGTCCCAAGCTCATCA-3′) and primer 2 (5′-GCTCTAGACTTGATCTCTCTCAAGAAGGA-3′) were used. To analyze the *pot1-2* allele, we used primer 1 and primer 3 (5′-TACCTCGAGCTAGATTAGGCTATCAGAGA-3′). DNA from individual whole plants was extracted as described (Cocciolone and Cone, 1993). TRF analysis was performed using *Tru1*I (Fermentas, Hanover, MD) restriction enzyme and a [^{32}P] 5′ end-labeled $(T_3AG_3)_4$ oligonucleotide probe (Fitzgerald *et al*, 1999). Subtelomeric TRF analysis was conducted using a 2R probe (Shakirov and Shippen, 2004), or a probe for 1L generated with 5′-ACGCTTGT CATCTCATCTCT-3′ and 5′-CGGGATCTTTGGTGTTTCTC-3′. Telomere fusion PCR and PETRA were performed as described (Heacock *et al*, 2004). TRAP assays were conducted on plant tissues as described in Fitzgerald *et al* (1996), using protein extracts prepared from a single wild-type or *pot1* inflorescence, unless otherwise indicated. For the anaphase, spreads were prepared from pistils and stained with DAPI (4′,6′-diamidino-2-phenylindole), as discussed in Riha *et al* (2001).

Antibodies, Western blotting, and IP

The P1-R polyclonal antibody was raised in rabbits against full-length recombinant AtPOT1 protein expressed in *E. coli* (Covance). The P1-P1 and P1-P2 peptide antibodies were raised in rabbits against the N′-CSDENRRHHQVLLTLEDST and N′-AAYPWQVEDFCS DENRRHHQVLLT peptides, respectively, and affinity purified (Covance). Western blots were conducted with primary antibodies and peroxidase-conjugated light chain-specific mouse anti-rabbit secondary antibodies (Jackson Immunoresearch). For IP of endogenous proteins from suspension culture, protein extracts were prepared in buffer containing 50 mM Tris–HCl, pH 7.4, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM DTT, and protease inhibitors (Roche). For IP of endogenous proteins from 4-day-old seedlings, protein extracts were prepared according to Fitzgerald *et al* (1996). Extracts were diluted at 1:5 in buffer W100 (20 mM TrisOAc, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 200 mM NaCl, 100 mM KGlu, 1% NP-40, 0.5 mM Na deoxycholate, 1 mM DTT), precleared with protein A agarose (Pierce), and subjected to IP with POT1 antibodies. Following IP, the beads were washed three times with buffer W300 (same as buffer W100, but 300 mM KGlu) and two times with TMG buffer (10 mM TrisOAc, pH 8.0, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT), and used for TRAP assay. In peptide competition experiments, a 100× molar excess of the P1-P1 peptide or AtPOT2 peptide (N′-DDYKFLRIQDAF KALHLHVNC) was added during the IP step. For salt stability experiments, the NaCl concentration in the input was adjusted by addition of 5 M NaCl to suspension culture extracts. For IP of recombinant proteins, AtPOT1 and AtPOT2 were expressed in rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-labeled methionine. Following IP, the signal was quantified using ImageQuant Software. IP efficiency was calculated as a ratio of the immunoprecipitated signal versus input.

Cell culture synchronization and FACS

MM2d *Arabidopsis* cell suspension culture was maintained as described in Menges and Murray (2002). For synchronization, the original protocol was employed with the following modifications: 100 ml of 4-day-old culture was divided into five 20 ml aliquots, diluted at 1:5 with fresh medium and then blocked in G1/early S-phase for 21 h with 12 μg/ml of aphidicolin (AG Scientific). Cells were filtered through Miracloth (Calbiochem), washed twice with 500 ml of fresh media, and resuspended in 100 ml of fresh media. Aliquots were taken at various time points for DNA content analysis and immediately frozen. DNA content was analyzed by flow cytometry. The cultured cells were chopped with a razor blade, resuspended in homogenization buffer, filtered through a 20 μm nylon mesh, treated with 10 μg/ml of RNase A, and stained with 50 μg/ml of propidium iodide. Samples were run on a Becton-Dickinson FACSCalibur at 488 nm and analyzed using CellQuest (Becton-Dickinson) and ModFit LT (Verity) programs.

ChIP

ChIP was performed essentially as described by Leibfried *et al* (2005), with some modifications. The equivalent of 100 ml of 4-day-old unsynchronized or synchronized cell suspension culture (∼3 g of dry material) was fixed for 1 h in 1% formaldehyde, followed by quenching for 10 min in 125 mM glycine. After vacuum filtration through Miracloth, cells were resuspended and washed in 500 ml of PBS, and filtered again before storage at −80°C. Cells were ground in liquid N_2 and then ChIP was performed using P1-R or P1-P2 antibody at a 1:100 dilution. The elution products were subjected to slot blot (Hybond N+, Amersham) and hybridized using a $(TTTAGGG)_4$ telomeric probe. Blots were stripped and rehybridized with a combination of radiolabeled 5S (5′-TTGCAGAATCCCGTGA ACCATCGAGT-3′) and 18S rDNA (5′-TGGAGCCTGCGGCTTAATTT GACTCA-3′) oligo-probes to monitor the specificity of IP. For quantification, the fold enrichment was determined as a ratio of the hybridization signal obtained with the POT1 antibody versus the preimmune sera control. rDNA sequences were used as a negative control.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Note added in proof

Since three different groups previously published on *Arabidopsis* POT-like genes and gave them different names (Kuchar and Fajkus, 2004; Shakirov *et al*, 2005; Tani and Murata, 2005), it is our joint decision to employ a unifying nomenclature that more closely follows the general trend in the telomere field. Therefore, At2g05210 (AtPOTl throughout this article) will hereafter be designated AtPOTla, while At5g06310 (AtPOT2) will be AtPOTl b.

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