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Development patterns of telomerase activity in barley and maize

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Abstract

Eukaryotic chromosomes terminate with specialized structures called telomeres. Maintenance of chromosomal ends in most eukaryotes studied to date requires a specialized enzyme, telomerase. Telomerase has been shown to be developmentally regulated in man and a few other multicellular organisms, while it is constitutively expressed in unicellular eukaryotes. Recently, we demonstrated telomerase activity in plant extracts using the PCR-based TRAP (Telomeric Repeat Amplification Protocol) assay developed for human cells. Here we report telomerase activities in two grass species, barley and maize, using a modified, semi-quantitative TRAP assay. Telomerase was highly active in very young immature embryos and gradually declined during embryo development. The endosperm telomerase activity was detectable, but significantly lower than in the embryo and declined during kernel development with no detectable activity in later stages. Telomerase activity in dissected maize embryo axis was several orders of magnitude higher than in the scutellum. Telomerase activity was not detected in a range of differentiated tissues including those with active meristems such as root tips as well as the internode and leaf base. The role of telomerase repression during differentiation and the relationship between chromosome healing and telomerase activity is discussed.

Introduction

Biochemically characterized DNA-dependent DNA polymerases are unable to fully replicate linear DNA molecules creating an ‘end-replicating problem’ [26, 31]. Evolutionary solutions to this problem in prokaryotes include genome circularization and a ‘terminal protein’-linked nucleotide primer [19]. Eukaryotic cells have evolved a different solution that involves a specialized reverse transcriptase-like enzyme, telomerase [12]. Telomerase uses its own RNA component as a template for addition of short, usually G-rich repeats onto chromosome ends. Stretches of the repeats synthesized by telomerase constitute the DNA component of the structure localized at the ends of eukaryotic chromosomes called the telomere [21, 25]. The telomere was originally defined as a protective cap for chromosome ends that is essential for chromosome stability. Recent studies emphasize the importance of the telomere for eukaryotic cells by demonstrating that the loss of a single telomere causes cell cycle arrest in yeast [29]. Broken chromosomes without a telomere are not maintained in cell division unless it is ‘healed’ by the addition of a new telomere. Telomerase was shown to perform the ‘healing’ process in humans [24] and *de novo* telomere formation in several species [22].

Telomerase activity has been identified in a number of lower and higher eukaryotes [5]. Interestingly, unicellular organisms express telomerase continuously, while in animals telomerase shows tissue and development specific expression patterns. In humans, the best studied organism, telomerase is primarily active in germ-line and embryonic tissues and cells [34] as well

as in most immortalized cell lines [24]. Telomerase was detected in a majority of human tumors and its reactivation may constitute an important event for continuous tumor cell growth [7, 17]. Telomerase activity is practically absent in normal somatic human tissues although, since the development of the highly sensitive PCR-based assay TRAP [17], reports of low enzyme activity in some somatic tissues have appeared [3]. Lack of telomerase activity in human somatic tissues is reflected by the shortening of terminal restriction fragments (TRF) in differentiated tissues compared to germ-line cells [1, 6]. This observation led to the hypothesis that telomere lenght and telomerase activity may be used as markers of the replicative history and proliferative potential of cells [13]. The role of telomere length in cellular senescence has gained significant interest and support in recent years [2, 23, 33], although a causal relationship has not been proven. Another issue unders scientific debate is the role of telomerase activity in cancer progression and immortalization [11]. Recently, immortalized human cells without detectable telomerase activity have been reported [4] and some notable exceptions to the rule of telomerase positive tumor cells and negative somatic cells are known [3, 17].

Compared to the very extensive ciliate and animal telomerase research, the plant kingdom is barely represented in the literature, in spite of the fact that the telomere concept was developed in a plant (maize) system [21] and the first higher eukaryote telomere was cloned from a plant, *Arabidopsis thaliana* [28]. Telomerase could be inferred as a mechanism for telomere maintenance in plants based on homology of plant terminal repeats with the consensus sequence for telomere repeats in other eukaryotes [18]. Recently we demonstrated that telomerase activity was also present in higher plant extracts [14]. Telomerase activity was detected in barley immature embryo, carpel and anther, but not in leaf blades. Previously we reported that telomere length in barley is regulated as a function of the developmental stage [16] suggesting that barley telomerase may be repressed in differentiated barley tissue. Telomerase activity was recently reported in several dicotyledonous plants and the activity was correlated with differentiation [10]. Here we report the results of a semi-quantitative, modified TRAP (Telomere, Repeat Amplification Protocol) assay of telomerase activity in two grass species, barley and maize. The role of telomerase repression during differentiation and the relationship between chromosome healing and telomerase activity is discussed.

Materials and methods

Plant material

Barley (*Hodeum vulgare* L. cv. Steptoe) plants were grown at 18 °C and 16 h light, while maize (*Zea mays* W22 inbred) plants were grown at 20 °C and 16 h light. Extracts were prepared from ca. 100 mg tissue as previously described [14].

Barley tissues. Various leaf parts and developmental stages were analyzed including the leaf blade (cell elongation region) and base (cell division region) from 1-week-old seedlings, as well as the blade and ligule of the flag leaf. The top node, basal internode fragment (including node junction) and middle internode part were also analyzed. Root tips and elongation zone were excised from 2-day-old seedlings. The apical (floral) meristems were ca. 5 mm long, while young inflorescences were 2 cm long. Young kernels 3–4 days after pollination (DAP) were bulked. Embryo and endosperm were isolated at 10 DAP (stage 1), 17 DAP (stage 2) and 24 DAP (stage 3). Embryos were also analyzed from germinating seeds (imbibed in water for 6 h and germinated overnight).

Maize tissues. Leaf blade was excised from the top leaf of a flowering plant. Young seeds, 7 DAP, were bulked. Embryos and endosperm were excised from kernels at 14 DAP and 25 DAP. The 25 DAP embryos were separated into embryo axis and scutellum as well as analyzed whole.

TRAP assay

The TRAP assay was as previously described [14] with several modifications introduced to increase sensitivity and linearity. The Telomerase Substrate (TS) forward primer was replaced by an Altered Telomerase Substrate (ATS) primer (5′-GATCCACGTCGAGCAGAGTT-3′) modified at the 5′ end to match the T_m of the reverse (Tel-5′-AAACCCTAAACCCTAAACCCTAAACCC-3′) primer. Annealing temperature was increased to 57 °C. The most important modification was the use of an Internal Standard (IS) in the PCR amplification [35]. Our IS was based on the ITAS standard described in detail by Wright *et al.* [35]. The ITAS sequence (20 attograms (ag, 10^{-18} g)) was amplified with ATS and CX (5′-CCCTTACCCTTACCCTTACCCTAA-3′) [17] primers and the PCR product was cloned into a

pUC19 vector containing 2.5 plant telomere repeats. The resulting insert was amplified with ATS and Tel primers. The amplification product (ca. 160 bp) was purified by PAGE, eluted in water, ethanol-precipitated and diluted to 10 ag/ml. The IS (15 ag per 50 μl reaction) was added to the PCR amplification step of the TRAP assay at hot start [14]. TRAP products were resolved on a 10% polyacrylamide gel (BioRad).

Data analysis

Polyacrylamide gels with TRAP products were analyzed using AMBIS Radioanalytic System and the software provided by the manufacturer. Only the middle 50% of the lane was scanned to avoid interference from the neighboring lanes. TRAP ladders were read from the first (49 bp) product up to just below the IS band which was read separately. Standardized telomerase activities are expressed as the ratio of the TRAP ladder and IS signals.

Results

TRAP assay

The TRAP assay exploits the capacity of the telomerase in a tissue or cell extract to synthesize telometric repeats by elongation of a non-telomeric oligonucleotide, the altered telomerase substrate (ATS). These telomerase products are then amplified in a polymerase chain reaction employing the ATS and Tel oligonucleotides as the upstream and downstream primers, respectively [14, 17, 35]. By separating the PCR products on a polyacrylamide gel, the 7 bp addition products generated by the telomerase are visualized as a ladder of DNA fragments. The ladder is a consequence of the enzyme pausing after each cycle of repeat addition.

Recent success in improving the linearity of TRAP assay for human cell extracts by incorporating an internal standard [35] prompted us to include this modification in our assay. To test the improved assay, we analysed telomerase activity at various protein concentrations (over 2 orders of magnitude) and telomerase extension times.

Maise immature embryo telomerase was readily detectable at a very low protein concentration (6 ng per reation) and the shortest extension time (5 min) (Figure 1A). Increasing the amount of protein by tenfold resulted in a stronger ladder and a weaker IS band, with the most apparent signal intensity increase in the higher molecular weight TRAP products. This was most obvious with 600 ng of protein (lane 1) where most of the signal was detected around the IS band. Only a several fold difference in the TRAP signal intensity was observed among telomerase ladders obtained with a 100-fold protein and 6-fold extension time ranges (Figure 1A and 1C). The IS signal, on the other hand, varied profoundly among the samples showing a good inverse correlation with protein concentration and time. Plotting standardized telomerase activity (the ratio of TRAP signal to IS signal) against protein concentration showed good linear relationship for all extension times (Figure 1B). The most linear relationship between protein concentration and standardized telomerase activity was observed for a 30 min extension and this time was used throughout this study. We believe that the reason for the best assay performance at the longest extension time is that the IS band is most clearly separated from the telomerase ladder at this stage (Figure 1A). With shorter extension times the IS signal overlaps with the telomerase ladder resulting in underestimation of telomerase activity at the higher protein concentrations. The benefit of using the IS was apparent when the results of the standardized telomerase activity were compared with non-standardized activity (Figure 1C). Without the IS, the 10 min extension time gave only a 3-fold increase in the TRAP signal for a 100-fold increase in the protein assayed. The relationship was even worse for 5 min and 30 min extension times.

The modifications to our assay described here resulted in much better quantification of the telomerase activity compared to previous results [14]. Nevertheless, the same qualitative differences between telomerase-rich and telomerase-poor tissues were observed. Thus, we are confident that the results reported here reflect biologically meaningful differences in telomerase activity in various plant tissues.

Telomerase activity in developing barley and maize seed and apical meristems

Various developmental stages of barley and maize seeds and seed parts were analyzed. Very young seeds, 3–4 DAP, had very high enzyme activity and the activity was reduced during embryo development (Figure 2A). Mature embryos, including germinating embryos, had very low telomerase activity (Figure 2A). Analysis of embryo and endosperm from 3 stages of barley (Figure 2B) and 2 stages of maize seed development (data not presented) showed that the embryo

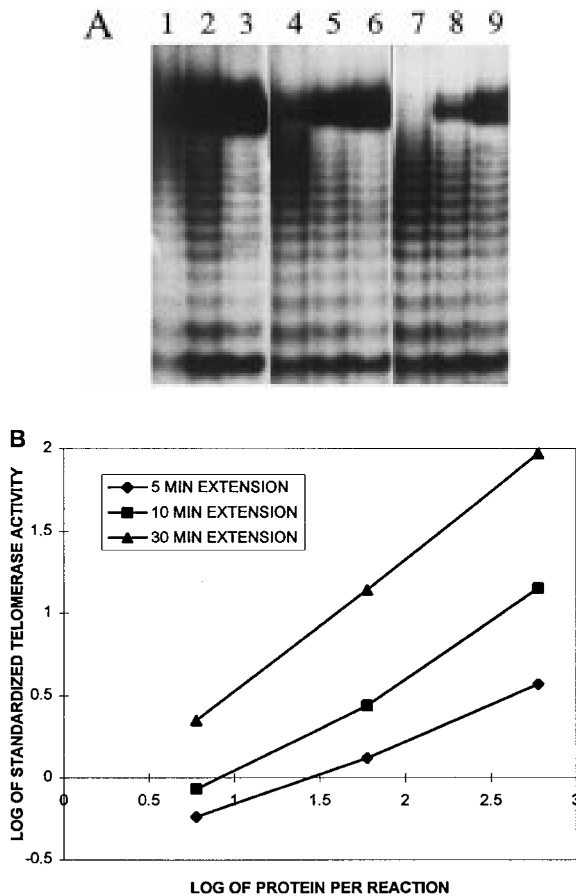

Figure 1. Internal Standard (IS) improves TRAP assay performance in plant extracts. A. TRAP assay amplification products using maize immature embryo extract. Lanes 1–3, 5 min extension reaction; lanes 4–6, 10 min extension reaction; lanes 7–9, 30 min extension reaction. Lanes 1, 4 and 7, 600 ng total protein; lanes 2, 5, and 8, 60 ng total protein; lanes 3, 6 and 9, 6 ng total protein. The bottom band is 49 bp and the top, heavy band, representing the Internal Standard (IS), is 160 bp. Note that the IS band in lane 7 is very weak, indicating that the *Taq* polymerase has become limiting due to the very extensive telomerase extension products outcompeting the IS. B. Relationship between standardized telomerase activity and protein concentration at three extension times. For all extension times telomerase activity correlated well with protein concentration. C. Relationship between unstandardized telomerase activity and protein concentration at three extension times. Telomerase activity is underestimated at higher protein concentrations and longer extension times.

telomerase activity is 3–10 times greater than from the corresponding endosperm. Telomerase activity was not detected from late endosperm development stages (beyond 24 DAP) in either species (Figure 2B, Figure 3). The maize embryo from the late stage could be dissected into a scutellum and an embryo proper. A dramatic difference in telomerase activity was observed for those tissues (Figure 3). Precise quantit-

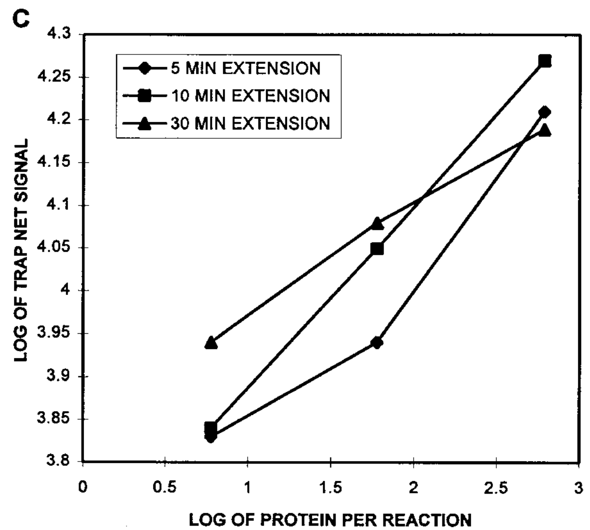

Figure 1. Continued.

ative comparisons were not possible since the internal standard was not detectable for embryo axis due to the very high telomerase activity. However, judging from the signal intensity of the TRAP ladder for 6 ng embryo axis protein (12 700 cpm) and 600 ng of the scutellum protein (9500 cpm), we estimate about two orders of magnitude difference in telomerase activity between those two tissues.

The barley inflorescence meristem showed considerable telomerase activity while the immature inflorescence (ca. early meiosis stage) had very little telomerase activity (Figure 4). Reduction of enzyme activity was not caused by telomerase/*Taq* polymerase inhibition as determined by mixing experiments (data not presented).

Telomerase is repressed in all differentiated tissues as well as root tips of barley

We searched for telomerase activity in numerous barley and maize tissues beyond the one previously described [14]. Leaf tissues analyzed included leaf blade, first leaf base and ligule area of the flag leaf. Stem samples included node, base of the last internode (around node junction) and middle part of the last internode. Telomerase activity was not detected over a 60 ng to 6 μg protein range. All the telomerase negative extracts were tested for the presence of telomerase and/or *Taq* polymerase inhibitors by mixing them with standard amount (600 ng of protein) of telomerase positive barley embryo extract (Figure 5). No significant inhibition

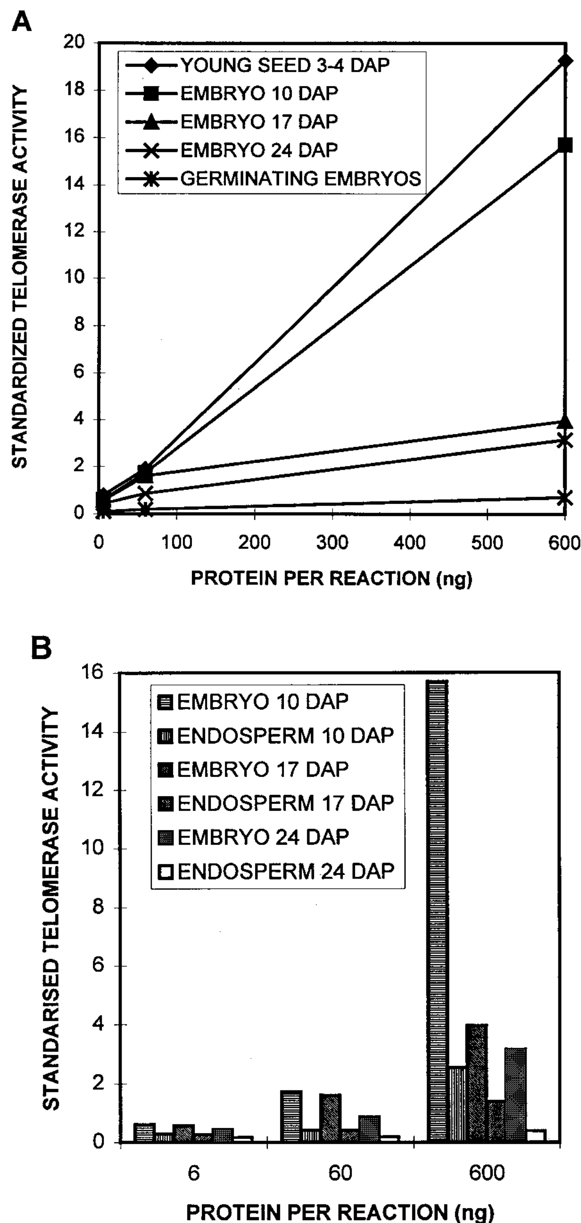

Figure 2. Telomerase activity during barley seed development. A. Extracts from five stages of embryo development were analyzed by standardized TRAP assay using three 10-fold dilutions (from 6 ng to 600 ng total protein) of each extract. Young seed (3–4 DAP); Stage 1 (10 DAP); Stage 2 (17 DAP); Stage 3 (24 DAP); Overnight germinated mature embryos. B. Standardized telomerase activity in embryo and endosperm during seed development. Embryo and endosperm extracts from Stage 1 (10 DAP), Stage 2 (17 DAP), and Stage 3 (24 DAP) were analyzed using standardized TRAP assay using three 10-fold dilutions (from 6 ng to 600 ng total protein) of each extract.

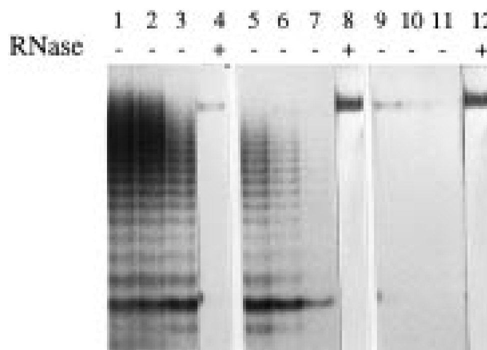

Figure 3. Telomerase activity in maize kernels. Maize kernels (25 DAP) were dissected into embryo axis (lanes 1–4), scutellum (lanes 5–8) and endosperm (lanes 9–12) and extracts assayed by TRAP at 3 protein concentrations: 600 ng total protein (lanes 1, 4, 5, 8, 9 and 12); 60 ng total protein (lanes 2, 6 and 10); 6 ng total protein (lanes 3, 7 and 11). Lanes 4, 8 and 12 represent samples treated with RNAse prior to TRAP assay.

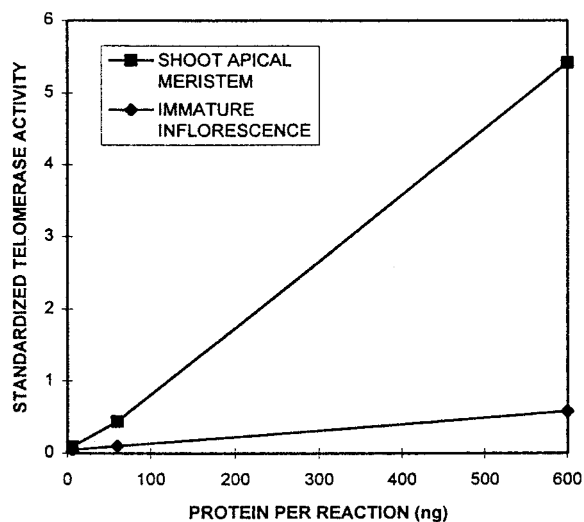

Figure 4. Telomerase activity is reduced during barley inflorescence development. Extracts from shoot apical meristem and immature inflorescence were analyzed using TRAP assay at three protein concentrations (6, 60 and 600 ng of total protein per reaction).

of telomerase/*Taq* polymerase was observed in any of the extracts analyzed.

Two root segments were analyzed: a terminal 3–4 mm fragment (root tip) containing the root apical meristem and the cell elongation zone. Neither extract showed telomerase activity across protein concentrations ranging from 60 ng to 6 μg. We can not exclude a very low telomerase activity in the root cell elongation zone because of very high nuclease activity of this extract. Even diluted elongation zone extract (600 ng of protein per reaction) strongly affected the TRAP lad-

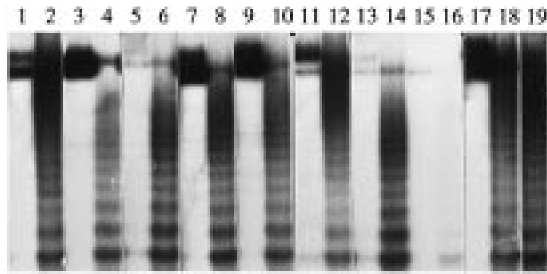

Figure 5. Telomerase not detected in differentiated tissues. Mixing experiments with telomerase-negative and -positive extracts demonstrated that the lack of TRAP products in differentiated tissues is not caused by telomerase/*Taq* polymerase inhibitors. Lane 1, barley leaf blade, 3 μg protein; lane 3, young barley leaf base, 3 μg protein; lane 5, barley ligule, 600 ng protein; lane 7, barley node, 600 ng protein; lane 9, barley node-internode junction, 600 ng protein; lane 11, barley internode, 3 μg protein; lane 13, barley root tips, 600 ng protein; lane 15, barley root elongation zone, 600 ng protein; lane 17, maize leaf blade, 600 ng protein. Lanes 2, 4, 6, 8, 10, 12, 14, 16: as above, except that barley embryo (stage 2) extract, 600 ng protein, was added to each differentiated tissue extract. Lane 19, barley embryo (stage 2), 600 ng protein.

der signal in the mixed root/embryo extract (Figure 5). Root tip extract showed much weaker inhibition of the TRAP ladders since good signal was obtained even with 3 μg of protein from this tissue when mixed with the telomerase positive extract.

Discussion

We have developed a sensitive and linear plant telomerase assay by adapting the human TRAP assay [17, 27]. The previously published assay [14] was improved by changing the forward primer (ATS) to match the T_m and reduce the complementarity with the reverse primer (Tel). An IS was developed and included in all assays. Incorporating an IS represented a very important step towards a fully quantitative and reliable telomerase activity assay, in complete agreement with the conclusions reached from similar modification of the human telomerase assay [35].

Telomerase activity patterns in maize and barley suggest that the enzyme is progressively repressed during differentiation. The enzyme is very active during early embryo development, decreasing rapidly (2–3 weeks). This activity reduction coincides with embryo differentiation. Maize embryos enabled us to analyze the scutellum and embryo proper separately, demonstrating that the embryo axis has about 100-fold higher telomerase activity than the scutellum. At present we can not determine if the whole embryo axis is expressing high telomerase activity or if it is confined to specific regions.

Apart from the developing seed (embryo and young endosperm) and generative tissues (anther and carpel), telomerase was detected only in the flowering shoot apical meristem. The flowering meristem had very high telomerase activity declining to a trace in the immature inflorescence around the early meiosis stage. All other tissues, various leaf and root parts, failed to show detectable telomerase activity in multiple experiments and with various protein concentrations.

Telomerase appears to be absent from these tissues since mixing experiments with telomerase-positive extracts showed no significant telomerase and/or *Taq* polymerase inhibition. There was an appreciable effect of nucleases in certain tissues (root elongation zone, for example) but even nuclease-rich extracts did not inhibit the positive signal in mixing experiments at protein concentrations of 600 ng to 3 μg. Lack of telomerase activity in the root apical meristem is particularly intriguing, especially since high levels of telomerase activity was reported for 3-day old root tips of soybean [10]. The root tip is a truly meristematic tissue and in many anatomic and developmental aspects resembles the shoot apical meristem [9]. However, the function of root apical meristem in plant development is markedly different from shoot apical and inflorescence meristems. It is tempting to speculate that telomerase in plants (at least barley and maize) is primarily active in the population of cells that are likely to contribute to germ-line development. Lack of telomerase in root tips and intercalary meristems of leaves (ligule area) and stem (internode base) argues that the telomerase activity is not necessarily a characteristic of all meristematic tissues. However, we can not exclude the possibility that in these tissues only a few cells have an active telomerase. The number of cells with an active telomerase would have to be about two orders of magnitude less than in the tissues with demonstrated telomerase activity.

We have previously reported that the barley telomere restriction fragment length was progressively shortened during embryo and inflorescence development [16]. These data correlate well with the telomerase activity levels reported here. The telomere restriction fragments from leaves were the shortest, consistent with no detectable telomerase activity in leaf tissue. However, telomere length also decreased in older stages of embryo development, although low telomerase activity was present. A similar phenomen-

on (low telomerase activity in tissues with telomere shortening) was reported for man [3]. It was suggested that only a small proportion of cells in the population analyzed have active telomerase while the majority of cells experience telomere reduction leading to an apparent telomere shortening for the whole population. Our observation that in the developing maize embryo telomerase is present primarily in the embryo proper with almost no activity in the scutellum, which accounts for a majority of the embryo tissue mass, supports this explanation. Additionally, it is becoming apparent that the regulation of telomere length in the cell is not a simple result of telomerase activity and incomplete DNA replication by DNA dependent polymerases [5]. Nuclease activities and telomere binding proteins are most likely additional important players in the process, especially, since telomerase itself seems to posses endonuclease activity [5].

There are profound differences between plants and animals in their developmental patterns and cell division activities [15]. Also, recent reports show that the cell proliferation controls in plants and animals may be very similar but they may interact differently during development [8]. Assuming a role for telomerase and telomere length in the control of DNA replicative capacity and senescence of the cell, one could expect significantly different patterns of telomere length and telomerase activity regulations between plant and animal kingdoms. The data reported here for telomerase activity and the results published previously show some remarkable similarities between plant and animal systems [10, 14, 16]. The most apparent similarity is in the enzyme's activity in the embryo and germ line [20, 34] and down regulation of telomerase activity as well as telomere shortening during the differentiation process. The root apical meristem with undetectable telomerase activity and the low telomerase activity in early endosperm development does not conform to the general picture. Some exceptions to the rule of telomerase negative differentiated cells and telomerase positive dedifferentiated cells have been identified also in animals [3, 4]. However, the general relationship between high telomerase activity in embryonic and germ-line cells versus low activity in differentiating cells, seems to be well established.

Differences in the telomerase expression levels between embryo and endosperm deserves a comment. At the beginning of the telomere concept, Barbara McClintock observed differences in broken chromosome behavior between maize embryo and endosperm. Broken chromosomes were going through the so called breakage-fusion-bridge cycles in the endosperm while chromosomes wre 'healed' in the embryo tissue. Reports indicating similar differences in 'healing' abilities in wheat [32] and barley [30] have been published and differential telomerase activity was postulated [30]. Our data support the hypothesis that telomerase is responsible for chromosome healing in barley and maize, and possibly other plant systems. Telomerase activity detected in the early stages of endosperm development in both maize and barley suggest that either the level of activity or its timing is insufficient for effective chromosome healing in the endosperm.

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