Arabidopsis POT1A interacts with TERT-V(I8), an N-terminal splicing variant of telomerase

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Summary

Chromosome integrity is maintained via the actions of ribonucleoprotein complexes that can add telomeric repeats or can protect the chromosome end from being degraded. POT1 (protection of telomeres 1), a class of single-stranded-DNA-binding proteins, is a regulator of telomeric length. The *Arabidopsis* genome contains three *POT1* homologues: *POT1A*, *POT1B* and *POT1C*. Using yeast two-hybrid assays to identify components of a potential POT1A complex, we retrieved three interactors: the N-terminus of the telomerase, a protein kinase and a plant-specific protein. Further analysis of the interaction of POT1 proteins with telomerase showed that this interaction is specific to POT1A, suggesting a specific role for this paralogue. The interaction is specific to the N-terminal region of the telomerase, which can be encoded by splicing variants. This interaction indicates possible mechanisms for telomerase regulation by alternative splicing and by POT1 proteins.

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Key words: Telomere, Protein-protein interaction

Introduction

Telomeres are the ends of linear eukaryotic chromosomes. Their main function is to maintain genome integrity by replicating chromosome ends and protecting them from exonucleolytic degradation and mutual fusions (reviewed in Blackburn, 2001). Telomeres also have a role in coordinating chromosome segregation and in regulating the expression of subtelomeric genes (reviewed in Harper et al., 2004; Rusche et al., 2003). Telomeric DNA is composed of double-stranded (ds) G-rich repeats of a 6-8 bp sequence, extending into a G-rich single-stranded (ss) overhang. In several organisms, long telomeres have been reported to fold back and form loops (Cesare et al., 2003; de Bruin et al., 2000; Griffith et al., 1999) in which the ss overhang invades the dsDNA. The formation and the role of these structures have yet to be elucidated, but it is thought that they might protect telomeres from being degraded or extensively extended by telomerase (reviewed in de Lange, 2004). Telomerase is required for telomere extension in most somatic cells and in some cancerous cells, allowing the addition of telomeric repeats to counterbalance the attrition effect due to replication of linear chromosome by DNA polymerase. Apart from the direct addition of telomeric repeats by the telomerase, telomere integrity is maintained through the binding of specific protein complexes (reviewed in Smogorzewska and de Lange, 2004).

POT1 (protection of telomeres 1) proteins are important for telomere-end protection because they cap the 3′ end. They are recruited to telomeres by direct binding to the ssDNA via a conserved DNA-binding motif – the oligonucleotide/oligosaccharide binding folds (OB folds) – and by interacting with other components of the telomeric complex, such as TPP1 in human cells (reviewed in (Smogorzewska and de Lange, 2004). The family of POT1-related proteins include the ciliate telomere-end binding proteins (TEBPs) (Gottschling and Zakian, 1986), *Schizosaccharomyces pombe* Pot1 (Baumann and Cech, 2001), mammalian POT1 (Baumann and Cech, 2001; Hockemeyer et al., 2006; Wei and Price, 2004; Wu et al., 2006), fungal NimU (Pitt et al., 2004), plant POT1-like proteins (Shakirov et al., 2005) and the distant orthologues such as *Saccharomyces cerevisiae* Cdc13p (ScCdc13p) from budding yeast (Nugent et al., 1996). Deletion studies have shown that POT1 serves an essential role in protecting the chromosome end against telomere loss. Deletion of Cdc13p in *S. cerevisiae* is lethal because of the inability of the cells to prevent telomere loss and to prevent resection of the C-rich strand of the chromosome (Booth et al., 2001). In *S. pombe*, cells lacking Pot1 showed telomere attrition and the few escaping cells displayed chromosome re-circularisation (Baumann and Cech, 2001). POT1 is also essential in chicken DT40 cells (Churikov et al., 2006) and, in mice, MEF cells that are null for *POT1A* are not viable (Hockemeyer et al., 2006). NimU, an orthologue of POT1, from *Aspergillus nidulans* was identified in a screen for mitotically defective mutants (Pitt et al., 2004), and $NimU^{POT1}$ deficiency led to chromosome mis-segregation, suggesting a role for the fungal Pot1 in mitotic checkpoint control. In *Arabidopsis*, overexpression of truncated forms of the proteins lead to deregulation of telomere length and chromosome instability (Shakirov et al., 2005).

POT1-related proteins appear to act as both positive and negative regulators of telomere length. Analysis of a class of

mutants with less-dramatic effects in budding yeast showed that the Cdc13 protein can be both a positive and a negative regulator of telomere length (Evans and Lundblad, 2000; Chandra et al., 2001; Pennock et al., 2001). Similarly, in human cells, POT1 has opposing roles in regulating telomere length. Overexpression of human POT1 led to telomere lengthening in telomerase-positive cells (Colgin et al., 2003), suggesting that POT1 positively regulates telomerase. However, other studies highlight a potential negative role of POT1, because overexpression of a dominant-negative form of the protein lacking the N-terminal DNA-binding domain also led to telomerase-dependent telomere lengthening (Loayza and De Lange, 2003). Additionally, RNAi downregulation of POT1 also led to telomere lengthening (Veldman et al., 2004). However, these studies did not completely deplete POT1 proteins from the telomeres so that POT1 could still be recruited to the G-overhang by interacting with another component of the telomeric complex, TPP1 (Liu et al., 2004; Ye et al., 2004).

In vitro studies gave insight into how POT1-like proteins could have these opposing roles. POT1 proteins have the ability to regulate telomere length by controlling telomerase access to the telomeres. After extension of the telomeres by the telomerase, the sequence produced (in human cells) corresponds to TTAGGGTTAG, which is also the binding site of human POT1 (Lei et al., 2004). In addition to sequestering the 3′ end of the telomeric DNA, human POT1 could also inhibit the telomerase activity by steric hindrance, preventing the binding of the RNA subunit of telomerase to the DNA (Kelleher et al., 2005; Lei et al., 2005). In another in vitro study (Lei et al., 2005), the conditions in which human POT1 could 'switch on and off' the telomerase and act as a positive regulator by recruiting telomerase or as a negative regulator by preventing access of the telomerase to its substrate was further defined. The authors also proposed that ssDNA bound to human POT1 could facilitate telomerase-dependent telomere extension (Lei et al., 2005). However, no direct interaction between human POT1 (or POT1-like protein) and the catalytic subunit of the telomerase TERT has been shown to date. Alternatively, the interaction could be mediated through another factor, such as human EST1A (SMG6), a homologue of the budding yeast Est1p that mediates interaction between the telomerase Est2p and the yeast homologue of human POT1, Cdc13p (Taggart and Zakian, 2003). This factor would displace human POT1 from its position either by sliding or by a dissociation/re-binding mechanism. However, it remains unclear exactly how human POT1 can act both as a positive and negative regulator in vivo.

In this paper, we report the physical interaction of an *Arabidopsis* POT1 protein, POT1A, with an N-terminal peptide of the catalytic subunit of the telomerase TERT encoded by a 5′ mRNA splicing variant. This is the first report of a direct interaction between these two regulators and might provide a mechanism by which POT1 regulates telomerase activity.

Results

The POT1 protein family in *Arabidopsis* contains three genes

Using NimU^{Pot1} (Pitt et al., 2004) as a query in a PSI-BLAST search, several *POT1* genes could be identified in plants. Two *POT1*-like genes were annotated in the *Arabidopsis* genome: *POT1A* (*At5g05210*) and *POT1B* (*At5g06310*). During the course of our study, both *POT1A* and *POT1B* were shown to be involved in telomere regulation in *Arabidopsis* (Kuchar and Fajkus, 2004; Shakirov et al., 2005; Tani and Murata, 2005). We found an additional locus in *Arabidopsis* encoding a potential POT1 protein (*At2g04395*) that appeared to have arisen as a partial duplication of the *POT1A* locus and was shown to be transcribed by 5′ and 3′ RACE (Accession number AY464640). We refer to this gene as *POT1C* (Accession number BN000975). Sequence alignments of *POT1C* with the two other *POT1* genes showed that the protein is 60% identical to the N-terminus of POT1A. In rice, only a single sequence could be identified as a *POT1* orthologue (locus OSJNBa0016O02.10).

Identification of POT1-interacting proteins

As a first step towards understanding the function of POT1, we searched for proteins interacting with POT1 proteins using a yeast two-hybrid screen. To facilitate cloning of Gateway-ready open reading frames (ORFs), compatible two-hybrid vectors were generated by converting the existing Matchmaker III vectors (Clontech) pGADT7 and pGBKT7 to the Gateway system (Fig. 1) and used to clone *AtPOT1* genes in translational fusion with either the activation domain (pAD-) or to the binding domain (pBD-) of GAL4.

Because POT1 proteins have been reported to bind DNA (Baumann and Cech, 2001), we first checked whether POT1A could self-activate the yeast two-hybrid system. Diploids containing either pGADT7/pBD-POT1A or pGBKT7/pAD-POT1A were unable to grow on SD-LWH and SD-LWHA, indicating that POT1A alone cannot activate gene expression in this system (data not shown). Similar results were obtained for POT1B. The ciliate TEBP proteins heterodimerise in vivo and chicken POT1 protein has been reported to dimerise in yeast (Horvath et al., 1998; Wei and Price, 2004). However, growth tests using suitable diploids expressing these fusion proteins indicated that POT1 proteins are unable to homo-dimerise or heterodimerise in yeast (data not shown).

To identify interacting proteins, we screened an expression library by mating the AH109 strain containing the library with Y187 transformed with pBD-POT1A. Positive clones were selected on SD-LWHA plates and the library plasmids rescued into *Escherichia coli* for further analysis. From a total of 1.4×10^7 clones screened, we isolated five putative interacting clones (Table 1). Of these, only three were confirmed to be able to interact with POT1A as fusions with both the DNA-binding and the activation domain of GAL4 (Fig. 2). These three positives include an N-terminal region of the telomerase, a CBL-interacting protein kinase (CIPK21) and a plant-specific protein of unknown function. Furthermore, these interactions were specific to POT1A, because no growth occurred when tested with POT1B or the telomeric protein KU80 (Fig. 2).

TERT-V(I8), a variant form of TERT, interacts with POT1A

Telomerase and POT1-related proteins both bind to telomeres, where they interact functionally and genetically (Lendvay et al., 1996; Smogorzewska and de Lange, 2004). The yeast two-hybrid results indicate that they also interact physically. To define the interaction domain, we tested different parts of the TERT protein for their ability to interact with POT1.

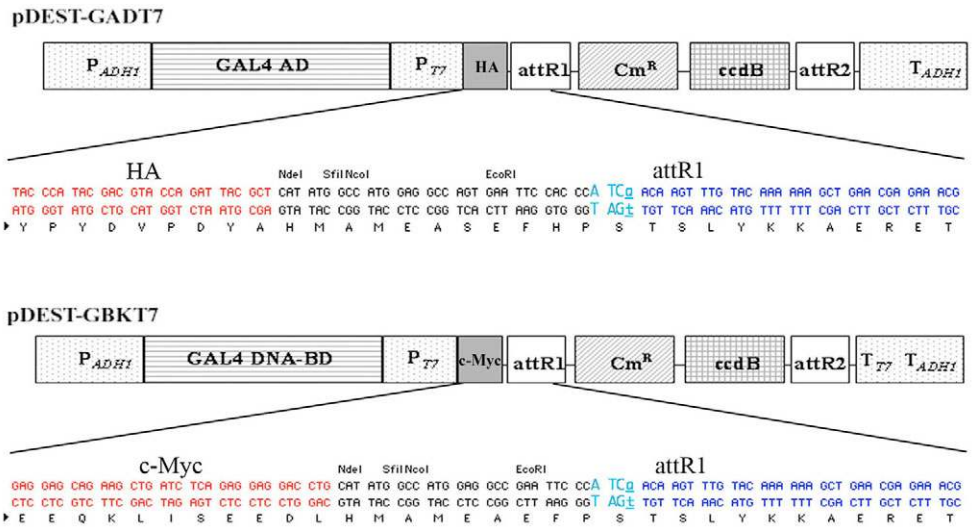

Fig. 1. Schematic of the pDEST-GADT7 and pDEST-GBKT7 expression cassettes. The Gateway cassette [composed of the recombination sites *attR1* and *attR2*, the chloramphenicol resistance gene (Cm^R) and the *ccdB* gene] was inserted into the *Sma*I site of the yeast two-hybrid vectors pGADT7 and pGBKT7. LR recombination with the target gene replaces the region between the *attR1* and *attR2* recombination sites to create in-frame fusions with either the GAL4 activation domain (GAL4 AD) fused to the HA-tag in the case of pDEST-GADT7 or the GAL4 binding domain (GAL4 BD) fused to the c-Myc tag for pDEST-GBKT7. The promoter from ADH1 (P_{ADH1}) and the ADH1 terminator (T_{ADH1}) were used to control the expression of the gene fusion in yeast, and the T7 promoter (P_{T7}) was used for in vitro expression. Nucleic acid sequence and predicted amino acid sequence of the junctions between the original yeast two-hybrid vectors and the gateway cassette are given below each vector.

The sequence of the *TERT* clone isolated from the library mapped to the 5′ part of the *TERT* (*At5g16850*) gene model. The inferred intron-exon structure of the library clone differs from that of the gene model, retaining the sixth and the eighth intron and terminating with a polyA tail part way through intron 8. We refer to this N-terminal variant as *TERT-V(I8)* (*TERT* variant in intron 8, accession number AM384991, supplementary material Fig. S1). Retention of intron 6 is predicted to introduce a stop codon 18 bp after the end of exon 6 (amino acid RILQWF-stop), resulting in a shorter version of TERT protein (supplementary material Fig. S1; from amino acids 1 to 323 plus six novel amino acids; 38.3 kDa, pI 9.69). This N-terminal peptide includes several conserved motifs (Xia et al., 2000), including the GQ motif (amino acids 115 and 233) and part of the linker (supplementary material Fig. S1).

TERT-V(I8) interacts with POT1A in vitro

To verify the yeast two-hybrid results, we investigated whether the two proteins could interact in vitro. For this we made use of the yeast two hybrid vectors that allow the in vitro expression of proteins fused to myc or HA tag using the T7 promoter (Fig. 1). The in-vitro-expressed proteins, therefore, do not contain the activation or binding domains of the GAL4 transcription system and allow the interaction to be assessed in the absence of possible interference by the GAL4 domains. HA-TERT-V(I8) and myc-POT1A were expressed separately in a coupled transcription/translation system based on the rabbit reticulocyte lysate system. We also used an ‘empty’ vector, pGBKT7, containing the myc tag as a control. The HA-TERT-V(I8) protein was radio-labelled with [^{35}S-methionine] (Fig. 3, lane 1), and the myc tag and myc-POT1A proteins (Fig. 3, lanes 2, 3) were detected using antibodies against the myc epitope. The proteins were mixed before immunoprecipitation with anti-myc bound to protein A-sepharose beads (Fig. 3, lanes 3, 4). Immunoprecipitation of TERT-V(I8) was found to be POT1A-dependent (Fig. 3, lane 6) because no TERT-V(I8) was detected in absence of myc-POT1A protein (Fig 3, lane 5). These results demonstrate the ability of the POT1A and TERT proteins to interact in vitro.

POT1A specifically interacts with the telomerase variant TERT-V(I8) or with the corresponding sequence of the protein, TERT$^{1\text{-}323}$

Because POT1A interacts with a variant of TERT, we checked whether POT1A could also interact with other parts of the protein. Selected deletion derivatives (Fig. 4A) were tested for interaction with POT1A using the yeast two-hybrid assay. TERT$^{1\text{-}323}$ (first six exons of the gene; amino acids 1-323)

Table 1. Putative interactors of *Arabidopsis* POT1A

Putative interactors of pBD-POT1A	Interactions with pAD-POT1A	Description
(1) At5g16850	Confirmed	TERT
(2) At5g57630	Confirmed	CIPK21
(3) At1g29040	Confirmed	Unknown, plant specific
(4) At5g67385	Not confirmed	Putative phototropism responsive protein
(5) At1g67930	Not tested	Similar to Golgi transport complex

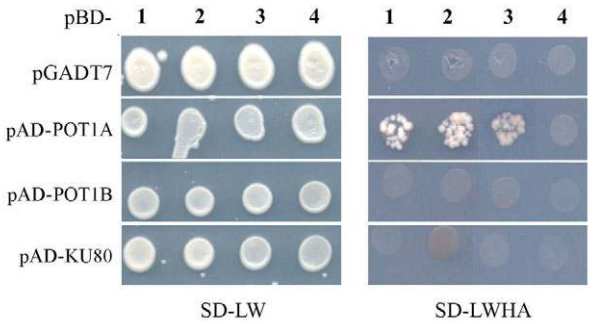

Fig. 2. Identification of POT1A-interacting proteins from the yeast two-hybrid screen. Growth tests of diploid yeast strains on SD-LW (non-selective) and SD-LWHA (selective) media. The activation domain (AD)-fusion protein genotypes of diploids are given on the left of the panel, whereas the binding domain (BD)-fusion protein genotypes (top) are as follows: 1, TERT; 2, CIPK21; 3, At1g29040; and 4, At5g67385.

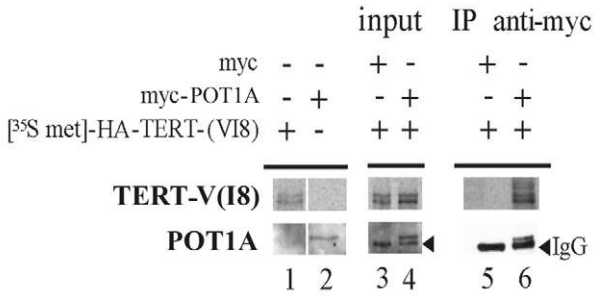

Fig. 3. POT1A interacts with TERT-V(I8) in vitro. The presence (+) or absence (–) of particular components is indicated above: myc, translation mix using control vector, pGBKT7 as template; myc-POT1A, translation mix using POT1A cloned in pDEST-GBKT7; and [^{35}S-met]-HA-TERT-V(I8), translation mix using TERT-V(I8) cloned in pDEST-GADT7. The upper panel [labelled AtTERT V(I8)] is an autoradiograph revealing the presence or absence of the TERT fusion protein. The lower panel (labelled POT1A) is a western blot revealing the presence or absence of the POT1A fusion protein. Heavy chain IgGs are indicated by arrowheads. Lane 1, radiolabelled [^{35}S-met]-TERT-V(I8) synthesis reaction; lane 2, POT1A synthesis reaction (myc-tagged); lanes 3 and 4, input: the mixed proteins after an overnight incubation with antibodies and protein A beads, correspond to 0.2% of the total amount of protein used for immunoprecipitation; lanes 5 and 6, IP anti-myc: the proteins remaining bound to the protein A beads after immunoprecipitation.

differs from TERT-V(I8) only in that it lacks the six amino acids encoded by the retained intron 6. TERT$^{1\text{-}413}$ is encoded by exon 1 to exon 8 (amino acids 1-413), and the C-terminal part of TERT, TERT$^{414\text{-}1123}$, is encoded by exon 9 to exon 12 (amino acids 414-1123). Only those cells containing POT1A in combination with both TERT-V(I8) or TERT$^{1\text{-}323}$ were able to grow on media depleted of histidine and adenine (Fig. 4B), indicating that the interaction was specific to the N-terminal domain. The level of activation of the *lacZ* reporter gene, as judged by a β-galactosidase assay, was on average 2.38-fold higher for TERT-V(I8) and 1.60-fold for TERT$^{1\text{-}323}$ than in the control. This suggests that the interaction with TERT$^{1\text{-}323}$ is 30% reduced compared with the TERT-V(I8) variant. A longer N-terminal derivative of TERT (Fig. 4B) was unable to interact with POT1, as judged both by growth characteristics and by *lacZ* activation. One reason for the apparent lack of interaction – failure to express the fusion protein – was eliminated for most of the deletion derivatives by western blotting (Fig. 4C). However, we failed to detect the C-terminal domain or the full-length TERT, so we cannot exclude the possibility that these proteins are not expressed in yeast.

The TERT gene is alternatively spliced

The truncation study above suggests that the N-terminal domain of TERT has higher binding affinity for POT1 than longer versions, and that this domain might be expressed as a result of alternative splice-site selection. Alternative splicing of TERT genes is a widespread phenomenon in animals (reviewed in Cong et al., 2002) and has also been reported in rice (Heller-Uszynska et al., 2002; Oguchi et al., 2004). To establish whether alternative splicing also occurs in the *Arabidopsis TERT* gene, we performed reverse transcriptase (RT)-PCR on mRNA extracted from rapidly dividing tissue (inflorescences) and slowly dividing tissue (rosette leaves) using primers specific for the beginning of the ORF and at different downstream locations (Fig. 5A). Consistent with multiple mRNA species in dividing tissues, RT-PCR with several different primer combinations produced amplification products of variable molecular weight in addition to the expected product (Fig. 5B). To determine the precise sites of alternative splicing, we cloned and sequenced RT-PCR products from each primer set (Fig. 5C). This indicated that alternative splicing sites are located in or after exon 5, generating stop codons close after the alternative splicing site (annotated as * in Fig. 5). Using primer sets 1 and 6, we found that two out of the three clones sequenced were identical to TERT-V(I8), whereas the third one corresponded to unspliced mRNA. Consensus intron donor and acceptor sites are conserved for both splicing variants at introns 5 and 7 (Table 2). Taken together with the yeast two-hybrid results, this suggests that TERT could be regulated by alternative splicing to produce a variant protein comprising an N-terminal domain with increased POT1-binding capabilities.

Nuclear localisation of TERT-V(I8)

If TERT and POT1 interact in vivo, then one prediction is that they should both be present in the same cellular compartment. In humans and in yeast, POT1 and telomerase are both located in the nucleus (Baumann et al., 2002; Bunch et al., 2005; Mattern et al., 2004; Wei and Price, 2004). It has recently been shown that human POT1 is also localised in the cytoplasm and that its localisation in the nucleus is controlled by the interacting protein TPP1 (Chen et al., 2007). Using the PSORT server, no particular motifs could be detected in the *Arabidopsis* POT1 proteins, but nuclear localisation signals were present in telomerase (certainty of 0.980) (see also Sykorova et al., 2006). This prediction would agree with the nuclear localisation observed for the human and yeast proteins (Etheridge et al., 2002; Teixeira et al., 2002). Localisation of GFP fusion proteins was assessed by transient expression in *Arabidopsis* Col-0 cells and by BY-2 stable transformation (Koroleva et al., 2005). GFP alone, histone H3-like

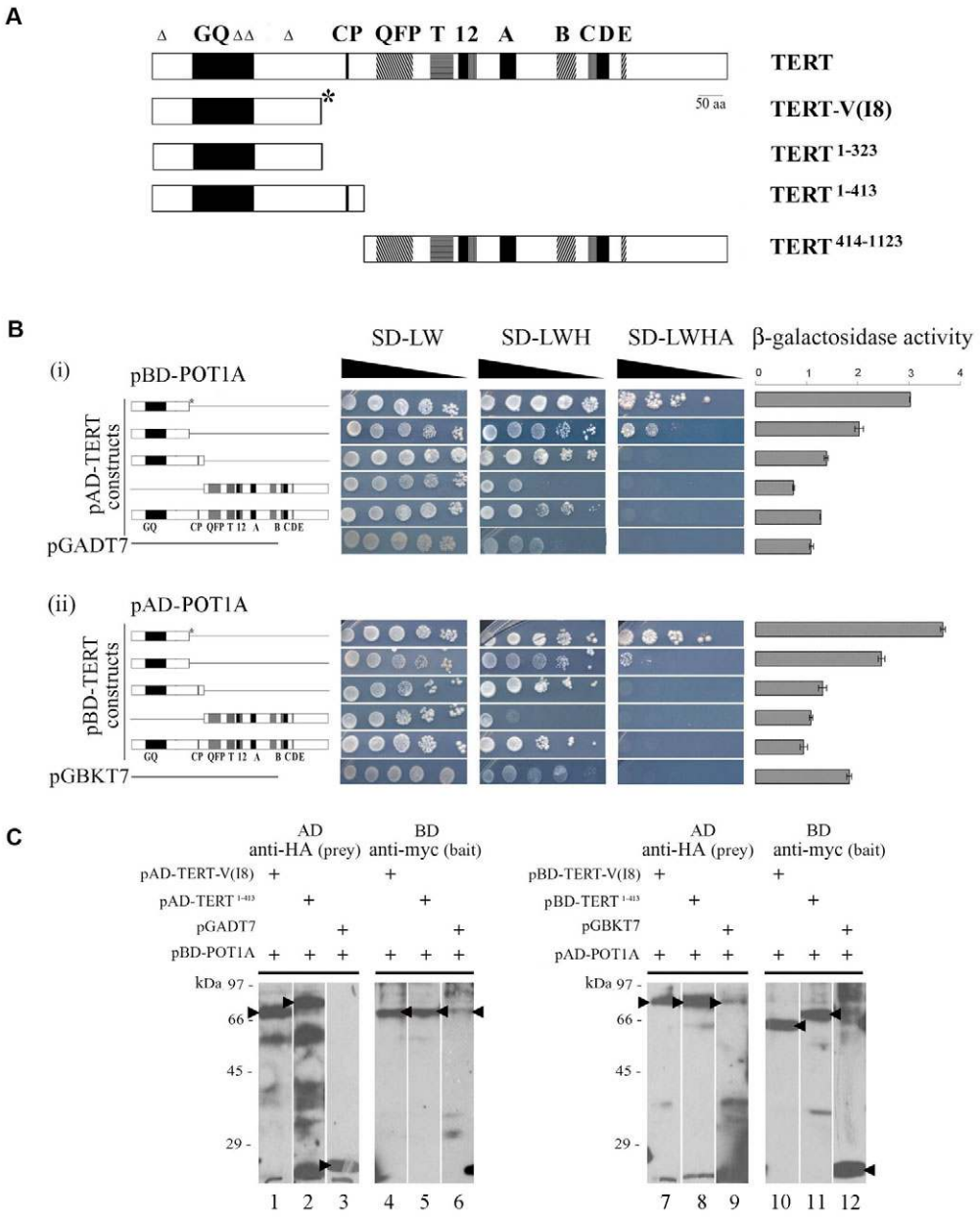

Fig. 4. Mapping of the interaction between POT1A and TERT.
(A) Structure of the *Arabidopsis* TERT protein, variants and truncations. Motifs in TERT (GQ, CP, QFP, T, 1, 2, A, B, C, D and E) were located according to Fitzgerald et al. (Fitzgerald et al., 1999) and Xia et al. (Xia et al., 2000). Putative nuclear localisation signals (NLS) are shown as triangles. The prediction of the peptide encoded by the *TERT-V(I8)* mRNA variant is shown and the stop codon is marked (*). Truncation constructs of TERT are shown below and correspond to amino acids 1-323 ($TERT^{1-323}$); 1-413 ($TERT^{1-413}$); and 414-1123 ($TERT^{414-1123}$).
(B) Quantification of the interaction between POT1A [as pBD fusion (i) or as pAD fusion (ii)] and various telomerase peptides [shown in the schematic from top to bottom: TERT-V(I8), $TERT^{1-323}$, $TERT^{1-413}$, $TERT^{414-1123}$, TERT, pGADT7 (control empty vector)]. The central panel shows serial dilutions of diploid cells on non-selective (SD-LW), low-selective (SD-LWH) and high-selective (SD-LWHA) media. The panel on the right-hand side shows the results of a β-galactosidase liquid culture assay, in Miller units, obtained from two separate experiments, each containing three replicates. Bars represent the standard errors.
(C) Western blot analysis of protein expression in diploid cells. The genotype of the cells is indicated above the blots. Lanes 1-3 and 7-9 were probed with anti-HA antibodies; lanes 4-6 and 10-12 were probed with anti-myc antibodies. Arrowheads indicate the positions of expected molecular masses for the fusions tested.

(At4g40030, H3-like) – a centromeric histone CENH3 [whose localisation was shown to correspond to centromeric DNA by immunofluorescence (Talbert et al., 2002)] – and KU80 [involved both at telomere regulation and DNA recombination (Tamura et al., 2002)] were used as controls.

GFP alone localised both in the cytoplasm and in the nucleus (Chytilova et al., 2000). H3-like::GFP localised to the nucleus and uniformly stained euchromatin during both interphase and mitosis (supplementary material Fig. S2). The heterochromatin marker CENH3::GFP specifically marked heterochromatin knobs and centromeric regions, as shown by counterstaining with DAPI (supplementary material Fig. S2). The putative telomeric protein, KU80, was observed both in the cytoplasm and in the nucleus, as reported previously (Tamura et al., 2002) (Fig. 6A), and was very similar to both POT1A and POT1B (Fig. 6A). To assess whether the POT1 proteins were tightly bound to any particular structure, cells were treated with detergent and the DNA was stained with DAPI (Fig. 6B). The GFP-tagged POT1 fusion proteins in the nucleus were resistant to detergent extraction, whereas the cytoplasmic-located GFP-POT1 proteins were not. GFP fusions with both TERT-V(I8) or $TERT^{1-413}$ were observed in the nucleoli and sometimes also in the nucleoplasm (Fig. 6C). These results, therefore, indicate that the two proteins locate in the same sub-cellular compartment and therefore have the potential to interact in vivo.

Discussion

In this paper we have identified a physical interaction between POT1A and the N-terminal domain of TERT. These proteins interact both in the yeast two-hybrid system and in vitro, and they co-localise to the same sub-cellular compartment – the

nucleoplasm. Therefore, they have the potential to interact in vivo. The N-terminus of TERT is conserved and mediates the recruitment of telomerase-associated proteins, the function of which is critical for the activity of TERT in vivo (reviewed in Collins, 2006). Our results suggest that this domain is also responsible for recruiting POT1A, providing a possible mechanism whereby their direct physical interaction might contribute to the regulation of telomerase function.

Different functions for POT1 proteins in *Arabidopsis*

We show that POT1A interacts with two other proteins, in addition to telomerase. One of the interactors has an unknown function and database searches failed to identify an orthologue in species other than plants or to define conserved domains. Another interactor, CIPK21, suggests that POT1A functions in an additional pathway. CIPK21 is a kinase belonging to a large family in *Arabidopsis* of which several members were shown to be involved in Ca^{2+} signalling. Besides a conserved kinase domain and a plant-specific NAF domain, CIPKs possess a third motif similar to the yeast DNA-damage signalling kinase Chk1p (CHEK1 in humans) (Ohta et al., 2003). Further experiments are needed to verify the function of CIPK21 in DNA-damage signalling. It is possible that POT1A is involved in this pathway, as indeed are many other telomeric proteins (reviewed in d'Adda di Fagagna et al., 2004; Gallego and White, 2005).

These interactions appear to be specific for only one member of the POT1 family. Our data suggests that there are at least three members of the POT1 family in *Arabidopsis*: two characterised members *POT1A* and *POT1B* (Kuchar and Fajkus, 2004; Shakirov et al., 2005; Tani and Murata, 2005), which encode full-length proteins, and a third member resulting from a partial duplication of the *POT1A* locus. Despite having a similar protein sequence (Shakirov et al., 2005) and sharing the presence of the N-terminal OB fold to bind the ssDNA, the two full-length proteins appear to have different roles in telomere biology: overexpression of truncations of the two proteins lead to distinct phenotypes, highlighting a role of POT1A in telomere length homeostasis and of POT1B in chromosome-end capping (Shakirov et al., 2005). Our results support this conclusion because POT1A-interacting proteins were unable to interact with POT1B. Specifically, TERT-V(I8) interacted with POT1A but not with POT1B. Interestingly, the two mouse POT1 genes, *Pot1a* and *Pot1b*, are not redundant and have different functions, with the Pot1a paralogue required to repress DNA damage at telomeres and Pot1b to regulate the amount of ssDNA at the 3′ terminus (Hockemeyer et al., 2006; Wu et al., 2006). Our study indicates that plant POT1 proteins also differ in the spectrum of proteins with which they interact. It will be interesting to define the interacting partners of POT1B.

POT1A binds to the N-terminal regulatory region of TERT

TERT proteins are well-conserved and contain several domains whose functions are being extensively investigated (reviewed Autexier and Lue, 2006). The RNA-binding domain (RBD; includes the conserved motifs CP, QFP and T) and the RT domains located in the middle of the protein are essential for enzyme activity both in vitro and in vivo. The distal N- and C-terminus domains are important for regulation and are also conserved. The N-terminal domain contains a conserved GQ motif and a flexible linker with nuclear localisation signals that are required for the enzyme

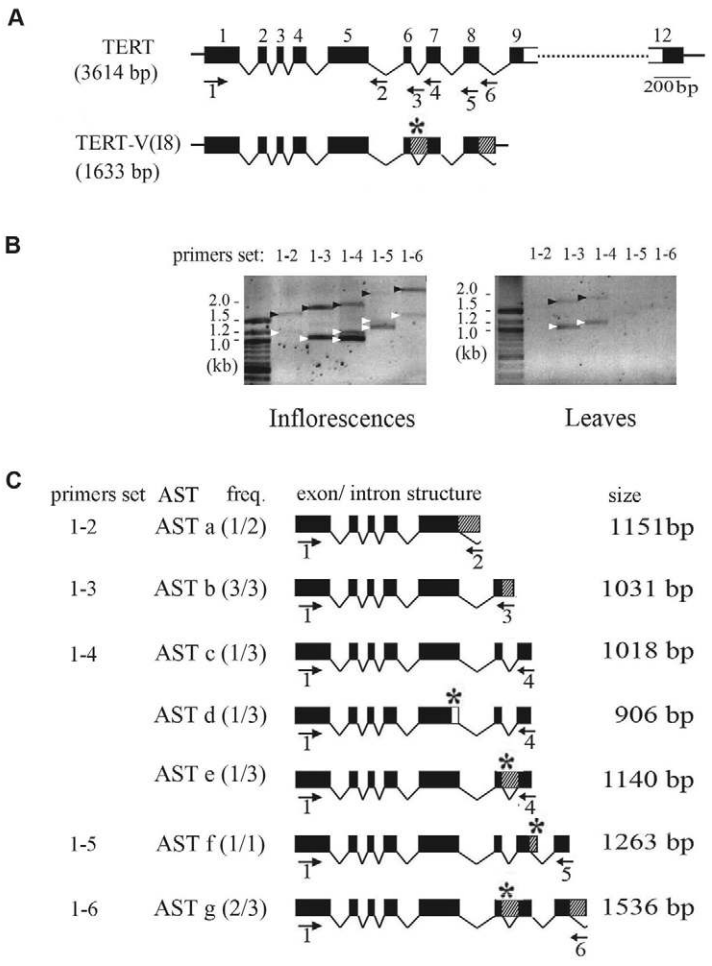

Fig. 5. Alternative splicing of the *Arabidopsis TERT* gene. (A) Schematic indicating the predicted intron/exons in the full-length *TERT* gene and those of the *TERT-V(I8)* variant. Size of the transcripts is given in bp. Exons are represented as black boxes and introns as striped boxes. Scale (bp) is given in the upper right corner. Numbered arrows show the location of the primers used in the RT-PCR experiments (see B). (B) RT-PCR analysis of the 5′ end of TERT transcripts from inflorescences and leaves using the sets of primers as shown below the *TERT* gene in A. Black arrowheads indicate amplification of unspliced transcripts, and white arrowheads indicate spliced and alternatively spliced variants. (C) Schematic representation of the alternative splicing transcript (AST) sequences obtained after RT-PCR using the different primers sets as shown for each AST (ASTa-ASTg). Ratios given in brackets represent the frequency of each variant after sequencing. For primer sets 1-2 and 1-6, the remaining clones sequenced corresponded to unspliced mRNA. Sizes of each variant are given in bp. Retained introns are represented as striped boxes and the partially deleted exon 5 is shown as a white box.

Table 2. Conservation of donor and acceptor splicing sites

Intron #	Donor site	Acceptor site
1	ACAGAGAGA**AG/gt**aagattct	agtttat**cag/**ATTGTTGAAA
2	ATATGATA**AG/gt**gaattctt	cctttga**cag/**TATGATCAGT
3	TCTCAAGCG**G/gt**ttgttctc	ttgtttat**ag/**GTCGGTCATG
4	AAGCATAAGC**/gt**gagtctca	catggtat**ag/**GGACATTGTC
5	TGTAAAGC**AG/gt**aatgttcg	ttttttgt**ag/GC**TAAACAGG
misspl. 5	AGTACAAAT**G/gt**aaagtctc	
6	CCACCAAACC**/gt**atccttca	tgatata**cag/**ACATTTTAAA
7	CTATTTGCTT**/gt**aagtcatc	tgatttg**cag/**ATACCATTCA
misspl. 7	ATTTATTCTT**/gt**aagttctt	
8	GACTTCTC**AG/gt**tgatattt	tgtatta**cag/**AGTTCAAGGA

Exon sequences are given in capitals and introns in lower case. Consensus sequences at donor and acceptor sites are shown in bold. Alternative donor splicing sites (misspl.) at intron 5 and intron 7 corresponding to alternative splicing variants d and f (see Fig. 5) are shown.

localisation. In addition, this domain can bind the telomerase RNA subunit, TR, because mutations led to a small reduction in TR association (Friedman and Cech, 1999; Moriarty et al., 2002), and can also bind to telomeric DNA (Xia et al., 2000). Mutagenesis of the GQ domain of yeast and human TERT abolished telomerase function in vivo, despite the enzyme still being catalytically active in vitro. The regulation of the enzyme activity in vivo is mediated via recruitment of telomerase-associated proteins (reviewed in Autexier and Lue, 2006). Our results suggest that the N-terminal domain also plays a role in binding POT1 and highlights two possible mechanisms by which TERT could bind to POT1.

The interaction might be specific to the N-terminal region of TERT that corresponds to the GQ domain and part of the linker, because increasing the size of the N-terminal region decreased the ability to interact. This suggests that the POT1-binding surface of the N-terminal domain could be inaccessible in the complete protein and is only revealed in truncated splice variants. We speculate that this interaction could provide a novel regulatory mechanism for controlling plant telomerase activity or its access to the telomere and, given the widespread occurrence of splice variants in TERT genes from other species, might be a general regulatory mechanism. Alternatively, but not mutually exclusive with the first model, the POT1-binding surface could be differentially accessible, depending on post-translation modification of the TERT protein (Fig. 7A)

The first model predicts that alternative splicing actively regulates TERT activity (or access to telomeres) by producing a biologically active N-terminal fragment that interacts with other telomere proteins or telomerase RNA, and thereby modifies their activity. Inactive and dominant-negative versions of mammalian TERT have previously been reported to arise as a result of alternative splicing (Saeboe-Larssen et al., 2006; Ulaner et al., 1998; Weinrich et al., 1997; Wick et al., 1999). Our data indicates that a similar process also occurs in plants and can produce similar 'inactive' versions with dramatically altered binding partners. Therefore, a possible role for the TERT-V(I8) splicing variant could be to displace POT1A from the telomeres, allowing the full-length functional telomerase to bind and to synthesize telomeric repeats (Fig. 7A).

The second model predicts that POT1A interacts in vivo with the full-length telomerase but that the domain mediating

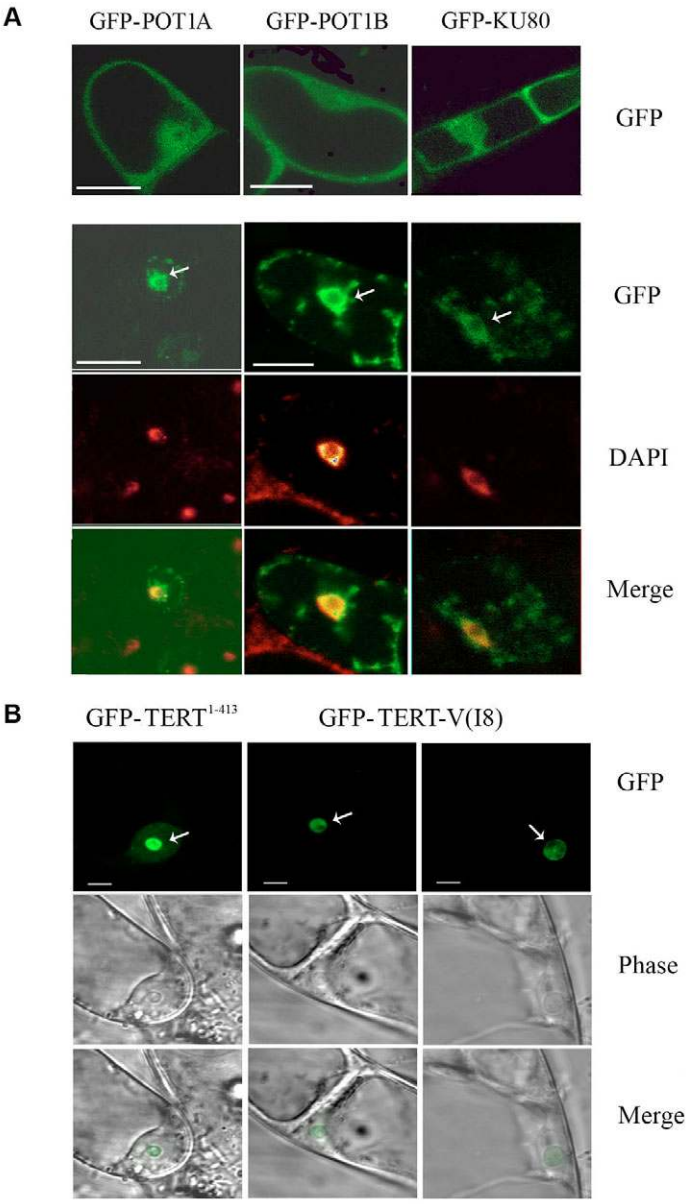

Fig. 6. Localisation of the *Arabidopsis* POT1 proteins. (A) POT1A, POT1B and KU80 localise both to the cytoplasm and to the nucleus of *Arabidopsis* Col-0 cells. Top panel: localisation of telomeric POT1A, POT1B and KU80 fused to GFP in living cells. Bottom panel: *Arabidopsis* Col-0 cells transiently transformed with POT1A, POT1B and KU80 fused to GFP were extracted with detergent. Localisation of the proteins is shown in the GFP panel. Arrows indicate nuclei. The chromatin was stained with DAPI (DAPI panel) and co-localisation of the signals obtained with GFP and DAPI are shown in the merge panel. (B) Nuclear localisation of TERT-V(I8) and of a TERT truncation fused to GFP in *Arabidopsis Col-0* cells. The two GFP fusions label the nucleoli (arrows) and the entire nuclei in some cells. Bars, 4 μm.

the interaction is normally hidden within the protein structure. Supporting this hypothesis, the TEN domain, which corresponds to the first 200 amino acids of *Tetrahymena* TERT, is soluble and resistant to protein degradation, in contrast to longer parts of the protein (Jacobs et al., 2005; Xia et al., 2000).

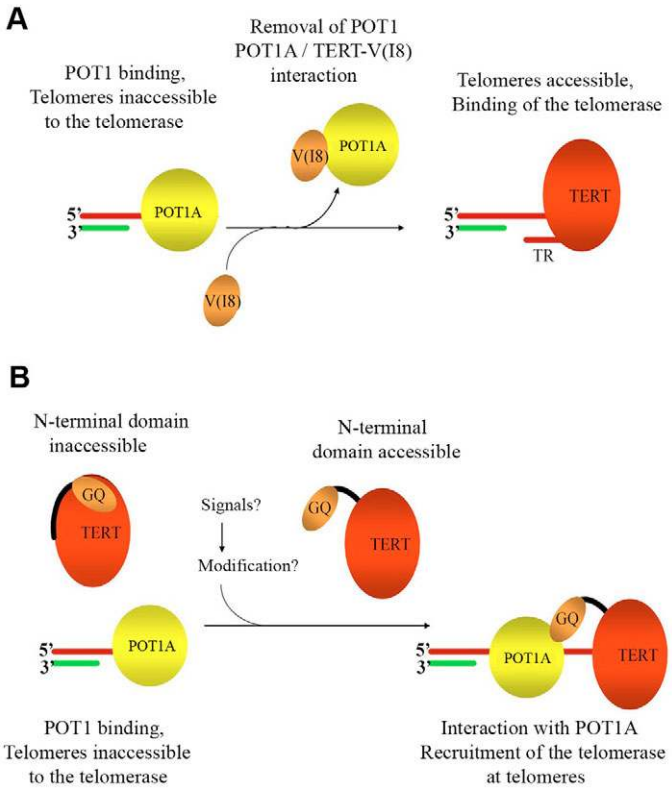

Fig. 7. Conceptual models for POT1-TERT interaction in telomere synthesis. (A) Alternatively spliced TERT, TERT-V(I8) [V(I8)], acts to displace POT1A bound to telomeres, and this protein interaction allows TERT to be recruited to telomeres, where it can synthesise DNA repeats. (B) The GQ domain of TERT is hidden within the protein structure and is inaccessible to POT1A interaction. Following signals or modifications that trigger conformational changes of the TERT protein, the GQ domain of TERT become accessible to POT1A and the interaction mediates the recruitment of TERT to telomeres.

Moreover, recent structural studies have shown that the N-terminal domain is folded separately from the rest of the protein (Jacobs et al., 2005). Post-translational modifications of TERT in vivo could release the N-terminal domain necessary for binding POT1A (Fig. 7B) and this might serve as a recruitment mechanism to tether telomerase to its DNA substrate. Such an interaction would resemble the situation in budding yeast, in which the telomerase-associated protein Est1p bridges the telomerase complex ($Est2p^{TERT}$-$TLC1^{TR}$) to Cdc13p, the budding yeast equivalent of POT1 (Evans and Lundblad, 1999; Qi and Zakian, 2000; Zhou et al., 2000). EST1 was also identified, by sequence similarity, in humans (Reichenbach et al., 2003; Snow et al., 2003), in fission yeast (Beernink et al., 2003) and in plants (Snow et al., 2003), but EST1 does not interact with either TERT or POT1A, as assessed by the yeast two-hybrid assay (R.R. and J.H.D., unpublished results). It is possible, however, that the interaction with the telomerase requires its RNA component, as is the case for the yeast Est1p (Seto et al., 2002; Zhou et al., 2000). Alternatively, the interaction between POT1A and the N-terminal domain of the TERT could bypass the requirement for Est1p. Recent studies in humans have shown that the human POT1-interacting protein TPP1 is orthologous to the β subunit of TEBP and is able to recruit telomerase, thus modulating telomerase activity (Xin et al., 2007; Wang et al., 2007). However, orthologues of TPP1 have not been described in *Arabidopsis* and therefore different mechanisms might have evolved to cope with this.

Regulation of telomerase activity by associated proteins is an important mechanism by which telomere length is maintained in somatic and also in cancerous cells. In vitro studies indicate that POT1 can regulate telomerase activity (Kelleher et al., 2005; Lei et al., 2005), but the mechanism was unclear. Our results suggest that this could involve a direct physical interaction between POT1 and the N-terminal regulatory domain of TERT.

Materials and Methods

Reverse transcriptase-PCR and cloning

PolyA RNA was isolated from *Arabidopsis thaliana* flower buds or rosette leaves using the Dynabeads mRNA kit (Dynal, Bromborough, UK). Typically, one inflorescence bud or one rosette leaf was ground in 100 μl of lysis buffer (Dynal), centrifuged for 1 minute at 16,000 ***g*** and 80 μl was used to bind to 20 μl of Dynabeads. The beads were then used to perform the reverse transcription (Omniscript Quiagen, Valencia, C.A.). 4 μl of the RT-mix was used as template for a PCR reaction (KOD Hot Start DNA polymerase, Novagen) in a volume of 50 μl and 10 pmole of specific primers.

For gene amplification, PCR primers were designed based on the predicted gene models available at TAIR (*POT1A*, *At2g05210*; *POT1B*, *At5g06310*). A list of primers and of their sequences is given in Table 3. Gateway Technology (Invitrogen) was used to generate entry clones using a two-step PCR procedure. The first set of primers, corresponding to half of the recombination site *attB1* or *attB2* and to the gene, are Pot1A-forward and Pot1A-reverse (amplifying a fragment of 1404 bp); a second PCR reaction added the full-length recombination sites, following Invitrogen's instructions. The PCR products were cleaned through PCR column purification (QIAQuick, PCR purification, Quiagen) and recombined into pDON207 by BP reaction. Plasmids were analysed by sequencing (Chromas, version 1.45, http://www.mb.mahidol.ac.th/pub/chromas/chromas.htm). Subsequent LR reactions were generated as recommended by Invitrogen.

Table 3. Primers used

Primer names	Sequence 5′ to 3′
POT1A-forward	AAAAAGCAGGCTTAATGGCGAAGAAGGAGAGTCC
POT1A-reverse	AGAAAGCTGGGTGATGAAGTAGTCTAGTACCAAAGATTCTG
TERT-forward	AAAAAGCAGGCTTCATGCCGCGTAAACCTAGACATCG
TERT-reverse	AGAAAGCTGGGTCATAATTCAACTTCCACAGCGAAGAAGAG
TERT V(I8)-reverse	AGAAAGCTGGGTCCC AAAGTCTATAAAGTAGCAACTGTG
$TERT^{1-323}$-reverse	AGAAAGCTGGGTCTGTGGTTTGGTGG TATAACTG
$TERT^{1-413}$-reverse	AGAAAGCTGGGTCCTGAGAAGTCGTTCCGGACTTCAATGC
$TERT^{414-1123}$-forward	AAAAAGCAGGCTCCATGAGTTCAAGGAAGGCAAAAAGC
TERT-intron5-reverse	CGAATTTGAAGGTAATGAAGTGTCAGGAA
TERT-intron6-reverse	CCATCAAGCTATCGTTGCTACAAGTC
TERT-exon7-reverse	CAGAGCAGTTGGGCCTCAAGG

The telomerase cDNA was amplified from the clone AF135454, a gift of Katsunori Tamura and Hideo Takahashi from the University of Tokyo (Oguchi et al., 1999) using the Gateway-compatible primers TERT-forward and TERT-reverse (Table 3). *TERT-V(I8)* was sub-cloned from pGADT7-rec using TERT-forward and TERT-V(I8)-reverse. Primers were designed to generate combinations of different truncations using two-step PCR and subsequent cloning was performed as described above using the Gateway Technology. For the N-terminal region, $TERT^{1\text{-}323}$ was generated using the primers TERT-forward and $TERT^{1\text{-}323}$-reverse; $TERT^{1\text{-}413}$ using primers TERT-forward and $TERT^{1\text{-}413}$-reverse; and the C-terminal part of the gene, $TERT^{414\text{-}1123}$, using primers $TERT^{414\text{-}1123}$-forward and TERT-reverse (Table 3).

For RT-PCR experiments to detect alternative splicing at the 5′ of the telomerase gene, reverse transcription was performed as above and 35 cycles of PCR amplification were performed using the primers shown in Fig. 5: Primer 1: TERT-forward; 2: TERT-intron5-reverse; 3: TERT-intron6-reverse; 4: TERT-exon7-reverse; 5: $TERT^{1\text{-}413}$-reverse; and 6: TERT-V(I8)-reverse. PCR products obtained from inflorescence tissue were cloned in pGEMT-easy and the corresponding sequence obtained after sequencing using universal M13F/R primers.

Yeast two-hybrid plasmids, screening and interaction testing

The two-hybrid vectors pGADT7 and pGBKT7, based on the GAL 4 system (Matchmaker III, Clontech Laboratories), were converted to the Gateway Technology by inserting the Gateway frame B cassette at the *Sma*I site of both vectors. The orientation and reading frame of the insert were confirmed by sequencing. The converted plasmids were called pDEST-GADT7 and pDEST-GBKT7 and are available from the ABRC DNA stock centre. Entry clones (containing full length or truncations) were used in recombination reactions to transfer genes to the pDEST-GADT7 and pDEST-GBKT7 vectors. The products, referred to as pBD-X or pAD-X, were transformed into *S. cerevisiae* AH109 or Y187 strains (Matchmaker III, Clontech Laboratories). Expression of fusion proteins in yeast were confirmed by western blotting of whole-cell extracts using antibodies against the haemagglutinin antigen HA (HA.11 antibody CRPinc, Berkerley, CA) and c-myc (Clontech) epitope tags according to the manufacturer's instructions. A gene library in pGADT7-rec vector, made using mRNA isolated from inflorescence tissues of *A. thaliana*, was a gift of Hans Sommers' laboratory (Max Plank Institute for Plant Breeding Research, Köln, Germany). Screening and interaction studies were carried out by mating compatible strains of yeast, following Clontech's instructions. Selection on media depleted of leucine, tryptophan, histidine and adenine, and liquid were performed following the manufacturer's recommendations.

In vitro protein expression and co-immunoprecipitation

POTA and TERT-V(I8) were expressed in the in vitro TNT T7-coupled transcription/translation Reticulocyte Lysate System (Promega) following the manufacturer's recommendation. *TERT-V(I8)* in pDEST-GADT7 was used as a template for the in vitro transcription/translation reaction and was radio-labelled using [^{35}S]-methionine. *POT1A* fused to a myc tag in pDEST-GBKT7 was expressed using non-radioactive methionine and, as a control, we used an 'empty' vector, pGBKT7, that should produce a short polypeptide containing the myc tag. Half of the total reaction of [^{35}S met]-TERT-V(I8) (50 μl) was mixed with either the myc tag alone or POT1A-myc, 50 μl of protein A sepharose and 10 μl of anti-myc monoclonal antibody (clone 9E10, AbCam) in 500 μl of binding buffer [100 mM HEPES pH7.5, 50 mM KCl, 5% (v/v) glycerol, 15 mM EGTA, 1 mM DTT, 0.1% Triton-X100, 1 tablet mini complete EDTA free protease inhibitor (Roche)] and incubated overnight at 4°C. After four washes of 5 minutes each in binding buffer, the sepharose resin was re-suspended in 15 μl of 1× loading buffer and proteins denatured for 3 minutes at 80°C. The resin was spun for 30 seconds and the supernatant separated on a 10% polyacrylamide SDS gel. After transfer, the membrane was exposed to storage phosphor-screen imager (Molecular Dynamics) and the radio-labelled TERT-V(I8) detected using a Typhoon 9200 scanner (Amersham Biosciences). Western blots to detect proteins were performed using antibody against the epitope monoclonal anti-myc 9E10. The secondary antibody was against IgG of mouse and coupled to horseradish peroxidase (DAKO).

Localisation

Entry clones were used as templates for LR reactions to sub-clone the corresponding genes in fusion with the GFP (pBin-GFP N or C, a gift from Ben Trevaskis, MPI, Germany). *Agrobacterium* cells and *Arabidopsis* cell suspension cells were transformed as described previously (Koroleva et al., 2005). Tobacco BY-2 cells stably expressing the GFP fusion were generated as previously described (Koroleva et al., 2004). Cells were permeabilised with detergent (Triton X-100 0.01%) and DNA stained with 1 μg/ml DAPI (4′-6-diamidino-2-phenylindole, Sigma). Analysis of the transformants was carried out with a 63× oil immersion objective on a confocal laser-scanning microscope (Leica SP and SP2; Leica microsystems, Wetzlar, Germany). GFP was excited at 488 nm from an Argon Ion laser and the emitted light was detected between 500 and 550 nm. To image DAPI images, sequential scans were taken between GFP and DAPI, with DAPI excited by 351-363 nm light from an Argon Ion (UV) laser and the emitted light detected between 420 and 470 nm. All images acquired were processed with Adobe Photoshop (7.0) and ImageJ programmes. ImageJ is a public-domain image-processing programme written by Wayne Rasband and is available at http://rsb.info.nih.gov/ij/. Overlay images of GFP and DAPI were created using Adobe Photoshop.

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