Compromised telomere maintenance in hypomethylated *Arabidopsis thaliana* plants

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ABSTRACT

Telomeres, nucleoprotein structures at the ends of linear eukaryotic chromosomes, are important for the maintenance of genomic stability. Telomeres were considered as typical heterochromatic regions, but in light of recent results, this view should be reconsidered. Asymmetrically located cytosines in plant telomeric DNA repeats may be substrates for a DNA methyltransferase enzyme and indeed, it was shown that these repeats are methylated. Here, we analyse the methylation of telomeric cytosines and the length of telomeres in *Arabidopsis thaliana* methylation mutants (*met 1-3* and *ddm 1-8*), and in their wild-type siblings that were germinated in the presence of hypomethylation drugs. Our results show that cytosine methylation in telomeric repeats depends on the activity of MET1 and DDM1 enzymes. Significantly shortened telomeres occur in later generations of methylation mutants as well as in plants germinated in the presence of hypomethylation drugs, and this phenotype is stably transmitted to the next plant generation. A possible role of compromised *in vivo* telomerase action in the observed telomere shortening is hypothesized based on telomere analysis of hypomethylated telomerase knockout plants. Results are discussed in connection with previous data in this field obtained using different model systems.

INTRODUCTION

Telomeres are specialized nucleoprotein structures, usually formed by minisatellite DNA repeat sequences located at the ends of linear eukaryotic chromosomes. Telomeres are essential for maintenance of genomic integrity, compensating for the replicative loss of DNA at chromosomal termini and distinguishing natural chromosome ends from chromosome breaks [reviewed in (1)]. The basic functions of telomeres are performed in chromatin context. Telomere repeats and adjacent subtelomeric regions are associated with histones and non-histone proteins, including a number of telomere-specific proteins.

Regulation of gene expression and chromatin structure via epigenetic mechanisms has been convincingly documented using many model organisms [reviewed in (2)]. Two kinds of modifications of macromolecules are crucial for epigenetic regulation: DNA methylation and modifications of histone proteins [reviewed in (3)]. Furthermore, regulatory roles of small and non-coding RNAs have been established (4). These mechanisms modulate the dynamics of chromatin structure governing activation/repression of resident genes, e.g. in response to developmental and environmental stimuli. The general epigenetic landscape in plants is considerably more varied compared with that in animals. (i) Cytosines located in CG, CHG (H = A, T, C) and asymmetrical CHH sequences can be methylated in plant genomes, while predominantly CG methylation and a lower level of non-CG methylation during specific developmental phases were reported in animal cells [(5), reviewed in (6)]. Methylation of asymmetrically localized cytosines in plant telomeric CCCTAAA sequences was reported in

Arabidopsis thaliana (7,8) and in tobacco (9). (ii) Genes encoding enzymes catalysing DNA demethylation during specific developmental phases and at specific genomic loci were identified in *Arabidopsis* (10,11), while in mammalian cells, demethylation is linked to base excision repair processes (12). (iii) The plant-specific RNA polymerases IV and V (13,14) catalyse the synthesis of RNA molecules involved in RNA-directed DNA methylation pathways.

The involvement of epigenetic mechanisms in the regulation of telomere homoeostasis is a popular research topic and studies in this field have been carried out using various animal models. In these cells, telomeric tracts, as well as adjacent subtelomeric regions, are maintained in a heterochromatic state associated with heterochromatin-specific histone modification. Nevertheless, in recent studies using mouse embryonic fibroblasts, association of telomeres with both the heterochromatin-specific (H3K9me3) and the euchromatin-specific (H3K4me3) epigenetic marks were reported, although the H3K4me3 loading was lower than that of H3K9me3 (15), and the level of heterochromatic marks was surprisingly low at telomeres in human fibroblasts (16) and T-cells (17). While mammalian telomeres lack CG sequences, the natural substrate of known mammalian DNA methyltransferases, in human somatic cells, the subtelomeric repeats are CG-rich and methylated (18,19). The importance of subtelomeric DNA methylation and heterochromatin-specific modifications of telomeric histones for telomere homoeostasis was reported in both human and mouse cells, where loss of heterochromatin-specific modifications led to significant lengthening of telomeres and in some cases, to an increase in telomere recombination (20–25). In contrast to these studies, Roberts *et al.* (26) reported that telomere lengths were not affected in mouse epigenetic mutants, challenging the idea of epigenetic control of telomere homoeostasis in mammalian cells.

In the classic model, and similarly as in mammalian cells, plant telomeres were viewed as heterochromatic loci (27). A more recent study, however, characterized *A. thaliana* telomeric chromatin as an intermediate heterochromatin possessing both hetero- and euchromatin-specific histone modifications (8). Vaquero-Sedas *et al.* (28) even concluded that telomeres of *A. thaliana* exhibit predominantly euchromatic features, while subtelomeres and interstitial telomeric sequences are of a heterochromatic nature.

Other factors possibly involved in telomere homoeostasis are telomeric transcripts [telomeric repeat containing RNA (TERRA)], the discovery of which has challenged the long-standing opinion that telomeres are transcriptionally inert (29). Although intensively studied, connections between TERRA and telomerase activity/telomere homoeostasis are far from clear. TERRA and transcription of telomeres do not affect telomere lengths, and TERRA does not inhibit telomerase activity *in vivo* in human cancer cells (30). Conversely, telomere length-dependent inhibition of telomerase activity *in vitro* by TERRA (31), binding of TERRA to the RNA subunit of telomerase (hTR) and partially to the telomerase catalytic subunit (hTERT) in human cells (32), and telomerase-independent telomere shortening induced by

up-regulation of TERRA in *Saccharomyces cerevisiae* (33), have been reported. In plants, the presence of TERRA transcripts has been reported in *Arabidopsis* (8) and in tobacco BY-2 cells (9).

Here, we examine the length and methylation of telomeres in *A. thaliana* methylation mutants (*met 1-3* and *ddm 1-8*), and in wild-type (wt) plants that were germinated in the presence of hypomethylation agents. The *MET1* gene encodes the DNA methyltransferase (34) responsible for maintaining methylation of cytosines located in CG sequences. Proper *MET1* gene function is crucial for the maintenance of the general epigenetic pattern, including CG and non-CG methylation, histone modifications and chromatin structure (35). The *DDM1* gene encodes a protein with similarities to the SWI/SNF family of chromatin remodelling factors (36). A mutation in this gene leads to a significant decrease in the overall level of DNA methylation (37).

Zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidine-2-one, ZEB] is a cytidine analogue (38) that is stable in aqueous solution and has relatively low toxicity [reviewed in (39)]. Its hypomethylation effect is similar to that of the traditionally used cytidine analogues, 5-azacytidine and 5-aza-2′-deoxycytidine (40). These compounds are incorporated into DNA during replication and form stable complexes with DNA methyltransferases, thereby reducing their methylation activity. In the model plants *A. thaliana* (41), and *Nicotiana tabacum* (9), ZEB-induced loss of DNA methylation was described.

DHPA [(S)-9-(2,3-dihydroxypropyl)adenine] (42) is a competitive inhibitor of *S*-adenosyl-L-homocysteine hydrolase, the enzyme that degrades *S*-adenosyl-L-homocysteine, a by-product of transmethylation reactions and a potent inhibitor of all methyltransferases, to homocysteine and adenosine. A number of experimental studies have been carried out using this drug for epigenetic modifications, including its application in plant systems (9,43–46).

We observe distinct telomere shortening in chemically hypomethylated plants and in later generations of methylation mutants. The shortening of telomeres neither correlates with the change in transcription of the telomerase reverse transcriptase (*AtTERT*) gene nor with telomerase activity assayed *in vitro*. Hypotheses considering *in vivo* processes involved in telomere shortening and the role of telomerase in the maintenance of shortened telomeres are presented.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana seeds of the Columbia-0 ecotype, *DDM1* (At5g66750) mutant (*ddm1-8* strain, SALK000590) and *TERT* (At5g16850) mutant (*tert−/−*, SALK061434) were purchased from the Nottingham Arabidopsis Stock Centre (47); seeds of the mutant plant with a T-DNA insertion in the *MET1* gene [At5g49160, *met1-3* strain, (48)] were kindly provided by Dr Ales Pecinka (GMI, Vienna, Austria). Primers for genotyping are shown in Supplementary Table S1. Seeds were placed on half strength Murashige–Skoog (½ MS, Duchefa) agar

plates and grown under cycles of 8 h light (illumination 100 mmol m^{-2} s^{-1}), 21°C and 16 h dark, 19°C. Plants were grown in soil under the same light/dark conditions favouring leaf growth; 1-month-old plants were then cultivated using 16 h/8 h light/dark cycles, accelerating flowering and seed development. Leaves were harvested from 2-month-old plants.

Arabidopsis thaliana Columbia-0 and *tert*−/− seeds were germinated for 7 days on agar plates containing ½ MS medium supplemented with ZEB (Sigma) or DHPA at concentrations of 100 and 250 μM. Plants were then grown in soil as described earlier.

Analysis of telomere lengths by the terminal restriction fragment method

Analysis of telomere length by the terminal restriction fragment (TRF) method is based on the digestion of genomic DNA by a frequently cutting restriction endonuclease without a recognition site in the G-rich telomeric sequences. After hybridization with a radioactively labelled telomeric oligonucleotide probe, the signal corresponds to non-digested telomeric tracts (plus subtelomeric regions up to the first restriction site upstream of the telomeres). Analyses were performed as previously described (49,50).

Analysis of cytosine methylation in telomeric repeats

Bisulfite conversion of genomic DNA was carried out using the EpiTect Bisulfite Kit (Qiagen). During the bisulfite treatment, non-methylated cytosines are converted to uracils and amplified as thymines in the subsequent PCR, while 5-methylcytosines are resistant to this reaction (51). To analyse the methylation of all cytosines located in telomeric repeats, we followed the protocol described in (8), with modifications (9). Bisulfite-modified DNA (300 ng) was transferred to a Hybond XL membrane (GE Healthcare) by vacuum dot blotting (Bio-Rad Dot Blot). Membranes were hybridized overnight at 42°C with [^{32}P] labelled oligonucleotide probes in ULTRAhybTM–Oligo Hybridization Buffer (Ambion). A 'loading' probe (CCCTAAA)$_4$ was used as a loading normalizer, and the 'degener' probe (TTAGRRT)$_4$, where R = A or G, detected telomeres where the third cytosine of the (ACCCTAA)$_n$ repeat was methylated, and other cytosines were either methylated or non-methylated. After washing under low stringency conditions (twice at 50°C for 30 min in 2× SSC, 0.1% SDS), hybridization signals were visualized using the FLA7000 phosphoimager (FujiFilm) and analysed using the MultiGauge software (FujiFilm).

Methylation of telomeric cytosines located in the proximal part of the 1L chromosome arm telomere was analysed as described in (8). PCR primer sequences for amplification of the fragment containing telomeric repeats are shown in Supplementary Table S1. PCR was carried out using DyNAzymeII DNA Polymerase (Finnzymes) in a cycle consisting of initial denaturation (2 min), 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, followed by final extension (72°C/8 min). PCR products were cloned using a TOPO TA Cloning Kit

(Invitrogen) and sequenced (Macrogene). Methylation of cytosines was analysed by CyMATE software (52) in lengths of ∼390 bp, where the sequencing signals were clear. Statistical evaluation was done by one-way weighted analysis of variance (ANOVA), a statistical tool used for simultaneous analysis of differences between two or more means. Seventeen clones from three Columbia plants, 11 clones from two *ddm1-8* G2 plants, 12 clones from two *met1-3* G2 plants and 4 clones from a segregated wt plant were analysed. Samples were taken as independent.

To analyse relative methylation of cytosines located in terminal and internal telomeric repeats, high-molecular weight DNA was isolated and digested by Bal31 nuclease as described (53,54). DNA in agarose plugs was either digested by Tru1I (MseI) restriction endonuclease (Fermentas/Thermo Fisher Scientific) and analysed by TRF, or isolated by QIAEX II Gel extraction Kit (Qiagen), modified by sodium bisulfite, spotted by vacuum blotting onto nylon membrane and hybridized with radioactively labelled 'loading' and 'degener' probes as described earlier.

RESULTS

Methylation of cytosines in telomeric repeats is significantly lower in hypomethylated plants

Telomere length and methylation of cytosines in telomeric repeats were assayed in hypomethylated *A. thaliana* var. Columbia-0 plants in which hypomethylation was induced by (i) loss of function of the gene essential for the maintenance of a stable methylation pattern in *met1-3* and *ddm1-8* mutants and (ii) germinating seeds of wt plants in the presence of the hypomethylation drugs, ZEB or DHPA (Figure 1). To verify the loss of DNA methylation in mutant plants and in seedlings germinated in the presence of hypomethylation drugs, methylation of cytosines in a 180-bp centromeric repeat was investigated using the methylation-sensitive restriction endonuclease HpaII (see Supplementary Methods). Methylation was significantly lower in leaves of mutant plants and in seedlings germinated in the presence of hypomethylation drugs (Supplementary Figure S1), as previously reported (41,48). In leaves of wt plants segregated from the *met1-3* mutant background, the methylation was almost comparable to samples without a mutant history, although in some individuals bands evidencing the non-methylated cytosines in the CCGG sequence motif were visible (Supplementary Figure S1A). In mature leaves of DHPA-treated plants that were grown in soil without the drugs, methylation returned to control levels (Supplementary Figure S1B), consistent with the transient hypomethylation effect described for this drug (44). Variable patterns of centromeric repeat methylations were observed in plants germinated in the presence of ZEB and even in the progenies of plants affected by 250 μM ZEB, although in these cases, the levels of non-methylated cytosines in CCGG sequences were significantly lower than those in ZEB-treated seedlings (Supplementary Figure S1B).
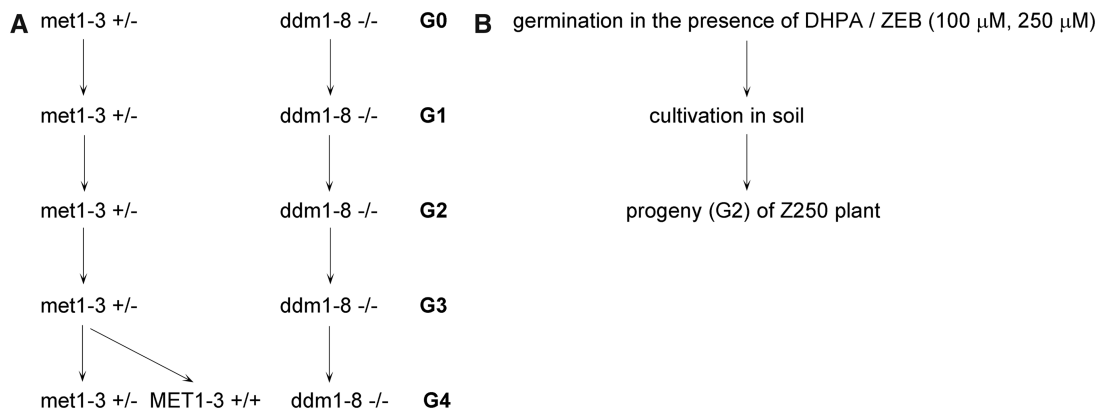
A met1-3 +/−   ddm1-8 -/-   **G0**   **B** germination in the presence of DHPA / ZEB (100 µM, 250 µM)

met1-3 +/−   ddm1-8 -/-   **G1**   cultivation in soil

met1-3 +/−   ddm1-8 -/-   **G2**   progeny (G2) of Z250 plant

met1-3 +/−   ddm1-8 -/-   **G3**

met1-3 +/- MET1-3 +/+   ddm1-8 -/-   **G4**

Figure 1. Schematic picture of the experimental strategy. (**A**) *met1-3* and *ddm1-8* mutant plants were propagated and genotyped in each generation. Material for analyses was collected from *ddm1-8−/−* homozygous individuals and *met1-3+/−* heterozygous plants; *met1-3−/−* homozygous mutants were selected with extremely low frequency and did not grow to the reproductive stage (48). G0 of *ddm1-8* plants represents the T3 progeny of the original accession. In the fourth generation (G4), segregated wt plants (*MET1-3+/+*) were selected for analysis of telomere length. (**B**) *Arabidopsis thaliana* seedlings were germinated in the presence of hypomethylation drugs DHPA or ZEB for 7 days. Plants were then cultivated in soil, and after 9 weeks, leaves were collected for analysis. Progenies (G2) of plants that had been treated with 250 µM ZEB (Z250) were grown for telomere length analysis.

To analyse cytosine methylation in telomeric repeats regardless of their chromosomal position, bisulfite-converted DNA was spotted onto nylon membrane and hybridized with a radioactively labelled probe that detected methylated and partially methylated telomeric repeats ('degener' probe). Relative differences between signals from *A. thaliana* wt plants compared with methylation mutants demonstrated a loss of methylated telomeric cytosines within the mutant background (Figure 2). A similar pattern was observed when populations of seedlings germinated in the presence of 250 µM DHPA and 250 µM ZEB were analysed—a distinctly lower hybridization signal using the 'degener' probe in hypomethylated seedlings as compared with the control non-treated sample. Hypomethylation did not persist in mature leaves of drug-germinated plants or in wt plants segregated from the *met1-3* mutant background; in these samples, methylation of telomeric cytosines recovered to the level of control/wt plants (Figure 2).

Results obtained from the above analyses provide information about methylation of cytosines in telomeric repeats in terminal (in telomeres) and internal (in ITSs) chromosomal locations within the *A. thaliana* genome. Because ITSs represent a relatively large proportion of all telomeric repeats [20–70%, depending on the methodology used and sequence accuracy, (55,56)], we followed the approach described in (8) and carried out a detailed analysis of methylation in a specific telomeric region; the proximal part of the 1L chromosome arm telomere. According to bisulfite genomic sequencing data from wt Columbia leaves, almost 60% of all asymmetrically located cytosines and >60% of cytosines located in perfect telomeric repeats are methylated (Figure 3A and B). These data demonstrate, at least for this specific region, the presence of methylated cytosines in *A. thaliana* telomeres. In both *met1-3* and *ddm1-8* methylation mutants, the number of methylated cytosines in asymmetrical sequences, within and outside of telomeric repeats, is significantly lower compared with Columbia wt leaves (Figure 3). In wt segregated from the *met1-3*

mutant, methylation recovered almost to the Columbia wt level, although a reduced level of methylation was observed in cytosines located outside of perfect telomeric repeats. Based on these data, methylation of cytosines in telomeric repeats appears to be dependent on the activity of enzymes that maintain a stable methylation pattern.

Although dot-blot analyses revealed cytosine hypomethylation in telomeric repeats of DHPA- and ZEB-treated seedlings (Figure 2, middle panel), no such trend was observed for cytosines located in the proximal 1L chromosome arm telomere; methylation patterns of asymmetrically located cytosines within and outside of perfect telomeric repeats, in treated seedlings (Supplementary Figure S2) and in leaves of plants germinated in the presence of drugs (not shown), were similar to controls.

Bearing in mind previous contradictory data revealing the presence (7,8) or absence (28) of methylation of telomeric cytosines in *A. thaliana*, we aimed to determine the relative level of methylation at terminally located telomeric repeats. To selectively degrade telomeres, high-molecular weight DNA from *A. thaliana* leaves was digested by Bal31 exonuclease. Telomere shortening by Bal31 was verified by TRF analysis (Figure 4A); a significant loss of the telomere-specific signal during Bal31 digestion was observed, while signals from ITSs were comparable in Bal31-treated and non-treated samples. DNA was isolated from agarose plugs, modified by sodium bisulfite, spotted onto nylon membrane and hybridized with radioactively labelled 'loading' and 'degener' probes (Figure 4B). The reduction in the telomere-specific signal from the loading probe in Bal31-digested samples (to about one half as compared with the non-digested control) demonstrated the loss of terminal telomeric repeats. Comparison of the hybridization signals obtained using the 'degener' probe relative to the 'loading' probe did not reveal any statistically significant differences and showed a relatively stable methylation of telomeric cytosines during BAL31 digestion (Figure 4C); this demonstrated that methylation of cytosines in telomeres and in interstitial telomeric repeats was similar.
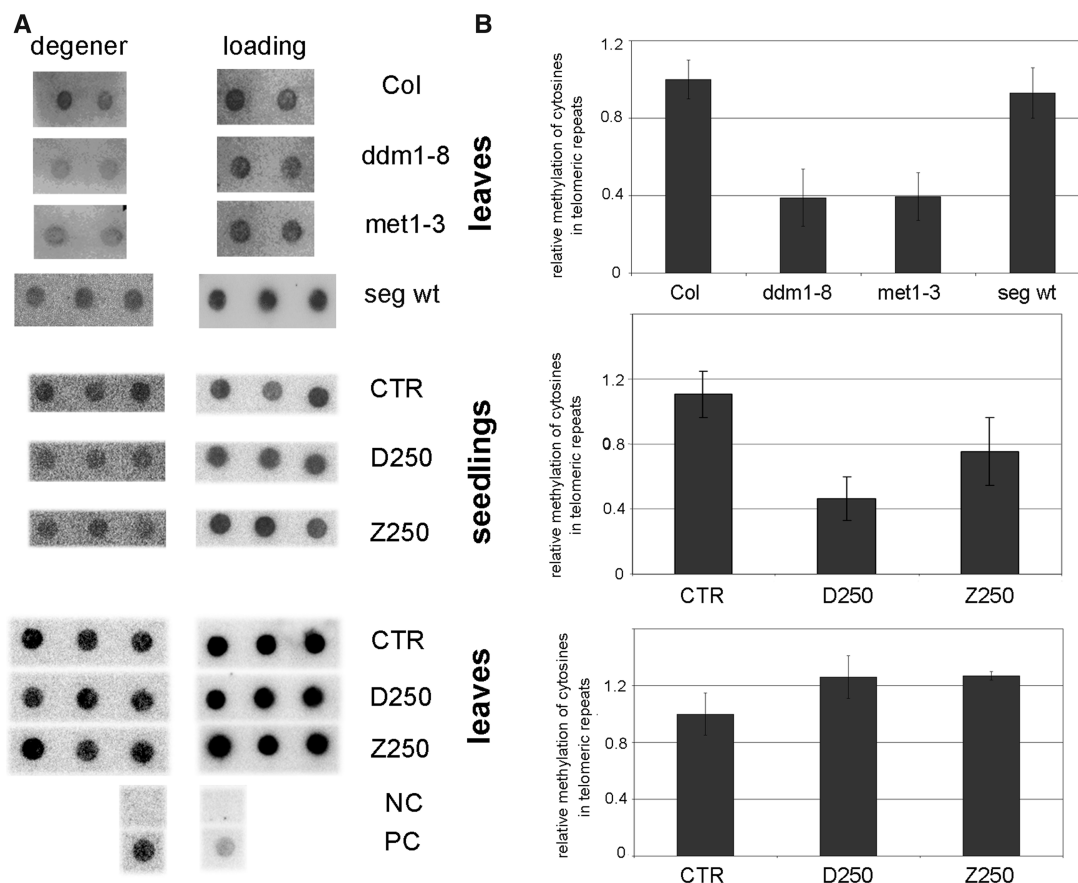
Figure 2. Analysis of general methylation of telomeric cytosines. (**A**) About 300 ng of bisulfite modified DNA isolated from plant tissues was spotted onto the membrane and hybridized with the radioactively labelled 'degener' probe (TTAGRRT)$_4$, R = A or G, which generated a signal from methylated and partially methylated fractions, and the 'loading' probe (CCCTAAA)$_4$ complementary to the G-strand of telomeres, which was used for normalization. Top panel: for analysis of *ddm1-8* and *met1-3* methylation mutants, leaves of G2 plants were collected from 10 individuals, two representatives are presented; 10 Columbia wt (Col; two presented) and 7 wt plants segregated from the *met1-3* mutant (seg wt; three presented) were analysed. Middle panel: analysis of seedlings grown on control medium (CTR), medium supplemented with 250 µM DHPA (D250) and 250 µM ZEB (Z250); three biological replicates were analysed. Bottom panel: analysis of mature leaves from three representative plants grown from drug-treated seedlings. NC, negative control, DNA from the plasmid pUC19; PC, positive control, non-converted genomic DNA isolated from *A. thaliana* leaves. (**B**) Relative methylation of telomeric cytosines in hypomethylated *A. thaliana* tissues. Hybridization signals were evaluated by the MultiGauge software (FujiFilm), and expressed as the 'degener'/'loading' ratio. Signals of Columbia leaves and control non-treated samples (CTR) were arbitrary taken as 1 in respective analyses.

Telomere shortening in hypomethylated *A. thaliana* plants and in plants with a history of hypomethylation

Stable telomere length is important for genomic stability. We determined the lengths of telomeric repeats to establish whether *A. thaliana* genomic hypomethylation influences telomere homoeostasis. In Columbia wt plants, telomeres were ∼3 kb long with some variations between individuals (Figure 5). Up to the second generation (G2) of methylation mutants, telomeres were maintained at about wt lengths, although some individuals with shorter telomeres were observed, even in G1 and G2. In G3, telomeres of mutants were markedly shortened by ∼500–700 bp in *met1-3* plants and even more than 1 kb in some *ddm1-8* individuals. Telomere shortening continued into the next generation (G4) although in this case, plants with shortened telomeres as distinct as in G3 were not observed (Figure 5A).

Telomeres in plants that had been treated by hypomethylation drugs during germination were also distinctly shorter, and the shortening was more pronounced in ZEB-treated plants. While telomeres of plants germinated in the presence of DHPA were ∼500 bp shorter compared with non-treated controls, in ZEB-treated individuals, shortening by more than 1 kb was observed (Figure 5B). Telomere shortenings in plants that were grown in the presence of different concentrations of methylation inhibitors (100 and 250 µM) were similar, thus independent of the drug concentrations used.

To investigate possible recovery from telomere shortening induced by genome hypomethylation, telomere lengths were analysed in wt plants segregated from the *met1-3* mutant background, and in the progenies of plants treated with 250 µM ZEB (Figure 1). Telomeres in both groups remained markedly shorter (Figure 5A and B), demonstrating meiotic stability of this phenotype.

Involvement of telomerase in telomere shortening of hypomethylated *A. thaliana* plants

Telomerase is an enzyme responsible for the elongation of telomeres, and loss of its function leads to progressive
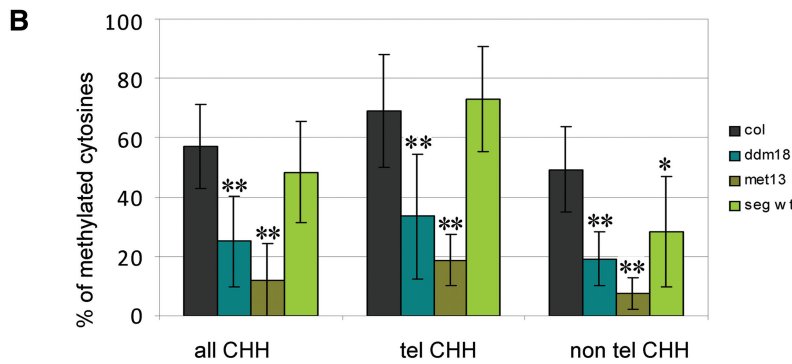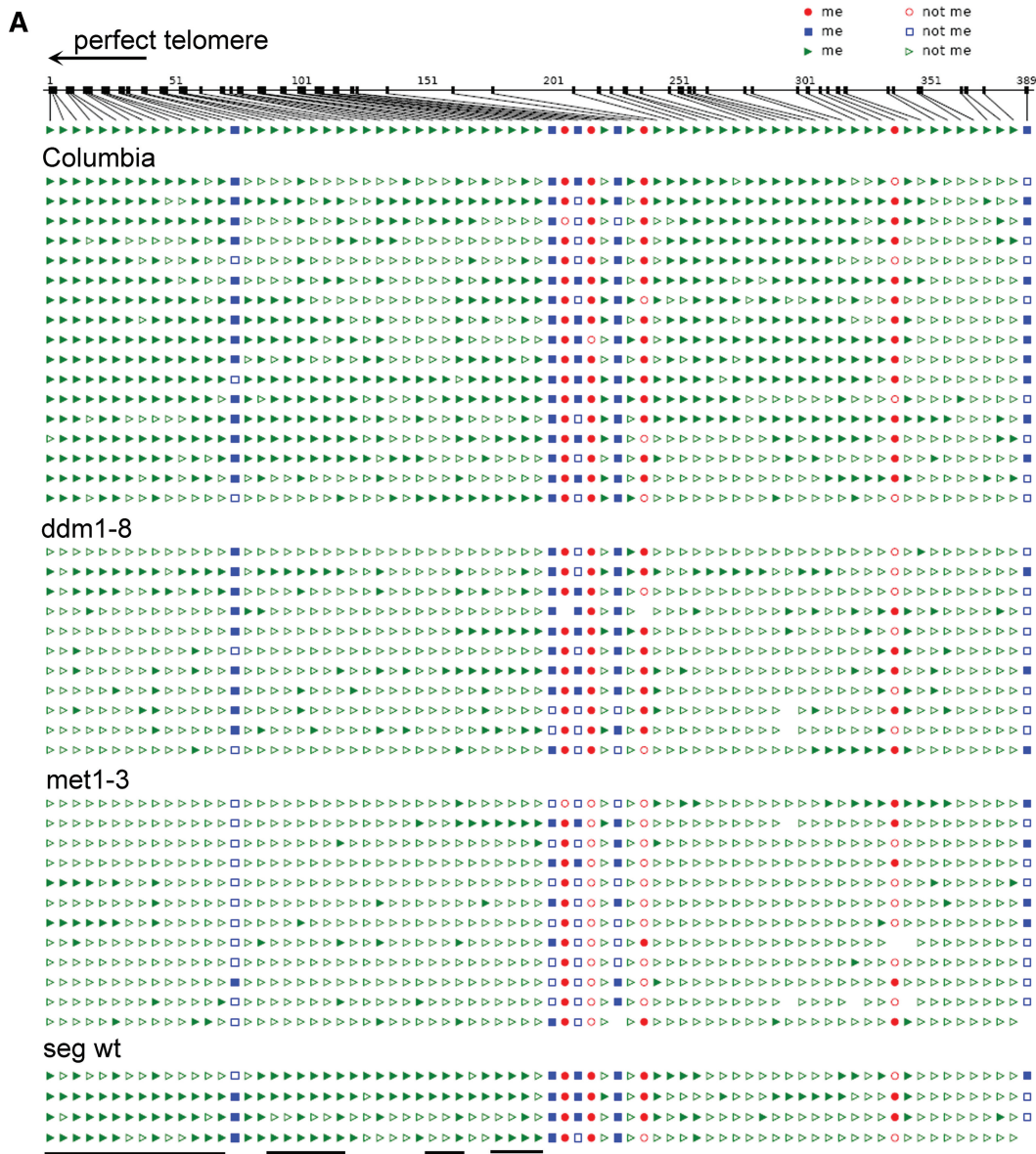
Figure 3. Analysis of cytosine methylation in the proximal part of the 1L telomere arm. (**A**) Distribution of methylated cytosines along the 389-bp region, which is delimited by primers derived from the subtelomeric region and a specific insertion into the 1L telomere (8). Seventeen clones from three Columbia plants, 11 clones from two *ddm1-8* G2 plants, 12 clones from two *met1-3* G2 plants and 4 clones from a segregated wt plant (seg wt) were analysed. Red circles, CG methylation; blue squares, CHG methylation; green triangles, CHH methylation; filled symbols, methylated cytosine; empty symbols, non-methylated cytosine. Positions of cytosines located in perfect telomeric repeats are delimited by the black lines below the figure. The arrowhead determines the direction to the perfect telomere. (**B**) Graphical representation of the level of all methylated cytosines located in non-asymmetrical sequences (all CHH), in perfect telomeric repeats (tel CHH) and outside these repeats (non-tel CHH). Standard deviations reflect variability between clones. The level of methylated cytosines is significantly lower in methylation mutant clones (**$P < 0.01$ for all sequence contexts). In the segregated wt, methylation of cytosines outside of telomeric repeats (non-tel CHH) is reduced (*$P < 0.05$).
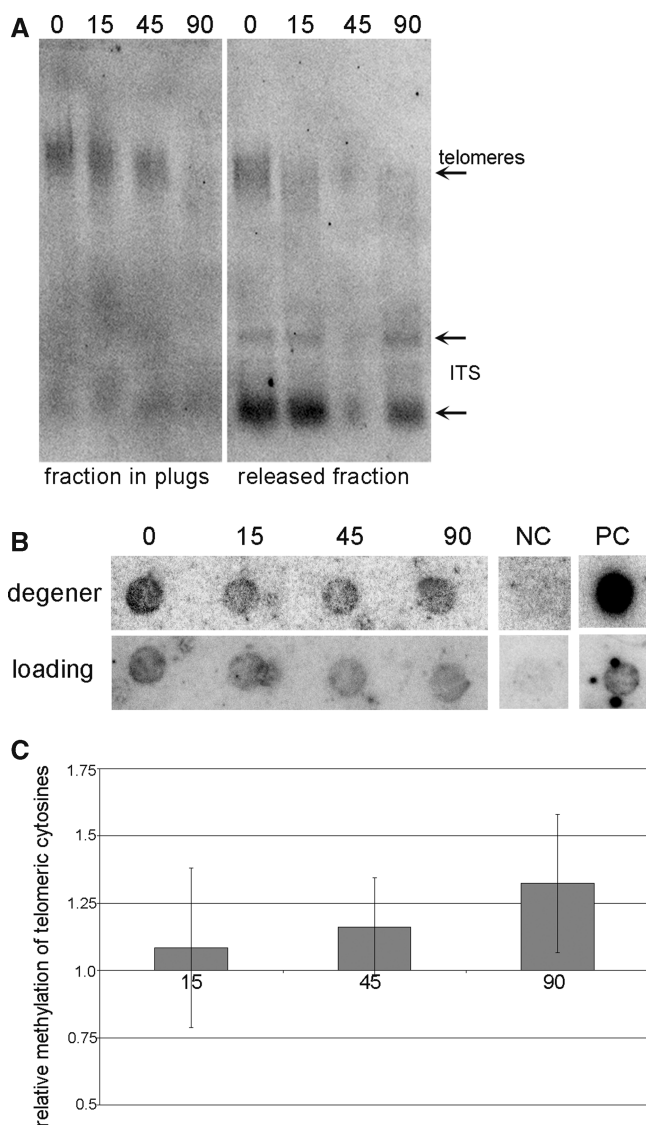
Figure 4. Analysis of relative methylation of cytosines in telomeric repeats. (**A**) High-molecular weight DNA in agarose plugs was digested by Bal31 exonuclease and analysed by TRF. The fraction retained in agarose plugs and the fraction released into the solution were analysed separately. The time of Bal31 digestion (in min) is marked above the lines. The positions of telomeres and ITSs are depicted by arrowheads. (**B**) DNA isolated from agarose plugs was modified by sodium bisulfite and analysed by Southern hybridization with radioactively labelled 'loading' and 'degener' probes. The time of BAL31 digestion (in min) is depicted. NC, negative control, DNA from the plasmid pUC19; PC, positive control, non-modified genomic DNA isolated from *A. thaliana* leaves. (**C**) Relative methylation of telomeric cytosines in Bal31-digested samples. Hybridization signals were evaluated by the MultiGauge software (FujiFilm), and expressed as the 'degener'/'loading' ratio. The ratio in BAL31 non-digested samples was arbitrary taken as 1. Five independent experiments were evaluated.

telomere shortening during subsequent plant generations (50,57). To determine whether telomerase dysfunction is involved in the process of telomere shortening observed in later generations of *met1-3* and *ddm1-8* mutants, and in plants germinated in the presence of hypomethylation drugs, telomerase activity and transcription of the gene encoding the telomerase protein subunit (*AtTERT*) were

analysed in buds, i.e. in tissue with high telomerase activity (58). Telomerase activity and *AtTERT* gene transcription were assayed in generations G2 and G4 of *met1-3* and *ddm1-8* relative to the wt (see Supplementary Methods). Neither telomerase activity nor *AtTERT* transcription was significantly affected in any of the mutants (Supplementary Figure S3). Correspondingly, in plants germinated in the presence of hypomethylation drugs, telomerase activity and transcription were at control levels in telomerase-positive tissues investigated, in 7-day seedlings and in buds (data not shown). Therefore, in *A. thaliana* plants, neither *AtTERT* transcription nor telomerase activity (as examined by assay *in vitro*) was sensitive to hypomethylation.

In addition to telomerase, other proteins involved in telomere homoeostasis in *A. thaliana* have been described. Nevertheless, transcription of six selected genes coding for such proteins [telomerase RNA subunits *AtTER1*, *AtTER2* (59), *AtPOT1a* (60), *ATPOT1b* (61,62), *AtTRB1* (63), *AtCTC1* (60)] was at wt levels in buds collected from G2 and G4 generations of *met1-3* and *ddm1-8* mutants, as well as in buds of plants treated with hypomethylation drugs (data not shown). In addition, analysis of TERRA transcripts (see Supplementary Methods) using primers specific for 2R, 3L and 5L chromosome arms (8) did not reveal significant differences between individuals germinated in the presence of 250 µM DHPA or 250 µM ZEB, except for slightly elevated levels of TERRA derived from the 3L chromosome arm (Supplementary Figure S4).

Due to the complexity of the process of telomere maintenance, it is tricky to obtain unambiguous information on possible *in vivo* involvement of telomerase in telomere shortening. We analysed telomere lengths in plants with loss of telomerase function; in *tert−/−* mutants (50,57), germinated in the presence and absence of 250 µM ZEB. According to the working hypothesis, if telomere shortening in hypomethylated *A. thaliana* plants is dependent on telomerase action, then the lengths of telomeres in hypomethylated *tert−/−* mutants would be similar to those in *tert−/−* mutants germinated in control medium because active telomerase is entirely absent here and therefore cannot be modified by hypomethylation during germination. If any telomerase-independent factor participates in telomere shortening, both effects will be additive and presumably telomeres will be even shorter in hypomethylated *tert−/−* mutant plants. As shown in the TRF profiles in Figure 6, telomere lengths in hypomethylated *tert−/−* plants remained the same as those in mutants germinated in control medium. Although these experiments suggest involvement of telomerase in the observed telomere shortening in hypomethylated plants, they do not identify directly a specific underlying step in telomerase *in vivo* regulation.

DISCUSSION

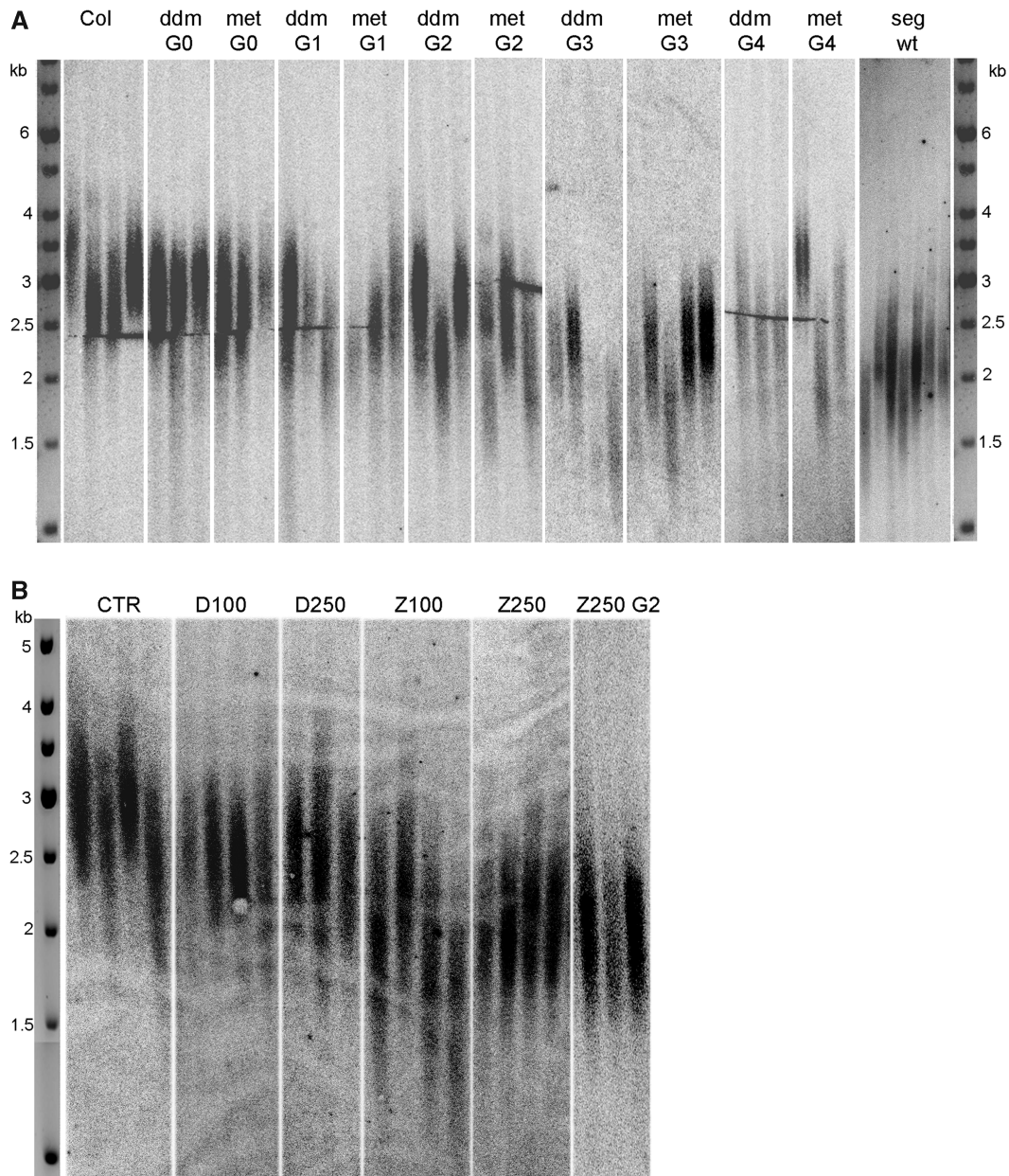The long-established view of plant telomeres as heterochromatic loci was challenged by the recent data

Figure 5. Telomere lengths in hypomethylated *A. thaliana* plants. Lengths of telomeres were analysed by the TRF method in methylation mutants (**A**) and in plants germinated in the presence of hypomethylation drugs (**B**). (A) G0–G4 generations of *ddm1-8* and *met1-3* mutant plants and plants without T-DNA insertion segregated from *met1-3* G3 mutants (seg wt, for details, see Figure 1) were analysed. (B) CTR, control non-treated *A. thaliana* plants; D100, plants germinated in the presence of 100 μM DHPA; D250, plants germinated in the presence of 250 μM DHPA; Z100, plants germinated in the presence of 100 μM ZEB; Z250, plants germinated in the presence of 250 μM ZEB; Z250 G2, progenies of plants germinated in the presence of 250 μM ZEB.

demonstrating the presence of both heterochromatin- and euchromatin-specific epigenetic modifications of telomeric histones in *A. thaliana* (8). Following an independent study by another group (28), telomeric sequences were hypothesized to have a euchromatic character for *A. thaliana* telomeres and a heterochromatic character for interstitial telomeric sequences. Even in the field of mammalian telomeric chromatin, where data on the importance of heterochromatin-specific modifications for telomere stability were collected mainly using mouse models [reviewed in (64)], the general doctrine of telomeric heterochromatin was challenged by observations in

human cells (16,17). Contrary to the universality of the genetic code, there is no unique or generally valid epigenetic code, and data obtained in different model systems are required to shed more light on the epigenetic nature of telomeric chromatin and to distinguish between specific features of a model system and more general principles.

Methylation of telomeric cytosines is dependent on the activity of MET1 a DDM1 enzymes

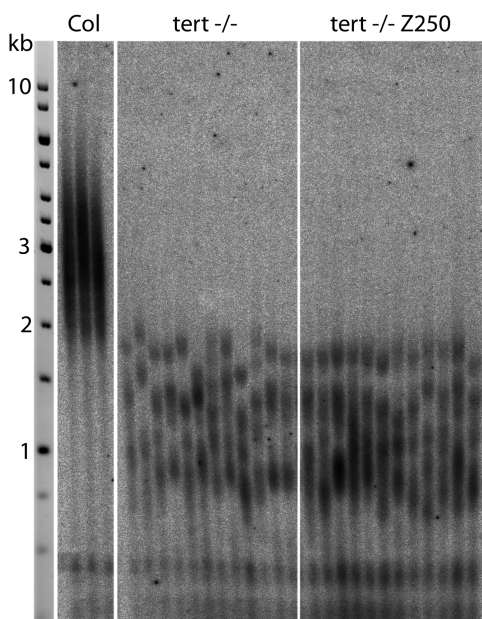*Arabidopsis thaliana* is an excellent model organism for epigenetic studies due to the accessibility of mutant line

Figure 6. Telomere lengths in hypomethylated telomerase mutant (*tert−/−*) plants. Lengths of telomeres were analysed by TRF in leaves of *tert−/−* plants and *tert−/−* plants germinated for 7 days in the presence of 250 μM ZEB (*tert−/−* Z250).

collections, including lines with loss-of-function of important epigenetic factors. On the other hand, hypomethylation induced by chemical drugs may be a relatively easy way to disturb an established epigenetic landscape in a broad range of model organisms. Such compounds were used in a pilot study analysing the epigenetic regulation of telomeres in cultured cells of *N. tabacum* (9).

In our experiments, cytosine methylation, including cytosines in telomeric repeats, was significantly lower in mutant plants with loss of function of enzymes (MET1 and DDM1) that are essential for the maintenance of a stable pattern of methylation. After restoration of the wt allele in a *met1-3* mutant background the methylation in both symmetrically (centromeric repeat; Supplementary Figure S1A) and asymmetrically (telomeres; Figures 2 and 3) located cytosines returned almost to the wt pattern. This agrees with a recent observation that loss of methylation in genic regions is persistent in segregated wt while methylation in another type of sequence, transposable elements, recovers to wt level, suggesting site-specific regulation of DNA methylation (65). Similarly, treatment of *A. thaliana* plants with hypomethylation drugs during germination led to a decreased level of telomeric and centromeric cytosine methylation in seedlings, i.e. during the developmental stage when plant tissue is in direct contact with the drug (Figure 2; Supplementary Figure S1B). During cultivation of plants in soil without drugs, DHPA-induced hypomethylation was completely reversed (Figure 2; Supplementary Figure S1B), as was observed in previous experiments using tobacco plants and cell cultures (44,45). Although the hypomethylating effect of ZEB was also reported as transient (41), in our experiments, distinct

hypomethylation of cytosines located in CCGG sequences was detectable even in the next plant generation (Supplementary Figure S1B). This may be due to a higher concentration of ZEB or a different *A. thaliana* ecotype used in our study. Nevertheless, our data agree with that of 5-azacytidine-induced stable hypomethylation of symmetrically located cytosines, as observed in repetitive sequences in tobacco plants (66). This is also consistent with similar hypomethylation mechanisms of both 5-azacytidine and ZEB drugs.

Although in the leaves of methylation mutants loss of methylation of asymmetrically located cytosines in the proximal part of the 1L telomere arm was demonstrated (Figure 3), no changes were detected in drug-treated seedlings (Supplementary Figure S2). This difference may be attributed to different methods of induction of the hypomethylation state (chemical versus genetic).

Even though it was demonstrated that cytosines located in perfect telomeric repeats in the proximal part of the telomere on the 1L chromosome arm are methylated [(8) and Figure 3; Supplementary Figure S2], and that the extent of this methylation is dependent on the activity of MET1 and DDM1 enzymes (Figure 3), questions remain about the level of true (terminal) telomere methylation in *A. thaliana* (28). Our analyses of the relative methylation of cytosines located in CCCTAAA sequences of Bal-31 digested DNA, i.e. in samples depleted of telomeres (Figure 4), demonstrated that cytosines in telomeres and in interstitial telomeric sequences are methylated to similar extents. The finding that cytosines in *A. thaliana* true telomeres are methylated is consistent with the data on drug-induced hypomethylation of telomeric cytosines in tobacco (9) where significant levels of intrachromosomal telomeric repeats were not found (67).

Compromised telomere maintenance in hypomethylated plants raises questions on underlying mechanism

In our previous work, we demonstrated that in *Arabidopsis,* DNA methylation was not significantly involved in the developmental regulation of transcription of the telomerase catalytic subunit (68). Correspondingly, transcription of the telomerase catalytic subunit and telomerase activity *in vitro* were not affected in later generations of *met1-3* and *ddm1-8* mutants nor in plants treated with hypomethylation drugs during germination (Supplementary Figure S3). Analysis of telomerase activity by *in vitro* TRAP assay only provides information on whether active telomerase is present in the extracted material. The absence of an additive effect of hypomethylation and disruption of telomerase activity (Figure 6) suggests (though indirectly) a possible involvement of telomerase in the observed telomere shortening. Although telomerase activity is essential for telomere elongation, the complex process of telomere maintenance involves numerous factors participating in regulation of telomerase activity *in vivo* which cannot be reflected by the used *in vitro* assays. In principle, the genome hypomethylation may affect telomere homoeostasis by three ways: (i) directly via modulation of telomerase
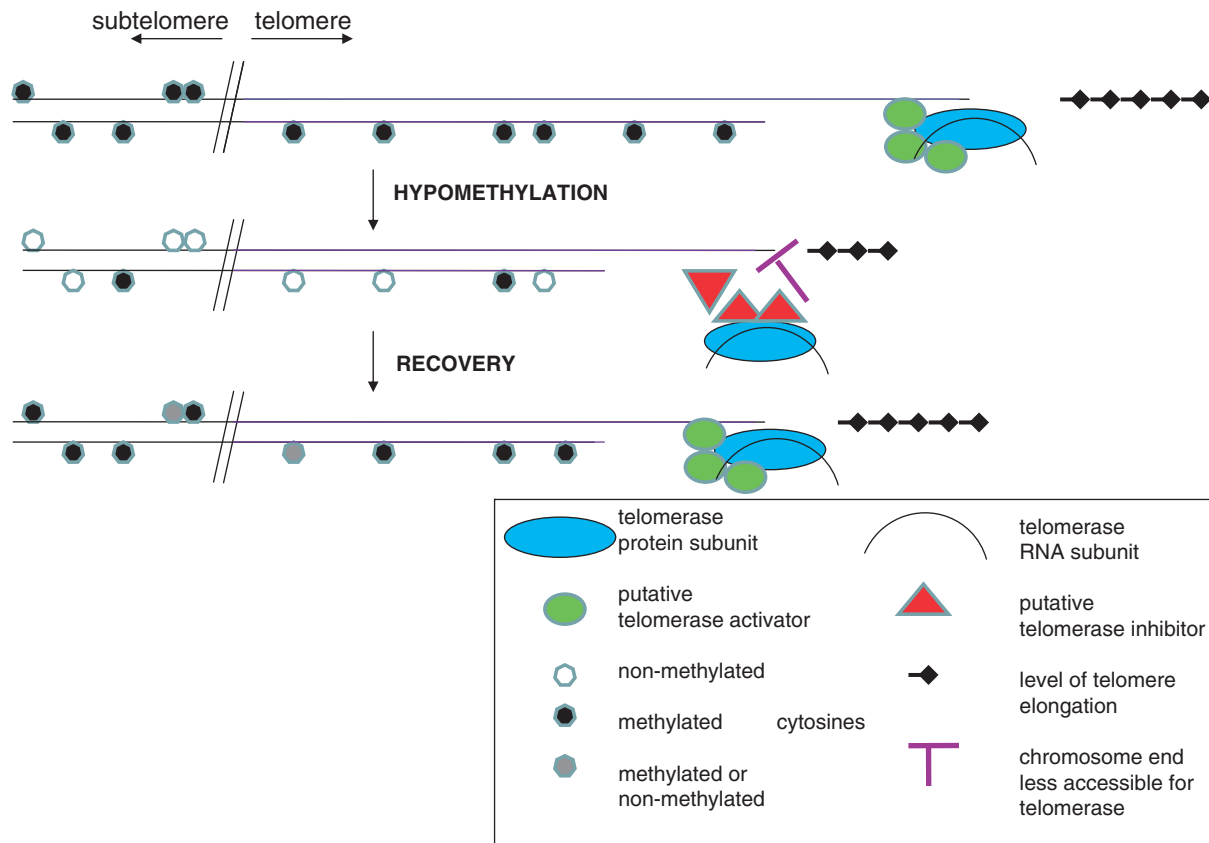
Figure 7. Schematic depiction of the role of telomerase in the process of telomere shortening and in the subsequent propagation of telomeres. Under normal circumstances (top), telomerase is active in telomerase-positive tissues and maintains the length of telomeres. Complex changes in hypomethylated genomes (middle) lead to compromised telomere maintenance (manifested as telomere shortening), which may be caused either by factors modulating telomerase action *in vivo* (e.g. the restricted accessibility of chromosome ends or the absence/presence of not yet-identified telomerase activators/inhibitors) or by telomerase-independent process(es). In wt segregated from the *met1-3* mutant and in the progenies of plants germinated in the presence of ZEB (bottom), telomeres remain shortened and are maintained at the stable length sufficient for normal cell functions by telomerase. The interface between telomeric and subtelomeric regions is marked by an oblique double line.

transcription (and consequently activity)—which was ruled out [Supplementary Figure S3; (68)]; (ii) by compromised function of telomerase 'holoenzyme' complex, which includes, in addition to both core telomerase subunits, a group of telomerase-associated protein factors affecting, e.g. telomerase recruitment, intracellular trafficking and localization and (iii) indirectly by a restricted accessibility of telomere chromatin to telomerase.

In animal models, telomere lengthening and increased recombination of telomeres were observed as consequences of the loss of heterochromatin-specific epigenetic modifications, including DNA methylation [(22) and reviewed in (64)]. On the other hand, the presence of critically short telomeres was correlated with hypomethylation of subtelomeric regions (20) or hypomethylation throughout the genome (69). Varied effects described in different animal model systems, and strikingly, quite opposite effects observed between plant and animal systems, are remarkable. Nevertheless, in telomere/telomerase biology and in epigenetics, considerable differences between plant and animal models do exist at many levels, and epigenetic modulation of telomere length—interlinking these two fields—thus logically reflects organism-specific features of both.

In our previous study, telomere lengths were stable, and telomerase activity was elevated in cultured tobacco cells treated with ZEB or DHPA (9), contrary to the observations in *Arabidopsis* where telomerase activity *in vitro* is not changed but telomeres are significantly shorter. These results suggest distinctions in pathways of epigenetic regulation of telomeres and telomerase, even between different model plants. The observed differences may be associated with distinct features of genomes and epigenomes of these plants, in particular a high content of ITSs, a lower global methylation and heterochromatin level (e.g. the absence of subtelomeric chromatin blocks), and a short telomere size in *A. thaliana* compared with *N. tabacum*.

Shortened telomeres are maintained over plant generations

Analysis of telomere lengths in wt plants segregated from the *met1-3* mutant, and in progenies of plant germinated in the presence of 250 μM ZEB reveals that shorter telomeres are maintained through meiosis. Similar results were obtained using mouse embryonic stem cells impaired in DNA methyltransferases function, but with elongated telomeres (22); a partial increase in subtelomeric DNA methylation was observed after re-introduction of the

methyltransferase gene, but the telomeres remained long. These data indicate that epigenetic stress—loss of DNA methylation—is necessary for implementation of telomere length change, but not for its maintenance. Moreover, telomere shortening in hypomethylated *A. thaliana* is not too dramatic or critical, and very probably does not lead to genomic or chromosomal instabilities (70). Thus telomerase simply maintains the same telomere length as present in parental cells (Figure 7). This scenario is supported by the considerable variability in telomere length, not only between different plant species [i.e. *A. thaliana* ecotype Columbia 3–5 kb, and *N. tabacum* 20–150 kb (71)] but also between different ecotypes of *A. thaliana*; striking variations in telomere length between individuals of the Wassilewkija ecotype have been documented. Crossings between Wassilewskija plants with short and long telomeres showed that telomere length in the progeny was determined by the parental telomere length (72).

Our data demonstrate that similarly to the other repetitive sequences in the plant genome, telomeres are under epigenetic control. Continuation of these studies using different model plants with distinctive telomeric distributions (i.e. *A. thaliana* with a significant proportion of telomeric repeats in interstitial telomeric sequences versus *N. tabacum* with a predominant fraction of telomeric repeats located in telomeres) may reveal new knowledge about specific and general features of plant telomeric chromatin.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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