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The *Arabidopsis* Pot1 and Pot2 Proteins Function in Telomere Length Homeostasis and Chromosome End Protection

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Pot1 (*p*rotection *o*f *t*elomeres 1) is a single-stranded telomere binding protein that is essential for chromosome end protection and telomere length homeostasis. *Arabidopsis* encodes two Pot1-like proteins, dubbed AtPot1 and AtPot2. Here we show that telomeres in transgenic plants expressing a truncated *AtPot1* allele lacking the N-terminal oligonucleotide/oligosaccharide binding fold (P1ΔN) are 1 to 1.5 kb shorter than in the wild type, suggesting that AtPot1 contributes to the positive regulation of telomere length control. In contrast, telomere length is unperturbed in plants expressing the analogous region of AtPot2. A strikingly different phenotype is observed in plants overexpressing the AtPot2 N terminus (P2ΔC) but not the corresponding region in AtPot1. Although bulk telomeres in P2ΔC mutants are 1 to 2 kb shorter than in the wild type, these plants resemble late-generation telomerase-deficient mutants with severe growth defects, sterility, and massive genome instability, including bridged chromosomes and aneuploidy. The genome instability associated with P2ΔC mutants implies that AtPot2 contributes to chromosome end protection. Thus, *Arabidopsis* has evolved two Pot genes that function differently in telomere biology. These findings provide unanticipated information about the evolution of single-stranded telomere binding proteins.

Telomeres are the essential protein-DNA structures at the ends of linear eukaryotic chromosomes whose primary functions are to facilitate complete replication of the chromosome terminus and to sequester it from DNA repair machinery and exonucleolytic attack (5, 7, 11). In most organisms, the DNA component of telomeres consists of tandem repeats of simple G-rich sequences that terminate in a single-stranded 3′ extension. The telomere can fold back on itself to form a t-loop, where the 3′ G-overhang invades the duplex region of the telomere to create a displaced loop consisting of single-stranded G-rich repeats (18). During S phase, the t-loop is thought to unfold, allowing telomerase access to the G-overhang for telomere length maintenance (50). The G-overhang associates with single-stranded specific proteins (42, 50). The first G-strand binding protein identified, *t*elomere *e*nd *b*inding *p*rotein (TEBP), was found in the hypotrichous ciliate *Oxytricha nova*. TEBP is a heterodimer of α and β subunits that binds tenaciously to the 3′ terminus of the G-overhang (35) via four oligonucleotide/oligosaccharide binding folds (OB folds) (21). The OB fold is a structurally conserved feature also associated with single-stranded telomere binding proteins in fungi and vertebrates (45). The *Saccharomyces cerevisiae* protein, Cdc13p, is the best characterized of this class of proteins (12, 42, 50). A multifunctional protein, Cdc13p binds the single-stranded G-overhang and provides telomere end protection, facilitates telomerase recruitment and repression, and coordinates telomeric G- and C-strand synthesis through interactions with lagging-strand replication machinery (50).

A distant relative of TEBP called Pot1 was recently found in *Schizosaccharomyces pombe* (3). SpPot1 shares weak sequence similarity with TEBP at its N terminus and, like Cdc13p, assumes an OB fold that facilitates specific recognition of telomeric DNA (25, 26). Deletion of *SpPot1* leads to immediate and catastrophic loss of telomeric repeats and, to some extent, erosion of subtelomeric DNA. *SpPot1* mutants missegregate their chromosomes and ultimately fail to divide. Cells that survive the loss of *SpPot1* undergo chromosome circularization (3). In a genetic screen for mitotic mutants, a sequence homologue of Pot1 was identified in *Aspergillus nidulans* (33). As with *SpPot1* mutants, *AnPot1* deficiency results in severe mitotic defects, as well as chromosome missegregation. These data imply that Pot1 from fungi is involved in chromosome end protection.

Pot1 orthologs have also been identified in vertebrates (4, 49) and are implicated in both telomere length regulation and chromosome end protection (10, 29, 47). Both human and chicken Pot1 proteins localize to telomeres in vivo (4, 49). Although human Pot1 (hPot1) binding is reduced in cells that have lost the G-overhang, hPot1 appears to associate with telomeres primarily through interactions with proteins that bind along the length of the duplex (29). The C-terminal domain of hPot1 interacts with the TTP1 protein linking hPot1 to the TRF1/TIN2 complex (28, 53). In several studies, overexpression of full-length hPOT1 resulted in lengthened telomeres in some, but not all, human tumor cell types, suggesting that Pot1 is a positive regulator of telomere length (1, 10, 28). However, in another study overexpression of a dominant-negative C-terminal fragment of hPot1 led to extensive telomerase-dependent telomere lengthening (29), implying that hPot1 serves as a negative regulator of telomere length. In support of this conclusion, RNA interference-mediated knock down of hPot1 resulted in longer telomeres (47, 51, 53), and recent in vitro studies suggest that hPot1 negatively regulates telomerase activity (24). hPot1 has also been implicated in chromosome

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end protection (47). Together, these data indicate that hPot1, like Cdc13p, is a multifunctional protein.

Here we investigate the role of Pot proteins in *Arabidopsis thaliana*. *Arabidopsis* has emerged as a powerful system for telomere biology (38). A genetically tractable higher eukaryote, *Arabidopsis* harbors short telomere tracts (2 to 5 kb in the Columbia ecotype) (40) and displays an unusually high tolerance to the genome instability that accompanies telomere dysfunction (37). Although relatively little is known about telomere-associated proteins in plants, *Arabidopsis* is distinguished from many other model organisms in that it harbors two Pot1-like genes (4). In this study we show that AtPot1 and AtPot2 are ubiquitously expressed at low levels. We also present data from transgenic plants establishing a function for both proteins in telomere length maintenance and a role for AtPot2 in chromosome end protection. These findings argue that the known functions of single-stranded telomere binding proteins have been segregated into two distinct polypeptides in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials, construction of *AtPot1* and *AtPot2* mutant alleles, and transformation. Wild-type *Arabidopsis* seeds were purchased from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, Ohio), cold-treated overnight at 4°C, and then placed in an environmental growth chamber and grown under a 16-h light/8-h dark photoperiod at 23°C. To obtain constructs for overexpression, regions of *AtPot1* and *AtPot2* cDNAs corresponding to the full-length, ΔC (amino acids 1 to 175), and ΔN (amino acids 176 to 467 for AtPot1 and 176 to 454 for AtPot2) polypeptides were amplified by PCR and then inserted into a binary vector pCBK05 (39) to allow expression from a 35S cauliflower mosaic virus promoter. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101. Transformation of wild-type plants was performed by the in planta method (39). T1 primary transformants were selected on 0.5 Murashige and Skoog basal medium supplemented with 20 mg/liter of phosphinothricine (Crescent Chemical), genotyped, and analyzed by reverse transcription-PCR (RT-PCR) for transgene expression.

cDNA synthesis. Total RNA was extracted from 0.1 to 0.5 g of plant tissue using Tri Reagent solution (Sigma). *AtPot1* and *AtPot2* cDNAs were synthesized from total leaf RNA using Superscript III reverse transcriptase (Invitrogen). Primers complementary to the stop codons of each cDNA were incubated with 2 μg of total RNA in the supplied buffer at 65°C for 5 min. Reverse transcription was carried with 100 U of Superscript III at 55°C for 50 min. RNA was degraded with RNase H (USB). The coding regions of *AtPot1* and *AtPot2* were then amplified with Turbo *Pfu* polymerase (Stratagene). PCR products were cloned and verified by sequencing.

TRF analysis, TRAP assays, cytogenetics, and fusion PCR. DNA from individual whole plants was extracted as previously described (9). Terminal restriction fragment (TRF) analysis was performed with Tru1I (Fermentas) restriction enzyme and ^{32}P 5′ end-labeled $(T_3AG_3)_4$ oligonucleotide as a probe (14). Telomere repeat amplification protocol (TRAP) assays were performed on plant tissues as previously described (13). Anaphase spreads were prepared from pistils and stained with DAPI (4′,6′-diamidino-2-phenylindole) as previously described (37). Telomere fusion PCR was performed as previously described (20). Briefly, DNA from mutant or wild-type plants was PCR amplified using primers for unique subtelomeric sequences that were directed toward telomeres. PCR products were resolved on an agarose gel, transferred to a nitrocellulose membrane, and hybridized with ^{32}P 5′ end-labeled $(T_3AG_3)_4$ oligonucleotide probe.

Nucleotide sequence accession numbers. The cDNA sequences of *AtPot1* and *AtPot2* have been deposited in the GenBank database under accession numbers AY884593 and AY884594, respectively.

RESULTS

Identification of two *Pot* genes in *Arabidopsis*. Two genes encoding putative Pot1 orthologs from *A. thaliana* were previously identified using the *S. pombe* Pot1 protein sequence (4) as the query in a BLAST search of the *Arabidopsis* genome database. *Arabidopsis* genes At2g05210 and At5g06310 showed strong similarity with the N-terminal Telo_bind_N domain, pfam02307, (32) of the *O. nova* TEBP α subunit and with the corresponding regions in *S. pombe* and human Pot1 proteins (Fig. 1A). The *Arabidopsis* genes were designated *AtPot1* and *AtPot2*, respectively. RT-PCR analysis of mRNA from leaves, flowers, roots, stems, and callus revealed that *AtPot1* and *AtPot2* mRNAs are ubiquitously expressed, although both transcripts are present in low amounts (Fig. 1B). This expression pattern differs from *AtTERT* mRNA, which is confined to proliferating tissues (13). Human Pot1 mRNA is alternatively spliced (4), and recent RT-PCR analysis suggests that *AtPot1* and *AtPot2* are also subject to alternative splicing (44). However, the variant splicing in *Arabidopsis* is not tissue specific, and all but one alternatively spliced form of the *AtPot1* and *AtPot2* mRNAs are predicted to result in prematurely truncated proteins. The functional relevance of these truncated isoforms is unclear.

The N termini of both AtPot1 and AtPot2 are predicted to assume two OB folds (M. Lei, personal communication). The OB-fold domains in AtPot1 and AtPot2 are 31% identical and 47% similar to each other (Fig. 1C). Outside this domain, the identity is 39% (55% similarity). As expected, both *Arabidopsis* proteins are more closely related to the mammalian orthologs than to Pot1 proteins from fungi. The N terminus of AtPot1 is 17% identical and 33% similar to the corresponding region of hPot1, while the N terminus of AtPot2 is 17% identical and 29% similar. Outside of this region, the similarity is lower (Fig. 1C). Notably, both *Arabidopsis* genes encode proteins that are significantly smaller in size than their mammalian and fungal counterparts.

Overexpression of *AtPot1* and *AtPot2* in transgenic *Arabidopsis*. Pot1 proteins from vertebrates and fission yeast specifically bind telomeric DNA in vitro (3, 30). To determine whether AtPot1 and AtPot2 display affinity for the plant telomeric DNA sequence, we expressed recombinant forms of the *Arabidopsis* proteins in *Escherichia coli* and in baculovirus. Most of the protein was insoluble, despite the application of many different expression conditions; however, the small fraction of soluble AtPot1 and Atpot2 protein that could be obtained displayed specific, but very weak, affinity for single-stranded plant telomeric DNA (data not shown). This observation suggests that AtPot1 and AtPot2 have the capacity to associate with telomeric DNA. Unfortunately, the inability to obtain sufficient quantities of soluble recombinant protein has stymied further biochemical analysis.

To investigate the function of AtPot1 and AtPot2, we employed a transgenic approach. When this study was initiated, no transfer DNA disruption lines were available for either gene in the annotated databases of several major *Arabidopsis* insertional mutagenesis facilities. Moreover, our attempts to reduce AtPot1 and AtPot2 protein levels by RNA interference were unsuccessful. Therefore, we examined the consequences of overexpressing full-length AtPot1, AtPot2, or C-terminal and N-terminal truncation derivatives. This strategy has been previously employed to investigate the function of human Pot1 (10, 29).

The C-terminal fragments of AtPot1 and AtPot2, dubbed P1ΔN (residues 176 to 467) and P2ΔN (residues 176 to 454), respectively, roughly correspond to the hPot1(ΔOB) construct

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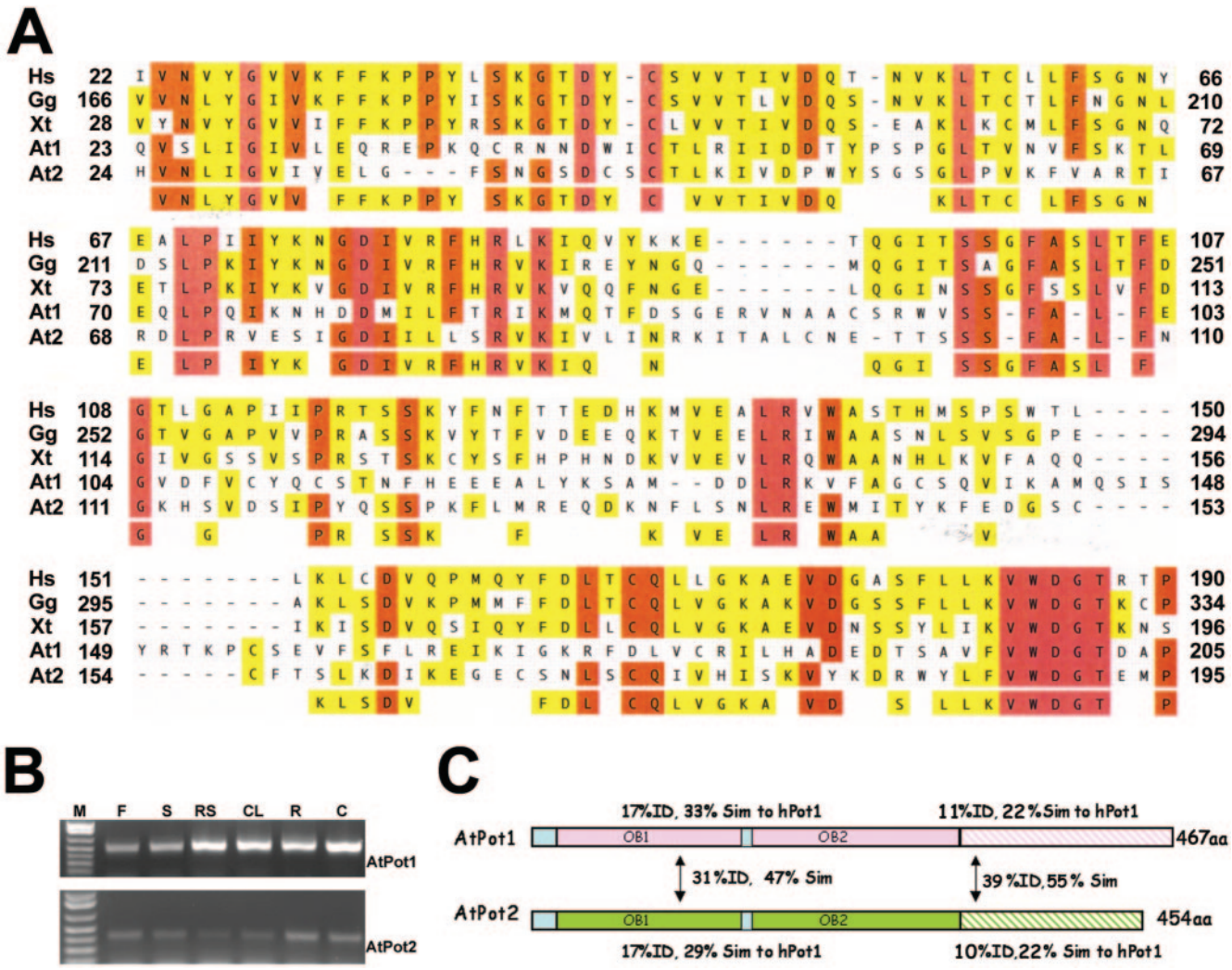

FIG. 1. Two Pot genes in *Arabidopsis*. (A) Amino acid alignment of N-terminal portions of Pot1 proteins. Hs, *Homo sapiens*; Gg, *Gallus gallus* (chicken), Xt, *Xenopus tropicalis* (accession number NP_998876); At1, AtPot1; At2, AtPot2. Residues conserved in at least two sequences are highlighted in yellow. Residues conserved in at least four sequences are highlighted in red. Residues conserved in all five sequences are highlighted in pink. A consensus sequence is shown below the alignment. (B) RT-PCR of AtPot1 and AtPot2 mRNAs in different *Arabidopsis* tissues. M, molecular weight markers; F, flowers; S, stems; RS, rosette leaves; CL, cauline leaves; R, roots; C, callus. (C) Sequence similarity between AtPot1 and AtPot2 proteins and human Pot1 protein.

(29). The N-terminal AtPot1 and AtPot2 fragments, P1ΔC and P2ΔC (residues 1 to 175), span the remainder of the proteins and contain the first OB fold. Previous studies indicate that the corresponding region in *S. pombe* Pot1 is sufficient for telomeric DNA binding in vitro (25).

To ensure robust expression in transgenic plants, the AtPot1 and AtPot2 constructs were cloned downstream of the cauliflower mosaic virus 35S promoter and were transformed into *Arabidopsis*. Overexpression of transgene mRNA was verified in all the transformants by RT-PCR with N- and C-terminal primer pairs (see Fig. 3A and 4A) (data not shown). Ectopic expression of full-length AtPot1 or AtPot2 proteins yielded no defects in growth or development, and telomeres were indistinguishable from wild-type controls as assayed by TRF analysis (Fig. 2A and B). The modest variability in telomere length associated with overexpression of full-length AtPot1 and AtPot2 falls within the wild-type range of 2 to 5 kb for *Arabidopsis* plants of the Columbia ecotype (40). The longest telomeres in the wild type typically span 4.0 to 5.5 kb, and the shortest are 1.6 to 2.5 kb (36, 40). The precise range is largely determined by the size of the telomere tract in the parent (40).

Transgenic plants overexpressing the AtPot1 N terminus (P1ΔC) or the AtPot2 C terminus (P2ΔN) also had wild-type appearance and showed no telomere perturbations when subjected to TRF analysis (Fig. 2C and D). Although the mRNAs for these constructs were highly expressed in transgenic plants, we could not verify that this was true for the corresponding polypeptides. Therefore, the lack of a phenotype in transgenic lines expressing full-length AtPot1, AtPot2, P1ΔC, or P2ΔN may reflect poor expression of these proteins. Alternatively, overexpression of these constructs may simply have no detrimental consequences for *Arabidopsis*.

Plants overexpressing P1ΔN and P2ΔC, by contrast, displayed reproducible, aberrant phenotypes, and these plants

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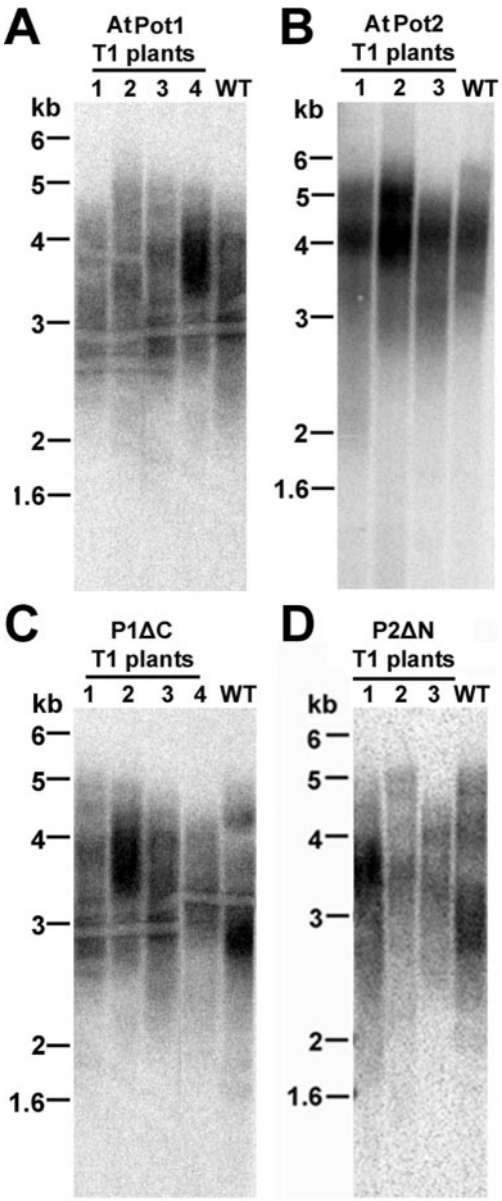

FIG. 2. Overexpression of full-length AtPot1 and AtPot2 or the P1ΔC and P2ΔN derivatives in *Arabidopsis* does not alter telomere length. TRF analysis was performed on DNA extracted from whole primary transformants (T1) shown by RT-PCR to express full-length AtPot1 (A), full-length AtPot2 (B), P1ΔC (C), or P2ΔN (D). Telomere length is 2 to 5 kb in wild-type (WT) *Arabidopsis* plants of Columbia ecotype (40).

were evaluated further. All of the plants overexpressing P1ΔN (n = 14) were fertile and morphologically indistinguishable from the wild type. While TRF analysis revealed that many primary transformants (T1) had wild-type telomere length (Fig. 3B, lanes 2 and 4), a significant percentage (~21%) displayed telomeres that were 1 to 1.5 kb shorter than wild type. In such cases, the longest telomeres averaged 3.5 kb (Fig. 3B, lanes 1 and 3). Subsequent generations of self-pollinated mutants displayed either very limited (≤300 bp) or no additional telomere shortening (Fig. 3C and data not shown), indicating that a new set point for telomere length was reached. Interestingly, in a subset of second generation progeny (T2), telomeres occupied a much broader size range, suggesting that a fraction of telomeres had returned to the wild-type length (Fig. 3C, lanes 4, 5, 6, and 8), even though RT-PCR indicated that these plants continued to express the transgene mRNA (data not shown). A similar unstable phenotype is associated with clonal human cell lines overexpressing full-length hPot1 (10). The high degree of sibling-to-sibling variation in telomere length observed with P1ΔN mutants in T2 is not seen in wild-type *Arabidopsis* (40) and implies that the loss of telomeric DNA is reversible. We conclude that AtPot1 contributes to telomere length homeostasis, and since telomere shortening was detected, our data suggest that AtPot1 is a positive regulator of telomere length.

Overexpression of P2ΔC results in severe morphological defects and telomere dysfunction. As with P1ΔN mutants, the majority of P2ΔC transformants (n = 52) were normal in appearance. However, a substantial fraction (~10%) showed distinct morphological defects at 2 to 3 weeks of age (Fig. 4B and C). Such plants exhibited a "terminal" morphological phenotype similar to that of late-generation (G_{7-9}) telomerase-deficient plants (37), with delayed growth (Fig. 4B) and flowering time and abnormally small rosette leaves that were wrinkled and curled down (Fig. 4C). Although numerous small siliques formed, they were sterile and produced no seeds (data not shown). Several attempts to make reciprocal crosses of the mutants to the wild type failed, consistent with severe anomalies in both male and female reproductive systems.

Overexpression of P2ΔC also resulted in an altered telomere phenotype in plants that displayed morphological defects. TRF analysis of such mutants revealed telomeres that were significantly shorter than in the wild type, with some transformants losing between 1 and 2 kb of telomeric sequence in a single generation (Fig. 4D, lanes 1, 3, and 5). As with P1ΔN, not all primary P2ΔC transformants had shortened telomeres (Fig. 4D, lanes 2, 4, and 6); however, this phenotype was reproducible and exclusively associated with mutants that displayed morphological defects and sterility.

The telomere shortening associated with P2ΔC and P1ΔN overexpression is not due to inactivation of telomerase. TRAP assays revealed that telomerase is biochemically active in both mutants (Fig. 5). Furthermore, relative to first-generation telomerase mutants, which lose 200 to 500 bp of telomeric DNA (37), depletion of telomeric DNA in P2ΔC and P1ΔN transgenic plants is greater by 2- to 3-fold and 1.5-fold, respectively (Fig. 3 and 4). Thus, the telomeres in both mutants appear to suffer additional replication defects or are exposed to nuclease attack.

In *Arabidopsis* the severe morphological abnormalities associated with dysfunctional telomeres are accompanied by genome instability (37). Therefore, transgenic plants were subjected to cytogenetic analysis of mitotic cells in pistils. No cytogenetic defects were observed in plants overexpressing full-length AtPot1, full-length AtPot2, P1ΔC, P2ΔN, or P1ΔN (Table 1 and data not shown), consistent with their wild-type appearance. Cytogenetic analysis was also performed on three of the P2ΔC mutants with morphological defects that were capable of producing flowers. In each case, we detected profound genome instability. Up to 8% of all mitotic figures con-

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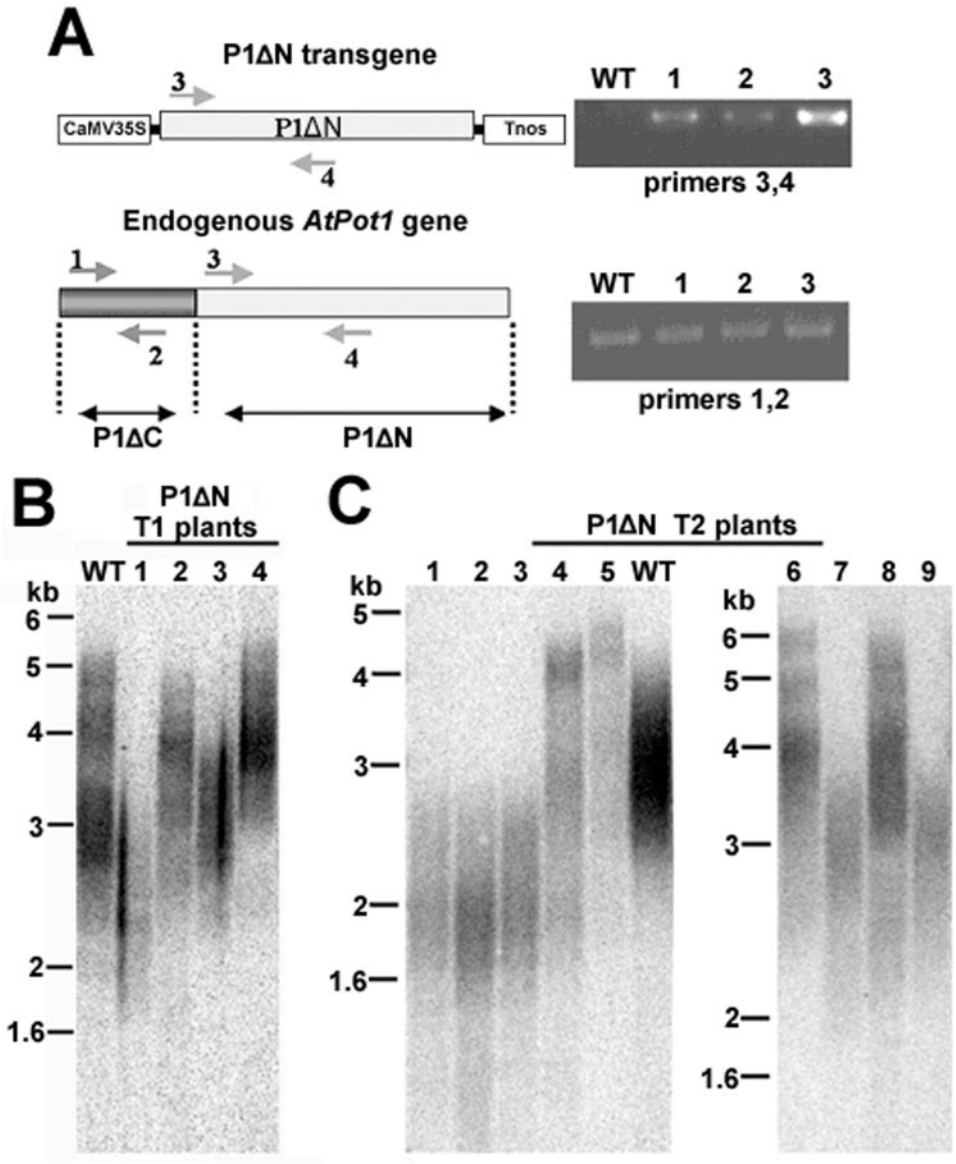

FIG. 3. Overexpression of P1ΔN leads to telomere shortening. (A) Schematic diagram of the P1ΔN transgene and the corresponding endogenous *AtPot1* allele (left); RT-PCR results of P1ΔN expression in transgenic plants 1 to 3 from panel B (right). Primers 1 and 2 are specific for the endogenous *AtPot1* mRNA. After 40 cycles, *AtPot1* mRNA is amplified in all plants equally well (lower panel, lanes 1 to 3, and WT). Primers 3 and 4 amplify both the P1ΔN transgene and the endogenous *AtPot1* allele. After 20 PCR cycles, P1ΔN mRNA is amplified only in the transgenic plants (upper panel, lanes 1 to 3), but not in the wild-type plant (upper panel, WT), confirming that the P1ΔN transgene is overexpressed. (B and C) TRF analysis of P1ΔN mutants. Examples of P1ΔN primary transformants (T1) displaying wild-type telomere lengths (lanes 2 and 4) or shortened telomeres (lanes 1 and 3) are shown (B). Analysis of the second-generation (T2) progeny from transformants 1 (lanes 1 to 5) and 3 (lanes 6 to 9) from panel B (C). For most T2 plants, no additional telomere shortening was observed (lanes 1 to 3, 7, and 9), but in some siblings telomere tracts were extended to resemble the wild type (lanes 4 and 5 and lanes 6 and 8).

tained anaphase bridges (Fig. 6B and C; Table 1), consistent with the formation of dicentric chromosomes as a result of end-to-end telomere fusions. Remarkably, 50% of all anaphases with bridged chromosomes contained two or more fusions (Fig. 6D and E), and in several cells a majority of the chromosomes appeared to be fused (Fig. 6F). In some instances, large DNA fragments, possibly entire chromosomes, lagged behind (Fig. 6G). Many mitotic cells displayed other defects, including unequal chromosome segregation that would result in aneuploidy in daughter cells (Fig. 6H). Similar mitotic abnormalities, including increased chromosome instability and segregation errors, are associated with *A. nidulans Pot1* mutants (33) and human cells with knocked-down hPot1 expression (47).

To determine whether the cytological defects associated with P2ΔC mutants involve dysfunctional telomeres, we performed telomere fusion PCR using primers directed outward from the unique subtelomeric sequences found on *Arabidopsis* chromosomes (20). In this assay, PCR products are generated when telomeres form covalent associations (Fig. 7A). PCRs

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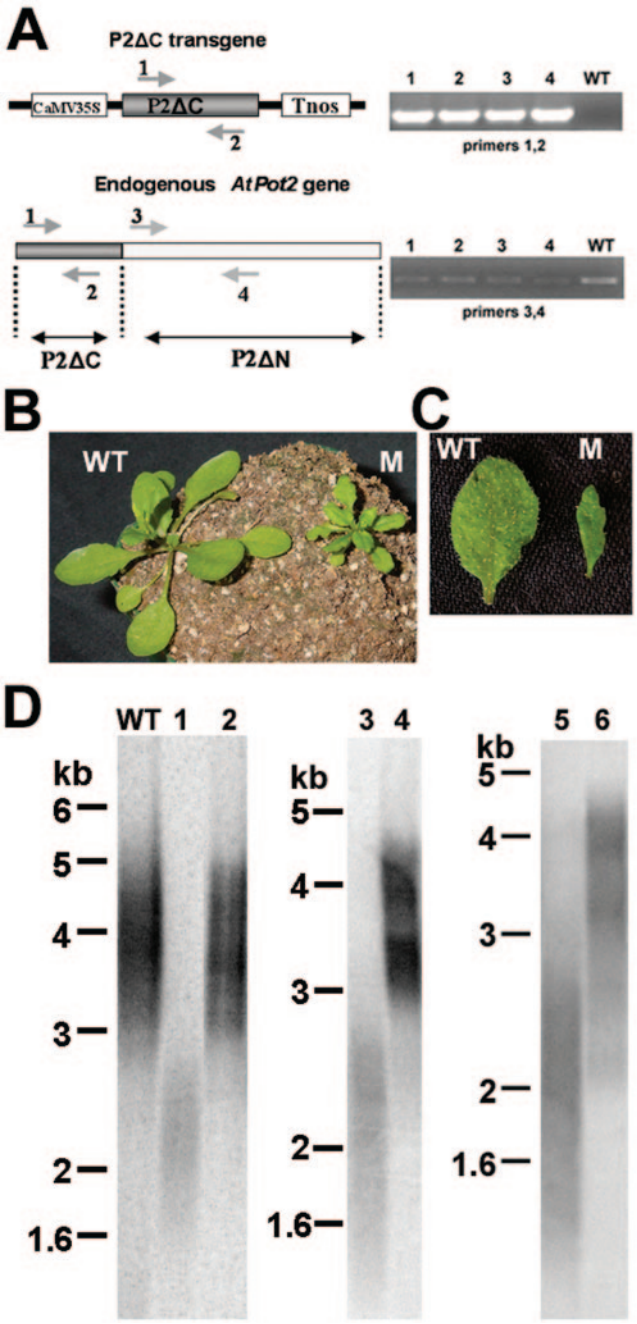

FIG. 4. Morphological defects and telomere shortening in mutants overexpressing P2ΔC. (A) Schematic of the P2ΔC transgene and the corresponding endogenous *AtPot2* allele (left); RT-PCR analysis of P2ΔC expression in transgenic plants 1 to 4 from panel D (right). RT-PCRs were conducted as described in the legend of Fig. 3. (B and C) Growth and developmental defects in P2ΔC mutants. (B) At 2 to 3 weeks of age, growth of several independent transgenic mutants (M) was delayed relative to wild-type (WT). (C) Such mutants displayed severe defects in leaf development. (D) TRF analysis of P2ΔC transformants. Primary transformants (T1) with a wild-type appearance displayed wild-type telomere length (lanes 2, 4, and 6), whereas plants that exhibited morphological defects had shortened telomeres (lanes 1, 3, and 5).

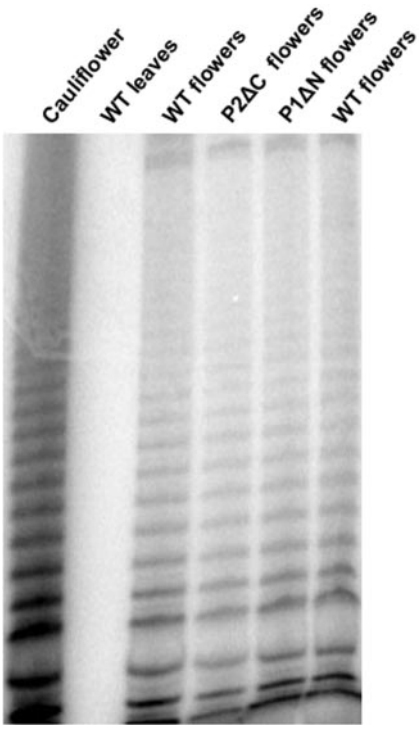

FIG. 5. TRAP assays of wild-type and mutant *Arabidopsis*. Extracts from cauliflower inflorescence and wild-type *Arabidopsis* flowers serve as positive controls, and wild-type *Arabidopsis* leaves serve as a negative control for telomerase activity. P2ΔC and P1ΔN mutants have wild-type levels of telomerase activity in vitro.

were performed with seven different primer combinations directed at subtelomeric sequences. Primer binding sites ranged from 200 bp (right arm of chromosome 5 [5R]) to 950 bp (3R) upstream of the telomere repeats. Strikingly, in contrast to PCRs with DNA from G_7 *tert* mutants, which display a comparable number of anaphase bridges to the most severely affected P2ΔC mutants (37) (Fig. 7B), only a few PCR products were generated from P2ΔC DNA with a subset of primer combinations (Fig. 7B). The rare products that could be cloned from these reactions (i.e., left arm of chromosome 3 [3L]+3R reaction) contained DNA derived from centromeric regions, which harbor a short stretch of telomeric DNA sequence. This observation implies that anaphase bridges in P2ΔC mutants do not involve intact telomeres. We speculate that the failure to amplify chromosome fusion junctions using subtelomeric prim-

TABLE 1. Frequency of anaphase bridges in *AtPot1* and *AtPot2* mutants

Genotype	No. of analyzed pistils	No. of anaphases		
		With bridges	Total scored	% Bridges
WT	4	0	291	0
P1ΔN	4	0	307	0
P2ΔC				
Plant 1	2	9	122	7.4
Plant 2	2	11	145	7.6
Plant 3	1	4	51	7.8

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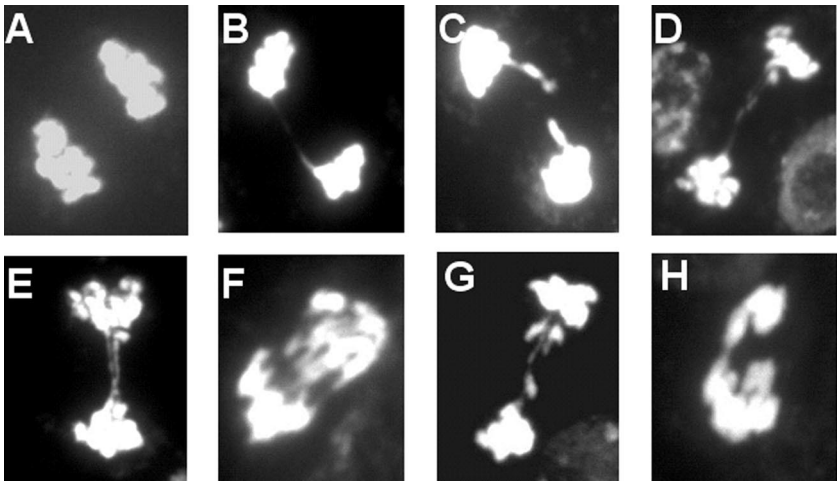

FIG. 6. Cytogenetic defects in mutants overexpressing P2ΔC. Cytogenetic analysis was performed on actively dividing mitotic tissues of pistils from wild-type (A) or P2ΔC mutants (B to H) using DAPI. Anaphase bridges, lagging chromosomes, and aneuploidy are evident in P2ΔC mutants.

ers reflects extensive nucleolytic processing of telomeres prior to end joining. Chromosomal termini in *S. pombe pot1* mutants lose more than 5 kb of DNA prior to circularization (3). If this occurs in *Arabidopsis* P2ΔC mutants, the binding sites for the primers used in our assay would be absent. Alternatively, chromosomes in *Arabidopsis* P2ΔC mutants could be held together by noncovalent interactions.

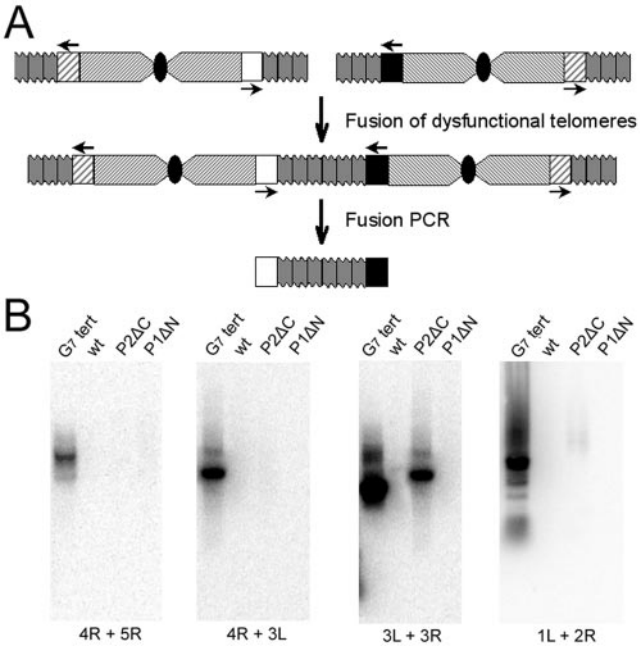

FIG. 7. Telomere fusion PCR analysis of P2ΔC and *tert* mutants. (A) Schematic diagram of telomere fusion PCR. Primers specific to unique subtelomeric sequences (white, black, and diagonally striped boxes) are directed toward telomeres and will only amplify a product if telomeres form covalent attachments with each other. (B) Primers specific for 4R and 3L, 4R and 5L, 4R and 5R, 3L and 3R, 3L and 5R, 5R and 5L, and 1L and 2R were used in the assay to amplify chromosome fusion products from DNA extracted from *tert*, wild-type, P2ΔC, and P1ΔN plants.

Taken together, our data argue that AtPot2 contributes to the protective cap on the chromosome terminus and that loss of this function triggers telomere shortening, chromosome fusions, and massive genome instability.

DISCUSSION

Single-stranded telomere binding proteins are found in a diverse array of single-celled and multicellular eukaryotes. Cdc13p from *S. cerevisiae* provides the best-studied example. Its functions include essential contributions to telomere length homeostasis and chromosome end protection, roles that are mediated through a plethora of protein interactions at the telomere (12, 50). Other proteins from this group include TEBP from *O. nova* and the Pot1 proteins from *S. pombe*, *A. nidulans*, chicken, and humans. Although it is not possible to explore the role of TEBP in vivo since *Oxytricha* is not a genetically tractable organism, TEBP's exquisite specificity for the 3′ OH on the G-overhang (21) argues strongly for a function in telomere end protection. Like TEBP, *S. pombe* Pot1 specifically binds single-stranded G-rich telomeric DNA, albeit not with a correspondingly strong preference for the 3′ OH (3, 25). Analysis of *S. pombe* and *A. nidulans Pot1* deletion mutants has established a critical role for these proteins in chromosome end protection (3, 33). Moreover, recent data suggest that SpPot1 may also contribute to telomere length regulation (46), and in vivo studies show that it is capable of binding differentially to its telomeric DNA substrate in a manner that can expose or block the terminus from elongation by telomerase (27). hPot1 has also been implicated in both telomere length regulation (10, 29) and chromosome end protection (47). Here, we establish that *Arabidopsis* harbors two Pot proteins and that both contribute to telomere biology.

AtPot1 contributes to telomere length homeostasis. Our data provide strong evidence that the AtPot1 protein plays a role in telomere length regulation. First-generation transgenic plants overexpressing the AtPot1 C terminus (P1ΔN) harbor telomeres that are up to 1.5 kb shorter than the wild type. In contrast to telomerase mutants, telomere length does not de-

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cline further in subsequent generations. Instead, in several second-generation transformants, many telomeres returned to wild-type length, indicating that AtPot1 influences telomere length homeostasis. Since the truncated AtPot1 polypeptide (P1ΔN) lacks the OB fold-containing N terminus, its effect is unlikely to be mediated by direct DNA binding. One possibility is that P1ΔN dislodges AtPot1-interacting proteins involved in telomerase recruitment, thereby disturbing telomere length homeostasis and tipping the balance in favor of telomere shortening. Alternatively, as has been shown for the human P1(ΔOB) protein (29), this truncated *Arabidopsis* polypeptide may remain associated with telomeres via protein-protein interactions, directly influencing the telomere-counting mechanism proposed to regulate the length of the telomere tract (31, 43). Although one can envision ways in which AtPot1 could act as a negative regulator of telomere homeostasis, the simplest explanation is that AtPot1 is a positive regulator of telomere length. AtPot2 may also contribute to the positive regulation of telomere length, as P2ΔC mutants display shorter telomeres. However, our data suggest that AtPot1 may be specialized to serve this role.

One surprising outcome of our study is that telomeres in plants overexpressing P1ΔN suffer the opposite fate of telomeres in human cells that overexpress the corresponding region of hPot1 [P1(ΔOB)] (24, 29). Disparate behavior of telomere proteins is not without precedent. The yeast Rif1 protein regulates telomere length in wild-type cells (19), while human Rif1 is only present at dysfunctional telomeres (41). Similarly, Ku deficiency in yeast leads to telomere shortening (6, 17, 34) but in *Arabidopsis* culminates in significant telomere elongation (16, 39). Most notably, no sequence homologues for hPot1 interacting factors TTP1 and TIN2 exist in the *Arabidopsis* and *S. pombe* genomes. Thus, a different ensemble of Pot-associated proteins remains to be discovered in these organisms.

AtPot2 functions in chromosome end protection. Our data implicate AtPot2 in chromosome end protection. A significant fraction of primary transformants overexpressing the AtPot2 N terminus (P2ΔC) resemble terminal generation telomerase-deficient mutants with severe morphological defects, sterility, and a high incidence of anaphase bridges (37). It is possible that P2ΔC competes with the endogenous AtPot2 for telomeric DNA, dislodging AtPot2 altogether or reducing the affinity of AtPot2-interacting factors involved in chromosome end protection. A more definitive understanding of the mechanism of both AtPot1 and AtPot2 dominant-negative alleles will require localization of these proteins and their full-length counterparts to telomeres in vivo.

The phenotype of P2ΔC mutants is remarkably similar to loss-of-function *pot1* mutants in *S. pombe* (3, 33). Both mutants experience an immediate onset of cytogenetic defects including chromosome missegregation and chromosome fusion. Both lose telomeric DNA. However, unlike the situation in the *S. pombe* deletion strain, where the entire telomere tract and several kilobases of subtelomeric DNA are lost within 10 cell generations (3), bulk telomeres decline by only 1 to 2 kb in *Arabidopsis* P2ΔC mutants and do not reach the critically shortened threshold defined for *Arabidopsis tert* mutants (20). Interestingly, however, we failed to amplify chromosome fusion junctions containing telomeric DNA in P2ΔC mutants. One interpretation of these data is that the subset of chromosomes with dysfunctional telomeres that are targeted for end-joining reactions are first subjected to extensive exonucleolytic degradation.

The relatively modest decline of bulk telomere length in P2ΔC mutants may reflect a limited opportunity for nuclease action on *Arabidopsis* telomeres, as telomeres are confined to the nucleolus throughout most of the mitotic and meiotic cell cycles (2, 15). Alternatively, the presence of endogenous AtPot1 and AtPot2 may offer partial protection to the chromosome terminus. A third possibility is that AtPot2 exerts its capping function in concert with a TRF2-like protein. hPot1 associates with TRF2, a protein essential for chromosome end protection in human cells (51, 52). A number of TRF-like proteins have been described in *Arabidopsis* (8, 22, 23). One or more of these could act in the same pathway with AtPot2 to facilitate chromosome capping.

Evolution of Pot proteins. The long delay in the identification of single-stranded telomere binding proteins in multicellular eukaryotes is due to the rapid divergence of this class of proteins. *Arabidopsis* may not be unique in possessing more than one *Pot1* gene. Database searches reveal the presence of two relatively similar *Pot1* genes in mouse, and the ciliate *Euplotes crassus* genome encodes two highly divergent Pot1-like proteins (48). Moreover, analysis of plant expressed sequence tag databases showed that several species harbor multiple *Pot* genes that, like the *AtPot1* and *AtPot2*, exhibit marked sequence divergence (E. Shakirov and D. Shippen, unpublished data). The striking sequence divergence of the *Arabidopsis Pot1* and *Pot2* genes, coupled with the genetic data presented here, argue that these proteins make distinct contributions to telomere biology. Hence, further analysis of these proteins should provide useful insight into the functions and evolution of single-stranded telomere binding proteins.

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