

Tobacco GTBP1, a Homolog of Human Heterogeneous Nuclear Ribonucleoprotein, Protects Telomeres from Aberrant Homologous Recombination

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Telomeres are nucleoprotein complexes essential for the integrity of eukaryotic chromosomes. Cellular roles of single-stranded telomeric DNA binding proteins have been extensively described in yeast and animals, but our knowledge about plant single-strand telomeric factors is rudimentary. Here, we investigated *Nicotiana tabacum* G-strand-specific single-stranded telomere binding proteins (GTBPs), homologs of a human heterogeneous nuclear ribonucleoprotein. GTBPs bound specifically to the plant single-stranded (TTAGGG)₄ telomeric repeat element in vitro and were associated with telomeric sequences in tobacco BY-2 suspension cells. Transgenic plants (*35S:RNAi-GTBP1*), in which *GTBP1* was suppressed, exhibited severe developmental anomalies. In addition, the chromosomes of *35S:RNAi-GTBP1* cells displayed elongated telomeres, frequent formation of extrachromosomal telomeric circles, and numerous abnormal anaphase bridges, indicating that *GTBP1* knockdown tobacco plants experienced genome instability. *GTBP1* inhibited strand invasion, an initial step in interchromosomal homologous recombination. We propose that *GTBP1* plays a critical role in telomere structure and function by preventing aberrant interchromosomal telomeric homologous recombination in tobacco.

INTRODUCTION

Telomeres are unique nucleoprotein structures that protect the extreme termini of linear eukaryotic chromosomes. They are composed of tandemly repeated G-rich DNA sequence elements (TTAGGG in vertebrates and TTTAGGG in higher plants) along with nonhistone telomere binding proteins (Blackburn, 1991; Collins, 2000; Shore, 2001). Telomere binding proteins play an essential role in telomere architecture. Therefore, without properly functioning telomere binding proteins, telomeres are destabilized and cells undergo senescence, apoptosis, or the ageing process (Blackburn, 2001; Blasco, 2005). Telomere binding proteins are classified into two groups based on their binding modes. Human TRF1/PIN2 and TRF2 and yeast Rap1 and Taz1 are double-stranded telomere binding proteins (Chong et al., 1995; Billaud et al., 1997; Smogorzewska and de Lange, 2004), whereas yeast Cdc13p and human POT1 are single-stranded specific telomeric binding factors (Nugent et al., 1996; Baumann and Cech, 2001). Cdc13p and POT1 are typified by their association with telomeric DNA through an oligonucleotide-oligosaccharide binding fold (OB-fold). In humans, POT1, TRF1, and TRF2, together with TIN2, TPP1, and Rap1, form a telomere-protein complex shelterin (de Lange, 2005). In addition, heterogeneous nuclear ribonucleoproteins (HnRNPs) A1 and D can bind


the single-stranded telomere sequences (Ishikawa et al., 1993; LaBranche et al., 1998). HnRNP A1 is a positive telomere length regulator, as *HnRNP1 A1*-deficient mouse cells harbor shorter telomeres than do normal cells, and complementation of *HnRNP1 A1* expression in mutant cells restores normal telomere length (LaBranche et al., 1998).


Although single-strand-specific telomere binding factors are largely unstudied in higher plants relative to those in humans and yeast, identification and some cellular aspects of these proteins have recently been elucidated. POT1a and POT1b were identified in *Arabidopsis thaliana* by their sequence homology with *Schizosaccharomyces pombe* POT1 (Shakirov et al., 2005). Transgenic *Arabidopsis* plants, which overexpressed the truncated N-terminal region of *POT1b*, exhibited extensive erosion of telomeres, nonspecific chromosome fusions, and severe morphological defects (Shakirov et al., 2005). This indicated that POT1b contributes to telomere stability by capping the ends of chromosomes. POT1a interacted with the active telomerase complex, and a knockout mutation of *POT1a* caused a massive decrease in telomerase activity and gradual shortening of telomeres over generations, suggesting its positive role in telomere length homeostasis (Surovtseva et al., 2007). However, unlike yeast and vertebrate POT1 proteins, recombinant At POT1 proteins have no detectable single-strand telomere binding activity in vitro (Shakirov et al., 2009). In addition, nuclear extracts prepared from *pot1a* and *pot1b* T-DNA insertion mutants displayed no changes in single-strand-specific telomere binding activity. These results raise the possibility that POT1 proteins are not major single-stranded telomeric binding proteins in *Arabidopsis* (Shakirov et al., 2009).

In addition to POT1-like proteins, several putative single-stranded telomeric binding proteins were identified in higher

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plants. From tobacco (*Nicotiana tabacum*) bright yellow-2 (BY-2) suspension-cultured cells, Hirata et al. (2004) detected a DNA-protein complex in gel retardation assays using a ^{32}P -(TTTAGGG)₄ repeat single-stranded probe. The binding protein was purified, and its partial amino acid sequence was determined. RT-PCR with degenerate primers resulted in the isolation of a tobacco cDNA encoding a 36-kD protein (G-strand-specific single-stranded telomere binding protein 1 [GTBP1]) containing two RNA recognition motifs (RRMs) (Hirata et al., 2004). With the aid of affinity chromatography followed by matrix-assisted laser-desorption ionization time of flight mass spectrometry analysis, two single-strand telomere binding proteins (STEP1 and WHY1) were isolated from *Arabidopsis* (Kwon and Chung, 2004; Yoo et al., 2007). The binding of STEP1 to telomeric DNA inhibited telomerase-mediated telomere elongation in vitro (Kwon and Chung, 2004). Although a T-DNA insertional mutation of *WHY1* did not result in detectable abnormal phenotypes, *why1* mutant plants contained longer telomeres, whereas *WHY1*-overexpressing plants showed shortened telomeres and decreased telomerase activity. Thus, both STEP1 and WHY1 might be involved in the regulation of telomere length in *Arabidopsis*. However, physiological roles of these putative tobacco and *Arabidopsis* telomeric proteins remain to be elucidated.

In this study, we isolated two additional *GTBP* paralogs (*GTBP2* and *GTBP3*) from tobacco. In vitro gel retardation assays indicated that the three *GTBP* paralogs interacted specifically with the single-stranded TTTAGGG element. RT-PCR with gene-specific primers revealed that the mRNA level of *GTBP1* was much higher than those of *GTBP2* and *GTBP3*. Chromatin immunoprecipitation (ChIP) analysis showed that *GTBP1* was associated with telomeric sequences in BY-2 cells. We investigated a possible physiological role of *GTBP1* via RNA interference (RNAi)-mediated gene knockdown. The T0 and T1 35S:RNAi-*GTBP1* transgenic tobacco plants showed severe developmental abnormalities. In addition, chromosomes of the transgenic cells displayed longer telomeres, frequent formation of extrachromosomal telomeric circles (t-circles), and one or more abnormal anaphase bridges, indicating that *GTBP1* knockdown plants experienced genome instability. *GTBP1* inhibited strand invasion, an initial step for interchromosomal recombination. Based on these results, we propose that *GTBPs* play important roles in telomere structure and function in tobacco plants.

RESULTS

Isolation and Characterization of Three *GTBP* Paralogs in Tobacco

The HnRNP homolog *GTBP1* was previously identified in tobacco BY-2 cells (Hirata et al., 2004) and encodes a 36-kD protein with two RRM motifs. With total RNA from BY-2 cells, we performed RT-PCR using degenerate oligonucleotides designed from the conserved region of the RRM motifs (Figure 1A; see Supplemental Table 1 online). The PCR products of ~300 bp encoded three homologous partial *GTBPs*, one of which corresponded to *GTBP1*. Thus, the products were referred to as *GTBP1*, *GTBP2*, and

GTBP3. Total recombinant λ DNA was prepared from the λ -uni-Zap II tobacco flower cDNA library. The complete coding regions of *GTBP2* and *GTBP3* were obtained through 5'- and 3'-rapid amplification of cDNA ends (RACE) using the λ DNA as a template with primers corresponding to the 5'- and 3'-ends of the library vector sequence and the internal regions of the partial *GTBP* cDNA clones (Figure 1A). The coding regions of *GTBP1* (Hirata et al., 2004), *GTBP2*, and *GTBP3* encode 346 (36.2 kD), 337 (35.8 kD), and 339 (36.0 kD) amino acid proteins, respectively. Each *GTBP* contains two RRM motifs in the N-terminal region similar to human HnRNP A1. *GTBP2* and *GTBP3* are 98% identical to each other, and 87 and 88% identical to *GTBP1*, respectively (Figure 1B; see Supplemental Figure 1 online). Thus, *GTBP2* and *GTBP3* are more closely related to each other than either is to *GTBP1*. The three *GTBPs* are 58 to 63% identical to the homologous dicot *Arabidopsis* (At3g13224) and monocot rice (*Oryza sativa*; Os08g0492100) proteins, whose cellular functions are unknown (Figure 1B). The Nt *GTBP* paralogs are relatively less identical (30 to 32%) to human hnRNP A1 and hnRNP D.

Genomic DNA was isolated from mature tobacco leaves, digested by *EcoRV* and *HindIII*, and hybridized with ^{32}P -labeled *GTBP1*, *GTBP2*, or *GTBP3* probes. These hybridizations detected two to four bands with each enzyme digestion (see Supplemental Figure 2 online). To examine the expression of each *GTBP* mRNA, RT-PCR was conducted with gene-specific primers (Figure 1A; see Supplemental Figure 3 online). In BY-2 suspension-cultured cells, all three *GTBP* genes were ubiquitously expressed during a 9-d culture period (Figure 1C). The *GTBP* genes were highly expressed in mature tobacco stems and roots, while their expression gradually decreased as leaf tissues matured. Notably, the level of *GTBP1* mRNA was markedly higher than those of *GTBP2* and *GTBP3* regardless of the different cell and tissue types, indicating that *GTBP1* was the predominantly expressed paralog in tobacco (Figure 1C). Taken together, the three *GTBP* paralogs do not represent a cloning artifact, but rather are encoded by a gene family.

Nt *GTBPs* Bind Plant Single-Stranded Telomeric Repeats with Sequence Specificity

GTBP1, *GTBP2*, and *GTBP3* were expressed in *Escherichia coli* as fusion proteins with maltose binding protein (MBP). The purified proteins were incubated with a ^{32}P -labeled single-stranded (TTTAGGG)₄ repeat in the presence or absence of various competitors and analyzed by gel retardation assays. The *GTBP* proteins produced a single DNA-protein complex. Increasing amounts of the proteins (0.1 to 0.9 μg) generated more intense shifted bands (Figure 2A). The binding affinities of all three *GTBPs* were largely disrupted when a 50-fold excess of unlabeled plant telomeric element (PT4) was included in the reaction mixtures. By contrast, nonspecific DNA or telomeric repeats from other species, including human (HT4) and *Caenorhabditis elegans* (CT4), did not exert any inhibitory effects on the interaction of Nt *GTBPs* with PT4 (Figure 2B). *GTBPs* did not bind double-stranded telomeric elements at detectable levels. Thus, *GTBPs* bound specifically to single-stranded plant telomeric repeats in vitro.

Figure 2C also shows that all three *GTBPs* were able to form shifted bands only with the three-repeat or longer TTTAGGG

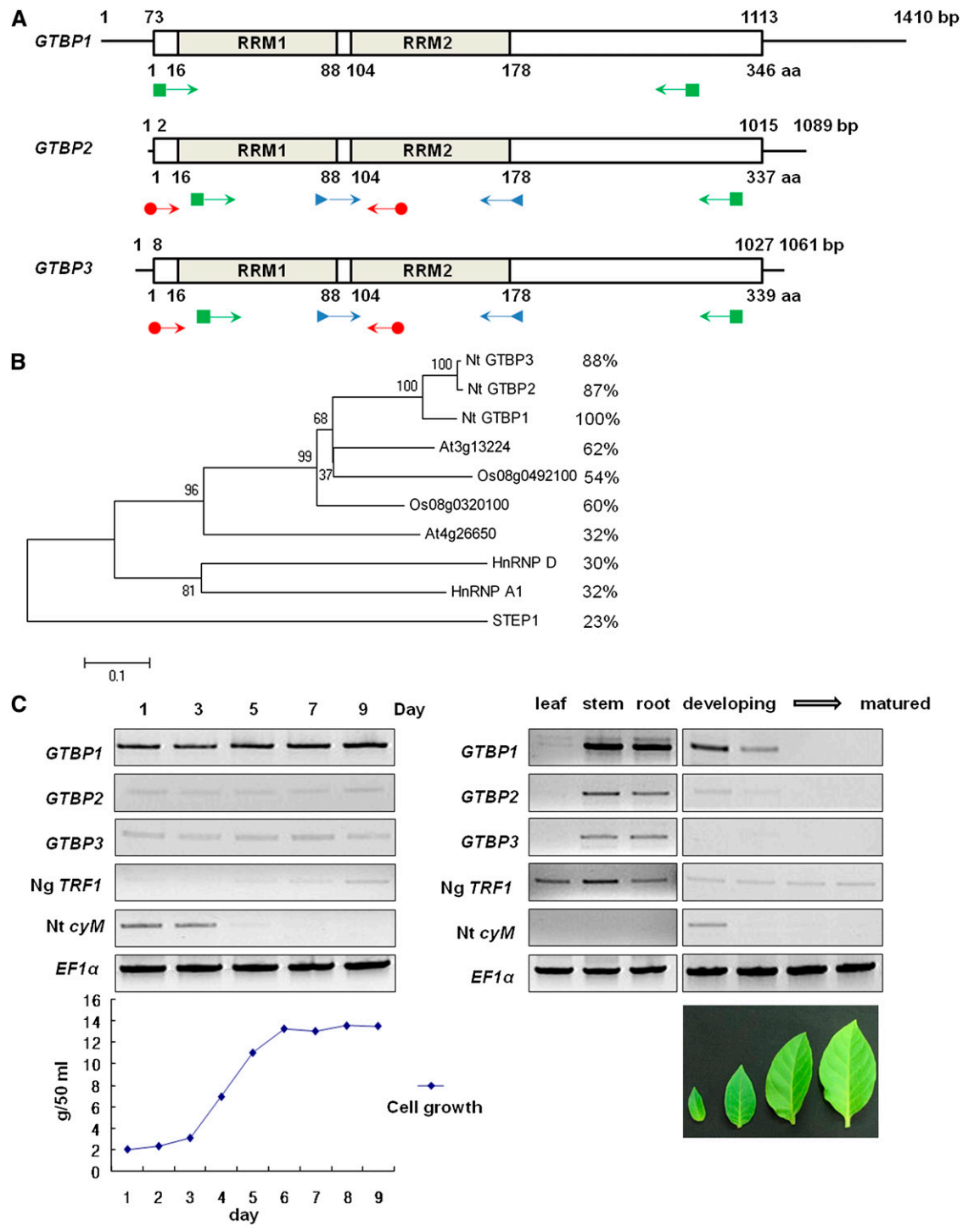


Figure 1. Structure and Expression of Nt *GTBPs*.

(A) Schematic representation of three tobacco *GTBP* cDNAs and their predicted proteins. The solid lines show 5'- and 3'-untranslated regions. The bars depict coding regions. The RRM (RRM1 and RRM2) are indicated. PCR primers for partial cDNA cloning, 5'- and 3'-RACE, and RT-PCR are indicated by arrows with triangles, circles, and squares, respectively.

(B) Phylogenetic relationship of 10 *GTBP* homologs from tobacco, *Arabidopsis*, rice, and human. The tree was constructed using MEGA4 software with the neighbor-joining method and the summed branch length of the tree is 3.21911554. Amino acid identities of these proteins to *GTBP1* are shown in right side. A text file of the alignment used to generate this tree is available as Supplemental Data Set 1 online.

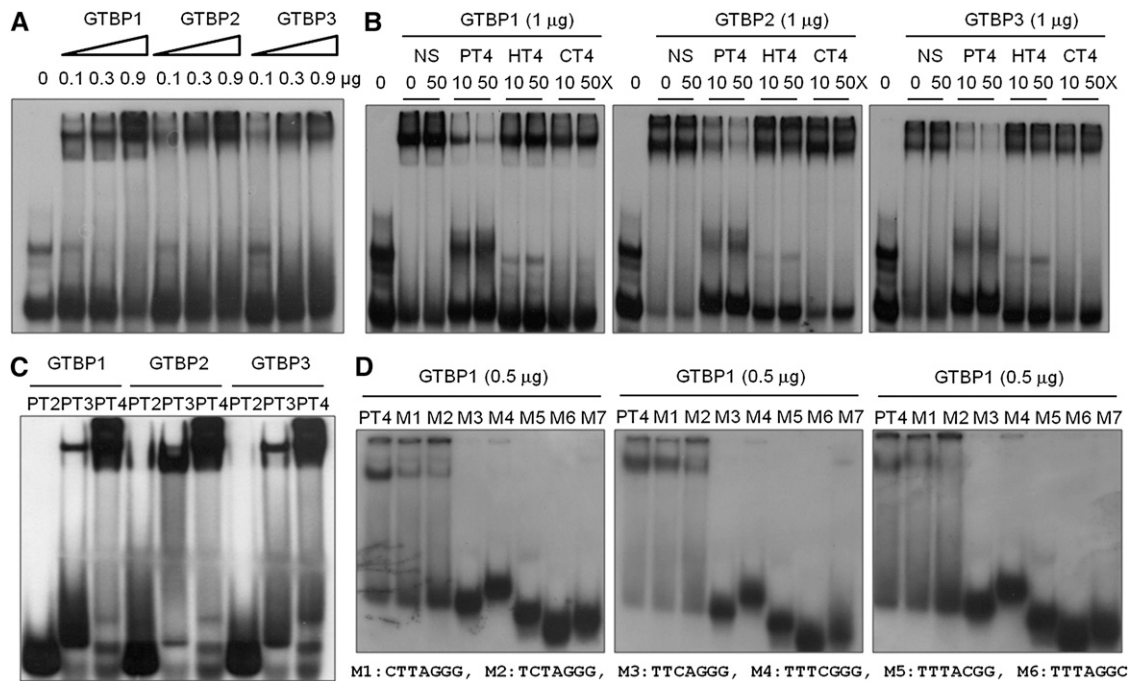


Figure 2. Nt GTBPs Bind Plant Single-Stranded Telomeric Repeats with Sequence Specificity.

- (A)** GTBPs bind plant single-stranded telomeric sequences. Various quantities (0, 0.1, 0.3, and 0.9 μg) of three Nt GTBP paralogs were incubated with radiolabeled single-stranded (TTTAGGG)₄ (PT4) repeats and analyzed in a gel retardation assay.
- (B)** Sequence-specific binding activity of GTBPs. Nt GTBPs (1 μg) were incubated with radiolabeled PT4 in the presence of a 10- or 50-fold excess of unlabeled plant (PT4), human (HT4), *C. elegans* (CT4), or nonspecific (NS) telomeric DNA as competitors. The first "0" lane of each gel contained radiolabeled PT4 without protein.
- (C)** Gel retardation assays with various telomere repeats. GTBPs (1 μg) were analyzed with radiolabeled PT2, PT3, or PT4 (containing two, three, or four repeats, respectively).
- (D)** Gel retardation assays with a series of single nucleotide mutants of PT4. Each mutant (M1 to M7) contained a single nucleotide substitution in the repeated sequence, as listed below the gels.

single-stranded telomeric element. The binding activities of GTBPs were markedly increased with four telomeric repeats (PT4) relative to those with three repeats (PT3), which suggested that, under our experimental conditions, three repeats were minimal telomeric sequences. In addition, substitutions of the first and second thymine nucleotides (T1 to C1 and T2 to C2) reduced the binding signals by $\sim 50\%$, whereas single mutations introduced to nucleotides between the third and seventh positions (T3 \rightarrow C3, A4 \rightarrow C4, G5 \rightarrow C5, G6 \rightarrow C6, or G7 \rightarrow C7) completely abrogated the DNA binding capacity (Figure 2D). Overall, these results indicate that all three Nt GTBP paralogs have similar binding specificities for plant single-stranded telomeric repeats *in vitro*.

GTBP1 Is Associated with Telomeric Sequences in Tobacco BY-2 Cells

ChIP assays were performed to provide *in vivo* evidence for the interaction of GTBPs with telomeric sequences. The hemagglutinin (HA)-tagged *GTBP1* fusion gene was transiently expressed in BY-2 suspension cells under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The cellular localization of HA-GTBP1 was examined by immunostaining using primary anti-HA antibody and secondary Cy3-conjugated anti-mouse antibody. The HA-GTBP1 localization signal was detected exclusively in the nucleus, indicating that the HA-GTBP1 fusion protein was effectively expressed in BY-2 cells (Figure 3A). The

Figure 1. (continued).

- (C)** Expression profiles of three *GTBP* paralogs. Total RNA was isolated from BY-2 cells over the course of 9 d (left gel) and from leaves, stems, and roots of 1-month-old tobacco plants (right gel) and analyzed by RT-PCR using gene-specific primers. Growth curve of BY-2 cells is shown in the bottom of left panel. Tobacco leaves that are in four different developmental stages are shown in the bottom of the right panel. Ng *TRF1* and Nt *cyM* were used as G-phase and M-phase markers, respectively. The level of elongation factor 1 α (*EF1 α*) transcript is shown as a loading control. [See online article for color version of this figure.]

genomic DNA-protein complex was prepared from wild-type and *35S:HA-GTBP1* BY-2 cells, fragmented by sonication, and subjected to immunoprecipitation using an anti-HA antibody. The coimmunoprecipitated DNA was then detected by hybridization with a ^{32}P -labeled (TTTAGGG) $_{70}$ repeat element. As shown in Figure 3B, telomeric fragments were pulled down by the anti-HA antibody in *35S:HA-GTBP1* BY-2 cells, while *HRS60*, a tandemly organized highly repeated DNA sequence in tobacco (Koukalova et al., 1993), was not pulled down by the antibody. In addition, there was no hybridization signal in wild-type cell-free extracts. These results support the notion that GTBP1 is associated with telomeric sequences *in vivo*.

Generation and Characterization of *35S:RNAi-GTBP1* Transgenic Tobacco Plants

Because *GTBP1* was predominantly expressed in all examined tobacco tissues (Figure 1C), *GTBP1* was chosen for further examination. To address the cellular functions of GTBP1, we employed an RNAi-mediated gene knockdown transgenic approach. Transgenic tobacco plants (*35S:RNAi-GTBP1*) with suppressed *GTBP1* were established by introducing a comple-

mentary hairpin-forming DNA fragment of the N-terminal (122 to 498 bp) or C-terminal (726 to 1070 bp) region of *GTBP1* cDNA under the control of the CaMV 35S promoter (Figure 4A). Several independent primary transformants were selected due to their kanamycin resistance. The presence of the transgene was confirmed by genomic DNA gel blot analysis (see Supplemental Figure 4 online). RT-PCR analysis showed that these independent RNAi transgenic lines contained markedly reduced levels of *GTBP1* mRNA compared with wild-type tobacco plants (Figure 4B).

As shown in Figure 4C, knockdown of *GTBP1* resulted in abnormal pleiotropic phenotypes in tobacco plants. The independent T0 *35S:RNAi-GTBP1* transgenic plants showed severe growth retardation, failed to produce healthy leaves and shoots, and exhibited premature senescence. Floral organs emerged earlier in *35S:RNAi-GTBP1* plants, but they usually fell off before maturation; thus, most transgenic plants were unable to produce seeds. In general, abnormal phenotypes of the *35S:RNAi-GTBP1* plants were less apparent during the earlier stages of development but became gradually more serious as plants matured. Among the various independent RNAi transgenic plants, lines #3, #5, #11, #12, #15, #16, and #17, which all contained multiple

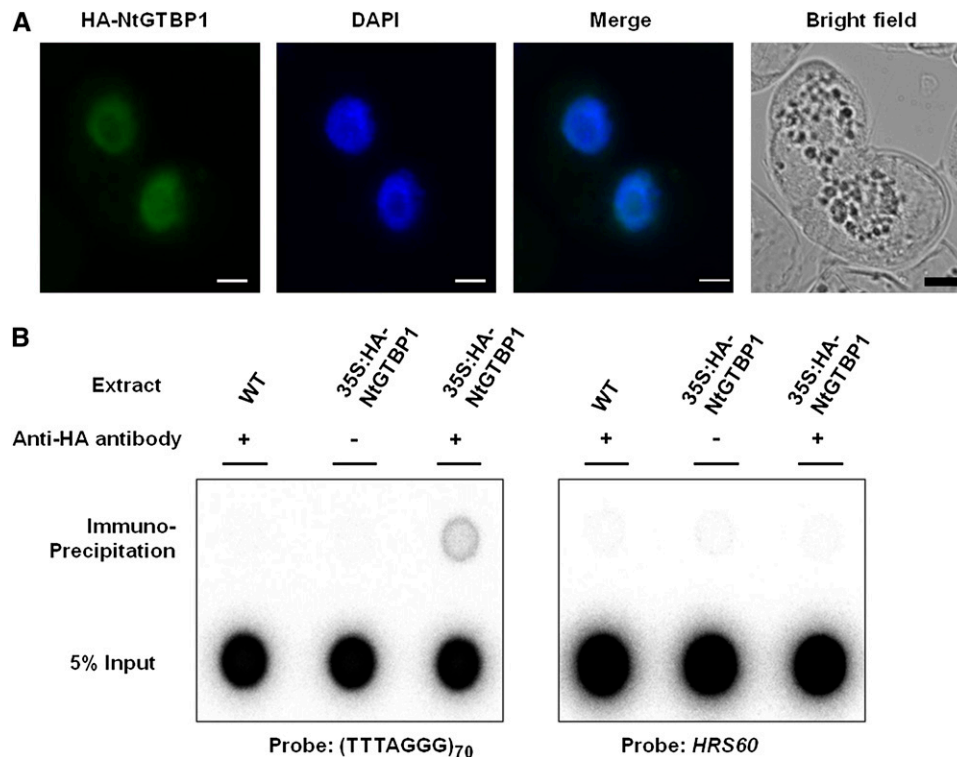


Figure 3. GTBP1 Is Associated with Telomeric Sequences in Tobacco BY-2 Cells.

(A) Nuclear localization of HA-GTBP1. *35S:HA-GTBP1* was transformed into BY-2 cells, and localization of HA-GTBP1 was examined by immunohistochemistry using an anti-HA antibody. The BY-2 cells were viewed by fluorescence microscopy under dark field and light field. The nuclei were stained with DAPI. Bars = 10 μm .

(B) ChIP assay. The genomic DNA-protein complex from wild-type and *35S:HA-GTBP1* BY-2 cells was fragmented by sonication and subjected to immunoprecipitation using an anti-HA antibody. The coimmunoprecipitated DNA was hybridized with ^{32}P -labeled (TTTAGGG) $_{70}$ or *HRS60* repeated tobacco DNA sequences.

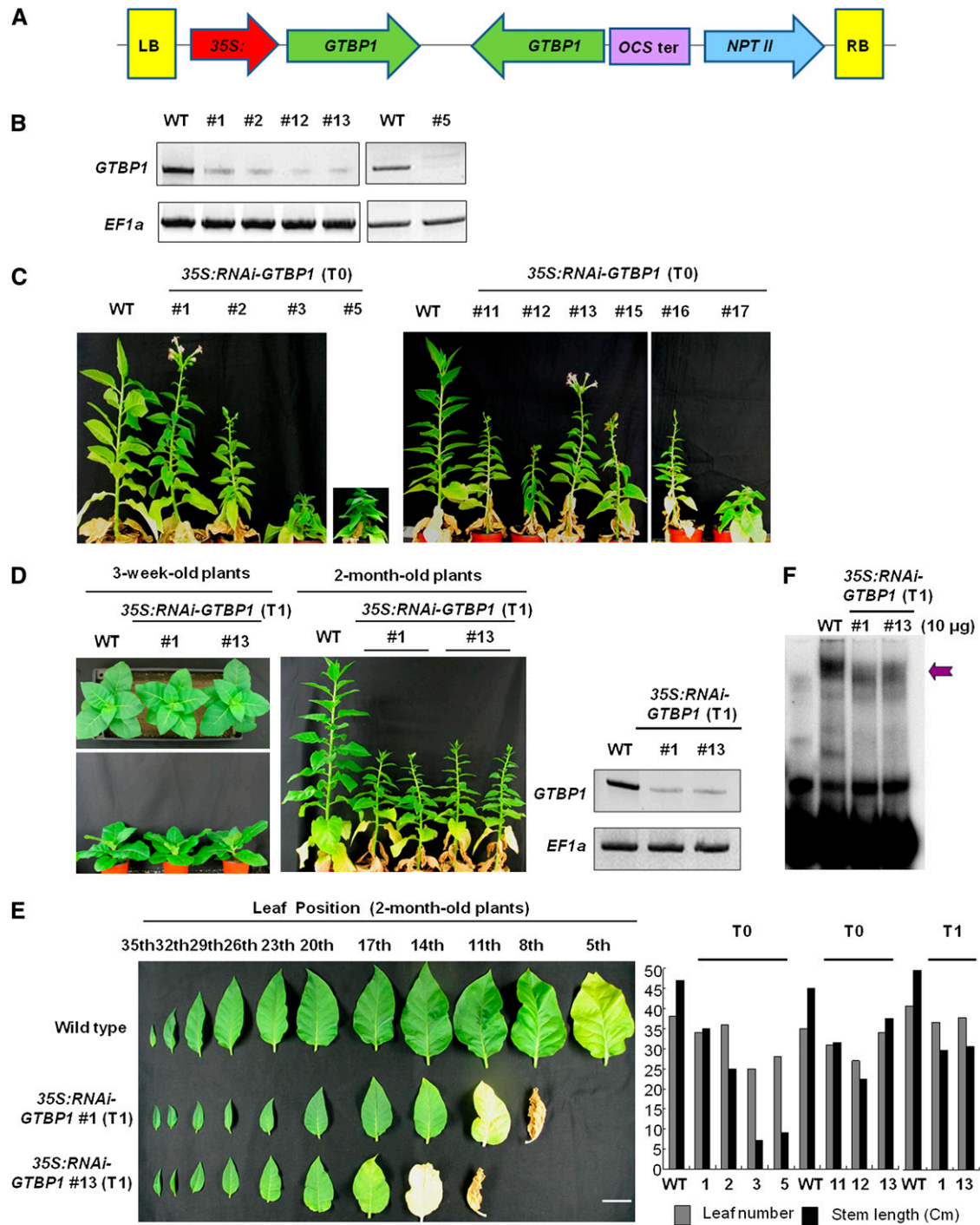


Figure 4. Construction and Characterization of *35S:RNAi-GTBP1* Knockdown Transgenic Tobacco Plants.

(A) Schematic structure of *GTBP1* RNAi binary vector construct. The *35S:RNAi-GTBP1* vector includes the inverted-repeat sequence of the N-terminal (122 to 498 bp) or C-terminal (726 to 1070 bp) regions of *GTBP1* cDNA. LB, left border; *OCS ter*, octopine synthase terminator; *NPTII*, neomycin phosphotransferase II; RB, right border.

(B) Repression of *GTBP1* mRNA in transgenic plants. Total leaf RNA from wild-type and independent T0 *35S:RNAi-GTBP1* lines was analyzed by RT-PCR. *EF1α* was used as a loading control.

(C) Gross morphology of 2-month-old wild-type and independent T0 transgenic lines grown under greenhouse conditions.

(D) Morphology of 3-week-old and 2-month-old wild-type and T1 RNAi transgenic lines (#1 and #13). *GTBP1* transcript levels were determined by

copies of the transgene (see Supplemental Figure 4 online), displayed more severe phenotypes and were sterile. Thus, we obtained subsequent T1 progeny from transgenic lines #1 and #13 that contained a single copy of the transgene with less severe phenotypes. The T1 #1 and #13 plants also displayed retarded growth (Figure 4D). Although leaf number in wild-type and T1 transgenic plants was similar, transgenic leaves were smaller and became senescent earlier than did wild-type leaves (Figure 4E). In addition, internode lengths in the RNAi lines were shorter relative to those in wild-type tobacco plants; hence, T1 *35S:RNAi-GTBP1* plants were ~50% as tall as wild-type tobacco plants (Figure 4D). Cell-free nuclear extracts were prepared from 1-week-old wild-type and T1 transgenic seedlings (#1 and #13), incubated with single-stranded four telomeric repeats (PT4), and subjected to gel retardation assays. The results revealed that the RNAi transgenic seedlings possessed decreased binding activities for the telomeric repeats compared with wild-type plants (Figure 4F). Collectively, the results in Figure 4 suggest that downregulation of *GTBP1* caused severe developmental anomalies in transgenic tobacco, which correlated with the reduction of binding activity to single-stranded telomeric repeats.

Telomere Lengthening and Formation of Extrachromosomal Telomeric Circles in *35S:RNAi-GTBP1* Transgenic Tobacco Plants

The massive abnormalities of the *35S:RNAi-GTBP1* transgenic tobacco plants may be due to defects of telomere metabolism. To investigate whether *GTBP1* participates in telomere regulation, telomere lengths were determined in wild-type, vector control, and *35S:RNAi-GTBP1* (lines #1 and #13) leaves. Telomeres in wild-type and vector control leaves were ~15 to 50 kb, which were consistent with previous studies (Fajkus et al., 1995; Yang et al., 2004). On the other hand, both #1 and #13 T0 transgenic lines displayed varied lengths of telomeres depending on the developmental stages of the leaf tissues. For example, in line #1, the seventh leaf contained normal telomeres (15 to 50 kb), whereas the 15th leaf contained telomeres ranging from 15 to 65 kb. Telomeres in the 27th leaf were even longer (15 to 75 kb) (Figure 5A, left panel). Similarly, the 15th and 27th leaves in the RNAi transgenic line #13 also had longer telomeres (15 to 60 kb) than did control plants. These results parallel our observation that aberrant morphology of the transgenic plants becomes progressively more apparent as the plants mature (Figure 4), suggesting that the abnormal phenotypes are associated with altered telomere lengths.

We then measured telomere length in T1 transgenic leaves (lines #1 and #13). Intriguingly, telomeres of the seventh leaves in the T1 generation were similar to the wild-type telomere ranges (15 to 50 kb) (Figure 5A, right panel). Subsequently, as the T1 transgenic tobacco plants matured, telomeres elongated to 15 to 75 kb in the 15th and 27th leaves, in which the elongation pattern was comparable to that of T0 transgenic plants. In addition, we noticed that, in both T0 and T1 transgenic lines, the lengths of the longer telomeres were more drastically changed than were the shorter telomeres; 50-kb telomeres were lengthened to 60 to 75 kb, while 15-kb telomeres were fairly constant in the transgenic plants (Figure 5A). Overall, these results suggest that knockdown of *GTBP1* resulted in increased telomere length, which was coupled with anomalous morphology, and that telomere length homeostasis is subject to developmental regulation. Furthermore, it is likely that unusually elongated telomeres in *35S:RNAi-GTBP1* transgenic leaves are reset to normal lengths after each generation.

Telomerase enzymatic activities are closely tied with the cell division program and are generally low in differentiated vegetative tissues (Fitzgerald et al., 1996; Riha et al., 1998; Yang et al., 2002). Because *35S:RNAi-GTBP1* leaves possess longer telomeres than do control leaves, we monitored telomerase activity in wild-type and RNAi transgenic leaves by telomere repeat amplification (TRAP) assays (Yang et al., 2002). In spite of elongated telomeres in *35S:RNAi-GTBP1* lines, the levels of telomerase activity were indistinguishable between wild-type and transgenic shoot apices and leaves (Figure 5B). Thus, we concluded that the longer telomeres found in the transgenic leaves are not due to increased telomerase activity.

In mammalian cells, a lack of functional TRF2 and Pot1 caused generation of extrachromosomal telomeric circles (t-circles) that arose from aberrant homologous recombination (HR) of the telomeres (Wang et al., 2004; Wu et al., 2006). Recently, Zellinger et al. (2007) detected increased formation of t-circles in *Arabidopsis ku70* and *ku80* mutant cells. Because *35S:RNAi-GTBP1* lines contained longer telomeres without enhanced telomerase activity, we considered the possibility that the elongated telomeres were subject to HR. To examine this possibility, we performed two-dimensional (2D) pulse-field gel electrophoresis to detect possible formations of extrachromosomal t-circles. Telomeres from both T0 and T1 *35S:RNAi-GTBP1* plants showed extrachromosomal arcs of open-circular and super-coiled double-stranded circles in addition to the linear telomeric arcs (Figure 5C). These t-circles were slightly detectable in the seventh leaves but became increasingly apparent in the 15th to 27th leaves, a pattern reminiscent of telomere elongation (Figure 5A). Thus,

Figure 4. (continued).

RT-PCR in T1 generation (shown at the right with *EF1 α* as a control).

(E) Morphological comparison of leaves from 2-month-old wild-type and T1 transgenic (#1 and #13) plants. Leaf number and stem length of wild-type and *35S:RNAi-GTBP1* (T0 and T1) tobacco plants are shown in the right panel. Bar = 3 cm.

(F) Single-strand telomere binding activities of wild-type and T1 RNAi transgenic seedlings. The cell-free nuclear extracts containing 10 μ g of protein were prepared from wild-type and T1 transgenic seedlings (#1 and #13), incubated with PT4, and analyzed by gel retardation assays as described in Figure 2. Arrow indicates protein-PT4 complex.

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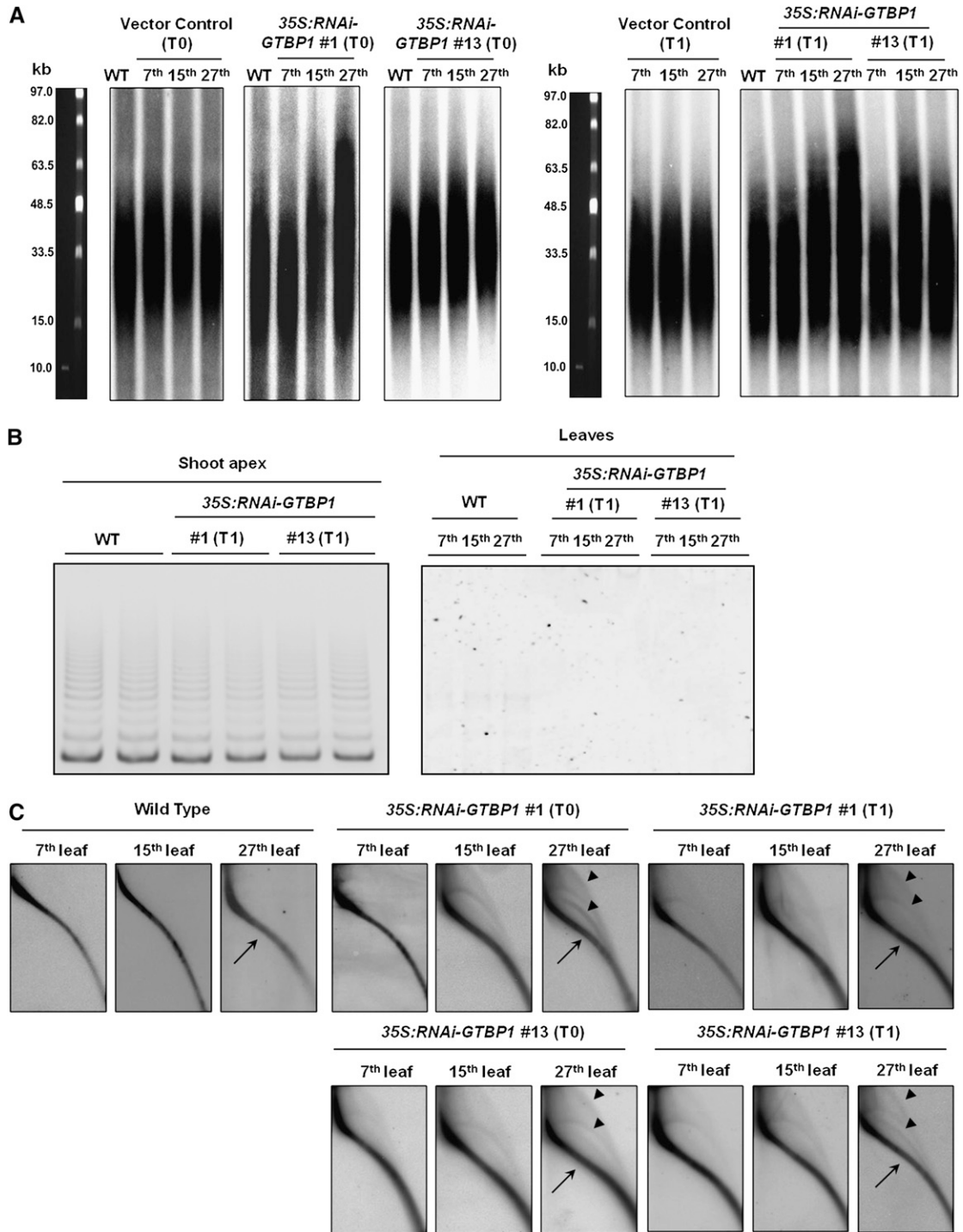


Figure 5. Telomere Deregulation in 35S:RNAi-GTBP1 Tobacco Plants.

(A) Telomere elongation in T0 and T1 transgenic plants. Genomic DNA was purified from the 27th leaves of wild-type plants and the 7th, 15th, and 27th leaves of T0 and T1 transgenic (#1 and #13) plants. After digestion with *TaqI*, DNA was subjected to TRF analysis by pulse-field gel electrophoresis.

(B) Telomerase activity in wild-type and T1 transgenic (#1 and #13) plants. The level of telomerase activity was examined in shoot apices and various leaves by PCR-based TRAP assays.

(C) Formation of extrachromosomal t-circles in T0 and T1 transgenic (#1 and #13) plants. Genomic DNA from the 7th, 15th, and 27th leaves of wild-type and T0 and T1 35S:RNAi-GTBP1 (lines #1 and #13) tobacco plants were subjected to TRF analysis by 2D pulse-field gel electrophoresis. Linear telomeric DNA and extrachromosomal t-circles are indicated by arrows and arrowheads, respectively.

t-circle generation appeared to coincide temporally and spatially with telomeric elongation and also with progressively worsening phenotypes observed in both T0 and T1 *35S:RNAi-GTBP1* plants. We did not detect such additional t-circles in wild-type tobacco leaves regardless of their developmental stages. These results indicate that knockdown of *GTBP1* resulted not only in telomere elongation but also in t-circle formation that may be due to the aberrant HR of the telomeres. Both of these events, in turn, caused impaired development of *35S:RNAi-GTBP1* tobacco plants.

***GTBP1* Knockdown Plants Exhibit a High Frequency of Anaphase Bridges in Anther Cells**

Most mutants that have impaired telomere stability inevitably suffer cytogenetic abnormalities that arise from end-to-end chromosome fusions (Shakirov et al., 2005; Vannier et al., 2006; Hong et al., 2007, 2010; Song et al., 2008; Surovtseva et al., 2009). To investigate whether repression of *GTBP1* also caused chromosome instability in tobacco, we examined wild-type and transgenic chromosomes during meiotic progression of anther cells. As expected, there was negligible anaphase bridge formation in wild-type tobacco meiotic cells (1 of 100) (Figure 6A). By contrast, T1 *35S:RNAi-GTBP1* meiotic cells presented abnormal anaphase bridges with frequencies of 55% (55 of 100) and 42% (42 of 100) in lines #1 and #13, respectively. Moreover, anaphase chromosomes in the transgenic cells contained double (14 to 17%) and multiple (8 to 19%) bridges in addition to single bridges (19 to 20%) (Figure 6A).

Fluorescence in situ hybridization (FISH) analysis revealed that anaphase bridges in *Arabidopsis tert* mutant and *rad50 tert* double mutant cells contained telomeric repeats, suggesting that the unusual chromosome-end fusions included telomeric repeats at the fusion points (Vannier et al., 2006). Similarly, Hong et al. (2007) showed that the anaphase bridges in *rice telomere binding protein1 (rtbp1)* loss-of-function mutant meiotic cells contained telomeric DNA. To examine whether the anaphase bridges in *35S:RNAi-GTBP1* chromosomes were due to telomere dysfunction, we conducted FISH using a (TTTAGGG)₇₀ telomeric DNA repeat probe (see Supplemental Figure 5 online). Single and multiple hybridization signals of the telomeric probe were detected with anaphase bridges in the *35S:RNAi-GTBP1* chromosomes (Figure 6B). The frequencies of positive FISH signals with anaphase bridges were 84% (21 of 25) and 76% (19 of 25) in lines #1 and #13, respectively (Figure 6B). Collectively, these results suggest that suppression of *GTBP1* indeed resulted in telomere malfunction, which is closely associated with the chromosome instability and abnormal phenotypes of *35S:RNAi-GTBP1* plants.

***GTBP1* Inhibits Telomeric Strand Invasion in Vitro**

In the process of interchromosomal telomeric HR, invasion of a 3'-overhang single-stranded telomeric sequence into the double-stranded telomeric region is considered to be a prerequisite step (Cesare and Reddel, 2010). In view of the single-strand-specific telomere binding activity of *GTBP1* and the high frequency of anaphase telomeric fusion in *GTBP1* knockdown plants, we

postulated that telomere binding of *GTBP1* affects the strand invasion process. To test this hypothesis, we performed a recently developed strand invasion assay for telomeric DNA repeats (Amiard et al., 2007; Zhang et al., 2007). The recombinant full-length (MBP-*GTBP1*¹⁻³⁴⁶) or deletion mutants (MBP-*GTBP1* Δ C1¹⁻¹⁹⁴, MBP-GRP1 Δ C2¹⁻¹⁷⁹, MBP-GRP1 Δ N Δ C1²⁴⁻¹⁹⁴, and MBP-GRP1 Δ N Δ C2²⁴⁻¹⁷⁹) of *GTBP1* were incubated with a ³²P-labeled single-stranded (TTTAGGG)₈ repeat probe with T-vector plasmid containing double-stranded (TTTAGGG)₇₀ telomeric repeats (Figure 7A). The protein-DNA complexes were treated with proteinase K and then analyzed by gel electrophoresis. The degree of invasion of the single-stranded telomeric probe into the plasmid was determined by the intensity of the shifted band. MBP-*GTBP1*¹⁻³⁴⁶ inhibited the invasion of single-stranded telomeric probe into the plasmid in a dose-dependent manner (Figure 7B). Approximately 80% of the single-strand invasion was inhibited by a saturating amount of MBP-*GTBP1*¹⁻³⁴⁶. Similar concentration-dependent inhibitory profiles were detected when the MBP-*GTBP1* Δ C1¹⁻¹⁹⁴ or MBP-GRP1 Δ C2¹⁻¹⁷⁹ mutants that retained high binding activities for single-strand telomere (Figure 7C) were included in the assay mixture. Notably, the MBP-GRP1 Δ N Δ C1²⁴⁻¹⁹⁴ mutant protein, which had reduced telomere binding activity, partially inhibited the strand invasion process. Finally, MBP-*GTBP1* Δ N Δ C2²⁴⁻¹⁷⁹ and MBP, which lack telomere binding activity (Figure 7C), both failed to inhibit strand invasion (Figure 7B). Therefore, binding activity of *GTBP1* was proportionally correlated with the degree of strand invasion inhibitory activity in vitro. These results argue that *GTBP1* may prevent aberrant interchromosomal telomeric HR by inhibiting telomeric strand invasion.

DISCUSSION

Telomeres shield the extreme ends of linear eukaryotic chromosomes. Both animal and plant telomeres form a stable t-loop, in which the 3' single-strand overhang is inserted into a double-stranded telomeric region so that telomeres can be protected and distinguished from double-strand break repair mechanisms (Griffith et al., 1999; Cesare et al., 2003). Single-strand telomere binding proteins, along with double-strand-specific factors, play critical roles in telomere maintenance. In mammals, POT1 is an essential factor for telomere protection as loss-of-function of *POT1* caused severe genomic anomalies due to telomere malfunction (Hockemeyer et al., 2006; Wu et al., 2006). In addition, animal POT1 participates in the negative regulation of telomere length homeostasis by inhibiting access of telomerase to telomeric ends (Kelleher et al., 2005). Higher plants also have homologs of animal POT1. *Arabidopsis* POT1b was shown to function in telomere protection (Shakirov et al., 2005), whereas POT1a, contrary to its animal homolog, appeared to work as a positive regulator for telomerase and telomere length regulation (Surovtseva et al., 2007). However, neither telomeric protein exhibited single-strand telomere binding activities. Therefore, it was postulated that POT1s may not be major single-stranded telomeric factors in *Arabidopsis* (Shakirov et al., 2009). These results prompted us to examine the cellular role for *GTBP* paralogs in tobacco plants.

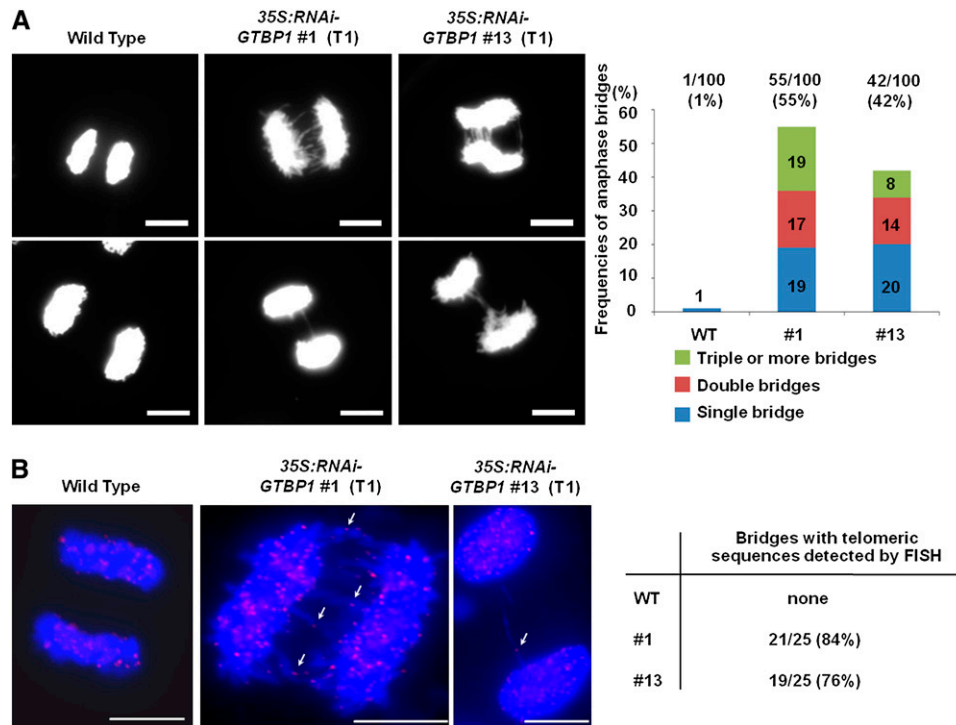


Figure 6. Formation of Anaphase Bridges in T1 *35S:RNAi-GTBP1* Transgenic Cells.

(A) Anaphase chromosomes from wild-type and T1 *35S:RNAi-GTBP1* (lines #1 and #13) anther cells. Anaphase chromosome spreads were obtained from anthers of wild-type and *35S:RNAi-GTBP1* transgenic lines (#1 and #13), stained with DAPI, and observed by fluorescence microscopy. Abnormal anaphase bridges were detected in transgenic meiotic cells. Frequencies of single, double, and multiple anaphase bridges are indicated in the right panel. Bars = 10 μ m.

(B) FISH analysis of anaphase chromosomes in wild-type and T1 *35S:RNAi-GTBP1* (lines #1 and #13) meiotic cells using a (TTAGGG)₇₀ repeat telomeric fluorescent probe. Chromosomal DNA was denatured and incubated with Texas red-dUTP-incorporated (TTAGGG)₇₀ telomeric probe. The chromosomes were counterstained with DAPI and observed using fluorescence microscopy. Arrows indicate telomeric signals at the fusion points of anaphase bridges. Percentages of anaphase bridges with telomeric sequences detected by FISH are indicated in the right panel. Bars = 10 μ m.

While three homologous tobacco GTBPs displayed similar modes of binding to single-stranded telomeric sequences *in vitro*, *GTBP1* was the predominantly expressed gene in tobacco (Figures 1 and 2). We noticed that the expression profile of Nt *GTBP1* was quite different from that of the double-stranded telomeric factor Ng *TRF1* in tobacco. Nt *GTBP1* was constitutively expressed in BY-2 suspension cells during the 9-d culture period, as opposed to Ng *TRF1*, whose expression was inversely correlated with cell division activity (Figure 1C; Yang et al., 2003). In addition, Nt *GTBP1*, but not Ng *TRF1*, was developmentally regulated during leaf development, suggesting that Nt *GTBP1* and Ng *TRF1* may have different roles in telomeres. Nt *GTBP1* was associated with telomeric sequences as evidenced by ChIP assay (Figure 3). The independent T0 and T1 *35S:RNAi-GTBP1* plants displayed severely aberrant phenotypes with retarded vegetative growth, premature senescence, and infertility, which were coupled with reduced binding activity to single-stranded telomeric sequences (Figure 4). Also, the *GTBP1* knockdown leaves contained longer telomeres with frequent formation of extrachromosomal t-circles (Figure 5). Thus, Nt *GTBP1* is a negative regulator of telomere length, which is opposite of the

role of At POT1a, but, at least in part, similar to mammalian POT1. This raises the tantalizing possibility that telomere length homeostasis could be regulated by the functional balance between two different single-stranded telomeric factors, POT1 and *GTBP1*, in plants. Furthermore, given that the *GTBP1* knockdown meiotic cells suffered frequent anaphase bridges that contained telomeric sequences at the fusion points, it is highly plausible that *GTBP1* plays a positive role in telomere stability (Figure 6). This view is further supported by *in vitro* results that *GTBP1* inhibited the single-strand invasion into double-stranded telomeric DNA that is a prerequisite step for interchromosomal telomeric HR (Figure 7). Therefore, it is reasonable to consider that *GTBP1* suppression-induced abnormal phenotypes are closely correlated with genomic instability due to the dysfunctional telomeres in the transgenic tobacco. With this in mind, we conclude that *GTBP1* is an essential component for both structure and function of tobacco telomeres.

It is worth noting that phenotypic severity, telomere elongation, and the frequency of t-circle formation in T0 RNAi transgenic plants became increasingly apparent as the plants grew to maturity (Figures 4 and 5). Intriguingly, these abnormalities

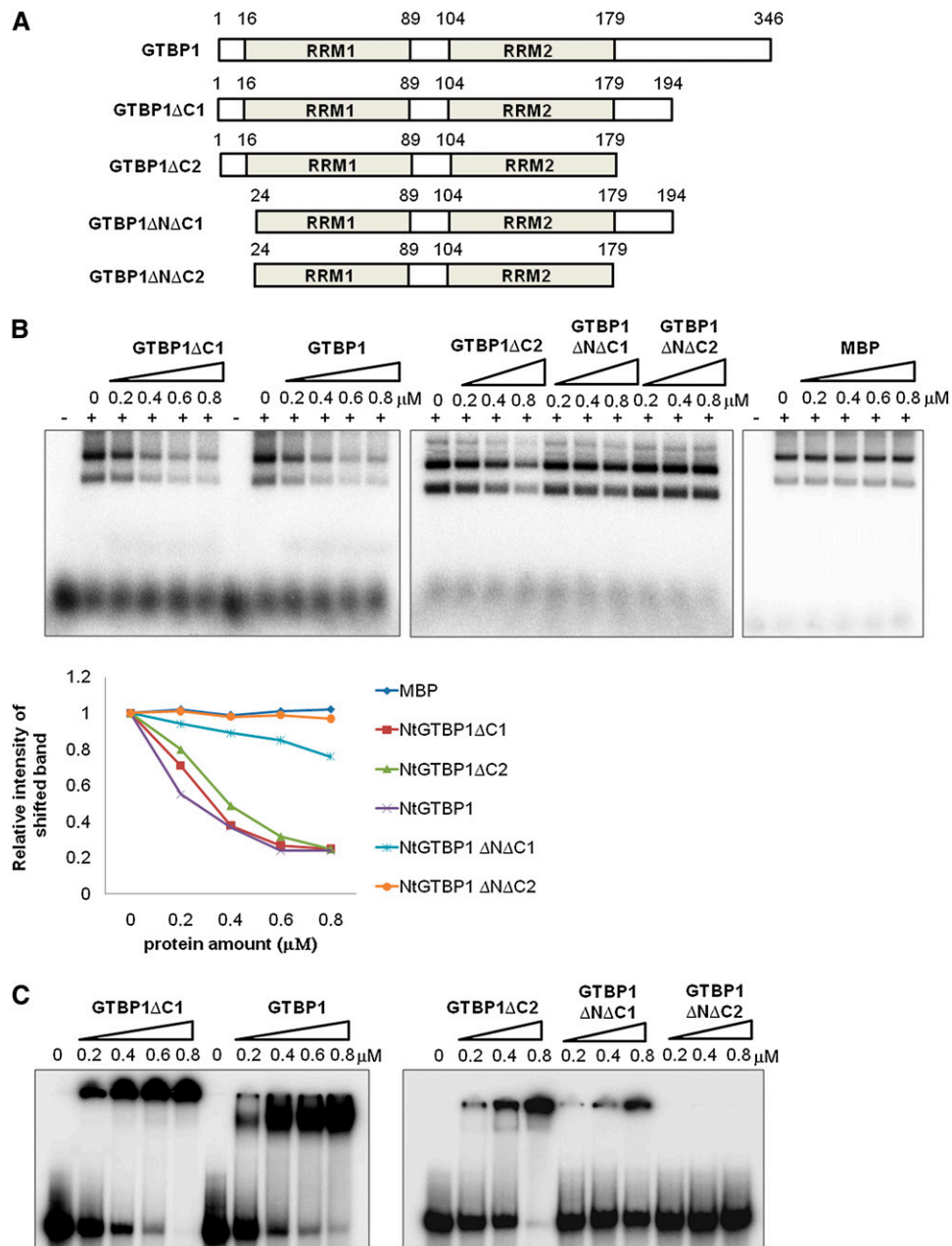


Figure 7. Nt GTBP1 Inhibits Telomeric Strand Invasion in Vitro.

(A) Schematic representation of full-length and deletion mutants of GTBP1.

(B) Strand invasion assay. Increasing concentrations of GTBP1 derivatives (0, 0.2, 0.4, 0.6, and 0.8 μM) were incubated with a ^{32}P -labeled single-stranded (TTTAGGG)₈ repeat probe with T-vector plasmid containing double-stranded (TTTAGGG)₇₀ telomere repeats. The degree of invasion of the single-stranded telomeric probe into the plasmid was determined by the shifted band intensity. The first “–” lane of each gel contained radiolabeled PT8 without protein. The graph in the bottom panel shows relative intensities of shifted bands.

(C) Binding activities of full-length and deletion mutants of GTBP1 to single-strand telomere sequences. Various concentrations (0, 0.2, 0.4, 0.6, and 0.8 μM) of GTBP1 derivatives were analyzed with radiolabeled single-stranded (TTTAGGG)₈ (PT8) repeats.

[See online article for color version of this figure.]

were restored to normal levels in the early stages of the T1 generation but then concomitantly reappeared when T1 transgenic plants were further grown. Thus, phenotypic properties were subject to developmental regulation and influenced by telomere length and architecture, with its severity being more serious in the later stage of development. In *Arabidopsis ku70* mutant cells, abnormally elongated telomeres were reset to a new length after three generations through the telomere rapid deletion process (Watson and Shippen, 2007). It was previously proposed that individual telomere tracts on homologous chromosomes were coordinately regulated through development in *Arabidopsis* (Shakirov and Shippen, 2004). Based on these results, we speculate that elongated telomeres were reset during the T0 to T1 generation of the *35S:RNAi-GTBP1* transgenic plants. However, due to the lack of properly functioning GTBP1, telomeres were again gradually lengthened and destabilized, which in turn caused gradual phenotypic defects.

Higher plants usually exhibit flexible phenotypes in response to changes in telomere length. For example, although the last five generations show multiple phenotypic problems, the telomerase-deficient *Arabidopsis tert* mutants could survive for up to 10 generations (Riha et al., 2001). Transgenic tobacco *35S:TRF1* cells, in which the double-strand binding protein Ng TRF1 was overexpressed, showed progressive reduction in cell viability with shortened telomeres over successive rounds of batch subculture (Yang et al., 2004). A rice *rtbp1* mutant that suppressed telomere binding protein 1 already contained markedly elongated telomeres in the G1 generation but showed gradually more defective phenotypes during the consecutive four generations (Hong et al., 2007). By contrast, knockdown of *GTBP1* resulted in immediate defective phenotypes, and most independent T0 plants were seriously impaired and sterile (Figure 4). The rapid onset of defective phenotypes was also identified in *Arabidopsis ctc1* (for conserved telomere maintenance component1), in which telomere length deregulation, recombination, and end-to-end fusion were strongly present in the first generation of loss-of-function mutant plants (Surovtseva et al., 2009). Thus, suppression of At *CTC1* and Nt *GTBP1* had more immediate effects on telomere stability than did suppression of Ng *TRF1* or Os *RTBP1*. This may be due to the existence of a gene family for Ng *TRF1* and Os *RTBP1* and/or to the more direct roles of Nt *GTBP1* and At *CTC1*. In this respect, it is of interest to note that knockdown of Nt *GTBP1* induced longer telomeres, and *Arabidopsis ctc1* mutants, by contrast, exhibited shorter telomeres. Therefore, defects in the *35S:RNAi-GTBP1* and *ctc1* plants might not be due to telomere length deregulation per se but to the structural instability that resulted from the absence of capping proteins. This notion is supported by the results that telomeres in the seventh leaves of T0 and T1 *35S:RNAi-GTBP1* plants displayed normal lengths but showed extrachromosomal t-circles (Figure 5C).

Approximately 10% of human cancer cells maintain their telomeres under telomerase negative conditions by alternative lengthening of telomeres (ALT) (Cesare and Reddel, 2010). ALT cancer cells are typified by enhanced t-circle formation, as ALT is initiated by telomeric HR (Wang et al., 2004). Likewise, *Arabidopsis tert ku70* double mutant callus cells displayed a large number of t-circles, which is typical of ALT (Zellinger et al., 2007).

Given that t-circles are frequently found in *35S:RNAi-GTBP1* lines and GTBP1 inhibits strand invasion in vitro, it is of keen interest to determine whether GTBP1 is a negative regulator of the ALT initiation process in tobacco. In conclusion, our data provide evidence that GTBP1 is an essential factor for telomere integrity and normal growth and development in tobacco, a dicot model crop plant.

METHODS

Plant Materials

Wild-type and *35S:RNAi-GTBP1* transgenic tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown on 0.8% agar for 7 d or on Murashige and Skoog (MS) medium containing 3% sucrose, vitamin B5 (12 mg/L), and 0.8% agar, pH 5.8, for 14 d in a growth chamber under a 16-h-light/8-h-dark photoperiod at 25°C. Tobacco seedlings were transferred to pots and moved to a growth room for further growth. The tobacco BY-2 suspension cell line was maintained in MS medium on a rotary shaker (150 rpm) at 27°C in the dark. Every 9 d, stationary phase cells (5 mL) were transferred to 45 mL of fresh medium and cultured (Yang et al., 2002).

Isolation of Three Full-Length Nt *GTBP* Paralogs

To isolate the cDNAs for tobacco *GTBP* paralogs, degenerate oligonucleotides corresponding to the amino acid sequence VEIKRTIP for the upstream primer and VEIKKA for the downstream primer were produced (see Supplemental Table 1 online). These amino acid sequences are conserved in RRM1s (Figure 1A). RT-PCR was performed with oligonucleotide primers and first-strand cDNA prepared from total RNA (2.0 µg) of log-phase BY-2 suspension-cultured cells using high-fidelity Ex-Taq DNA polymerase (Takara). PCR comprised 35 amplification cycles with an annealing temperature of 52°C for 45 s, an elongation temperature of 72°C for 1 min, and a denaturing temperature of 95°C for 30 s. PCR products were separated via agarose gel electrophoresis, and ~300-bp-long cDNA fragments were eluted from the gel and cloned into the pGEM-T Easy vector (Promega) for sequencing. Three homologous Nt *GTBP* cDNAs were identified and named *GTBP1* (GenBank accession number HM049166; Hirata et al., 2004), *GTBP2* (GenBank accession number HM049167), and *GTBP3* (GenBank accession number HM049168). To obtain full-length cDNAs, total recombinant λ DNA was prepared from the λ-uni-Zap II tobacco flower cDNA library (Kim et al., 2000) and used for RACE (Frohman, 1994) with primers corresponding to the 5'- and 3'-ends of the library vector sequence (T7 and T3 promoters) and the internal regions of the partial *GTBP* cDNA clones (see Supplemental Table 1 online).

Phylogenetic Analysis

Protein sequences were aligned (available as Supplemental Data Set 1 online) using ClustalW in Mega4 software (Tamura et al., 2007). The evolutionary relationship was analyzed by the neighbor-joining method (Saitou and Nei, 1987). The optimal tree that shows the sum of branch length was 4.37412444. Bootstrapping was performed with 20,000 replicates. The evolutionary distances were calculated by the Poisson correction method (Zuckerkanndl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site.

Genomic DNA Isolation and DNA Gel Blot Analysis

Leaf genomic DNA was isolated from tobacco plants as described previously (Yang et al., 2003). Genomic DNA (10 µg) was digested with

indicated restriction enzymes, blotted onto Hybond-N nylon membranes (Amersham), and hybridized with ^{32}P -labeled probes (Nt *GTBP1*, Nt *GTBP2*, and Nt *GTBP3* full-length cDNAs [Figure 1A], respectively, in Supplemental Figure 2 online and *NPTII* in Supplemental Figure 4 online).

RNA Extraction and RT-PCR

Total RNAs were extracted from BY-2 cells and various tobacco tissues as described previously (Yang et al., 2003). Total RNA (2 μg) was used for first-strand cDNA synthesis with MMLV reverse transcriptase (Promega). To investigate gene expression profiles, RT-PCR was performed with gene-specific primers (see Supplemental Figure 2 and Supplemental Table 1 online) using Ex-Taq polymerase (Takara). Amplification comprised 25 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C in an automatic thermal cycler (Perkin-Elmer/Cetus). PCR products were separated on 1.0% agarose gels and visualized with ethidium bromide under UV light.

Gel Retardation Assays

The full-length or various deletion mutants of *GTBP* cDNAs were generated using primers listed in Supplemental Table 1 online and inserted into the pMAL-c2x vector (New England Biolabs) using *Bam*HI and *Sal*I sites. MBP-GTBP fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography using amylose resin (New England Biolabs) as described by Yang et al. (2003). The cell-free nuclear extracts were prepared from 1-week-old wild-type and *35S:RNAi-GTBP1* seedlings as described previously (Lee et al., 2000). Gel retardation assays were performed with bacterially expressed recombinant proteins or nuclear extracts containing 10 μg proteins as described by Byun et al. (2008). Various telomeric DNA probes and competitors used in this study are listed in Supplemental Table 1 online.

Transformation of *35S:HA-GTBP1* Construct into BY-2 Cells

The 3xHA-tagged *GTBP1* cDNA was generated using HA tag-*Pst*I sense, HA tag-*Pst*I antisense, *GTBP1-Sal*I sense, and *GTBP1-Bam*HI antisense primers (see Supplemental Table 1 online) and cloned into the pCambia 1390 vector (Cambia), which contained the CaMV 35S promoter. The *35S:HA-GTBP1* construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. *Agrobacterium* was then coinoculated with actively dividing tobacco BY-2 suspension cultured cells for 3 d at 25°C with gently shaking (150 rpm). Transgenic BY-2 cells were selected on MS medium supplemented with 100 $\mu\text{g}/\text{mL}$ hygromycin and 100 $\mu\text{g}/\text{mL}$ carbenicillin. Expression and localization of HA-GTBP1 fusion protein were confirmed by immunostaining using primary anti-HA antibody (New England Biolabs) and secondary Cy3-conjugated anti-mouse antibody (Millipore) as described by Lee et al. (2009). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in mounting solution (Vector Laboratories).

ChIP Assays

An established ChIP assay protocol (<http://www.epigenome-noe.net/researchtools/protocol.php?protid=13>) was followed with some modifications. To cross-link genomic DNA with bound proteins, wild-type and *35S:HA-GTBP1* transgenic BY-2 cells were incubated with a 1% formaldehyde solution for 10 min. Cross-linking was quenched by the addition of glycine up to 150 mM. After harvesting cells by vacuum filtration through filter paper, chromatin was fractionated by sonication into fragments 0.2 to 1.0 kb in length in ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl). ChIP was performed by incubating sonicated cell extracts without (as a negative control) or with

1:100 diluted anti-HA antibody (New England Biolabs). Bound chromatin fragments were eluted with protein G agarose beads (Santa Cruz Biotechnology). Elution products were dot blotted onto Hybond-N nylon membranes (Amersham) and subjected to hybridization with ^{32}P -labeled (TTTAGGG) $_{70}$ repeat or *HRS 60* (Koukalova et al., 1993) probes.

Generation of *35S:RNAi-GTBP1* Knockdown Transgenic Tobacco Plants

GTBP1 cDNA encompassing 377 bp of the 5' end (from 122 to 498 bp) (RNAi lines #11, #12, #13, #15, #16, and #17) or 345 bp of the 3' end (from 726 to 1070 bp) (RNAi lines #1, #2, #3, and #5) was generated by primers listed in Supplemental Table 1 online and inserted into the pHANNIBAL vector (CSIRO) using *Xho*I, *Eco*RI, *Bam*HI, and *Hind*III sites in inverted orientations. The hairpin-producing cassette was then cloned into *Not*I site of binary vector pART (<http://www.pi.csiro.au/RNAi/vectors.htm>). The produced Nt *GTBP1* RNAi constructs were transferred to *Agrobacterium* strain LBA4404 by electroporation. Leaf discs of *N. tabacum* cv Samsun NN were cocultivated with *Agrobacterium* as described by Bae et al. (2009), and transgenic tobacco plants were generated on medium containing 200 mg/L kanamycin. The regenerated T0 plants were grown in a growth room under a 16-h-light/8-h-dark photoperiod at 25°C. The T0 transformants (lines #1 and #13) were self-fertilized, and seeds were collected. T1 plants were selected on MS medium containing 200 mg/L kanamycin. T0 and T1 transgenic plants were used for phenotypic analysis.

TRAP Assay and Telomere Repeat Fragment Analysis

TRAP assays, which measure telomerase enzyme activity, were performed as described by Yang et al. (2002). TRAP assays were performed in 50 μL of reaction mixture composed of 50 mM Tris-acetate, pH 8.3, 50 mM potassium glutamate, 0.1% Triton X-100, 1 mM spermidine, 1 mM DTT, 50 μM each deoxynucleotide triphosphate, 5 mM MgCl_2 , 10 mM EGTA, 100 ng/ μL BSA, and 0.5 unit Taq polymerase (Takara). For telomere repeat fragment (TRF) analysis, tobacco leaf genomic DNA (5 μg) was digested by *Taq*I restriction enzyme, separated by pulse-field gel electrophoresis using a CHEF-DRILL system (Bio-Rad), transferred to nylon membrane, and hybridized with a ^{32}P -labeled (TTTAGGG) $_{70}$ telomeric probe.

2D Pulse-Field Gel Electrophoresis

The *Taq*I-digested tobacco leaf genomic DNA (5 μg) was separated in a 0.5% agarose gel by pulse-field gel electrophoresis using a CHEF-DRILL system (Bio-Rad) at 1 V/cm for 25.5 h running time at an angle of 120° with switching times ramped from 1 to 6 s at 14°C. The second dimension electrophoresis was performed in a 1.1% agarose gel at 6 V/cm for 8.5 h with the same conditions. The gel was blotted onto a Hybond-N nylon membrane (Amersham) and hybridized to a ^{32}P -labeled (TTTAGGG) $_{70}$ telomeric repeat probe under highly stringent conditions. The blots were visualized by autoradiography at -80°C.

Cytogenetic Analysis and FISH

Anaphase chromosome spreads were obtained from anthers of wild-type and *35S:RNAi-GTBP1* transgenic lines (#1 and #13) and stained with DAPI as described previously (Hong et al., 2007) with minor modifications. Anthers were fixed in 1:3 (v/v) acetic acid:ethanol for 2 h, soaked in distilled water, and digested with 2% cellulose, 1.5% macerozyme, 0.3% pectolyase, and 1 mM EDTA, pH 4.2, for 1 h. After being squashed on slide glass with 60% acetic acid, digested anthers were dried on hot plate at 45°C. Chromosomal DNAs were stained with DAPI and observed by fluorescence microscopy (Axio imager; Zeiss).

Chromosomal DNA was denatured with 70% formamide at 70°C for 3 min. Samples were then dehydrated in a 70, 85, 95, and 100% ethanol series at -20°C for 3 min each as described by Hong et al. (2007). Texas red-dUTP (Invitrogen) incorporated (TTTAGGG)₇₀ telomeric probe was applied to the denatured chromosomal DNA in hybridization solution (50% [v/v] formamide, 10% [w/v] dextran sulfate, 5 ng/μL salmon sperm DNA, and 2× SSC), covered with a plastic cover slip, and incubated in a humid chamber at 37°C for 36 h. The chromosomes were counterstained with DAPI and observed using fluorescence microscopy with Qmax Blue and XF 102-2 filters (Omega Optical).

Strand Invasion Assays

Strand invasion assays were performed with a ³²P-labeled single stranded (TTTAGGG)₈ (PT8) repeat probe and pGEM-T Easy plasmid containing double-stranded (TTTAGGG)₇₀ telomeric repeats according to Amiard et al. (2007). The full-length or various deletion mutants of GTBP1 were constructed using primers listed in Supplemental Table 1 online and incubated with PT8 (0.25 ng) in invasion buffer (50 mM HEPES, pH 8.0, 0.1 mg/mL BSA, 1 mM DTT, 100 mM NaCl, and 2% [v/v] glycerol) for 15 min at 4°C. The plasmid (100 ng) was added to the reaction mixture and incubated for 15 min at room temperature. Reactions were stopped by the addition of stop buffer [10% (w/v) SDS, 6 μg proteinase K, and 25 ng (CCCTAAA)₄]. Samples were separated on 1.1% agarose gels in 0.5× TBE at 85 V at room temperature. Gels were dried and visualized by autoradiography. Strand invasion signals were quantified with a phosphor imager (Fujii). The degree of invasion of the single-stranded telomeric probes into the plasmids was determined by the shifted band intensity.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: tobacco GTBP1 (HM049166), GTBP2 (HM049167), and GTBP3 (HM049168); *Arabidopsis* STEP1 (NP194208); *Arabidopsis* proteins (At3g13224 and At4g26650); rice proteins (Os08g0320100 and Os08g0492100); and human HnRNP D (BAA09522) and HnRNP A1 (P09651).

Supplemental Data

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

Supplemental Figure 1. Sequence Analysis of Tobacco GTBP Paralogs.

Supplemental Figure 2. DNA Gel Blot Analysis of Tobacco DNA.

Supplemental Figure 3. Specificity of the Gene-Specific Primers.

Supplemental Figure 4. DNA Gel Blot Analysis of Wild-Type and 10 T0 35S:RNAi-GTBP1 Tobacco Plants.

Supplemental Figure 5. FISH Analysis of Meiotic Chromosomes in Wild-Type Tobacco Anther Cells.

Supplemental Table 1. Oligonucleotide Sequences Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Figure 1B.

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REFERENCES

- Amiard, S., et al. (2007). A topological mechanism for TRF2-enhanced strand invasion. *Nat. Struct. Mol. Biol.* **14**: 147–154.
- Bae, H., Choi, S.M., Yang, S.W., Pai, H.S., and Kim, W.T. (2009). Suppression of the ER-localized AAA ATPase NgCDC48 inhibits tobacco growth and development. *Mol. Cells* **28**: 57–65.
- Baumann, P., and Cech, T.R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* **292**: 1171–1175.
- Bilaud, T., Brun, C., Ancelin, K., Koering, C.E., Laroche, T., and Gilson, E. (1997). Telomeric localization of TRF2, a novel human telobox protein. *Nat. Genet.* **17**: 236–239.
- Blackburn, E.H. (1991). Structure and function of telomeres. *Nature* **350**: 569–573.
- Blackburn, E.H. (2001). Switching and signaling at the telomere. *Cell* **106**: 661–673.
- Blasco, M.A. (2005). Telomeres and human disease: Ageing, cancer and beyond. *Nat. Rev. Genet.* **6**: 611–622.
- Byun, M.Y., Hong, J.-P., and Kim, W.T. (2008). Identification and characterization of three telomere repeat-binding factors in rice. *Biochem. Biophys. Res. Commun.* **372**: 85–90.
- Cesare, A.J., Quinney, N., Willcox, S., Subramanian, D., and Griffith, J.D. (2003). Telomere looping in *P. sativum* (common garden pea). *Plant J.* **36**: 271–279.
- Cesare, A.J., and Reddel, R.R. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. *Nat. Rev. Genet.* **11**: 319–330.
- Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995). A human telomeric protein. *Science* **270**: 1663–1667.
- Collins, K. (2000). Mammalian telomeres and telomerase. *Curr. Opin. Cell Biol.* **12**: 378–383.
- de Lange, T. (2005). Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19**: 2100–2110.
- Fajkus, J., Kovarik, A., Kralovics, R., and Bezdek, M. (1995). Organization of telomeric and subtelomeric chromatin in the higher plant *Nicotiana tabacum*. *Mol. Gen. Genet.* **247**: 633–638.
- Fitzgerald, M.S., McKnight, T.D., and Shippen, D.E. (1996). Characterization and developmental patterns of telomerase expression in plants. *Proc. Natl. Acad. Sci. USA* **93**: 14422–14427.
- Frohman, M.A. (1994). On beyond classic RACE (rapid amplification of cDNA ends). *Genome Res.* **4**: S40–S58.
- Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. *Cell* **97**: 503–514.
- Hirata, Y., Suzuki, C., and Sakai, S. (2004). Characterization and gene cloning of telomere-binding protein from tobacco BY-2 cells. *Plant Physiol. Biochem.* **42**: 7–14.
- Hockemeyer, D., Danieis, J.-P., Takai, H., and de Lange, T. (2006). Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. *Cell* **126**: 63–77.
- Hong, J.-P., Byun, M.Y., An, K., Yang, S.-J., An, G., and Kim, W.T.

- (2010). OsKu70 is associated with developmental growth and genome stability in rice (*Oryza sativa* L.). *Plant Physiol.* **152**: 374–387.
- Hong, J.-P., Byun, M.Y., Koo, D., An, K., Bang, J., Chung, I.K., An, G., and Kim, W.T.** (2007). Suppression of *RICE TELOMERE BINDING PROTEIN1* results in severe and gradual developmental defects accompanied by genome instability in rice. *Plant Cell* **19**: 1770–1781.
- Ishikawa, F., Matunis, M.J., Dreyfuss, G., and Cech, T.R.** (1993). Nuclear proteins that bind the pre-mRNA 3' splice site sequence (UUAG/G) and the human telomeric DNA sequence d(TTAGGG)_n. *Mol. Cell. Biol.* **13**: 4301–4310.
- Kelleher, C., Kurth, I., and Lingner, J.** (2005). Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol. Cell. Biol.* **25**: 808–818.
- Kim, Y.S., Lee, J.H., Yoon, G.M., Cho, H.S., Park, S.W., Suh, M.C., Choi, D., Ha, H.J., Liu, J.R., and Pai, H.S.** (2000). CHRK1, a chitinase-related receptor-like kinase in tobacco. *Plant Physiol.* **123**: 905–915.
- Koukalova, B., Komarnitsky, I.K., and Kuhrova, V.** (1993). The distribution of tobacco HRS60 DNA repeated sequences in species of the genus *Nicotiana*. *Plant Sci.* **88**: 39–44.
- Kwon, C., and Chung, I.K.** (2004). Interaction of an *Arabidopsis* RNA-binding protein with plant single-stranded telomeric DNA modulates telomerase activity. *J. Biol. Chem.* **279**: 12812–12818.
- LaBranche, H., Dupuis, S., Ben-David, Y., Bani, M.R., Wellinger, R.J., and Chabot, B.** (1998). Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat. Genet.* **19**: 199–202.
- Lee, H.K., Cho, S.K., Son, O., Xu, Z., Hwang, I., and Kim, W.T.** (2009). Drought stress-induced Rma1H1, a RING membrane-anchor E3 ubiquitin ligase homolog, regulates aquaporin levels via ubiquitination in transgenic *Arabidopsis* plants. *Plant Cell* **21**: 622–641.
- Lee, J.H., Kim, J.H., Kim, W.T., Kang, B.G., and Chung, I.K.** (2000). Characterization and developmental expression of single-stranded telomeric DNA-binding proteins from mung bean (*Vigna radiata*). *Plant Mol. Biol.* **42**: 547–557.
- Nugent, C.I., Hughes, T.R., Lue, N.F., and Lundblad, V.** (1996). Cdc13p: A single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science* **274**: 249–252.
- Riha, K., Fajkus, J., Siroky, J., and Vyskot, B.** (1998). Developmental control of telomere lengths and telomerase activity in plants. *Plant Cell* **10**: 1691–1698.
- Riha, K., McKnight, T.D., Griffing, L.R., and Shippen, D.E.** (2001). Living with genome instability: Plant response to telomere dysfunction. *Science* **291**: 1797–1800.
- Saitou, N., and Nei, M.** (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
- Shakirov, E.V., McKnight, T.D., and Shippen, D.E.** (2009). POT1-independent single-strand telomeric DNA binding activities in *Brassicaceae*. *Plant J.* **58**: 1004–1015.
- Shakirov, E.V., and Shippen, D.E.** (2004). Length regulation and dynamics of individual telomere tracks in wild-type *Arabidopsis*. *Plant Cell* **16**: 1959–1967.
- Shakirov, E.V., Surovtseva, Y.V., Osbun, N., and Shippen, D.E.** (2005). The *Arabidopsis* Pot1 and Pot2 proteins function in telomere length homeostasis and chromosome end protection. *Mol. Cell. Biol.* **25**: 7725–7733.
- Shore, D.** (2001). Telomeric chromatin: Replicating and wrapping up chromosome ends. *Curr. Opin. Genet. Dev.* **11**: 189–198.
- Smogorzewska, A., and de Lange, T.** (2004). Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem.* **73**: 177–208.
- Song, X., Leehy, K., Warrington, R.T., Lamb, J.C., Surovtseva, Y.V., and Shippen, D.E.** (2008). STN1 protects chromosome ends in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**: 19815–19820.
- Surovtseva, Y.V., Churikov, D., Boltz, K.A., Song, X., Lamb, J.C., Warrington, R., Leehy, K., Heacock, M., Price, C.M., and Shippen, D.E.** (2009). Conserved telomere maintenance component 1 interacts with STN1 and maintains chromosome ends in higher eukaryotes. *Mol. Cell* **36**: 207–218.
- Surovtseva, Y.V., Shakirov, E.V., Vespa, L., Osbun, N., Song, X., and Shippen, D.E.** (2007). *Arabidopsis* POT1 associates with the telomerase RNP and is required for telomere maintenance. *EMBO J.* **26**: 3653–3661.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S.** (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596–1599.
- Vannier, J.-B., Depeiges, A., White, C., and Gallego, M.E.** (2006). Two roles for Rad50 in telomere maintenance. *EMBO J.* **25**: 4577–4585.
- Yang, S.W., Jin, E.S., Chung, I.K., and Kim, W.T.** (2002). Cell cycle-dependent regulation of telomerase activity by auxin, ABA and protein phosphorylation in tobacco BY-2 suspension culture cells. *Plant J.* **29**: 617–626.
- Yang, S.W., Kim, D.H., Lee, J.J., Chun, Y.J., Lee, J.-H., Kim, Y.J., Chung, I.K., and Kim, W.T.** (2003). Expression of the telomeric repeat binding factor gene *NgTRF1* is closely coordinated with the cell division program in tobacco BY-2 suspension culture cells. *J. Biol. Chem.* **278**: 21395–21407.
- Yang, S.W., Kim, S.K., and Kim, W.T.** (2004). Perturbation of *NgTRF1* expression induces apoptosis-like cell death in tobacco BY-2 cells and implicates NgTRF1 in the control of telomere length and stability. *Plant Cell* **16**: 3370–3385.
- Yoo, H.H., Kwon, C., Lee, M.M., and Chung, I.K.** (2007). Single-stranded DNA binding factor AtWHY1 modulates telomere length homeostasis in *Arabidopsis*. *Plant J.* **49**: 442–451.
- Wang, R.C., Smogorzewska, A., and de Lange, T.** (2004). Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell* **119**: 355–368.
- Watson, J.M., and Shippen, D.E.** (2007). Telomere rapid deletion regulates telomere length in *Arabidopsis thaliana*. *Mol. Cell. Biol.* **27**: 1706–1715.
- Wu, L., et al.** (2006). Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. *Cell* **126**: 49–62.
- Zellinger, B., Akimcheva, S., Puizina, J., Schirato, M., and Riha, K.** (2007). Ku suppresses formation of telomeric circles and alternative telomere lengthening in *Arabidopsis*. *Mol. Cell* **27**: 163–169.
- Zhang, Z., Fan, H.Y., Goldman, J.A., and Kingston, R.E.** (2007). Homology-driven chromatin remodeling by human RAD54. *Nat. Struct. Mol. Biol.* **14**: 397–405.
- Zuckerklund, E., and Pauling, L.** (1965). Evolutionary divergence and convergence in proteins. In *Evolving Genes and Proteins*, V. Bryson and H.J. Vogel, eds (New York: Academic Press), pp. 97–166.