# Chapter 23

# Structure and Maintenance of Chromosome Ends in Plants

**Jirí Fajkus, Ulrike Zentgraf**

## Introduction

Let us open this chapter by answering two basic questions: which features distinguish the terminal parts of plant chromosomes from their internal regions, and what is special about plant chromosome ends with respect to those in other eukaryotes?

Like the rest of the chromosome, its terminal regions consist of DNA complexed with various proteins. The fact that a vast majority or maybe all genome functions occur at the dynamic supramolecular nucleoprotein complex structures generally termed chromatin is often ignored and particular processes are simply attributed to particular DNA sequences or proteins. Current progress in telomere biology gives a good example of the necessity of a complex view covering at least several main players in the telomere field: DNA sequences of telomeres and of adjacent subtelomeric regions are associated in vivo with histones and various non-histone and telomere-specific proteins. Further, the most common tool for telomere maintenance, telomerase, functions as a ribonucleoprotein complex. The remarkable structural flexibility of telomeres shown mainly by in vitro and in situ experiments, the potential to adopt various kinds of local structures formed by 1 to 4 DNA strands stabilized by specific proteins, would suggest a higher degree of structural polymorphism of these apparently monotonous "non-coding" DNA domains. Of course it remains questionable whether one could not find a similar level of structural polymorphism in internal chromosome loci if they would be exposed to comparably focused research. Nevertheless, representing the natural physical ends of chromosomes which have to be recognized, treated and maintained in a way different from chromosome breaks, telomeres can be regarded as true specific chromosome functional units.

Compared to animal chromosomes, repetitive sequences commonly constitute a considerably larger fraction of plant chromosomes, suggesting that even chromosome regions formed predominantly by repeated DNA sequences like centromeres, telomeres and subtelomeres, could be more expanded on plant chromosomes. Further, contrary to the situation in most other higher eukaryotes, no true germline is set aside in plants in early embryogenesis, and both vegetative and generative parts are derived from meristems during growth and differentiation, implying that any change in the nuclear genome of meristem cells that has occurred during plant life, including telomere shortening, can be transmitted to sexual progeny.[1] In other words, the virtual totipotency of plant cells imposes higher demands on the regulation of telomere homeostasis. It can therefore be expected, that certain regulatory mechanisms which may have been abandoned or masked in most human and animal somatic cells can be found in plant cells. Although today's knowledge base of plant telomere biology is hardly comparable to those of human, mouse, protozoan or yeast, the possibility of applying the principles of functional genomics provides us with a chance not only to fill the present gap in the plant field, but also to bring original impulses to general telomere research.

*Telomeres and Telomerases: Cancer and Biology*, edited by Guido Krupp and Reza Parwaresch.

## DNA Sequences Constituting Plant Telomeres

Telomeres are always referred to as the ends of chromosomes, not excluding the title of this chapter. They may, however, be regarded as chromosome beginnings as well. Therefore I suggest to start this discussion from the beginning—from telomeres.

The first characterized plant telomeres were cloned from *Arabidopsis thaliana*[2] and it was shown that these are composed primarily of tandemly repeated blocks of the sequence 5'-CCCTAAA-3' and are heterogeneous in length (2—5 kb). Homologous telomeric sequences were observed in *Zea mays* (corn) and the same telomeric sequence, but forming considerably larger arrays (30-60 kb) was then found in *Lycopersicon esculentum* (tomato)[3] and later in a number of other plant species including *Hordeum vulgare* (barley),[4,5] *Oryza sativa* (rice)[6,7] and *Nicotiana tabacum* (tobacco).[8,9] In addition to a wide variation of telomere lengths between species and between varieties of one species, lengths can vary also within a single species; analysis of 22 inbred lines of maize showed that they varied more than 25-fold.[10]

The conservation of the *Arabidopsis*—type telomeric sequence among various plant species suggested similarity of the molecular mechanism of their replication. In analogy to the situation in lower eukaryotes, where the specific telomere terminal transferase (telomerase) activity was first identified,[11] the existence of plant telomerase was generally expected and, indeed, finally demonstrated independently by three research groups both by a direct assay[12] and by an indirect PCR-based TRAP assay[13,14] (see below).

Compared to the conservation of the TTTAGGG telomeric repeat among most higher plants, the telomeres of green algae are more variable. While those of *Chlorella vulgaris* are constituted from TTTAGGG repeats,[15] those of *Chlamydomonas reinhardtii* contain octamer TTTTAGGG sequence units.[16] In chlorarachniophytes, protists that have captured and maintained a green algal endosymbiont, the telomeres of the algal chromosomes are composed of a TCTAGGG motif unlike the nuclear telomeres which have a TTAGGG sequence.[17]

However, it appeared quite soon that in spite of the widespread presence of the highly conserved *Arabidopsis*-type telomeres or, more generally, of *oligonucleotide* (minisatellite) telomeres synthesized by telomerase, other strategies are also used within the plant kingdom. The first reports of this kind[18,19] showed that *Alliaceae* and some related liliaceous species lack tandemly repeated TTTAGGG sequences at their chromosome termini; instead, their chromosomes reveal highly repetitive satellite and/or ribosomal DNA sequences at the very ends. As both of these sequence types are very active in homologous recombination, they might contribute to the stabilization of chromosome termini via compensation of replication-mediated shortening. The alternative to recombination-mediated telomere maintenance could be the retrotransposition-based mechanism inferred from the enrichment of Ty1-copia retrotransposons in the terminal heterochomatin of *Allium* cepa chomosomes.[20] Recently, the lack of "typical" telomeres was reported also in Aloe, a large genus of ca. 300 species (family *Asphodelaceae*)[21] and although these studies of Aloe are not complete yet, the occurrence of ribosomal DNA at some chromosome ends, like chromosomes of Alliaceae was shown using in situ hybridisation.[22] Both *Aloe* and *Allium* belong to the Asparagales, the group of petaloid monocotyledons and it was therefore suggested that an absence of *Arabidopsis*-type telomeres may be characteristic of this group.[21]

The situation observed in the above plant genera has an analogy in the animal kingdom: in dipteran insects of the genus *Chironomus* telomeres are composed of long tandem repeats;[23,24] RNA transcribed from these units has been found and there is histochemical evidence that reverse transcriptase is co-localized with this RNA at the telomeres.[25]

Although it would be interesting and pleasant to open the debate on the long tandem repeat type of telomeres, their evolution and maintenance, this is the subject of another chapter of this book (see Biessmann et al). Returning to plants, it should just be noted that detailed studies on alternative telomeres in members of the Asparagales group are in progress. In analogy to the situation in *Chironomus*, transcripts of the telomere satellite repeat have been detected in *Allium* cepa (S_Korová, unpublished). When considering the fact that the other

long tandem repeat sequence constituting telomeres in *Allium* cepa is ribosomal DNA, a general role of transcription, and possibly of reverse transcription, in the maintenance of this class of telomeres is plausible and an analogy with the telomerase-reverse transcriptase mechanism could be considered.

Further, it remains to be shown whether species lacking "typical" telomeres also lack telomerase, as the enzyme may be present but inhibited. Evidence for the presence of telomerase inhibitors in plants comes from our observations which show that telomerase activity in cell extracts from dividing meristems is inhibited when extracts from quiescent leaf cells are added.[26] Preliminary experiments also show the presence of telomerase inhibitors in extracts of *Allium* seedlings [Fajkus et al, unpublished]. This, of course, does not exclude the possibility that constitutive silencing, mutation or complete loss of either the catalytic or RNA telomerase subunit genes is the primary cause of the loss of "typical" telomeres.

## Subdomain Structure of Chromosome Termini

### *Subtelomeres and Telomere-Associated Regions*

The above results obtained on *Allium* and *Aloe*, however, provoke an interesting question: since long tandem repeats, similar to those found in subtelomeres of most plants possessing typical telomeres, are able to perform telomere functions upon the evolutionary loss of the original telomerase-maintained telomeres, it is plausible that subtelomeric satellite or ribosomal DNA repeats could also function as a general back-up system of telomere maintenance. Besides this function, subtelomeres take part in sister chromatid exchange,[27] correct recognition of homologous chromosomes during mitosis and meiosis,[28,29] and function as a "buffer zone" between telomeres and internal chromosomal regions.[30] Thus, subtelomeres, although formed predominantly by "junk" DNA in most plants, appear to be much more interesting and important genome regions than it would appear in a superficial view. One can even regard the telomere and subtelomere as a single functional domain, especially in the light of the data presented in previous paragraphs. Nevertheless, from the schematic point of view (see Fig. 1) and for terminological reasons, when walking along the chromosome from telomeres in the centromeric direction the junction region between the telomere and the rest of the chromosome is termed the Telomere Associated Sequence (TAS), which can also be regarded as the most distal part of the subtelomere. The TAS region is typically composed of variant telomeric repeats and single- or low-copy non-coding sequences but alternatively, subtelomeric tandem arrays may be attached directly to the telomere without any linker sequence.[31] The subtelomere then continues and formally terminates at its centromere-proximal end by the first gene. However, it should be noted that the schematic structure of chromosome termini depicted in Fig. 1 is more or less valid, e.g., for genomes of tomato, tobacco, barley or rye.[3,9,32,33] while in smaller genomes like that of *A. thaliana* the subtelomere is typically composed of dispersed repeats and single-copy sequences including genes, while long tandem arrays of satellite repeats (other than ribosomal DNA genes) are in short supply. Therefore, in *A. thaliana* and similar genomes, the subtelomere can hardly be regarded as a "buffer zone" or "junk".

### *The Telomeric G-Overhang*

The very end of the telomeric G-rich strand forms a 3' overhang (further termed the G-overhang) on the chromosome terminus. In general, these overhangs represent substrates for telomerase, and consequently their presence and microstructure may be of key importance for certain levels of regulation of telomere maintenance. The length of the overhangs in two model plants studied so far, *Silene latifolia* (white campion) and *Arabidopsis thaliana*, is greater than 20-30 nucleotides. However, only about half of the telomeres in *Silene* seedlings possess detectable overhangs. The remaining telomeres either contain no overhangs or overhangs shorter than about 12 nucleotides. These findings suggest that incomplete replication of the lagging strand, rather than synthesis by telomerase, is the primary mechanism for G-overhang synthe-

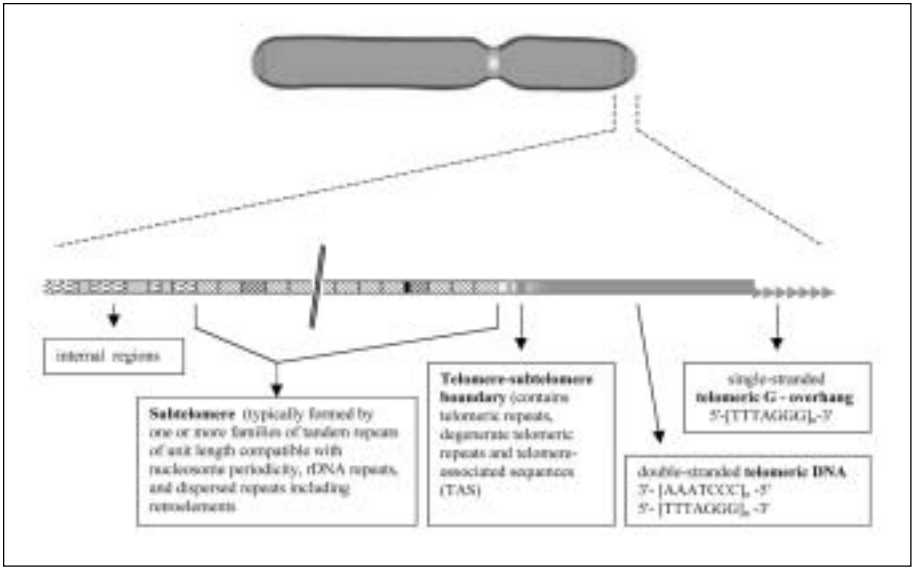


Figure 1. Plant telomeres are typically formed by a minisatellite repeat (TTTAGGG)n which in higher plants ranges from several kb to hundreds of kb in length. The very end of the telomere is constituted from the same sequence, but single stranded ( ). These overhangs are longer than 20-30 nt, but may be absent at more than 50% of plant telomeres.[34] A major part of the telomeric DNA is double-stranded ( ) and forms a specific chromatin structure (see below). At the boundary between telomere and subtelomere ( ), various sequences can be found: conserved telomere repeats may be mixed with degenerate telomeric sequences, and low- or single-copy intervening sequences. In a number of cases analyzed, this heterogeneous region is completely missing and the telomere is direcly attached to the subtelomere which typically consists of long arrays (hundreds of kb) of tandem repeats ( ) which are usually homogeneous and in a head-to-tail arrangement. Rarely, reversed orientation ( ) or interspersed sequences ( ) may also be present. Quite often, ribosomal RNA genes play the role of the major subtelomeric repeat. Subtelomeric DNA is packed in a condensed regular heterochromatin structure.

sis. G-overhangs were detected also in *Silene* seeds and leaves, tissues that lack telomerase activity; the fraction of detectable G-overhangs in leaves decreases to 35% compared to 50% in seedlings, possibly a result of their partial nuclease degradation in adult leaves which could act as a precursor for the catabolic processes accompanying leaf senescence.[34] Current models for telomere function assume that G-overhangs are found on both chromosome ends and that their association with specific end binding proteins is critical in allowing cells to distinguish natural ends from double-strand breaks.[35,36] A different interpretation of the data is that G-overhangs are simply a by-product of the DNA replication mechanism that must be masked to prevent chromosome instability or cell-cycle arrest,[34] an idea supported by the recent finding in human cells that accumulation of single-stranded G-telomeric DNA triggers p53-dependent cell-cycle arrest.[37] The identification of two distinct classes of telomere ends may reflect asymmetry or non-equivalency of the two telomere ends in plants arising from semiconservative replication; recent studies indicate that human chromosome termini are indeed asymmetric.[38,39]

Although the length of the G-overhang may be of great interest, its structure seems to be even more important. Although the G-rich telomeric single-strand is a favorite subject of in vitro studies on quadruplex (G4) DNA, the existence of this kind of structure has not yet been proved directly in vivo or in situ, although its existence, at least in other kingdoms, could be expected from the occurrence of specific proteins or antibodies recognizing this structure.[40-43]

The current alternative to a G4 structure is a t-loop structure (see another chapter of this book, xxx), in which the G-overhang is embedded within the double stranded part of the telomeric tract.[36] In either G4 or t-loop structures, the G-overhang is masked and made inaccessible to telomerase. The presence of either of these structures in plants, however, still remains to be shown.

In the formation of either of these structures or maybe some yet-unknown structure of the G-overhang, end-specific telomere-binding proteins are probably involved in analogy to the situation in, e.g., *Oxytricha, Euplotes*, and *Xenopus*.[44-46] Such proteins have been therefore searched for also in plants due to their presumed role in telomere length regulation.

The first plant protein specifically binding to the G-strand overhang was characterised and its cDNA identified in *Chlamydomonas reinhardtii*.[47] This 34 kDa protein, called G-strand binding protein (GBP), binds two or more single-strand TTTTAGGG telomeric repeats. Although the protein includes two domains with extensive homology to RNA recognition motifs, it does not bind the cognate telomere RNA sequence $r(UUUUAGGG)_3$ or double-stranded telomeric DNA.

Among higher plants, the first telomere binding protein of 67 kDa was found in *Arabidopsis thaliana*.[48] This protein (ATBP1) binds ~~to~~in vitro the single-stranded as well as to the double-stranded $(TTTAGGG)_4$ oligonucleotides. Both kinds of DNA-protein complexes were salt-resistant and insensitive to RNase. True single-strand telomere binding proteins were identified by gel retardation in rice nuclear extracts (rice G-rich telomere binding proteins, RGBP)[49] and three types of complexes of RGBP with two or more single-stranded TTTAGGG repeats were detected, which showed sequence specificity, high-salt resistance and no affinity to either double-stranded telomeric DNA or the cognate $r(UUUAGGG)_4$ sequence. These complexes, however, do not show end-specificity, as they do not require a free 3'-end and show the same affinity to the single-stranded $(TTTAGGG)_n$ within a non-telomeric sequence.

Recently, nucleoprotein complexes with G-telomeric sequences were identified in nuclear extracts of telomerase-negative tissues of *Nicotiana tabacum* and *Silene latifolia*. They were specific to $(TTTAGGG)_n$ sequence and resistant to high salt concentrations and their formation caused species-non-specific inhibition of plant telomerase due to reduced accessibility of the G-overhang [26] (see below, paragraph "Telomere hoemeostasis").

## *The Double-Stranded Region of Telomeric DNA*

The double-stranded part of telomeric DNA represents the major part of the telomere. It should be noted that although its main portion is indeed in B-double-helix form, local structures like triplex or quadruplex DNA might also be formed inside the telomere under appropriate conditions (e.g., superhelicity). Like the situation in rat, mouse and human telomeres, the higher plants studied so far possess at least several kb of telomeric repeat but shorter telomeres (hundreds of bp in length) have been found in green algae, *Chlamydomonas reinhardtii*,[16] *Chlorella vulgaris*[15] and in a green algal endosymbiont residing inside an amoeboid host cell of chlorarachniophytes.[17]

At the level of chromatin structure it is highly probable that in the case of very short (e.g., algal) telomeres, both telomeric subdomains, i.e. the G-overhang and the double-stranded region, are packed into a telosome structure in analogy to the situation described in yeast.[50] In higher plants, possessing either several kb-long telomeres (e.g., *Silene latifolia*, *Arabidopsis thaliana*) or tens to hundreds of kb-long telomeres (*Nicotiana tabacum*), the majority of telomeric DNA is packed in nucleosomes [9,51] whereas telosome structure may still be present at the very end of these telomeres.[52]

When plant telomeric chromatin is digested by micrococcal nuclease (MNase), a short (but regular) nucleosome periodicity and extensive subnucleosomal fragmentation of short mono- and oligonucleosome-size particles is observed. This was interpreted as a consequence of nucleosome sliding on the short fragments of telomeric chromatin fibre in the absence of nucleosome positioning signals,[9] an interpretation proved later by nucleosome reconstitution

experiments on telomeric DNA templates consisting of 6-8 bp repeated sequences.[53] These findings correspond to results obtained on animal telomeric chromatin.[54-56] In long fibres of telomeric chromatin the nucleosomes have the standard spacing (of 15 DNA helical repeats in the case of plants,[9,51] and of 15-17 in all other cases) and apparently do not slide. This suggests that a special packing of the nucleosomes in the fiber, rather than its particular DNA sequence, determines the regularity and stability of the fiber. The simple telomere sequences could not possibly carry any nucleosome positioning signal, and yet the nucleosomes are apparently organized in a regular chromatin structure. The nucleosome repeat can, therefore, only be dictated by the chromatin structure itself. The data above are consistent with a continuous winding of the DNA in the telomeric chromatin in a parallel manner [57,58] around the stacked histone cores. A structure recently described and termed a columnar chromatin structure.[59] In this structure (Fig. 2), nucleosome linkers are deformed in the same manner as the deformable part of the nucleosome DNAs, which is not unusual considering that in both cases the DNA sequence is the same simple repeating motif. Octamer-to-octamer stacking contacts cooperatively stabilise the overall structure, preventing the whole nucleosome array from sliding. Since the stability of such a co-operatively formed structure increases with the number of telomeric nucleosomes involved, a certain minimal length of chromatin fibre may be required to achieve thermodynamic stability.

This model is consistent with the observation of expansion of shortly spaced chromatin structure from telomeres into adjacent telomere-associated regions, especially in the case of relatively short telomeres and/or the absence of a strong nucleosome-positioning signal in the telomere associated region.[51,60] The columnar structure[59] offers a relatively condensed arrangement of nucleosomes, but telomeric DNA remains relatively accessible for specific telomere-binding proteins, facilitating the necessary dynamics of telomeres in their diverse functions.

## Stable Telomere Maintenance in Plants

The results of studies on plant telomere dynamics during development illustrate probably better than other aspects of telomere biology both the analogies and differences between the plant and animal kingdoms.

### *Telomere Homeostasis in Ontogenesis*

As mentioned at the beginning of this chapter, in contrast to animals where the germ line is separated from the soma in early embryogenesis, plant gametes are derived from the vegetative shoot apical meristem after the development of vegetative organs, thus imposing higher demands on regulation of telomere homeostasis during ontogenesis. Although the body of data on telomere length regulation in plants is hardly comparable with that available in animals, a number of interesting results have been obtained in this field within the last years.

In work addressing the analysis of telomere lengths during development of barley (*Hordeum vulgare*), in vivo differentiation was found to result in a striking reduction of telomere lengths; while 80 kb long telomeres were observed in young embryos, telomeres of only about 20 kb were observed in leaves or mature inflorescences.[61] This result is in contradiction with the presumption of telomere stability during ontogenesis. Moreover, the observed loss of about 50 kb of telomeric DNA in a few cell generations was far too rapid to be caused by incomplete end-replication in the absence of telomerase and the existence of an active mechanism of telomere shortening would have to be considered.[62] An alternative simpler explanation is that the integrity of the DNA preparations used in these experiments was not maintained sufficiently well for pulsed field gel electrophoretic analyses and, consequently, the centers of the resulting smeared telomere-specific hybridisation signals did not show the true telomere lengths. In the same work, telomere elongation up to about 300 kb was deduced during dedifferentiation in a long-term barley callus culture from the wide smears of telomere-specific signals.

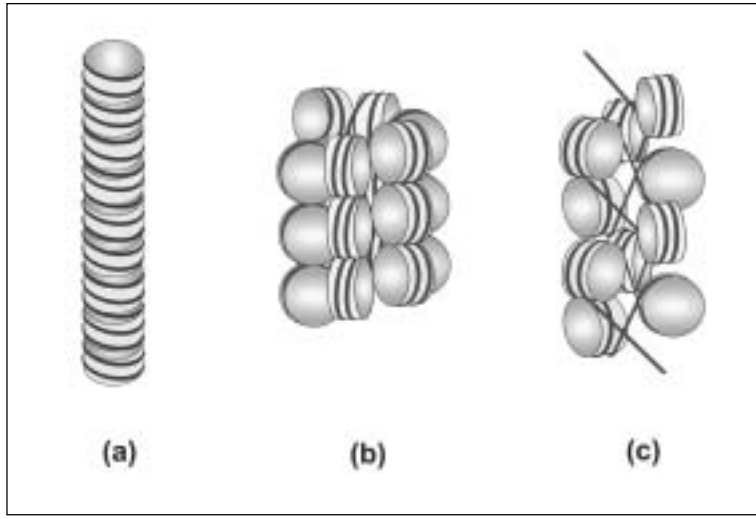


Figure 2. Comparison of the model of columnar structure of telomeric chromatin with other models of chromatin fiber architecture. (a) In the columnar model,[59] the DNA is continuously wound in a parallel manner around the stacked histone octamers and linkers are deformed in the same manner as the deformable part of the nucleosome DNAs. (b) In solenoidal models,[104] it is assumed that the chain of nucleosomes forms a helical structure with the axis of the core particles being perpendicular to the solenoid axis. The DNA entry-exit side faces inward towards the axis of the solenoid. The linker DNA is required to be bent in order to connect neighboring nucleosomes in the solenoid allowing partial face-to-face contact of consecutive nucleosomes . (c) In the zigzag model,[04] the linker DNA segments are straight and connect nucleosomes located on opposite sides of the fiber. Consecutive nucleosomes are not nearest neighbors. (Reprinted from Fajkus and Trifonov [59] with permission of Academic Press).

Since 1996, studies of telomere dynamics could be supplemented by analysis of telomerase activity which has been generally found to be associated with proliferating plant tissues or tissue cultures.[12-14,63] Soon after the initial reports on plant telomerase detection, studies appeared in which telomere lengths were measured in parallel with telomerase activity.

In our developmental study performed on a model dioecious plant, white campion (*Melandrium album*, syn. *Silene latifolia*), telomere lengths in different tissues and ontogenetic stages showed precise stability and did not change during plant growth and development. Telomerase activity correlated with cell proliferation in the tissues analyzed; Highest activity was found in germinating seedlings and root tips, whereas a 100-fold decrease in activity was observed in leaves and no activity in quiescent seeds. Telomerase was also found in mature pollen grains where it could be involved in DNA repair processes; in previous work, mutants possessing terminally deleted Y chromosome were prepared by irradiation of *S. latifolia* pollen grains[64] and the mitotic stability of this chromosome suggests its healing by telomeres. It is also plausible that a vegetative pollen nucleus undergoes a process of programmed breakage which involves new telomere formation, a process analogous to chromatin elimination or diminution during formation of vegetative nuclei in ciliated protozoa[65,66] and to development of somatic cell nuclei in nematodes.[67]

In contrast to the precise telomere length control during ontogenesis, callus cultures derived from *S. latifolia* leaves showed telomeres about 1 kb longer (3.0—5.5 kb) compared to the original leaves (2.5—4.5 kb). This result suggests that the telomere length regulation mechanism could be unbalanced during in vitro dedifferentiation.[68]

Telomere dynamics over repeated rounds of dedifferentiation and differentiation was also a focus of our study on tobacco cells[69] (see Fig. 3). Leaf tissues were used to initiate callus cultures, which were then induced to regenerate plants. The advantage of using tobacco is that the hybridization pattern of telomeres (terminal restriction fragments) is composed of discrete bands corresponding to 20—170 kb,[9, 70] which makes the detection of possible changes easier and more reliable. Moreover, the presence of discrete bands provides an internal control of DNA integrity. While no significant changes in the range of telomere lengths were observed in response to dedifferentiation and differentiation, there was a conspicuous increase in telomerase activity in calli compared to the source leaves, where the activity was hardly detectable. In leaves of regenerated plants, the telomerase activity fell to almost the same level as in the original plant, showing on the average 0.04% of the level in callus. The process was then repeated using the regenerants as the source material and in the second round of dedifferentiation and differentiation, telomerase activity again showed a similar increase in calli derived from regenerated plants and a drop in plants regenerated from these calli. Telomere lengths remained unchanged both in calli and in leaves of regenerants, although subtle changes in callus stages were observed as slightly diffuse bands of terminal restriction fragments. Thus despite dramatic changes in cell division rate and corresponding variations in telomerase activity during repeated dedifferentiation and differentiation processes, telomere lengths were stably maintained via the function of a regulatory mechanism which controls telomerase action to compensate exactly for replicative loss of telomeric DNA. This precise regulation of tobacco telomere maintenance may contribute to the relatively easy regeneration of tobacco plants from callus cultures.

To explore this regulatory mechanism, the effect on telomerase activity of protein extracts from nuclei of telomerase-negative tissues (leaves) was examined.[26] An inhibition of telomerase activity was found which was species-non-specific and was due to proteins which form salt-stable, sequence specific complexes with the G-rich telomeric strand and reduce its accessibility for telomerase. A candidate 40 kDa polypeptide was detected by SDS-PAGE after cross-linking the complex formed by extracts from tobacco leaf nuclei.

Stable telomere lengths (2.0 to 6.5 kb) were found also during developmental stages of *Arabidopsis thaliana*, including the transition from pre-senescent to senescent leaves, indicating, that contrary to mammalian cells (or more specifically, human cells), telomere length is not involved in differentiation and replicative senescence nor in post-mitotic senescence of *A. thaliana*.[71] In dedifferentiated cultured cells a slight increase in length could be seen in agreement with previous observations on *Silene latifolia*.[68] The nucleoprotein structure of the telomeric DNA was investigated by gel mobility shift assays, with synthetic oligonucleotides and nuclear protein extracts derived from four defined stages of post-mitotic leaf senescence. In all four stages, a highly salt-resistant DNA-protein complex of the 67 kDa protein (ATBP1,[48] see above) was formed with the G-rich telomeric strand as well as with double-stranded telomeric DNA. An additional protein of 22 kDa (ATBP2) was associated via protein-protein interactions with ATBP1 to form a higher-order complex exclusively during the onset of senescence. The interaction of this higher-order protein complex seems to be restricted to double-stranded telomeric DNA, and the defined period of ATBP1/ATBP2 complex formation with telomeric DNA probably indicates that ATBP2 is involved in the onset of post-mitotic leaf senescence.[71] Recently, a gene encoding an A. thaliana myb-like protein (AtTRP1) that binds telomeric GGTTTAG repeats has been cloned.[72] This protein binds in vitro to double-stranded DNA fragments containing five or more GGTTTAG repeats and is homologous to the Myb DNA-binding motifs of other telomere-binding proteins and similar to several initiator-binding proteins in plants. However, neither its function nor its relation to the previously characterized telomere binding proteins of *A. thaliana* is clear. For rice, a 70 kDa protein RTBP1 (rice telomere-binding protein1), has been cloned and characterized which also contains a Myb-like domain.[73] Whether this is a functional homolog of AtTRP1 has still to be elucidated (Yu et al., 2000). In addition, DNA-protein complex formation at the telomere seems to exhibit

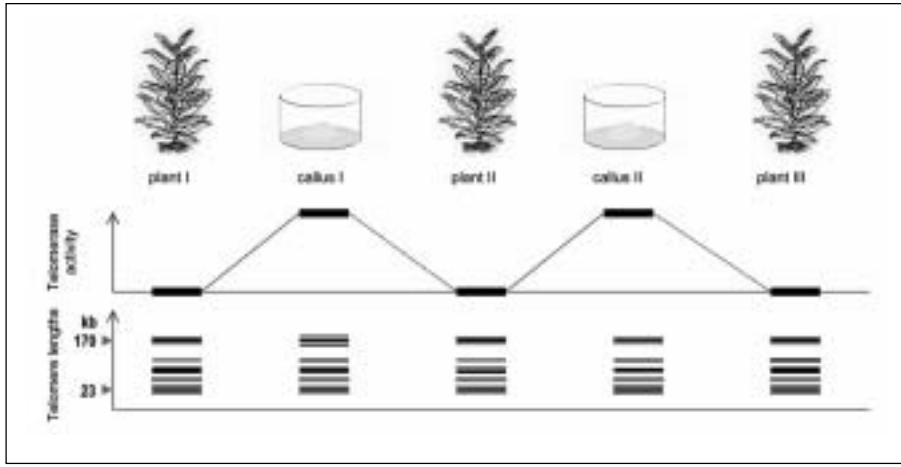


Figure 3. Schematic representation of an experiment illustrating tobacco telomere dynamics over repeated rounds of dedifferentiation (upon transfer to callus culture) and differentiation (plant regeneration from callus). Corresponding to increased cell proliferation in the callus, telomerase activity increases here in comparison to the source leaves, where zero or negligible activity is present. The pattern of terminal restriction fragments (telomeres) remains the same in the observed fragment lengths, but slightly differs in the intensity and sharpness of individual bands, possibly reflecting minor fluctuation of telomere length during in vitro cell culture.

developmental dynamics as it was shown for ATBP2 of *Arabidopsis*[71] or the single-stranded telomere binding proteins of *Vigna radiata* (mung bean).[74] Obviously, there is still a lot of work to be done to characterize the factors involved in the maintenance of the telomeres and to understand the telomere dynamic and its functional implication for the plant cells.

## *Plant Responses to Telomere Dysfunction*

Telomerase-deficient lines of *A. thaliana* have been generated by selfing plants heterozygous for a transferred DNA (T-DNA) insertion into the single AtTERT gene.[75] In the absence of telomerase, telomeres shortened by approximately 500 bp per generation, a rate 10 times slower than that seen in telomerase-deficient mice. This gradual loss of telomeric DNA may reflect a lower rate of nucleotide depletion per round of DNA replication, or the fewer cell divisions per generation. Nevertheless, progressive telomere shortening in the mutants, however slow, should ultimately be lethal. These mutants can survive up to 10 generations without telomerase and a phenotypic effect could not be observed until the sixth generation. Telomere lengths did not decrease steadily: in several mutants, a subset of terminal restriction fragments even increased in the fourth and fifth generation relative to previous generations suggesting the transient presence of a telomerase-independent mechanism of telomere extension. In the last five generations, increasing levels of cytogenetic damage and corresponding developmental anomalies in both vegetative and reproductive organs were observed. The mutants ultimately arrested at a terminal vegetative state, harboring shoot meristems that were grossly enlarged, disorganized, and in some cases dedifferentiated into a callusoid mass. Interestingly, late generation mutants had an extended life-span (compared to wild types) and remained metabolically active. Thus although an efficient telomere-maintenance mechanism is crucial for indefinite cell proliferation, plants, in contrast to metazoans, do not respond to telomere dysfunction by cell senescence and programmed cell death which may reflect the unusual plasticity of plant development and genome organization.[76]

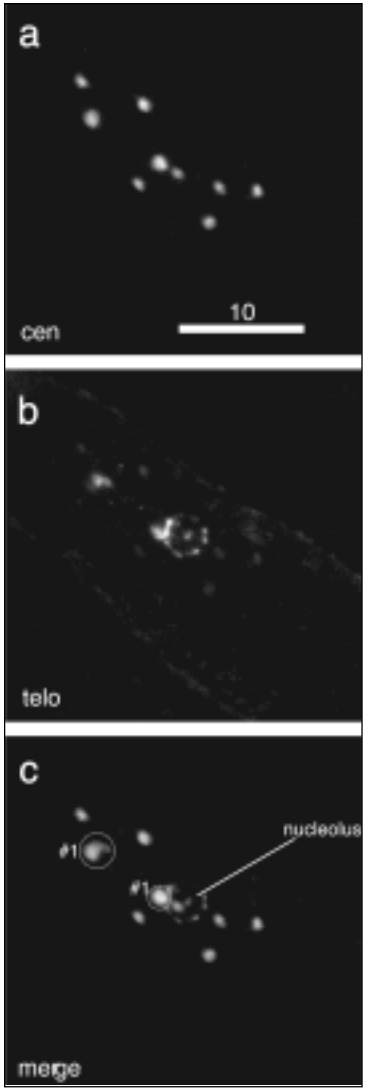


Figure 4. Arrangement of centromeres and telomeres in Arabidopsis interphase nuclei. The picture shows a root hair nucleus after a dual color in situ hybridisation using (a) fluorescein-labeled cen, a centromere-specific oligonuclotide, and (b) Cy3-labeled telo, a telomere-specific oligonucleotide, as probes; (c) merge of cen and telo. The telomere-similar sequences in the pericentromeric heterochromatin of chromosome 1 [100] are detected by the telo oligonucleotide and localize in close proximity to two of the cen signals. The other telomeres surround the nucleolus (Buchenau et al., in preparation)

## *Coupling of Telomerase with the Cell Cycle*

Another important question in both plant and animal telomere biology is the relation of telomerase activity to the cell cycle. In the first report on this question in plants, a group of Japanese authors showed that telomerase activity in synchronized tobacco BY-2 cells is largely restricted to early S phase, indicating cell-cycle dependent regulation. The appearance of telomerase activity was not affected by the S phase blockers aphidicolin (which inhibits DNA polymerase alpha) or hydroxyurea (which inhibits ribonucleotide reductase), but was prevented by olomoucine, an inhibitor of Cdc2/Cdk2 kinases that blocks the G(1)-S cell cycle transition. Tobacco telomerase activity was also shown to be inducible by phytohormone auxin, which promotes cell cycle progression.[77]

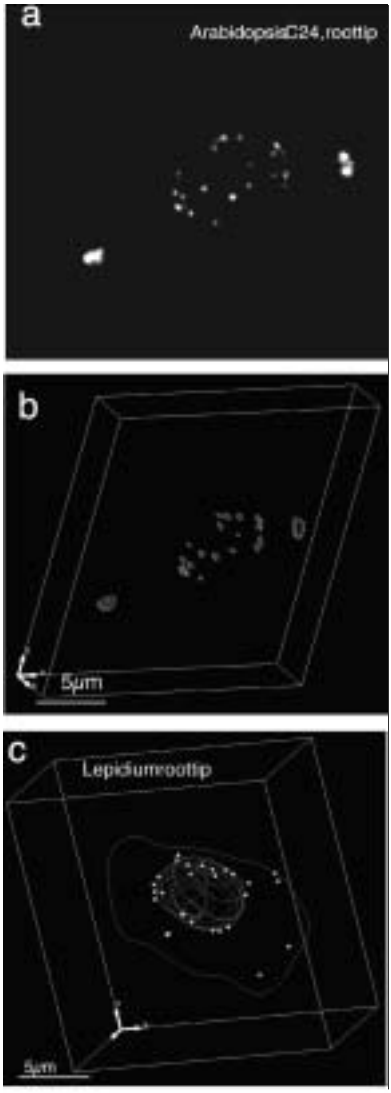


Figure 5. Arrangement of the telomeres in interphase nuclei of Arabidopsis and Lepidium root cells. (a) in situ hybridization of Arabidopsis root hair nucleus with Cy3-labeled telo, a telomere-specific oligonucleotide, as probe; (b) 3-dimentional computer analysis of the same picture; (c) 3-dimentional computer analysis of the telomere distribution in a Lepidium root cell nucleus (Buchenau et al., in preparation).

## Plant Chromosome Healing

When chromosomes are broken, the breakpoints become highly unstable and acquire the ability to fuse with other broken ends to form stable monocentric terminal translocation chromosomes [76]. Another mechanism of stabilization of breakpoints is the phenomenon known as "healing", which involves the addition of telomere sequences at the breakpoints by telomerase.[79] Studies of this de novo telomere addition provide insight into the properties of the telomerase activity at the breakpoints. While the major part of the telomeric region at natural chromosome ends is synthesized by the conventional semiconservative replication machinery, de novo added telomeres reflect the action of telomerase itself. Analysis of the healed breakpoints in the nuclear organizer region of wheat deletion chromosomes showed that the telomere sequences initiated from 2- to 4-nucleotide motifs in the original ribosomal

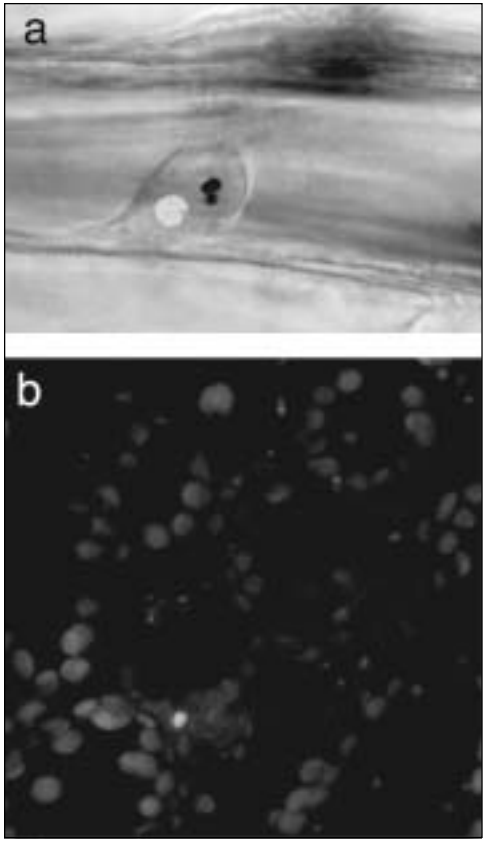


Figure 6. Localization of telomerase by transient transformation of Lepidium root cells (a) and Vicia faba epidermal cells (b) with a GFP::AtTERT construct by particle bombardment (Buchenau et al.). Green: GFP-TERT, red: chloroplasts, blue: cell border line, black spot: gold particle of bombardment (Buchenau et al., in preparation).

DNA sequence which are also found in telomeric sequences.[80,81] No specific sequences or structures were observed at or around the breakpoints. The newly synthesized telomeres were typically several hundreds of bp long and contained considerable numbers of atypical telomere sequence units, particularly TTAGGG, which is the common unit of mammalian telomere sequences.

Recent studies suggest that de novo addition of telomeres need not necessarily represent the final stage of chromosome repair. It has been shown that the tobacco suspension cell culture BY-2 undergoes genome fragmentation when incubated with 50 mM concentration of $CdSO_4$.[82] Until the day 3 the process is reversible, i.e., cell viability is not affected in spite of extensive fragmentation of about 95% of the chromatin into 50—200 kb domains. When the cells are then washed and transferred to $Cd^{2+}$- free medium, genome integrity is re-established in 2 days. Genome repair could be blocked by aphidicolin, the inhibitor of DNA polymerases a, d and e. Telomerase activity monitored in the course of the experiment showed a several-fold increase from day 1 until day 3 of the recovery phase, falling back to its normal level on day 4. A certain increase of telomerase activity could also be observed on day 4 of $Cd^{2+}$- treatment, beyond the "point of reversibility" when cells are no longer able to maintain viability and to repair their genome upon transfer to $Cd^{2+}$- free medium. These observations suggest that telomerase induction participates in the genotoxic-stress-response and, together with DNA polymerases, is involved in re-establishment of genome integrity [Fojtova et al., in preparation]. It should also be noted that de novo synthesized telomeric sequences could not

be detected in the recovered cells, suggesting either that their lengths are below the detection limit of about 1 kb, or that they appear only transiently at chromosome breaks during initial stages of the repair process. An attractive hypothesis can be proposed that de novo-synthesized telomeres could possibly function here (in analogy to one of the known roles of natural telomeres) as nuclear matrix- or nuclear lamina-associating regions. The reassembly of the extensively fragmented genome on the nuclear matrix would result in a decrease of genomic disorder and may contribute to correct repair via DNA-recombination and repair machinery. This makes sense considering that (1) the size of the DNA fragments corresponds to that of chromatin loops, and (2) chromosome breaks and recombination events preferentially occur at nuclear matrix attachment sites where DNA is bound to topoisomerase II.[85] It is plausible that contacts between the ends of chromatin loops and nuclear matrix proteins mostly survive the first (reversible) stage of cadmium- induced genome fragmentation and that these DNA-protein bonds provide a mechanism to conserve the nuclear position and function of individual loop domains. This maintenance of the loop organization of nuclear chromatin may be functionally more important than the simple integrity of genomic DNA and reconstruction of the genome which has been "broken into thousands of pieces" may hardly be possible without maintenance or re-establishment of chromatin loop attachment to the nuclear matrix.

## Nuclear Localization of Plant Telomeres

Not only the maintenance of the chromatin loop structure but also the overall three-dimensional architecture of the nucleus might be important for the integrity and function of the nucleus. Many cytological studies show that telomeres are not randomly distributed within the nucleus. Besides clustering and single end-to-end associations resulting in a linear array of chromosomes, telomere interaction with the nuclear envelope has been reported for different organisms like *Drosophila, Xenopus, Vicia faba, Pisum sativum* and *Saccharomyces cerevisiae*.[87] In yeast, the association of chromatin with the nuclear membrane helps to establish transcriptionally silent domains[88] which might also be a cause of telomeric silencing. Interestingly, the silencing Sir-complex (Sir2p, Sir3p, Sir4p) was found to redistribute from the telomeres to the nucleolus in ageing cells of *S. cerevisiae* thereby lengthening the yeast life span.[89,90] Another link between telomeres and the nucleolus is suggested by the in vitro-binding of human nucleolin to double-stranded telomeric DNA.[91] Whether the localization of telomeres at the nuclear envelope is also involved in the organisation of the functional nuclear domains and/or the specific nuclear territories occupied by chromosomes.[92,93] has still to be elucidated. However, nuclear and cell shape of *Tetrahymena* cells were strongly distorted, and DNA distribution within the nucleus became irregular when the telomeric repeat was mutated by altering the telomerase RNA template.[94] The proteins that mediate the interaction between the telomere and the nuclear envelope are not yet well characterized. In vitro, an interaction of lamin A and C with telomeric DNA has also been reported for human cells.[95] In yeast, which has no detectable nuclear lamina, an interaction between telomeres and the envelope is probably mediated by the Sir4 protein containing a coiled-coil domain with sequence similarity to nuclear lamins. The Sir4 protein coiled-coiled carboxy terminus interacts with Ku70 in a two hybrid system. Deletion of the genes encoding yKu70p or its partner yKu80p altered the subnuclear localisation of the yeast telomeres from few foci at the perinuclear rim to a dispersed organisation throughout the nucleoplasm.[96] Ku 86-deficient Chinese hamster ovary cells have abnormal nuclear morphology and nuclei assembled in Ku70 depleted *Xenopus* egg extracts show irregular interactions between the nuclear lamina and chromatin.[97,98] This indicates that an interaction between Ku70/80, lamins and chromatin, especially the telomeric chromatin, is involved in the formation of a proper nuclear architecture. There is also evidence that the telomeres directly interact with the nuclear membrane via a 70 kDa membrane protein.[99]

Reconstruction and analysis of optical sections from *Arabidopsis thaliana* nuclei hybridized with a telomere-specific fluorescent probe led to the following picture: All nuclei showed two large and intense hybridisation signals that could clearly be distinguished from the numerous

smaller telomere signals (Fig 4; Buchenau et al., in preparation). These large signals correspond to the blocks of telomere-similar sequences located within the heterochromatic region near the centromere of chromosome 1.[100] The pericentromeric heterochromatin blocks of all chromosomes were always located at the periphery of interphase nuclei. The 3-dimensional arrangement of the telomeres within the *Arabidopsis* interphase nucleus differed from that of most other plants. A specific clustering of the telomeres directly at the nucleolar boundary was found (Figs 4 and 5). This arrangement of the telomeres seems to by specific for *Brassicaceae* for it also showed up in interphase nuclei of other *Brassicaceae* like *Lepidium sativum* (cress; Fig 5), *Sinapis alba* (mustard) and *Brassica oleracea* (cabbage) but not in other species like *Nicotiana tabacum* (tobacco), *Helianthus annuus* (sunflower), *Vicia faba* (bean), *Triticum aestivum* (wheat), *Secale cereale* (rye) or *Zea mays* (maize) (Buchenau et al., in preparation). The association of the telomeres with the nucleolar boundary was dependent on the age of the analysed tissue. It was most pronounced in the cells of young plants (<1 week) and in dividing and young cells of older plants (e.g., meristems and elongation regions of the root system). Here, almost all telomeres were located at the nucleolus. In contrast, cells that had stopped dividing, typically displayed a telomere distribution where a few signals are attached to the nucleolus while most of the telomeres have dispersed into the nuclear volume (Buchenau et al., in preparation). Remarkably, even in a random distribution, four out of twenty telomeres of a diploid cell are always associated with the nucleolus because the *Arabidopsis* rDNA clusters are located directly subtelomeric on chromosomes 2 and 4.[101] These results demonstrate specific developmentally regulated intranuclear positions of the *Arabidopsis* telomeres.

In accordance to the location of the telomeres, telomerase is also located at the nucleolus as it was shown for *Arabidospsis, Lepidium* and *Vicia* cells by transient particle transformation of GFP-fusion proteins with AtTERT (Fig 6; Buchenau et al., in preparation). How telomerase is directed to the nucleolus is still unclear. Remarkably, AtTERT contains a sequence motif – RRKQRK -with homology to a consenesus sequence (-RRQRR-) for nucleolus localization in animals.[102] The question, who was first, are telomeres associated with the nucleolus because telomerase is localized here or is telomerase localizes to the nucleolus because the telomeres are gathering at the nucleolar boundary, is open.

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