Protection of Telomeres 1 Is Required for Telomere Integrity in the Moss *Physcomitrella patens* [W]

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In vertebrates, the single-stranded telomeric DNA binding protein Protection of Telomeres 1 (POT1) shields chromosome ends and prevents them from eliciting a DNA damage response. By contrast, *Arabidopsis thaliana* encodes two divergent full-length POT1 paralogs that do not exhibit telomeric DNA binding in vitro and have evolved to mediate telomerase regulation instead of chromosome end protection. To further investigate the role of POT1 in plants, we established the moss *Physcomitrella patens* as a new model for telomere biology and a counterpoint to *Arabidopsis*. The sequence and architecture of the telomere tract is similar in *P. patens* and *Arabidopsis*, but *P. patens* harbors only a single-copy POT1 gene. Unlike At POT1 proteins, Pp POT1 efficiently bound single-stranded telomeric DNA in vitro. Deletion of the *P. patens* POT1 gene resulted in the rapid onset of severe developmental defects and sterility. Although telomerase activity levels were unperturbed, telomeres were substantially shortened, harbored extended G-overhangs, and engaged in end-to-end fusions. We conclude that the telomere capping function of POT1 is conserved in early diverging land plants but is subsequently lost in *Arabidopsis*.

INTRODUCTION

Telomeres are nucleoprotein complexes that physically cap the ends of eukaryotic chromosomes. Telomeric DNA promotes genome stability through elaborate interactions with a plethora of telomere-associated proteins. The evolutionarily conserved Protection of Telomeres 1 (POT1) protein is a key component of shelterin, the vertebrate telomere capping complex (de Lange, 2005). POT1 binds to the single-stranded G-rich region of the chromosome terminus via structurally conserved N-terminal oligonucleotide/oligosaccharide binding folds (OB-folds) (Lei et al., 2003, 2004). Through interactions with its binding partner TPP1 (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004), POT1 forms a bridge to the double-stranded region of the telomere tract. Deletion of *POT1* in *Schizosaccharomyces pombe* causes rapid telomere loss and cell death, suggesting that its main function is to protect telomeres from degradation (Baumann and Cech, 2001). In vertebrates, the most critical POT1 function appears to be its ability to block ATR (Ataxia Telangiectasia and Rad3 related)-dependent recognition of telomeric DNA as sites of DNA damage (Denchi and de Lange, 2007; Churikov and Price, 2008). POT1 also plays a role in both

telomere length regulation by modulating telomerase activity and in chromosome end protection by preventing nucleolytic attack of the C-rich telomeric DNA strand (reviewed in de Lange, 2009).

Arabidopsis thaliana is the reference species for plant biology; consequently, telomere research has focused on this model. Although orthologs of several yeast and vertebrate telomere-related genes have been described (Riha et al., 2001; Song et al., 2008), a technical obstacle for the functional characterization of other candidates is that T-DNA insertion mutants are currently unavailable for a substantial fraction of *Arabidopsis* genes (O'Malley and Ecker, 2010). Genetic analysis is also complicated by the multiple whole-genome duplications associated with Brassicaceae, the family to which *Arabidopsis* belongs (Schranz and Mitchell-Olds, 2006). For example, sequence homologs of the mammalian shelterin subunits TRF1 and TRF2 are encoded by at least six different *TRFL* (TRF-like) genes in *Arabidopsis*, which appear to be partially redundant (Chen et al., 2001; Karamysheva et al., 2004; Hwang and Cho, 2007). Furthermore, *Arabidopsis* encodes three divergent POT1 paralogues, including two full-length proteins, At POT1a and At POT1b, and a truncated isoform termed At POT1c (Shakirov et al., 2005; Rossignol et al., 2007).

This pattern of extensive gene duplication and divergence in Brassicaceae suggests that lessons learned from *Arabidopsis* may not always be applicable to telomere biology in other plants. Indeed, while POT1 is critical for telomere integrity in yeast and vertebrates, this is not true for At POT1a and At POT1b. Like the other Brassicaceae POT1 proteins examined to date, At POT1a and At POT1b exhibit no detectable binding to single-stranded telomeric DNA in vitro (Surovtseva et al.,

2007; Shakirov et al., 2009a). Notably, At POT1a physically associates with the telomerase RNP and is critical for telomere length maintenance in vivo (Surovtseva et al., 2007). Although initial experiments with overexpression of dominant-negative mutants suggested that At *POT1b* is involved in chromosome end protection (Shakirov et al., 2005), recent analysis of a null allele revealed that At POT1b is a negative regulator of telomerase activity and plays no significant role in telomere protection (E. Shakirov, A. Nelson, and D. Shippen, unpublished data). Thus, At POT1a and At POT1b have evolved to function in the telomerase pathway. To fully elucidate the spectrum of *POT1* functions in the plant kingdom, it will be necessary to examine the contribution of *POT1* in other plant species.

Mosses (bryophytes) belong to the oldest diverging clade of extant land plants, whose ancestors made the first move from aquatic to terrestrial habitats some 450 million years ago (Zimmer et al., 2007). Unlike its younger cousins angiosperms (flowering plants), the moss *Physcomitrella patens* possesses a remarkably efficient system of homologous recombination, which accounts for an unprecedented success rate in targeted gene replacement among higher eukaryotes (Quatrano et al., 2007). Gene targeting in *P. patens* is not only five orders of magnitude more efficient than in angiosperms, but also two orders of magnitude more efficient than in the embryonic stem cells of mice and comparable with that observed in *Saccharomyces cerevisiae* (Schaefer, 2002). Virtually any nonessential *P. patens* gene can be deleted, and the resulting moss phenotype can be screened within several weeks following transformation.

With its recently sequenced genome (Rensing et al., 2008) and excellent molecular and genetic tools (Quatrano et al., 2007), *P. patens* is poised to become the new green yeast of plant biology (Schaefer, 2001). Due to its phylogenetic position within the plant kingdom, *P. patens* also provides a unique opportunity to trace the evolution of telomere-related genes from the first primitive land plants to the most developmentally advanced angiosperms. Here, we describe the initial characterization of telomere biology in *P. patens* and show that this species possesses canonical plant TTTAGGG telomere repeats. However, unlike *Arabidopsis*, *P. patens* harbors only a single *POT1* gene. Deletion of Pp *POT1* results in catastrophic loss of bulk telomeric DNA, increased G-overhangs, and end-to-end chromosome fusions. These findings point to an essential role for Pp *POT1* in chromosome end protection similar to its yeast and vertebrate orthologs but distinct from *Arabidopsis*.

RESULTS

Telomere Length and Composition in *P. patens*

The length of the telomere tract varies considerably among different species of land plants and green algae, ranging from 0.5 kb in *Chlorella vulgaris* to over 150 kb in tobacco (*Nicotiana tabacum*; Fajkus et al., 1995; Higashiyama et al., 1995). In most *Arabidopsis* ecotypes, telomeres span 2 to 5 kb (Shakirov and Shippen, 2004). To evaluate telomere length in *P. patens*, terminal

restriction fragment (TRF) analysis was performed using a radioactive probe consisting of four canonical plant TTTAGGG repeats. The most commonly used isolate of *P. patens*, Gransden (Gd), harbored telomere tracts in the range of 0.6 to 3 kb (Figure 1A, lane 2). Telomeres in the second isolate, Villersexel (Vx-1), were slightly longer, in the range of 1 to 3.5 kb (Figure 1A, lane 3). Thus, *P. patens* telomeres are approximately twofold shorter than in *Arabidopsis* (Figure 1A, lane 1) but similar in length to the telomere tracts in another moss species, *Barbula unguiculata* (Suzuki, 2004). We verified that TTTAGGG-hybridizing repeats are located at chromosomal ends using *Bal*31 exonuclease. A true telomeric signal should disappear over time, while interstitial cross-hybridizing regions are resistant to enzyme treatment. With the exception of a single interstitial band (indicated by an asterisk), the bulk hybridization signal was lost by 30 min of *Bal*31
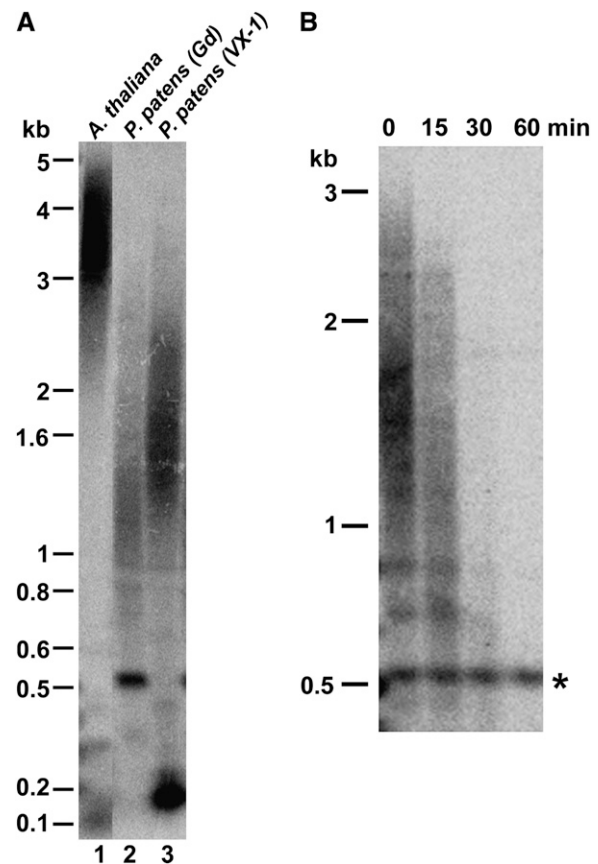

Figure 1. Moss Telomere Length Analysis.

(A) TRF analysis of telomeric DNA in *P. patens* isolates Gd (lane 2) and Vx-1 (lane 3). Molecular weight markers are shown on the left. Genomic DNA was digested with *Tru*II and hybridized with a plant telomere-specific (TTTAGGG)$_4$ probe. *Arabidopsis* DNA was used as a control (lane 1).

(B) *Bal*31 analysis of genomic DNA from *P. patens* isolate Gd. *Tru*II digestion of genomic DNA was performed without prior *Bal*31 treatment (0 min) or after various incubation periods with *Bal*31 exonuclease. Asterisk indicates cross-hybridizing interstitial telomeric DNA band that is not sensitive to *Bal*31 digestion for up to 60 min.

treatment (Figure 1B), confirming that these *Bal*31-sensitive TTTAGGG-hybridizing repeats are located at chromosome ends.

Identification of *P. patens* POT1

The publicly available *P. patens* genome database (http://moss. nibb.ac.jp/) was searched with the *Arabidopsis* POT1a and POT1b sequences as the queries using BLASTp and tBLASTn programs. Similar to most other plants analyzed to date (Shakirov et al., 2009b), *P. patens* harbors a single *POT1* gene, which encodes a 497–amino acid protein with 50 and 45% sequence similarity to *Arabidopsis* POT1a and POT1b, respectively. The Pp POT1 protein is predicted to contain two N-terminal DNA binding OB-folds with secondary structures similar to the human POT1 protein (Figure 2A). Like At *POT1a* and At *POT1b*, Pp *POT1* consists of 10 exons with conserved exon-intron junctions, indicating that *P. patens* and *Arabidopsis POT1* genes are indeed orthologous. As noted for other *P. patens* genes (Rensing et al., 2005), Pp *POT1* introns are on average twice as long as in *Arabidopsis*. RT-PCR revealed that Pp *POT1* is continuously expressed during gametophytic development (protonemata and gametophore tissues) (Figure 2B, lanes 1 and 2).

Characterization of Pp POT1 DNA Binding Activity

Although POT1 proteins from Brassicaceae have thus far failed to bind single-stranded telomeric DNA in vitro (Surovtseva et al., 2007; Shakirov et al., 2009a), a recent survey revealed that recombinant POT1 proteins from asparagus (*Asparagus offici-nalis*), maize (*Zea mays*), and the green alga *Ostreococcus lucimarinus* are capable of efficient telomeric DNA binding (Shakirov et al., 2009b). To examine the nucleic acid binding properties of Pp POT1, we expressed Pp POT1 in rabbit reticulocyte lysate (RRL) and performed electrophoretic mobility shift assays (EMSA) with radioactively labeled cocktails of telomeric oligonucleotides containing seven different permutations of either two (2TELO) or three (3TELO) plant TTTAGGG repeats (Figure 3A). *A. officinalis* POT1 (Ao POT1) served as a positive control (Figure 3A, lane 3) (Shakirov et al., 2009b). While no binding of Pp POT1 to the 2TELO probe was observed (Figure
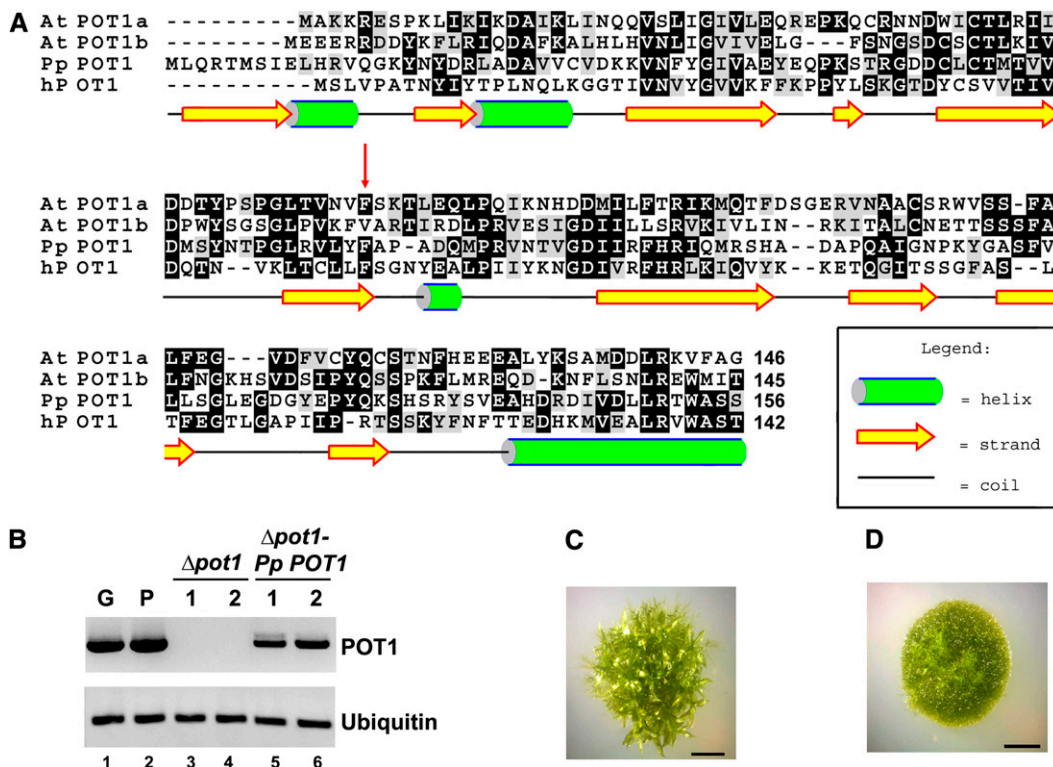

Figure 2. Identification and Analysis of *P. patens* POT1.

(A) Amino acid alignment of the predicted Pp POT1 OB1 region with *Arabidopsis* (At POT1a and At POT1b) and human POT1 proteins. The secondary structure of Pp POT1 OB1 was predicted with PsiPred software (McGuffin et al., 2000). Numbers indicate amino acid positions relative to the start codon. Red arrow indicates the position of a biochemically important F62 residue in human POT1 and the corresponding amino acid F74 in Pp POT1. Alignment was generated with MEGA 3 software (Kumar et al., 2004) and visualized in the BOXSHADE format.
(B) RT-PCR results of Pp *POT1* gene expression (top panel) in the wild type (lanes 1 and 2), Δ*pot1* (lanes 3 and 4), and Δ*pot1*-Pp-*POT1* complemented line (lanes 5 and 6). Ubiquitin (bottom panel) was used to normalize for RNA loading.
(C) and **(D)** General morphology of wild-type **(C)** and Δ*pot1* **(D)** colonies at the 4-week-old stage. The wild-type colony harbors multiple leafy-like gametophores, but only filamentous protonemata tissue is visible in Δ*pot1*.
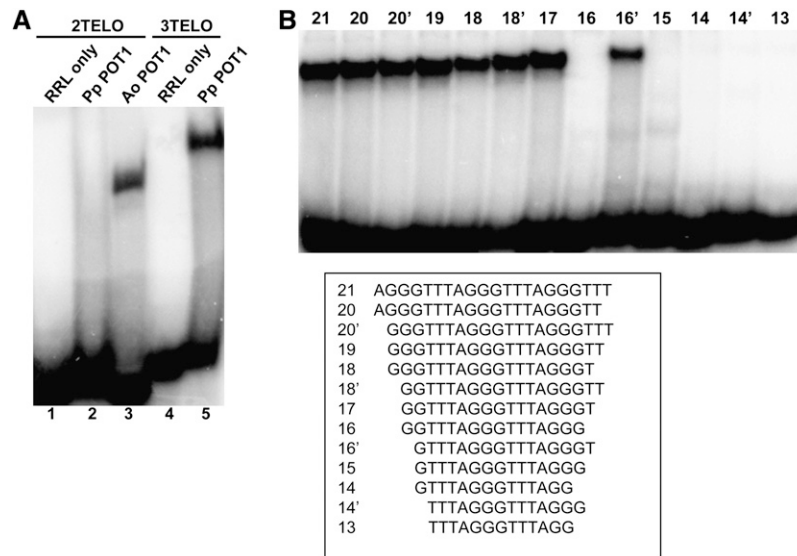
Figure 3. Characterization of Pp POT1 Interaction with Telomeric DNA in Vitro.

(A) EMSA was performed with a cocktail of seven ^{32}P-labeled oligonucleotides corresponding to two (2TELO) (lanes 1 to 3) or three (3TELO) (lanes 4 and 5) TTTAGGG repeats. Unprogrammed RRL reactions lacking external DNA template were used as negative controls (lanes 1 and 4), and a reaction with asparagus POT1 protein (Ao POT1) was performed as a positive control (lane 3). Pp POT1 binds 3TELO probe (lane 5) but not 2TELO probe (lane 2).
(B) Identification of the Pp POT1 minimum DNA binding site. Equal amounts of RRL-expressed Pp POT1 were incubated with the indicated radioactively labeled oligonucleotides (bottom panel), and protein-DNA complexes were separated by native PAGE.

3A, lane 2), Pp POT1 efficiently bound the 3TELO cocktail (Figure 3A, lane 5). As expected (Surovtseva et al., 2007; Shakirov et al., 2009a), POT1 proteins from *Arabidopsis* and several other vascular plants showed no detectable telomeric DNA binding (see Supplemental Figure 1 online). Of the seven individual permutations in the 3TELO cocktail probe, Pp POT1 bound (AGGGTTT)$_3$ most efficiently (see Supplemental Figure 2 online).

The minimum DNA binding site (MBS) in vertebrate POT1 proteins is highly conserved and consists of 10 to 12 nucleotides (Lei et al., 2004; Loayza et al., 2004; Wei and Price, 2004; He et al., 2006). To define the Pp POT1 MBS, we performed EMSA with (AGGGTTT)$_3$ and a series of single nucleotide truncations from either the 5′ or 3′ end of this substrate (Figure 3B). Removal of the first two nucleotides from both ends of the substrate (oligonucleotide 17) did not decrease binding. However, deletion of one additional nucleotide from the 3′ end, but not from the 5′ end of this substrate, completely abolished binding (Figure 3B, compare oligonucleotides 16 and 16′). Since Pp POT1 does not bind to two different oligonucleotides containing 15 nucleotides each (see Supplemental Figure 3A online), we conclude that its minimum tight binding substrate is the 16-nucleotide sequence, GTTTAGGGTTTAGGGT.

We next asked which domain(s) in the *P. patens* POT1 are required for MBS recognition. Like POT1 proteins from vertebrates and several plants (Lei et al., 2004; He et al., 2006; Wu et al., 2006; Shakirov et al., 2009b), both N-terminal OB-folds were necessary for telomeric DNA binding (see Supplemental Figure 3B online, lanes 4 to 7). Interestingly, the EMSA profile changed from a single band to a smear in the absence of Pp POT1 C terminus (see Supplemental Figure 3B online, compare

lanes 2 and 3), suggesting that this region (amino acids 323 to 497) modulates Pp POT1 interaction with DNA in vitro.

In mammalian and fission yeast POT1 proteins, an invariant Phe in the first OB-fold (F62 in human POT1; Figure 2A) is required for efficient DNA binding (Lei et al., 2004; He et al., 2006; Wu et al., 2006). To test if the corresponding residue (F74) is required for telomeric DNA interaction by Pp POT1, we performed EMSA with an F74A Pp POT1 mutant. The mutant protein failed to bind telomeric DNA in vitro (see Supplemental Figure 3B online, lane 1). Altogether, these data indicate that *P. patens* POT1 is a bona fide single-stranded telomeric DNA binding protein with structural and biochemical properties similar to POT1 proteins from yeast and vertebrates but distinct from *Arabidopsis*.

Morphological and Developmental Defects in the Δpot1 Strain

We generated a deletion of *P. patens POT1* (*Δpot1*) by replacing its complete open reading frame (ORF) with the hygromycin resistance gene *HptII* (see Supplemental Figure 4A online). The absence of the *POT1* ORF in hygromycin-resistant transformants was verified by PCR genotyping (see Supplemental Figure 4B online), DNA gel blot analysis (see Supplemental Figure 4C online), and by sequencing across the entire *Δpot1* locus. RT-PCR confirmed the absence of *POT1* mRNA in *Δpot1* (Figure 2B, lanes 3 and 4). Hereafter, we refer to *Δpot1* as a null mutant.

P. patens was able to grow vegetatively in the absence of *POT1*, suggesting that this gene is not essential for long-term moss survival. Nevertheless, the *Δpot1* strain displayed a

striking loss of developmental program. Although the juvenile protonemata filaments initially grew efficiently, aberrant morphological defects emerged and worsened progressively as the *Δpot1* advanced to more mature stages of development, ultimately culminating in complete sterility. The growth of gametophores (leafy-like tissue on top of the colony) was significantly delayed in the *Δpot1* strain (Figures 2C and 2D), but the overall number of emerging gametophores was reduced only slightly (see Supplemental Figure 5 online). Furthermore, upon cold treatment, which in wild-type *P. patens* induces the formation of sex organs, the female archegonium and the male antheridium never formed in *Δpot1*, explaining the sterility phenotype. These data indicate that *POT1* is necessary for the normal development of *P. patens*.

Telomere Length Maintenance Defects in Δpot1

We next asked if the developmental and morphological defects in *Δpot1* are accompanied by deficiencies in telomere maintenance. TRF analysis revealed a dramatic decrease (~72%) in the overall intensity of telomeric hybridization signal in *Δpot1* relative to the wild type, indicating that the majority of chromosome ends experienced a massive loss of telomeric DNA (Figure 4A). The average length of the remaining telomere tracts was reduced by ~200 bp (Figure 4B). *Bal*31 digestion of *Δpot1* DNA confirmed that the residual TTTAGGG hybridization signal was terminally located (see Supplemental Figure 6 online). Complementation with a wild-type Pp *POT1* gene (hereafter termed *Δpot1*-Pp-*POT1*) restored telomere length to wild-type, demonstrating that

telomere loss was the result of *POT1* deletion (see Supplemental Figures 7A and 8A online). As expected, RT-PCR confirmed that *POT1* mRNA expression was restored in the *Δpot1*-Pp-*POT1* line (Figure 2B, lanes 5 and 6). We conclude that POT1 is necessary for proper telomere length maintenance in *P. patens*.

Notably, the *Δpot1*-Pp-*POT1* construct did not complement *Δpot1* morphological and developmental defects. Although PCR genotyping of *Δpot1*-Pp-*POT1* strain confirmed the presence of the *POT1* gene in its original locus (see Supplemental Figure 4B online), DNA gel blot analysis indicated that additional copies of the complementation construct are present in the *Δpot1*-Pp-*POT1* strain (see Supplemental Figure 7B online). Thus, increased gene dosage may explain the persistence of developmental defects. Alternatively, the severe genome instability that arises from telomere uncapping (see below) may have resulted in irreversible aberrations in cellular pathways controlling moss morphology and development, as previously reported for *Arabidopsis* mutants with dysfunctional telomeres (Riha et al., 2001; Song et al., 2008; Surovtseva et al., 2009).

The TRF assay measures bulk telomere length changes. To explore the dynamics of individual telomere tracts in *Δpot1*, we employed the primer extension telomere repeat amplification (PETRA) assay originally developed to study *Arabidopsis* telomeres (Heacock et al., 2004). PETRA amplifies individual telomeric DNA tracts using PCR primers directed at the G-overhang and a unique subtelomeric sequence. Although the sequenced *P. patens* genome is not yet assembled into its 27 chromosomes, two unique subtelomeric regions (arbitrarily designated A and B; see Methods) were identified. PETRA reactions conducted with
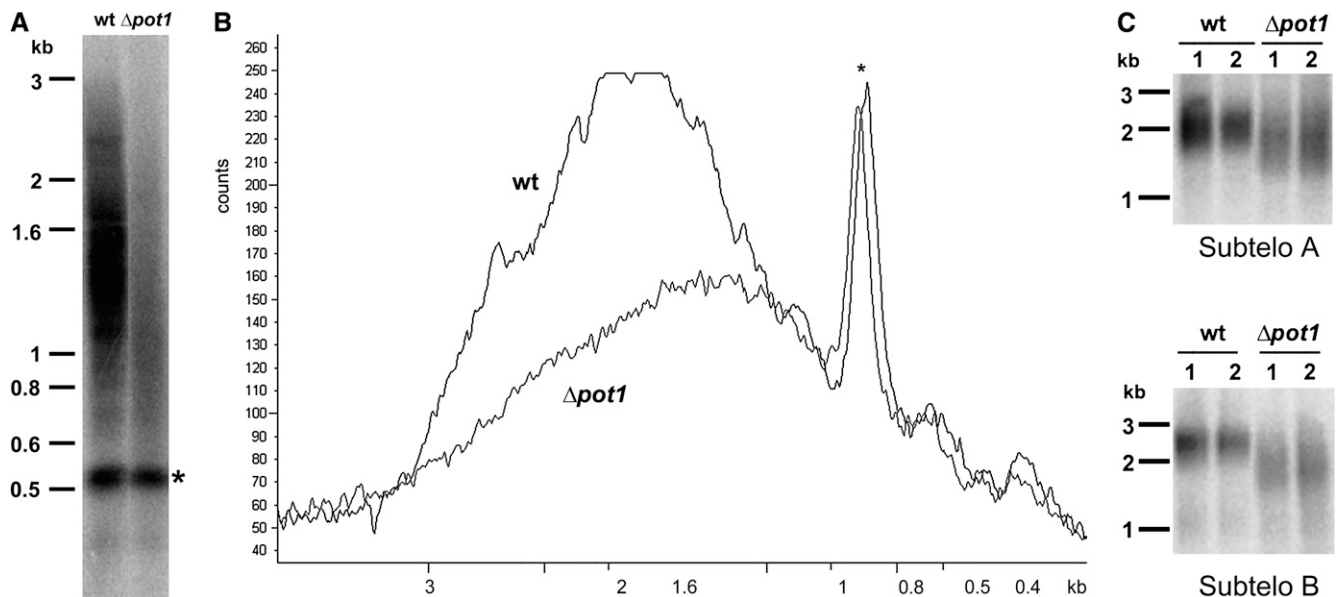

Figure 4. Defects in Telomere Length Regulation in *Δpot1*.

(A) TRF analysis of DNA from the wild type (wt) and *Δpot1*. The asterisk indicates an interstitial cross-hybridizing band used as a loading control.
(B) Quantification of results in **(A)** with ImageQuant software.
(C) PETRA results of telomere lengths on two individual chromosome arms, designated A and B, in the wild-type and *Δpot1* moss. A telomeric probe (TTTAGGG)$_4$ was used to detect TRF and PETRA signals. Molecular weight markers are shown on the left of each panel.

wild-type *P. patens* generated a smear spanning up to 0.5 kb (Figure 4C), but in *Δpot1*, both A and B telomeres were >200 bp shorter (Figure 4C). Importantly, the wild-type PETRA profile was restored in *Δpot1*-Pp-*POT1* (see Supplemental Figure 8B online).

Telomerase activity is reduced by ~10-fold in *Arabidopsis* plants null for At *POT1a* (Surovtseva et al., 2007). To determine if in vitro telomerase activity is altered in *Δpot1*, we performed the quantitative telomere repeat amplification protocol (Kannan et al., 2008) with protein extracts from wild-type and *Δpot1* strains. No statistically significant difference in enzyme activity was observed (see Supplemental Figure 9 online). We conclude that POT1 proteins make fundamentally different contributions to telomere maintenance in *Arabidopsis* and *P. patens*.

Defects in Telomere Architecture and Genome Stability in *Δpot1*

Mutations in telomere capping proteins can lead to increased G-overhang length, resulting from increased nucleolytic attack on the C-rich telomeric strand or uncoupled replication of the G-rich and C-rich strands (Linger and Price, 2009). We analyzed G-overhang status in *Δpot1* mutants using the in-gel hybridization assay. DNA gel blotting with a telomeric DNA probe was performed under native conditions to measure single-stranded telomeric DNA. The gel was rehybridized with the same probe under denaturing conditions to normalize for the total amount of double- and single-stranded telomeric DNA loaded. Relative to the wild type, the G-overhang signal in *Δpot1* increased by approximately eightfold (Figures 5A, lanes 1 and 3, and 5B). Exonuclease treatment confirmed that hybridization was associated with the chromosome terminus (Figure 5A, lanes 2 and 4). We conclude that *P. patens* POT1 is critical for proper chromosome end structure.

S. pombe and mammalian POT1 proteins are required to protect chromosomes from end-to-end fusions (Baumann and Cech, 2001; Veldman et al., 2004; He et al., 2006; Hockemeyer et al., 2006). By contrast, *Arabidopsis POT1a* and *POT1b* null mutants do not exhibit telomere fusions (Surovtseva et al., 2007; E.V. Shakirov, A.D. Nelson, and D.E. Shippen, unpublished data). To evaluate chromosome stability in *Δpot1*, we employed the telomere fusion PCR assay (TF-PCR) (Heacock et al., 2004). As with PETRA, TF-PCR uses unique subtelomere-specific PCR primers directed toward the chromosome terminus. If the target chromosome ends are covalently fused, the fusion junction will be amplified and detected by DNA gel blot analysis. As expected, no TF-PCR products were observed with DNA samples from wild-type *P. patens* or in the complemented *Δpot1*-Pp-*POT1* strain (see Supplemental Figure 8C online). However TF-PCR products were abundant in reactions with *Δpot1* DNA (Figure 6A). Interestingly, chromosome fusions were also detected in *Δpot1* PCR reactions containing only one subtelomere-specific primer (Figure 6B). Since *P. patens* spends most of its lifecycle in the haploid form, these data suggest that sister chromatids fuse in the absence of POT1.

Chromosome end maintenance defects worsen over time in mouse models deficient for either *mPOT1a* or *mPOT1b* (Wu et al., 2006; Hockemeyer et al., 2008). By contrast, we found no evidence of progressive telomere shortening or increased incidence of telomere fusions in long-term vegetatively growing *Δpot1* cultures. However, since relatively few cultured *P. patens* cells are proliferating (Cove et al., 2006), it is possible that the telomere deprotection phenotype would be exacerbated if the majority of *Δpot1* cells were forced to divide. To investigate this possibility, we subjected *Δpot1* cells to several consecutive rounds of colony regrowth from a single progenitor cell (protoplast) (Figure 6C). As predicted, the abundance of TF-PCR products was elevated in *Δpot1* cultures as the number of protoplasting and new colony formation events increased (Figure 6C). Taken together, our data indicate that POT1 is essential for telomere integrity and chromosome end protection in *P. patens*.
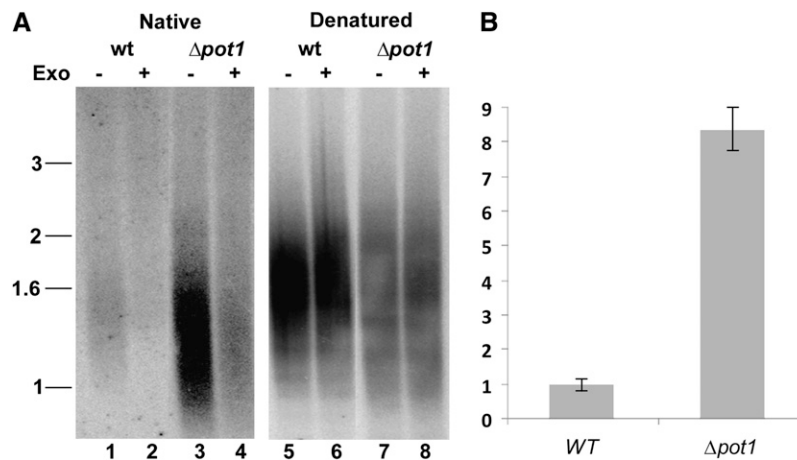

Figure 5. Increased G-Overhang Signals in *Δpot1* Mutants.

(A) In-gel hybridization results using a G-strand specific probe $(C_3TA_3)_4$ to detect telomeric DNA under native (lanes 1 to 4) and denaturing (lanes 5 to 8) conditions in the wild type (wt) and *Δpot1*. T4 DNA Polymerase (a $3' \rightarrow 5'$ exonuclease [Exo]) was added to reactions shown in lanes designated by (+).
(B) Quantification of the G-overhang signal in **(A)**. *Δpot1* signal intensity relative to the wild type is shown (error bars measured for $n = 4$, SD is $\pm$ 0.835).
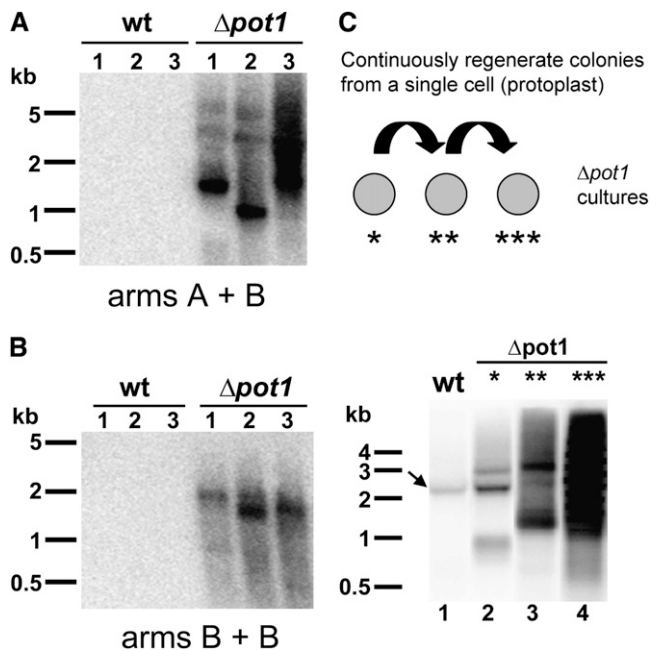
Figure 6. Accumulation of Chromosome Fusions in *Δpot1*.

(A) and **(B)** TF-PCR detects multiple instances of end-to-end chromosome fusions in *Δpot1* moss but not in the wild type (wt). TF-PCR results with subtelomeric primers specific for chromosome arms A and B and DNA from three individual colonies of the wild type or *Δpot1* **(A)**. TF-PCR results with one subtelomeric primer specific for chromosome arm B **(B)**. **(C)** Increased incidence of chromosome fusions in *Δpot1* cultures continuously regrown from a single protoplast. Top panel: Diagram of the experimental design. Bottom panel: TF-PCR results with DNA from wild-type (lane 1) and *Δpot1* colonies (lanes 2-4). *Δpot1* colonies were regenerated from a single cell through protoplasting once (lane 2), twice (lane 3), or three times (lane 4) (see asterisks in top panel). Arrow indicates a nonspecific PCR product detected in some wild-type samples. Molecular weight markers are shown on the left of each panel.

DISCUSSION

P. patens, a New Model System for Plant Telomere Research

The ability of eukaryotic cells to distinguish between double-stranded DNA breaks and natural chromosome termini is essential to avoid aberrant genome rearrangements and to ensure continued cell proliferation. The factors responsible for telomere stability are evolving rapidly (Linger and Price, 2009). In vertebrates, shelterin represses ATR- and ATM-dependent DNA damage pathways, preventing telomere-specific homologous recombination and nonhomologous end-joining reactions (de Lange, 2009). While only a subset of the shelterin components can be discerned in plant genomes, single-stranded telomeric DNA binding proteins are an essential component of the protective telomere cap in plants (Surovtseva et al., 2009) and many other model organisms (Gao et al., 2007; Raices et al., 2008; Linger and Price, 2009; Miyake et al., 2009). POT1 orthologs have been described in a wide variety of eukaryotic lineages, from ciliated protozoa and yeast to humans (Baumann and Cech,

2001; Baumann et al., 2002; Jacob et al., 2007). Despite substantial amino acid sequence conservation, POT1a and POT1b from *Arabidopsis* appear to have lost this critical chromosome capping function, raising the possibility that POT1 plays a fundamentally different role in the plant kingdom. The analysis of POT1 in the moss *P. patens* reported here rejects this hypothesis and indicates that in early land plant lineages, such as bryophytes, POT1 functions in a similar fashion to its fungal and vertebrate counterparts.

P. patens has the potential to emerge as a powerful new model for telomere biology. Like *Arabidopsis*, *P. patens* has short telomere tracts abutted by unique sequences on at least some chromosome ends. These characteristics allow detection of subtle defects in telomere maintenance and chromosome end protection using the highly sensitive PCR assays of PETRA and TF-PCR (Heacock et al., 2004). Like *Arabidopsis*, *P. patens* is genetically tractable, but it has the added advantage of a streamlined genome with much less redundancy. Besides a single-copy *POT1* gene, *P. patens* harbors only two *TRFL* genes (as opposed to six in *Arabidopsis*). Finally, *P. patens* has an exceptionally high rate of homologous recombination, and we exploited this property for targeted deletion of *POT1*. Our results not only provide direct genetic evidence of an evolutionarily conserved role for POT1 in chromosome end protection in plants, but they also demonstrate that *P. patens* can serve as a critical counterpoint to *Arabidopsis*, facilitating a deeper understanding of plant telomere composition and evolution.

Conservation of POT1 Functions in Eukaryotes

While the functions of POT1 differ between *Arabidopsis* and *P. patens*, together they encompass several of the roles previously ascribed to POT1 orthologs in mammals and fission yeast. Several studies indicated that mammalian POT1 proteins modulate telomerase action in vitro (Wang et al., 2007; Latrick and Cech, 2010; Zaug et al., 2010) and in vivo (Colgin et al., 2003; Loayza and de Lange, 2003). Although the details of how POT1 influences telomerase are likely to be organism specific, this function is conserved in plants, as illustrated by the phenotypes of *Arabidopsis POT1a*-deficient plants (Surovtseva et al., 2007). Similar to the budding yeast *ever-shorter-telomere* mutants (Lundblad and Szostak, 1989), *Arabidopsis POT1a* null mutants display progressive telomere shortening at a rate of ~200 to 500 bp per plant generation (Surovtseva et al., 2007). As previously described for plants lacking the telomerase catalytic subunit TERT (Riha et al., 2001), this gradual telomere erosion leads to a delayed onset of developmental and cell proliferation defects apparent only after multiple plant generations. By contrast, *P. patens Δpot1* mutants exhibit a rapid onset of telomere uncapping characterized by extensive telomere shortening, increased G-overhang length and chromosome fusions. These phenotypes are reminiscent of the situation in *S. pombe Pot1*-deficient cells, where chromosome ends rapidly lose all of their telomeric DNA and undergo chromosome circularization (Baumann and Cech, 2001). Thus, the function of *P. patens* POT1 is consistent with an essential role in chromosome end protection.

Like the surviving POT1-deficient *S. pombe* cells, which cannot undergo meiosis (Baumann and Cech, 2001), *P. patens*

Δpot1 cultures fail to undergo sexual reproduction and display a partial loss of developmental program. However, unlike *S. pombe*, *P. patens Δpot1* mutants do not lose all telomeric DNA and instead telomeres stabilize at a new, shorter length. This outcome may reflect the establishment of a new equilibrium between telomere-shortening activities and extension by telomerase. It is also possible that homologous recombination contributes to the maintenance of telomere tracts in *Δpot1 P. patens*, as proposed for telomerase-deficient *Arabidopsis* mutants (Watson and Shippen, 2007). Mouse POT1 proteins prevent recombination of telomeric DNA (He et al., 2006; Wu et al., 2006; Palm et al., 2009), and considering the naturally high rate of homologous recombination in *P. patens*, this alternative mode of telomere maintenance is certainly feasible.

Although we could detect chromosome fusions in long-term dividing *P. patens Δpot1* cultures, accumulation of these aberrant structures was slow, required multiple cell divisions, and did not result in complete senescence. A similar phenotype is associated with *POT1* deletion in chicken cells (Churikov et al., 2006), implying that some aspects of Pp POT1 function are more similar to vertebrate POT1 than to the *S. pombe* POT1. It is also possible that *P. patens* POT1 functions redundantly with another telomere protection factor, such as a homolog of the putative *Arabidopsis* double-stranded telomere binding protein TBP1 (Hong et al., 2007) or the newly identified plant CST complex (Surovtseva et al., 2009).

Evolution of POT1 Function in Plants

Our data underscore remarkable divergence of POT1 functions in lower versus higher plants and provide a framework for elucidating evolutionary changes responsible for the switch from telomere protection to telomerase regulation. We speculate that much of the functional divergence is reflected by changes in the nucleic acid binding properties of plant POT1 proteins. Only a subset of the plant POT1 proteins examined to date display single-stranded telomeric DNA binding activity in vitro (Surovtseva et al., 2007; Shakirov et al., 2009a, 2009b). Among those that do bind telomeric DNA, significant variation exists within their respective minimal DNA binding site. For example, while Pp POT1 and Ol POT1 (from green alga *O. lucimarinus*) prefer telomeric DNA substrates terminating with a T on the 3′ end, POT1 proteins from the flowering plants prefer oligonucleotides terminating in a G (Shakirov et al., 2009b). The length of the POT1 MBS also varies dramatically among different plant species, ranging from seven to 16 nucleotides (Shakirov et al., 2009b; this study). Previously described MBS sequences were reported in the size range 7 to 12 (Croy and Wuttke, 2006; Shakirov et al., 2009b), but here we show that the *P. patens* POT1 MBS is 16 nucleotides in length. The C terminus of Pp POT1 enhances telomeric DNA binding. It is possible that Pp POT1 oligomerizes through its C-terminal domain, accounting for its unusually long MBS. Notably, the *A. officinalis* POT1, whose MBS is only nine nucleotides, does not require the C-terminal domain for efficient DNA binding in vitro (Shakirov et al., 2009b). Alternatively, the extended minimum DNA binding site in Pp POT1 might reflect the contribution of an additional OB-fold in the C terminus, as has been postulated in

yeast Cdc13 and vertebrate POT1 (Theobald and Wuttke, 2004; Wei and Price, 2004).

The most compelling evidence of the rapid evolution of POT1 is illustrated by the *Arabidopsis* POT1 proteins. Recent data indicate that At POT1a and At POT1b physically interact with telomerase ribonucleoprotein complexes instead of telomeric DNA (Surovtseva et al., 2007; C. Cifuentes-Rojas, K. Kannan, L. Tseng, and D. Shippen, unpublished data). Thus, we speculate that the switch in plant POT1 proteins from binding telomeric DNA to telomerase fueled their evolution from telomere capping proteins into telomerase regulatory factors. Further analysis of POT1 in *Arabidopsis* and *P. patens* may more clearly elucidate the dynamic and evolving interactions between the chromosome terminus and the telomerase enzyme.

METHODS

Moss Techniques and Generation of *POT1* Deletion and Complementation Constructs

Growth of *Physcomitrella patens* isolates Gd and Vx-1 and protoplast generation and transformation were performed as described previously (Perroud and Quatrano, 2008). The gene deletion construct was designed to remove the full ORF of Pp *POT1* from start codon to stop codon. Genomic DNA (1.2 kb) immediately upstream of the start codon was PCR amplified with primers 5′-GCATCCTAGGCGTGTGATCCCGCAAT-3′ and 5′-GCATCTCGAGGAGAACAGACGATTATGTAAG-3′ and inserted 5′ to the hygromycin resistance *HptII* gene in the pBHRF vector (Schaefer et al., 2010), using *Avr*II and *Xho*I restriction enzymes. Similarly, 1.2 kb of genomic DNA immediately downstream of the Pp *POT1* stop codon was PCR amplified with primers 5′-GCATGCGGCCGCGAGCCAATTTTT-TTTTTGGCTTTG-3′ and 5′-GCATACTAGTATGAAGATAATAGTGC-3′ and inserted 3′ to the hygromycin resistance cassette using *Not*I and *Spe*I restriction enzymes. The final vector containing the hygromycin resistance cassette, flanked by the targeting *P. patens* genomic DNA sequence on both sides, was cut with *Avr*II and *Spe*I and introduced into wild-type *P. patens* isolate Gd by protoplast transformation (Schaefer and Zryd, 1997). Double crossing-over with this construct results in the complete deletion of *POT1* ORF, and the entire *Δpot1* locus was subsequently PCR amplified with primers 5′-GCTCATACAACAAGCACATT-GAC-3′ and 5′-CAACTTCATCCAACCATGCAG-3′, flanking the *POT1* locus, and sequenced to verify its absence.

To generate the complementation construct *Δpot1*-Pp-*POT1*, the entire Pp *POT1* ORF was PCR amplified with 5′-GAATTCTCTTGGA-AAGATGCGAGCGGCTGGTCTATGTTGCAGAGGACCATGTCG-3′ and 5′-GATCCTCGAGTCATCCCGGAAATCGTGTAC-3′ and cloned into the pBNRF vector (Schaefer et al., 2010) upstream of the G418 (kanamycin) resistance gene *NptII* using the same restriction sites. The targeting *P. patens* genomic DNA sequence on both sides of the complementation construct was the same as in pBHRF and contained the native Pp *POT1* promoter. *Δpot1* strain was transformed as described above. Stable transformants were selected on G418-containing medium and genotyped for the presence of the rescuing construct in the correct location. DNA gel blot analysis was performed with DIG-labeled PCR probes spanning the targeting sequences as described by Perroud and Quatrano (2008).

TRF, PETRA, and TF-PCR

P. patens DNA was extracted as described (Cocciolone and Cone, 1993). TRF analysis was performed using *Tru*II (Fermentas) restriction enzyme and a ^{32}P 5′ end-labeled $(T_3AG_3)_4$ oligonucleotide probe (Fitzgerald et al.,

1999). For the *Bal*31 exonuclease assay, 100 μg of *P. patens* genomic DNA was incubated with 30 units of *Bal*31 (New England Biolabs) or with water (0 min time point) in 1× *Bal*31 reaction buffer at 30°C. Equal amounts of sample were removed at specified intervals for 60 min. Reactions were stopped by the addition of 20 mM EGTA and heating to 65°C for 15 min. DNA in each sample was precipitated with isopropanol and ammonium acetate, followed by *Tru*II digestion and DNA gel blotting as described above. TF-PCR was performed essentially as described (Heacock et al., 2004) using primers 5′-CACTACATCGCTGGTCA-GAAACGA-3′ (specific for chromosome arm A, *P. patens* scaffold_2162) and 5′-GAAGGTATGTCATGGCCTCAAAGCT-3′ (specific for chromo-some arm B, *P. patens* scaffold_229). Primer B can also be substituted with primer B1 (5′-GATTGCACCATCATTGCCATCGCA-3′), which is lo-cated on the same scaffold 32 nucleotides closer to the start of the telomeric tract. Two modifications to the TF-PCR protocol were made: primer annealing was performed at 58°C, and PCR was run for 30 cycles. PETRA was performed for 19 cycles with primers A or B as described (Heacock et al., 2004). Radioactive signals were scanned by a Pharos FX Plus Molecular Imager (Bio-Rad Laboratories), and the data were ana-lyzed by either IMAGEQUANT software (Molecular Dynamics) or by Quantity One v.4.6.5 software (Bio-Rad). G-overhang analysis and site-directed mutagenesis were performed as described (Song et al., 2008). The quantitative telomere repeat amplification protocol procedure can be found in the Supplemental Methods online.

EMSA Assays

Expression of plant POT1 proteins in RRL and EMSA assays were conducted as described (Shakirov et al., 2009a). Briefly, each reaction (15 μL total volume) contained equal amounts (4 μL) of RRL-translated plant POT1 protein, 0.5 pmol of ^{32}P-labeled telomeric oligonucleotide, 3 μL of 5× DNA binding buffer (100 mM Tris-HCl, pH 7.8, 250 mM NaCl, 50 mM MgCl, 5 mM EDTA, 5 mM DTT, and 25% glycerol) and 1 μL each of Herring sperm DNA (50 μg/mL), DNA oligonucleotide 5′-TTAAT-TAACCCGGGGATCCGGCTTGATCAACGAATGATCC-3′ and RNA oligo-nucleotide (UUUAGGG)$_5$, which were added as nonspecific competitors (2.5 μM each) as described by Baumann et al. (2002). Reactions were incubated at room temperature for 15 min, and the complexes were separated on 5% polyacrylamide gel (acrylamide:bisacrylamide, 29:1) for 2 h at 150 V in 0.8× TBE at room temperature, dried, and exposed to phosphor imager screens. Screens were scanned by a Pharos FX Plus molecular imager, and signal intensity was quantified by Quantity One v.4.6.5 software.

RACE and RT-PCR Conditions

Total RNA was extracted from plant tissue using Tri Reagent solution (Sigma-Aldrich). Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) as described (Shakirov et al., 2005). To deduce the full-length Pp *POT1* cDNA, 5′ and 3′ rapid amplification of cDNA ends (FirstChoice RLM-RACE kit; Ambion) was performed accord-ing to the manufacturer's instructions. To analyze Pp *POT1* expression, RT-PCR was initially performed with control reactions for Ubiquitin gene expression as described (Harries et al., 2005) to normalize for RNA loading, followed by *POT1* RT-PCR with primers 5′-TCGACCCGG-GATGTTGCAGAGGACCATGTC-3′ and 5′-GATCCTCGAGTCATCCCG-GAAATCGTGTAC-3′, which span the entire ORF.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AY884593 (At POT1a), AY884594 (At POT1b), Q9NUX5 (h

POT1), and XP_001766873 (Pp Ubiquitin). Pp *POT1* cDNA was deposited into GenBank under accession number EU880302.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of DNA Binding Capacity of Recombinant Plant POT1 Proteins.

Supplemental Figure 2. Pp POT1 Efficiently Binds the AGGGTTT Permutation of the Plant Telomeric Repeat in Vitro.

Supplemental Figure 3. DNA and Protein Domain Requirements for Efficient DNA Binding of Pp POT1 Protein.

Supplemental Figure 4. Generation of the *Δpot1* Strain.

Supplemental Figure 5. Colony Morphology and Growth Defects in *Δpot1 P. patens*.

Supplemental Figure 6. *Bal*31 Analysis of Telomeres in the *Δpot1* Strain of *P. patens*.

Supplemental Figure 7. Generation of *Δpot1*-Pp *POT1* Complemen-tation Strain.

Supplemental Figure 8. Complementation of *Δpot1*-Specific Telo-mere Phenotypes in the *Δpot1*-Pp *POT1* Complementation Strain.

Supplemental Figure 9. In Vitro Telomerase Activity Levels Are Not Affected in *Δpot1* Mutants.

Supplemental Methods. Quantitative Telomerase Activity Assay (Q-TRAP).

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