J Plant Res (2007) 120:523–528
DOI 10.1007/s10265-007-0092-1

REGULAR PAPER

Comparative analysis of telomeric restriction fragment lengths in different tissues of *Ginkgo biloba* trees of different age

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Received: 11 February 2007 / Accepted: 6 April 2007 / Published online: 12 June 2007

Abstract Ginkgo trees of four different ages were selected as experimental material. Telomeric restriction fragment (TRF) lengths, as an indicator of telomere length, were determined for different tissues by Southern hybridization analysis. Statistical analysis was performed to compare two aspects of TRF length. By determining TRF lengths for different tissues for each age, a latent tendency was found. TRF length varied from short to long in these tissues in the order microspore < embryonal callus < leaf < branchlet. TRF lengths for leaf tissue and branchlet tissue were dissimilar for female and male mature trees, although this difference between TRF lengths for the two sexes was not statistically significant. Evaluation of TRF lengths for each tissue for trees of all four ages revealed TRF lengths increased with age to some extent. Different rates of change were found for leaf tissue and for branchlet tissue, although tendencies to increase were not linear for either. Finally, a simple mathematical model was formulated to describe the relationship between telomere length and age for *Ginkgo biloba* L.

Keywords Age · *Ginkgo biloba* L. · Telomere · TRF length (TRFL)

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Introduction

The term ''Telomere'' was initially used by Muller (1938). Telomeres are the structures located at the termini of linear eukaryotic chromosomes. At each end of all chromosomes are telomeric repeats (TRs) which comprise a variable length of tandem arrays of short G-rich repeats (Richards and Ausubel 1988; Ganal et al. 1991). The sequence which is a simple repeat of the heptanucleotide 5′-TTTAGGG-3′, has been found in most angiosperms, for example *Arabidopsis thaliana* (Richards and Ausubel 1988; Richards et al. 1992), maize (Burr et al. 1992), barley (Wang et al. 1991; Kilian and Kleinhofs 1992; Cox et al. 1993), and rice (Wu and Tanksley 1993). A group of monocotyledonous plants in the order Asparagales, forming a distinct clade in phylogenetic analysis, was reported previously to lack the ''typical'' Arabidopsis-type telomere repeat sequence $(TTTAGGG)_n$ (Fajkus et al. 2005). the variability of the interstitial sites and copy number of the Arabidopsis-type telomeric sequences also seemed to be a characteristic of gymnosperms (Murray et al. 2002). Schmidt et al. (2000) analyzed the telomere repeats of conifers by Southern hybridization and showed that the low copy number of $(CAC)_5$ and $(GACA)_4$ in conifers and *G. biloba* L. was consistent with the data for angiosperms, which indicated that the structure of large conifer genomes and the organization of simple sequence repeats were to some extent similar to those of other, higher, plants. The Arabidopsis-type TTTAGGG tandem repeats have also been found in the telomeres of *Ginkgo biloba* L. (Liu et al. 2005). The variability of the distribution and abundance of simple sequence repeats has been used for taxonomic studies and to explore the genetic similarity among species or breeding lines in agronomic crops.

Telomeres play essential roles in versatile cellular processes including DNA replication, cell cycle progression,

meiosis, and mitosis. After each cell division the length of telomeres will be shortened because of the inability of DNA polymerase to replicate linear DNA molecules completely. The highly conserved repeats (TTTAGGG) at the outermost ends of chromosomes of plants can form stable stretches which protect the linear chromosomes from exonucleolytic degradation. Nakamura recently investigated progressive telomere shortening in a normal human epidermis and in lingual epithelium during aging and concluded that the aging accompanying telomere length reduction occurred at different rates in the two tissues (Nakamura et al. 2002). In contrast, stable telomere lengths were observed in tomato leaves from four-week old and six-month-old plants (Broun et al. 1992). Telomere lengths did not change during plant ontogenesis and maintenance of telomere lengths was connected with telomerase activation in dividing cells (Riha et al. 1998). Flanary et al. (2005) discovered, furthermore, that in needle samples of *Pinus longaeva* the longest, mean, and shortest TRF lengths (TRFL) had a cyclical pattern of lengthening and shortening in size with age. They suggested that telomere length and telomerase activity may be essential in directly or indirectly regulating the life-span of the bristlecone pine.

In general, modern gymnosperms are representatives of an ancient group of plants first recorded as fossils in the Upper Devonian (350 million years ago) (Biswas and Johri 1997). Four classes are included—Cycadopsida, Ginkgopsida, Coniferopsida and Gnetopsida. The ginkgo tree, known as a ''living fossil tree'', is related to the group of plants classified as gymnosperms, or conifers. The genetic line of the ginkgo tree spans the Mesozoic era back to the Triassic period. Closely related species are thought to have existed for over 200 million years. Fifty million years ago, ginkgo trees spread all over the temperate regions of the world. *Ginkgo biloba*, the species native to southeast China, survived the millennia and has been preserved for its majestic beauty in Asian gardens. Except for the species included in the families mentioned above, all other species are extinct.

We must ask the following questions: how does telomere length change in different tissues and how does it vary with age? Can age be measured by TRFL analysis or not? We selected ginkgo trees of four different ages, i.e. ancient trees, mature trees, saplings, and seedlings as our experimental material and determined TRF lengths by Southern hybridization analysis in different tissues—leaves, branchlets, microspores, embryonal calli, and cambium. By analyzing TRF length and trends in its variation in these tissues of different age we attempted to build a mathematical model and describe the relationship between telomere length and the age of *Ginkgo biloba* L.

Materials and methods

Collection of the samples

The *Ginkgo biloba* L. trees of four different ages consisted of two ancient trees (over 700 years, one female and one male), eight mature trees (70 ± 10 years, four female trees and four male), eight saplings (7 ± 1 years) and nine seedlings (<1 year). Samples were collected from the Haidian district of Beijing (Table 1). Leaves, branchlets, microspores, embryonal calli, and cambium, from each were fixed in liquid nitrogen and stored at –80°C.

DNA extraction and Southern hybridization analysis

TRF length, as an indicator of telomere length, was determined by Southern hybridization analysis (Nakamura et al. 2002). Tree samples (i.e. leaves, branchlets, microspores, Embryonal calli, and cambium) were placed in a mortar and ground to a fine powder using a pestle and liquid nitrogen. Genomic DNA was prepared from each sample by the hot CTAB method (Rogers and Bendich 1988) and DNA samples were quantified by microclorimetric spectrophotography. DNA samples of approximately 20 μg were digested for 36 h with *Taq* I and the digestion products were loaded on to a horizontal 6.5 × 10 cm 0.8% agarose gel and electrophoresed in 1 × TAE buffer for approximately 3 h at 100 V at room temperature with buffer recirculation. Southern hybridizations were performed using the DIG High Prime DNA Labeling and Detection Starter kit II (Roche) with an end digoxigenin-labeled complementary telomere-specific oligonucleotide probe 5′-CCCTAAACCCTAAACCCTAA ACCC-3′. The membranes were exposed to X-ray film (Super RX, Fuji Medical X-ray Films) for 1.5 h at room temperature. An autoradiograph was developed by use of

Table 1 Tissue samples collected from four different age classes in *Ginkgo biloba* L. trees

Age	Leaf		Branchlet		Microspore	Embryonal Callus	Cambium
	♀	♂	♀	♂			
Ancient	√	√		√	√	√	
Mature	√	√	√	√	√	√	
Sapling	√		√				√
Seedling	√		√				

The sign ''√'' denotes samples obtained. The age of the ancient trees is over 700 years, that of the mature trees is approximately 70 years, that of the saplings is 7 years, and that of the seedlings is <1 year. As a cultural heritage, ancient ginkgo trees are protected so cambium and female branchlet samples were not available. The seedlings were too small to obtain cambium samples, so cambium samples could be obtained from the saplings only. Also, there is no sex differentiation in saplings and seedlings, so we could collect samples of leaves and branchlets only

D-72 developing solution for 2 min and was fixed with an acid fixing solution for 5 min.

Measurement of TRF length

The autoradiograph was scanned and imported as a ''tif''-format image for measurement of TRF length. Although other methods have been used to measure telomere length in acrylamide and pulsed-field gels (Allsopp et al. 1992; Feng et al. 1998) and directly in cells using fluorescent tags (Poon et al. 1999), an easy-to-use system for performing accurate, reproducible measurements on inexpensive constant field agarose gels was developed by Grant et al. (2001). In this work, the program for TRFL assay was determined by use of the mathematical model:

$$C_i = \frac{I_i - B_i}{L_i}$$

where C_i is the relative copy number associated with line i across the gel lane, I_i is the integrated grayscale intensity within line i, B_i is the background measurement for line i, and L_i is the DNA molecular weight at line i (Grant et al. 2001). The equation provides a correction for the background intensity (B) and for the binding of multiple copies of the telomeric probe, which scales with the size of the DNA (L) (Grant et al. 2001).

Statistical analysis

Measurement of TRF length for each sample was repeated three times by Southern hybridization. TRF lengths are reported as mean ± standard deviation. Two aspects of TRFL were compared:

we measured TRF lengths for different tissues of the same age; and

we measured TRF lengths for tissue of different ages.

Statistical analysis were performed by one-way analysis of variance using Matlab 2006a software (The MathWorks). Statistically significant differences were assumed when the p value was <0.05.

Results and discussion

TRF lengths for *Ginkgo biloba* trees

To determine telomere length in different tissues—leaves, branchlets, microspores, embryonal calli and cambium—TRFL analysis was performed on tissues from *Ginkgo biloba* of four different ages. Representative Southern hybridization images used for measurement and quantitation of TRF length are shown in Fig. 1. The hybridization signal for different samples represents the

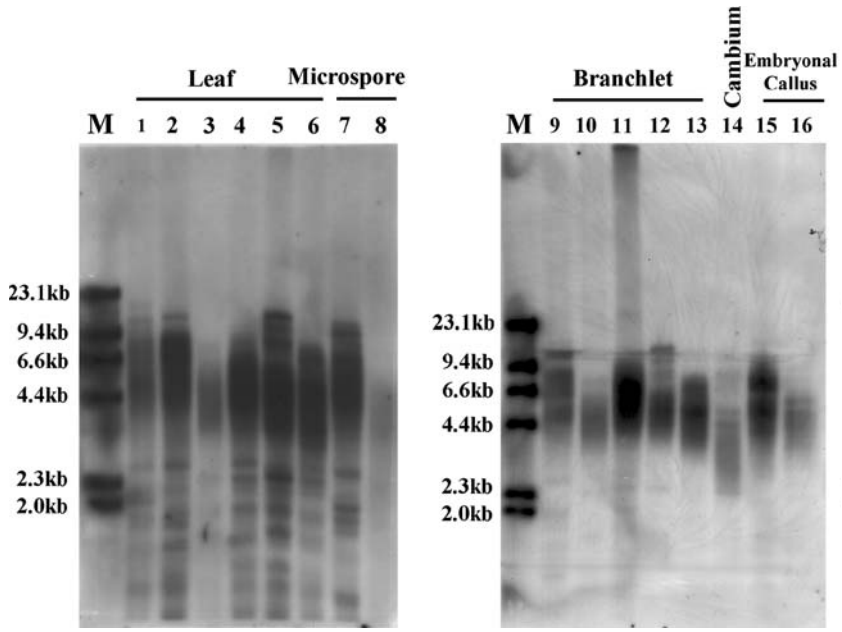

Fig. 1 Representative Southern hybridization images used for measurement and quantitation of TRF length. Lane M: DNA Molecular Weight Marker II, Digoxigenin-labeled (Roche). Leaf samples from lanes 1 to lanes 6: ancient male leaf (*lane 1*), ancient female leaf (*lane 2*), mature male leaf (*lane 3*), mature female leaf (*lane 4*), sapling (*lane 5*) and seedling (*lane 6*). Microspore samples from lane 7 to lane 8: ancient microspore (*lane 7*) and mature microspore (*lane 8*). Branchlet samples from lane 9 to lane 13: ancient male branchlet (*lane 9*), mature male branchlet (*lane 10*), mature female branchlet (*lane 11*), young branchlet (*lane 12*) and seedling branchlet (*lane 13*). Sapling cambium sample (*lane 14*). Embryonal callus samples were run in lanes 15 and 16: ancient embryonal callus (*lane 15*) and mature embryonal callus (*lane 16*)

telomeric regions. This result indicates that even ancient trees maintain their telomeres. Measurements of the location of peak intensity are not accurate if observed by eye, however, so an easy-to-use system which can be used to determine distributions by copy number and calculate statistics on telomeric regions (Grant et al. 2001) was used to measure TRF lengths of ginkgo trees. Statistical analysis was performed on TRFL and compared from the two aspects described above.

The TRF length in the different tissues of *Ginkgo biloba* L.

Comparison of TRF lengths for different tissues of each age (Fig. 2) revealed a tendency toward variation in these tissues. For ancient trees (Fig. 2a) the TRF length in microspore tissue was 4,330 ± 410 bp, that in embryonal callus was 4,700 ± 400 bp, that in leaf tissue was 5,090 ± 180 bp (female 5,090 ± 150 bp and male 5,090 ± 220 bp), and that male branchlet tissue was 5,220 ± 110 bp. For mature trees (Fig. 2b) the TRF length in microspore tissue was 3,820 ± 270 bp, that in embryonal callus was 4,360 ± 140 bp, that in leaf tissue was 4,720 ± 330 bp (female 4,770 ± 200 bp and male 4,670 ± 430 bp), and that in branchlet tissue was 4,970 ± 310 bp (female 5,040 ± 240 bp and male 4,910 ± 370 bp). For saplings (Fig. 2c) the TRF length in cambium tissue was 3,640 ± 300 bp, that in leaf tissue was 4,280 ± 120 bp, and that in branchlet tissue was 4,990 ± 400 bp. For seedlings (Fig. 2d) the TRF length in leaf tissue was 3,960 ± 270 bp and in branchlet tissue it was 4,510 ± 230 bp.

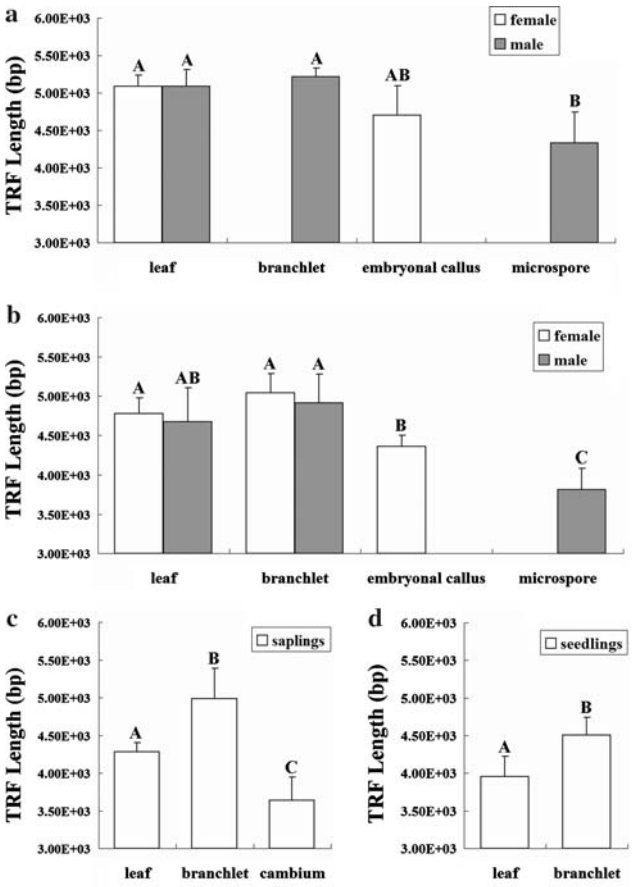

Fig. 2 TRF lengths in different tissues of different ages. With reference to sex differentiation in ancient (**a**) and mature (**b**) trees, TRF lengths vary from short to long in the order: microspore < embryonal callus < leaf < branchlet. In gender-undifferentiated classes of saplings (**c**), and seedlings (**d**) TRF lengths for leaves are also shorter than those for branchlets. *Different letters above the bars* are indicative of significance at the 0.05 level for the same age

With regard to sex differentiation in the ancient (Fig. 2a) and mature (Fig. 2b) trees, the results show that TRF lengths varied from short to long in these tissues in the order microspore < embryonal callus < leaf < branchlet. TRF lengths for microspores were statistically significantly ($p < 0.05$) less than for branchlets and leaves. In the gender undifferentiated classes of saplings (Fig. 2c) and seedlings (Fig. 2d), TRF lengths for leaves were significantly ($p < 0.05$) shorter than for branchlets. TRF lengths for cambium tissue were also significantly ($p < 0.05$) shorter in the saplings (Fig. 2c).

These results indicate that different tissues have tissue-specific TRF lengths and the same tendency was found for each age. Interestingly, for the mature trees (Fig. 2b) TRF lengths were also different for female and male leaf tissue and for branchlet tissue, but this difference was not statistically significant ($p > 0.05$). There are no apparent differences between TRF lengths in male and female samples of the model dioecious herbaceous plant, *Melandrium album* (Riha et al. 1998). As diaecious woody plants, young ginkgo trees grown under natural conditions begin to flower when they are 15–25 years old. In this study male and female ancient and mature trees could be easily distinguished from external morphological characteristics; for seedlings and saplings, however, there were no obvious phenotype characteristics enabling us to identify the sex. In the absence of information of a genetic basis for gender determination, the question arises: Could different telomere length in male and female plants become a standard for distinguishing between males and females in the early period of plant life? The means of making such a distinction has eluded researchers for decades.

TRF length for *Ginkgo biloba* L. of four different ages

By comparing TRF lengths for each tissue from trees of different ages it was found that average TRF lengths usually increase with age in many tissues; for different tissues, however, the trend in TRF length during the development of

ginkgo trees was not the same (Fig. 3). In leaf tissue TRF lengths for seedlings and saplings were significantly ($p < 0.05$) shorter than for mature and ancient trees. TRF length usually increased with age, but not in a linear, incremental way. The rate of growth of TRF lengths from seedling to mature tree was greater than that from mature to ancient tree (Fig. 3a). Unlike leaf tissue, differences between TRF lengths in branchlet tissue among ancient and mature trees and saplings was not statistically significant ($p > 0.05$), although all were significantly ($p < 0.05$) longer than for seedlings. TRF lengths from seedling to sapling increased sharply with age but they tended to vary slightly (incresed/decreased) with age at the stages from sapling to mature and from mature to ancient (Fig. 3b). For both microspore (Fig. 3c) and embryonal callus (Fig. 3d) tissues, which could only be obtained for the gender differentiated trees, TRF lengths also increased with age, although the difference between TRF lengths for ancient and mature trees was not significant ($p > 0.05$) for these tissues.

These results indicate that TRF length varies with development and even ancient ginkgo trees maintain their telomeres, suggesting there should be a balance between telomere length and telomerase activity or other factors in cell division. It is well known that, in the absence of other factors, for example telomerase, the length of telomeres would be shortened after each cell division because of the end-replication problem (Kilian et al. 1995). In the development of ginkgo trees, however, when their telomeres increase to an optimum size, their TRF lengths will be in homeostasis. This supports the results of Shakirov et al. (2004), who reported a model for telomere length homeostasis in the Columbia ecotype of *Arabidopsis*. Nevertheless, telomere elongation also was observed in some immortalized human cell lines in vitro (Bryan et al. 1995) which lacked telomerase activity, suggesting a telomerase-independent mechanism. Apart from the telomerase mechanism, divergence in the ALT (alternative lengthening of telomere) mechanism could involve replacement of minisatellite telomere repeats by other tandem repeats, probably the repeats that were the original subtelomeric repeats (Fajkus et al. 2005). Thus, *Ginkgo biloba* L., a species with a long life-span, may have two mechanisms for maintaining telomere length.

Conclusions

We measured TRF lengths for different tissues from *Ginkgo biloba* trees of four different ages. By comparing these TRF lengths we showed that different tissues contained TRF with tissue-specific lengths and the same trend was found in tissues of all ages. By comparing TRF lengths for each tissue from trees of different ages we concluded that TRF lengths vary with development and even ancient ginkgo trees maintain their telomeres; this suggests their telomere lengths are maintained or increased by

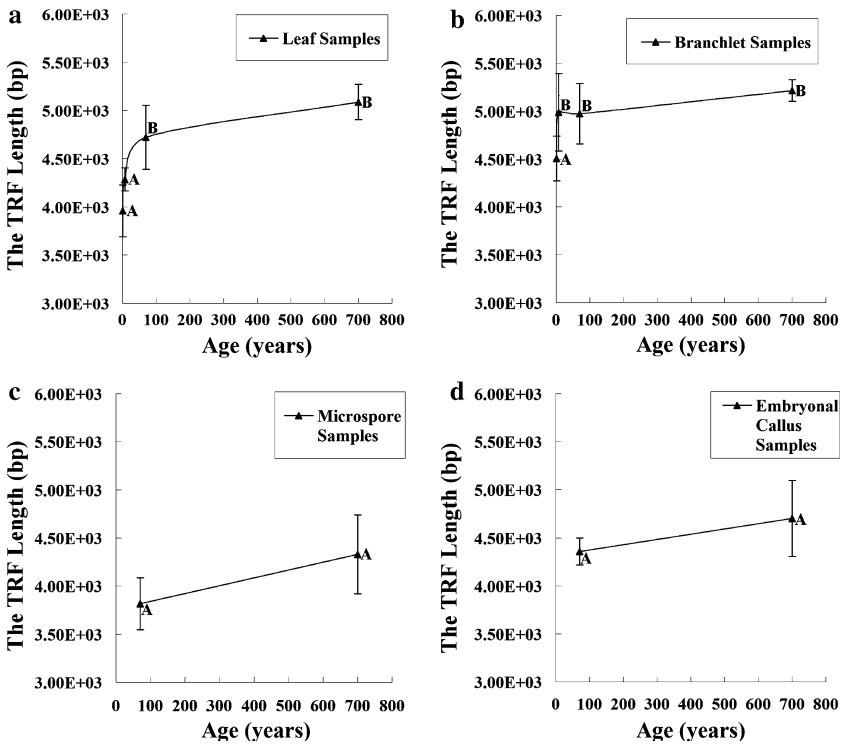

Fig. 3 Trend of variation of TRF length with age for different tissues. Overall, average TRF length increased with age in leaf (**a**), branchlet (**b**), microspore (**c**), and embryonal callus (**d**) samples. *Different letters* beside the *triangle markers* indicate significance at the 0.05 level for the same tissue

maintenance mechanisms (telomerase and/or ALT mechanism). On the basis of these results, we propose a relationship between telomere length and age for *Ginkgo biloba* L.:

$$L(x) = L_0 + \int [A(x) - R(x)]\mathrm{d}x$$

This equation is a mathematical model relating telomere length to age of *Ginkgo biloba* L. In the equation x is the age in years, $L(x)$ the TRF length, L_0 the initial TRF length, $A(x)$ the increase in length resulting from annual maintenance mechanisms (telomerase and/or ALT), and $R(x)$ is the decrease in length as a result of annual cell division. In the equation $L(x)$ can be measured by TRFL analysis. The expression $A(x) - R(x)$ is a variable and represents the annual change in TRF length.

With deeper understanding of telomere structure and telomere end maintenance systems it is highly probable the age of trees could be estimated by use of this model, rather than by use of traditional methods such as counting annual rings, ^{14}C techniques (Bonani et al. 1994), and CT scanning (Mettler et al. 2000).

Acknowledgments The authors thank Lu Yao, Xu Wang, and members of the Botany laboratory for support during data analysis and many helpful discussions, and Renyuan Zhu and Hongtao Wu for support during sample collection in the Wofo Temple of the Beijing Botanical Garden. The authors also thank Qingyin Zeng, Hailing Yang, and Pengjun Cheng for revising the manuscript. This work was supported by the National Natural Science Foundation of China (Grant No.30571473).

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