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Telomere Length Dynamics in Pearl Millet (*Pennisetum glaucum* [L.] R. Br.) as Observed at Different Developmental Stages

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Abstract: **In plants, the developmental dynamics of telomere length have only been studied in a few species to date. Contrasting results have been reported. To search for the pattern(s) operating in plants, a study of the telomere length was made in pearl millet. Telomere length in cells representing different developmental stages: a) embryo, b) leaves of 1-week-old seedlings, 1-month-old plants and boot leaf and c) germ cells (pollen) were compared. The presence of the consensus plant telomere repeat sequence (5′-TTTAGGG-3′) in pearl millet telomeres was first ascertained; the sensitivity of the sequences hybridizing with $(TTTAGGG)_4$ oligonucleotide to time course *Bal* 31 exonuclease digestions were studied on dot blots and gel blots. The exonuclease digestion kinetics revealed the presence of the consensus telomere repeat sequence in pearl millet telomeres. The average telomere length (ATL) was measured from autoradiograms of *Hae* III digested DNA, hybridized with labelled $(5'\text{-}TTTAGGG\text{-}3')_4$ oligonucleotide using "UVI band" software. No significant difference in the average telomere length was observed between the embryo, leaves of 1-week-old seedlings, boot leaf and pollen. The ATL in leaves of 1-month-old plants was slightly higher. The results of the present investigation and analysis of the reports in the other plants suggest that there is an occasional increase in telomere length in some telomeres but no significant decrease due to loss during DNA replication.**

Key words: **Average telomere length, pearl millet, *Pennisetum glaucum,* germ cells, somatic cells, telomere repeat sequence.**

Abbreviations:
ATL: average telomere length

Introduction

Telomeres, the physical ends of the chromosomes, are made up of a long stretch of direct tandem repeats of a short (6/7 bp) nucleotide sequence in the majority of the plant and animal species analysed (Richards, 1995). The telomeres tend to shorten progressively with successive cycles of DNA replication unless they are replenished. The telomere length rescue is largely accomplished by a telomerase – a ribonucleoprotein enzyme with reverse transcriptase activity. In humans, the telomerase was found to be active only in the germ (sperm) and embryonic cells. Therefore, the telomere length is maintained in sperm cells but is reduced with age in somatic cells (Broccoli and Cooke, 1993). In plants, there is no early divergence of the germ line; the vegetative as well as the reproductive tissues develop from the meristem – a mitotically active tissue. Telomerase activity has been detected in shoot and root meristems of some plants (Fitzgerald et al., 1996; Shippen and McKnight, 1998). Considering the plastic developmental mode operative in plants, it was predicted that the telomere length would not differ between somatic tissues formed at different ages of the plant life cycle or between the somatic tissues and the germ cells (Richards, 1995). This has been found in tomato (Broun et al., 1992), *Melandrium album* (Riha et al., 1998) and *Arabidopsis* (Fitzgerald et al., 1999). However, in barley a shortening of the telomere length was reported in the developing inflorescence as well as the embryo (Kilian et al., 1995). It was concluded that the pattern of telomere length maintenance in barley was similar to that observed in humans (Kilian et al., 1995). But in barley, the telomere length in the germ cells was not measured to definitively correlate the observed decrease in telomere length to aging (progression in cell generations). Moreover, the rate of decrease of telomere length in barley was much higher than could be explained as loss during DNA replication from the number of DNA replication cycles taking place (Shippen and McKnight, 1998). Thus, among the plant species investigated, the telomere length remained constant in tomato, *Arabidopsis* and *Melandrium,* all dicotyledonous plants, while it was variable in barley, a monocotyledon. It appears as though more than one pattern exists for upkeep of telomere length in different plants species, a plurality situation akin to man and mice patterns (Harley et al., 1990; Kipling and Cooke, 1990) among animals. To verify this, we studied the telomere length in another monocotyledon – *Pennisetum glaucum,* including the germ cells (pollen) in the analysis, along with the somatic tissues arising at different stages of the plant life cycle.

Materials and Methods

Plant material

Pearl millet inbred line Vg 272 was used in this study. Suraj, a hybrid variety of maize (*Zea mays* L.) and a local variety of onion (*Allium cepa* L.) were included as the positive and negative

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controls. Maize is reported to have 5′-TTTAGGG-3′ repeats in the telomeres (Burr et al., 1992); onion telomeres lack this consensus repeat sequence and are reported to be constituted by a 375 bp tandemly repeated satellite sequence and/or rDNA (Pich et al., 1996).

Pearl millet: 1) Embryo and endosperm: the mature seed was soaked in water overnight and the embryo and endosperm were separated with a dissection needle. 2) Pollen: Spikes with fully emerged styles were cut from the plant in the morning and the peduncle was immersed in a flask containing 3% sucrose solution. The flask was placed on a piece of butter paper with a table lamp focused on it to facilitate anthesis. The dehisced mature pollen was collected on the butter paper. 3) Leaves: leaves from 1-week-old seedlings, 1-month-old plants and the boot leaf (leaf subtending from the inflorescence) were collected. All the materials were frozen in liquid nitrogen and stored at – 20 °C till they were used for DNA extraction.

The top leaf of a 1-month-old plant of maize and the fleshy leaves of the bulb of an onion were collected and frozen as above.

The DNA from the pearl millet embryos and pollen was extracted using the Nucleon Phytopure kit (Amersham Pharmacia, USA) following the manufacturer's instructions. DNA from all other samples was extracted using the method described by Sharp et al. (1988). The integrity of the genomic DNA was checked by running on a gel.

Bal 31 exonuclease digestions were done with 15 units of the enzyme for 100 µg of DNA in a 200 µl volume, using the buffer supplied by the manufacturer (Amersham Pharmacia, USA) at 30 °C. Samples (30 µl) were drawn from the mixture at various time intervals and the reaction was terminated by the addition of 200 mM EGTA and heating at 72 °C for 10 min. Five µl (2.5 µg of initial DNA) of each sample were spotted onto a nylon membrane using a dot blot apparatus. The remaining 25 µl of each sample were ethanol precipitated and re-suspended in $T_{10}E_1$ buffer. It was digested with *Hae* III (Amersham Pharmacia) in the buffer supplied by the manufacturer at 37 °C for 16 h.

To separate the telomere arrays from genomic DNA, *Hae* III digestions were performed with 15 µg of the total genomic DNA, according to the manufacturer's instructions. The DNA was run on a 0.6% gel at 1 v/cm for 19 h, as described in maize telomere length analysis (Burr et al., 1992). Uncut Lambda phage DNA, Lambda *Hind III* digest and *Hae* III digested ØX 174 were used as molecular weight markers. The gels were stained in ethidium bromide (0.5 ug/ml in TAE buffer) to check for complete digestion. The DNA from the gels was transferred to membranes (Hybond N^+, Amersham Pharmacia) by alkaline transfer (Sambrook et al., 1989). A vacuum blot manifold was used for dot blotting the *Bal* 31 exonuclease digested samples. The blots were probed with 5′end-labelled telomere repeat sequence -d (5′-TTTAGGG-3′)$_4$ and strand-labelled DNA size markers. Post hybridization, the membranes were stringently washed and exposed to X-ray film (Kodak, USA) with a Du Pont Crone intensifying screen.

Calculation of average telomere length (ATL)

The telomere lengths were made through densitometric measurements, as previously used in barley (Kilian et al., 1995) and maize (Burr et al., 1992). The autoradiograms were scanned with a densitometer and each lane was divided into 30 grids. The optical density values integrating the volume and molecular weight were obtained for each grid with the "UVI Band" software (UVI Tech, Cambridge, UK). These values were entered on a Microsoft Excel spreadsheet. The average telomere length was calculated using the formula of Kruk et al. (1995). $ATL = \Sigma(MW_i \times OD_i) / \Sigma(OD_i)$, where OD_i is the densitometric output and MW_i is the length of DNA (in kb) at that position. This calculation assumes that DNA transfers at equal efficiency from all points in the gel and the number of target sequences (telomere repeats) per DNA fragment is proportional to the DNA length. Sums were calculated for the entire range over which the signal appeared. The ATL values were calculated from three autoradiograms of three independent DNA extractions for each tissue and were expressed as mean ± SE of the three measurements (Kilian et al., 1995).

Results

In the dot blots with genomic DNA, digested with *Bal* 31 exonuclease for various time intervals, probed with the consensus plant telomere repeat sequence -(TTTAGGG)$_4$, a decrease in the intensity of the signal was observed for up to 60 min (Figs. **1 a, c**). In the autoradiogram of the Southern blot of the *Bal* 31 + *Hae* III digests of the genomic DNA, probed with the telomere oligo, a progressive decrease in the average telomere length was observed (Figs. **1 b, d**). These results indicate that the consensus telomere sequence 5′-TTTAGGG-3′ is present in pearl millet telomeres.

In the autoradiograms with *Hae* III digests of DNA from different pearl millet tissues, maize and onion probed with the telomere repeat sequence, positive signals corresponding to the telomeres were observed as a streak both in maize (positive control) and pearl millet. No signal was observed in the lane with onion DNA (the negative control) (Fig. **2**). The ATL of maize, as measured from these autoradiograms, is 7.18 ± 0.2 kb. The size range over which the signal appeared and the ATL values in the pearl millet samples are given in Table **1**. No significant difference in ATL was observed between the germ cells (pollen) and the vegetative cells of the embryo, leaves from 1-week-old seedlings and the boot leaf. The mean of the ATL values of these four samples of pearl millet is ~ 23.8 kb. The ATL value in leaves from 1-month-old plants was ~ 28 kb; it was 5 kb higher than the mean of the ATL values of the other samples of pearl millet analysed.

In the lane with endosperm DNA, the signal appeared as a streak throughout (starting from the well at the bottom of the gel) in both the uncut and *Hae* III digested samples (Fig. **2**). Therefore, the ATL in this tissue could not be calculated. Nimmi (1991) reported a high degree of polyteny in the endosperm cells of pearl millet and that all endosperm cells are dead 20 days after pollination. The DNA from the endosperm of the mature seed from dead cells must have degenerated and thus appeared sheared in the gels.

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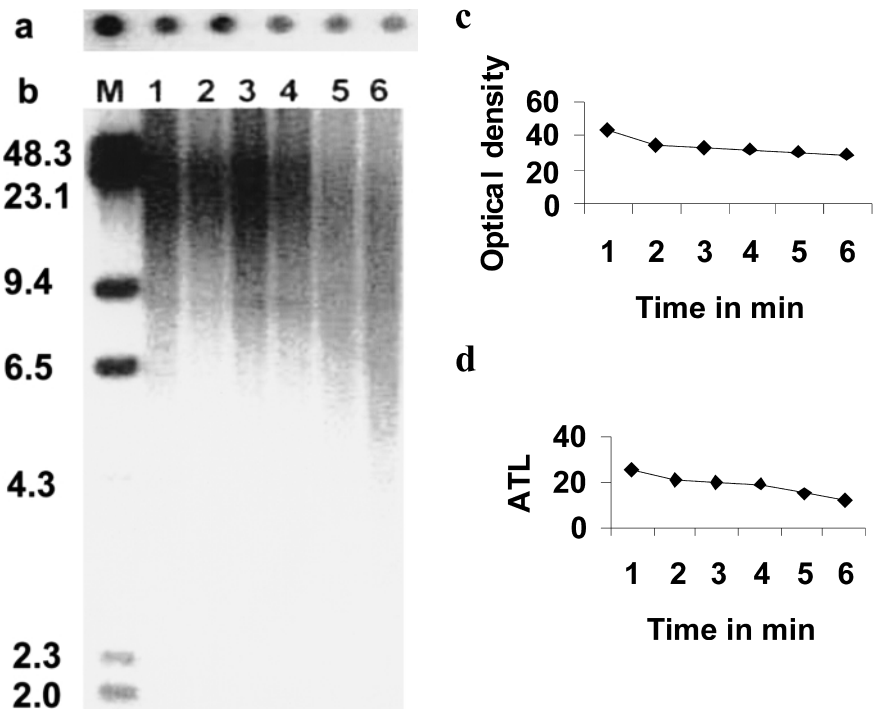

Fig. 1 (**a, b**) Respectively autoradiograms of dot blot of *Bal* 31 exonuclease digested DNA for 0, 5, 10, 20, 40 and 60 min (from left to right) and Southern blot of samples in **a** subsequently digested with *Hae* III from a 1-week-old seedling of *Pennisetum glaucum* (L.) R. Br. hybridized with $(5'\text{-TTTAGGG-}3')_4$ oligonucleotide sequence (representing tandem repeat sequence in plant telomeres), end-labelled with [γ-P^{32}] dATP. (**c**) The optical density values ($\times 10^7$) of the dots in blot **a** (values per 2.5 g DNA); the values decrease with time of *Bal* 31 nuclease digestion. (**d**) Average telomere length (ATL in kb) calculated from blot in **b**; it decreases with increase in time of *Bal* 31 nuclease digestion.

Table 1 Average telomere length in kb in different tissues (representing different developmental stages) of *Pennisetum glaucum* (L.) R. Br.

Tissue	Average telomere length*
Leaf (1-week-old seedling)	22.95 ± 1.02 (45.9 – 7.52)
Leaf (1-month-old plant)	28.20 ± 0.68 (45.9 – 7.46)
Boot leaf (leaf subtending the inflorescence)	23.9 ± 2.78 (45.9 – 7.52)
Pollen	24.34 ± 1.70 (45.9 – 7.52)
Embryo	24.02 ± 1.63 (45.9 – 7.52)

* Mean ± SE from three autorads; the values in the brackets indicate the range over which the signal of hybridization with the telomere repeat sequence $(5\text{-TTTAGGG-}3)_4$ appeared in the autoradiogram in Fig. **2**. The ranges in the other two autoradiograms are similar.

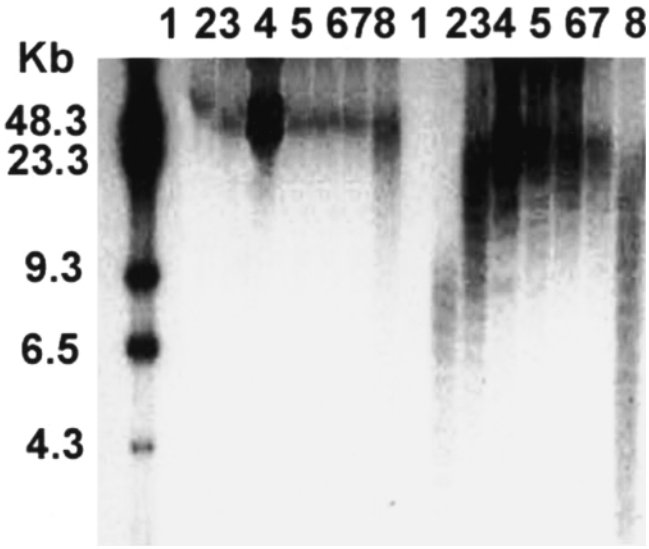

Fig. 2 Autoradiogram of the Southern blot of total genomic DNA (Lanes 1 – 8, left) and *Hae* III digested DNA (Lanes 1 – 8, right) from different tissues of *Pennisetum glaucum* (L.) R. Br. hybridized with the plant telomere repeat sequence $(5'\text{-TTTAGGG-}3')_4$ end-labelled with [γ-P^{32}] dATP. Lanes with the same numbers have DNA from the same tissue. Lane 1 has DNA from the fleshy leaves of a bulb of *Allium cepa* (negative control – *Allium* is reported to have satellite DNA in the telomeres [Pich et al., 1996]), lane 2 has DNA from the top leaf of a 1-month-old plant of *Zea mays* (Positive control – maize is reported to have a TTTAGGG repeat sequence in the telomeres [Burr et al., 1992]). Lanes 3 – 8 have DNA from *P. glaucum*. Lane 3, 1-week-old seedling; lane 4, 1-month-old seedling; lane 5, boot leaf (leaf subtending the inflorescence) from the main tiller; lane 6, pollen (male germ cells); lane 7, embryo; lane 8, endosperm. The sizes of the DNA size marker are in kb.

Discussion

In pearl millet, no significant difference in telomere length was observed between the germ cells and embryo, and the vegetative tissues of the seedling and the boot leaf. The size range over which the telomere sequence highlighted streaks appeared in the autoradiogram were similar in all these lanes. Though pulse field gel electrophoresis was not used in the present study, the electrophoretic conditions in which the gel was run would show size differences that are significant (such as the difference observed between sperm and somatic cells in humans). A similar result was reported in *Melandrium* (Riha et al., 1998), the only other plant species in which telomere length was estimated in both the somatic and germ cells. The pattern of telomerase activity in plants was found to be similar to that in humans (Fitzgerald et al., 1996; Fajkus et al., 1996, 1998; Heller et al., 1996; Riha et al., 1998; Shippen and McKnight, 1998), being active in reproductive tissues (carpels,

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anthers, pollen and embryo), and remaining inactive in vegetative tissues (leaves). Fitzgerald et al. (1999) observed, that "plants and animals have independently evolved mechanisms to repress telomerase in most non-reproductive organs". But the sharp difference in telomere length between the germ cells (sperm) and somatic cells noted in humans (Broccoli and Cooke, 1993) has not been observed either in *Melandrium* (Riha et al., 1998) or in the present investigation of pearl millet. This distinction between plants and humans, despite similar patterns of telomerase activity, may be due to the difference in their developmental patterns. The germ line in plants, in contrast to animals, is not differentiated early in their life history but is derived from structures (anthers and carpels) arising at the time of developmental maturity from meristems – the mitotically active tissues mediating growth in which telomerase is reported to be active. Therefore the vegetative cells and the germ cells progress through a similar number of cell generations from their time of initiation (from the meristem) to differentiation. From the slower rate of telomere length shortening in telomerase null mutants of *Arabidopsis,* Fitzgerald et al. (1999) also suspected that fewer nucleotides may be lost in *Arabidopsis* per round of DNA replication. If this were a general situation in plants, then the difference in telomere length resulting from the preferential activity of the telomerase in reproductive tissues compared to vegetative tissues may not be substantial enough to become discernable in the gels. The telomere length pattern observed in vegetative structures formed at different ages of the plant such as leaves on older and younger plants in tomato (Broun et al., 1992), in pearl millet in the present investigation, and in leaves, flower buds and siliques in *Arabidopsis* (Fitzgerald et al., 1999) has been found to be similar. Despite the absence of telomerase activity in vegetative tissues, such a status quo situation of telomere length may again be due to the plastic developmental pattern in plants. The cells of the leaves formed at different ages of the plant are all the same number of cell generations away from the meristematic tissue from which they arise.

In barley, Kilian et al. (1995) observed long telomeres in young inflorescences and embryos and nearly 20 kb shorter telomeres in leaves. They likened it to the situation in humans, where germ and embryo cells have conspicuously longer telomeres than somatic cells. In barley, a rapid decrease in telomere length was observed as the inflorescence and embryo matured (Kilian et al., 1995), the telomeres shortened by ~ 20 kb during these stages and equalled the telomere length in the cells of the leaves (Kilian et al., 1995). Shippen and McKnight (1998) estimated that such a large and swift decrease cannot be explained as due to the loss associated with successive cycles of DNA polymerase-mediated DNA replication in the absence of telomerase activity. Rather, it may be the outcome of an active excision mechanism. In fact, in barley, telomerase activity has been demonstrated in growing embryos (Heller et al., 1996), at a stage when a rapid decrease in telomere length was reported by Kilian et al. (1995). Evidence for operation of an active mechanism of telomere excision is found in wheat embryos: large extra-chromosomal fragments consisting of telomeric repeats have been isolated from dormant wheat embryos but not young embryos (Bucholc and Buchowicz, 1995). Large segments of telomeric heterochromatin were found in rye tissues in culture (Karp et al., 1992). These fragments are inferred to be the result of active excision from the telomeres (Shippen and McKnight, 1998). Thus it could be that the normal ATL value in barley is what is observed in the leaves, and the long telomeres in the immature inflorescence and young embryo may be the result of an increase in telomere length. The ATL value in the leaves of 1-month-old plants of pearl millet was also found to be slightly more than the rest of the tissues analysed. The upper range over which the hybridization signal appeared was similar to the other tissues, while there was a slight change in the lower range (Table **1**). An increase in telomere length of some chromosomes has also been observed in telomerase null mutants of *Arabidopsis* (Fitzgerald et al., 1999; Riha et al., 2001). Telomere extension was observed in *in vitro* cultured cells of barley (Kilian et al., 1995) and *Melandrium* (Riha et al., 1998). Fajkus et al. (1998) noted that telomere lengthening may not be the effect of *in vitro* culture conditions, as the telomere length remained stable in cultured tobacco cells. In *Arabidopsis,* the increase in telomere length of some telomeres in telomerase null mutants is believed to be due to the operation of alternate telomere maintenance mechanisms like recombination (Fitzgerald et al., 1999; Riha et al., 2001). Plants induce recombination pathways at a higher frequency than animals (Tovar and Lichtenstein,1992; Fitzgerald et al., 1999). The events related to telomere length increases (substantially on occasion) have yet to be studied. Whatever the mechanism, it appears that an occasional stochastic increase in telomere length of some telomeres may be prevalent in plants.

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