

SHORT COMMUNICATION

Katarzyna Heller · Andrzej Kilian
Mieczysław A. Piatyszek · Andris Kleinhofs

Telomerase activity in plant extracts

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Abstract Telomeres of most eucaryotes terminate in long stretches of short, guanine-rich repeats. Telomerase, a specialized enzyme with reverse transcriptase-like activity, has been shown to synthesize these repeats in many lower eucaryotes and several animal species. Although a sequence (TTTAGGG)_n that matches the eucaryotic consensus sequence T_x(A)G_y is present in several plant species, the activity and expression patterns of plant telomerase have not been reported. Here we document the presence of telomerase activity in plant tissues using a modification of the human Telomeric Repeat Amplification Protocol (TRAP) assay. Telomerase activity was detected in barley embryo, anther and carpel tissues and in immature seeds of *Arabidopsis thaliana*, but not in barley leaf tissue.

Key words Barley · *Arabidopsis thaliana* · Telomere · Chromosome

Introduction

Chromosome termini in many evolutionarily distant eucaryotes are maintained by telomerase (reviewed in Kipling 1995). This enzyme is a unique ribonucleoprotein with reverse transcriptase-like activity that synthesizes telomere repeats using its own RNA template. It appears that the most terminal sequences of eucaryotic chromosomes are thus determined by the

coding domain of the telomerase RNA component (Blackburn 1992).

Most of the eucaryotes tested to date have a similar telomere repeat sequence conforming to the consensus sequence T_x(A)G_y (Kipling 1995). This consensus sequence matches the telomere repeat sequence (TTTAGGG) of many plant species including *Arabidopsis thaliana* and barley (Richards and Ausubel 1988; Kilian and Kleinhofs 1992). In situ hybridizations using the (TTTAGGG)_n repeat as a probe have demonstrated the presence of this sequence at chromosome ends in diverse plant genera (Fuchs et al. 1995). Telomere sequence conservation suggests that the mechanism of maintenance of chromosome termini is similar in plants and other eucaryotes. However, telomerase activity, or any other mechanism of telomere maintenance in plants, has not been reported.

Telomerase activity was originally detected by a telomere substrate extension assay (Greider and Blackburn 1985). Recently, TRAP (Telomeric Repeat Amplification Protocol), a highly sensitive, PCR-based assay, has been developed for detection of human telomerase activity (Kim et al. 1994). TRAP has been successfully used for telomerase activity assays in other organisms, including yeast (Lin and Zakian 1995) and mouse (Prowse and Greider 1995; Bednarek et al. 1995), thereby proving its usefulness for non-human systems. In this report, the TRAP assay was adapted for detection of telomerase activity in higher plant tissues. Telomerase activity was detected in barley embryo, anther and carpel tissues, as well as in immature seeds of *A. thaliana* but not in barley leaf tissue.

Materials and methods

Plant material and preparation of extracts

Plants for extract preparation were grown in growth chambers at 18°C and with 16 h light. Immature embryos, 1–2 mm long, were

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K. Heller · A. Kilian¹ (✉) · A. Kleinhofs
Departments of Crop and Soil Sciences and of Genetics
and Cell Biology, Washington State University,
Pullman, WA 99164-6420, USA

M. A. Piatyszek
Geron Corporation, Menlo Park, CA 94025, USA

Permanent address:

¹ Department of Genetics, Silesian University,
40-032 Katowice, Poland

bulked from two spikes. Anthers and carpels were isolated from two or three spikes a few days prior to anthesis and bulked for lysis. Leaf tissue (100–200 mg) was taken from 7-day-old seedlings. Immature seeds of *A. thaliana* (strain Columbia) were bulked from 10–20 siliques. Tissues were placed in 1.5 ml Eppendorf tubes and ground with matching plastic pestles for 20 s in ice-cold lysis buffer. The lysis buffer was as described (Piatyszek et al. 1995), except that buffer pH was 8.3 instead of 7.5 and 16 units of PRIME RNase inhibitor (5 Prime-3 Prime) was added per ml of buffer. Extracts were processed and analyzed for protein concentration and alkaline phosphatase activity as described (Piatyszek et al. 1995). Protein concentrations were typically between 2.0–6.0 mg/ml, while alkaline phosphatase activity varied from 0.4 to 1.1 Sigma units/ml for carpel and leaf extracts, respectively.

Telomerase assay

In the standard TRAP assay telomerase extends an oligonucleotide primer TS (5'-AATCCGTCGAGCAGAGTT-3') by adding telomere repeats. The extension products are then amplified by PCR using TS as the forward primer and an oligonucleotide complementary to telomere repeats as the reverse primer (Kim et al. 1994). In this study Tel (5'-AAACCCTAAACCCTAAACCCTAAACCC-3') and CX (5'-CCCTTACCCTTACCCTTACCCTAA-3') were used as reverse primers for plant and human extracts, respectively.

Telomerase assay was performed as described (Piatyszek et al. 1995), except that (i) the PCR regime was changed to: 3 min of initial denaturation at 94°C, 31 cycles of each 94°C for 45 s, 50°C for 45 s and 72°C for 1 min; (ii) only [α -³²P]dCTP was used for radioactive labelling of PCR amplification products; (iii) the amount of primers was increased from 100.0 ng to 150.0 ng per reaction, and (iv) 0.5 U of PRIME RNase inhibitor was added to the reaction mixture. The last two modifications were introduced to minimize plant nuclease effects on the TS primer, extension products, and the telomerase template. To avoid PCR artifacts (primer dimer formation), a hot start was performed by adding *Taq* DNA polymerase, reverse primer and [α -³²P]dCTP at 80°C after TS primer extension by telomerase and initial denaturation of the reaction mixture.

To verify the authenticity of TRAP assay products, controls were performed as described (Kim et al. 1994), except that the heat treatment was done at 94°C for 5 min and 5 μ g/ μ l of RNase A were used. This higher concentration of RNase A was necessary to compensate for the RNase inhibitor added to the lysis buffer.

For the time course assay of telomerase activity, aliquots of barley embryo extract (1.2 μ g of protein) were incubated with TS primer in TRAP reaction mixture at 25°C. Reactions were stopped at various time points by 5 min incubation at 94°C, the reverse primer Tel, radioactive tracer and *Taq* polymerase were added and the TRAP assay was completed as described (Kim et al. 1994). TRAP products (14 μ l) were resolved by polyacrylamide gel electrophoresis (Piatyszek et al. 1995) and scanned with an AMBIS Radioanalytic Imaging System. To avoid interference by signal from neighboring lanes only the central 50% of each lane was quantified and the signal from the negative control lane was subtracted to yield the net value.

Nuclease activity test

Nuclease activities were analyzed by 30-min incubation of high molecular weight (> 25 kb) barley genomic DNA (500 ng) with extracts in TRAP reaction mixture (without dNTPs, T4g32p or primers). DNA degradation (determined by agarose gel electrophoresis) was highest for anthers and lowest for leaves (data not presented). The effect of the extracts on PCR amplification under TRAP assay conditions was tested by using three pUC19 recombinant plasmids as templates. In the control PCR reaction (no extracts added) fragments of approximately 100 bp, 200 bp and 300 bp were

amplified in a PCR reaction with M13 Forward and Reverse primers. Serial dilutions of extracts (6.0, 0.6 and 0.06 μ g of protein) were preincubated in PCR reaction mix for 30 min at 25°C prior to the PCR. The amplification products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Results and discussion

Human telomerase-positive (MDA-MB-231; breast adenocarcinoma) and -negative (HSF; normal human synovial fibroblast) cell extracts (Kim et al. 1994) were used as controls for the modifications introduced to the TRAP assay (see Materials and methods). A typical ladder pattern was observed using positive human extract with the first product at 40 bp and 6 bp spacing between bands (Fig. 1A). No amplification was noted with the negative human extract or with the negative controls for the telomerase-positive extract (Fig. 1A). Having proven the functionality of the assay for the human extracts, we performed all negative controls used earlier for the human telomerase assay (Kim et al. 1994) using the plant extracts. No signal was detected in any of the negative controls but data is presented only for routinely performed controls, i.e. heat denaturation and RNase treatment. Lack of signal in these two controls (Fig. 1A) demonstrated that functional protein and RNA components of barley telomerase are necessary for a positive signal in the TRAP assay (Fig. 1A).

To further verify that the positive signal derives from barley telomerase activity and to gain insight into the enzyme's processivity, a time course experiment with barley embryo extracts was performed (Fig. 1B). A positive relationship between TS primer extension time and signal intensity and distribution was apparent. Longer extension times resulted in a stronger signal and a shift of the signal distribution towards higher molecular weight (Fig. 1B). Additionally, heterodispersion of the signal was increased at longer extension times. It is particularly apparent for the longest extension time tested (40 min), where a smear is observed rather than discrete bands. We assume that nuclease degradation of the TS primer and extension products are responsible for the breakdown of discrete banding patterns.

Quantitation of TRAP products revealed a linear relationship between time of TS primer extension and net signal intensity (Fig. 1C), confirming that the patterns observed are *bona fide* telomerase products amplified by *Taq* polymerase.

Telomerase activity was detected in barley embryo, anther and carpel extracts but was absent in leaf extract (Fig. 2A). Heterodisperse bands, and a high molecular weight smear instead of distinct bands, are present in all TRAP products (Fig. 2A). The patterns obtained for all positive extracts seem to be a sum of telomerase and nuclease activities. This notion is

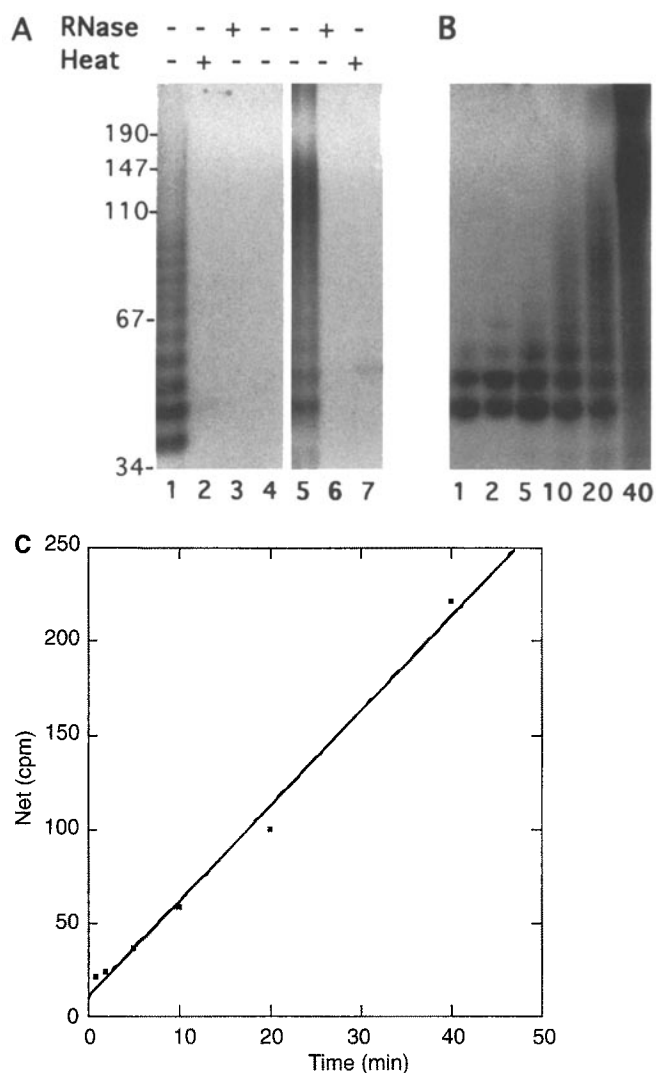


Fig. 1A–C TRAP assay for telomerase activity in human and barley extracts. **A** Specificity of the TRAP assay for telomerase activity in human (lanes 1–4) and barley embryo (lanes 5–7) extracts. Lane 1, human telomerase-positive (MDA-MB-231; breast adenocarcinoma) extract (1000 cell equivalents); lane 2, MDA-MB-231 extract pre-treated with heat; lane 3, MDA-MB-231 extract pre-treated with RNase; lane 4, human telomerase-negative (HSF; normal synovial fibroblast) cell extract (1000 cells equivalent); lane 5, barley immature embryo (1–2 mm) extract (600 ng protein); lane 6, heat-treated barley embryo extract; lane 7, RNase-treated barley embryo extract. Molecular sizes (in bp) of DNA marker (end-labelled *Hpa*II restriction fragments of pUC19) are indicated. **B** TRAP assay of barley immature embryo extract (1.2 μ g protein) in a time course experiment. Extension time (in min) is indicated for each lane. **C** Relationship between extension time and (from **B**) net cpm incorporated in the TRAP assay, as determined with the AMBIS imaging system

supported by the differences in amplification patterns observed in dilutions of the same extract. In all telomerase-positive extracts greater dilution resulted in a shift of amplification products towards higher molecular weight. The most drastic result was obtained for anthers, where nuclease activity was found to be the

highest (data not presented). Amplification products were not detected with 6 μ g of anther extract protein per reaction (Fig. 2A). The most diluted anther extract (60.0 ng of protein) gave the strongest signal, particularly apparent in the high molecular weight region of the gel. A similar relationship between protein concentration and distribution of amplification products was observed for carpel and embryo extracts. These results suggest nuclease degradation of the TS primer and extension products. The results obtained on evaluating the impact of plant extract on PCR using plasmid templates (Fig. 2B) and the degradation of radiolabelled TS primer by these extracts (data not presented) provide strong support for the hypothesis that plant nucleases are influencing TRAP patterns.

A strong positive signal was obtained with very dilute barley embryo extract (6 ng of total protein per assay). This result demonstrates the very high sensitivity of the TRAP assay, recently reported to approach the single-cell detection limit (Wright et al. 1995). Additionally, the positive signal obtained for immature seeds of *A. thaliana* (Fig. 2A) and tobacco cells in suspension culture (data not presented) suggests that TRAP assay, as described here, may be applicable for telomerase analysis in diverse plant species.

Failure to detect telomerase activity with a barley leaf extract is noteworthy. Analysis of data on telomere

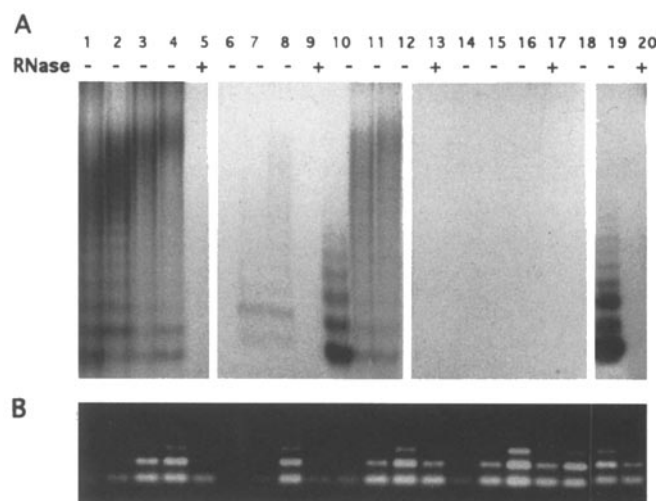


Fig. 2A, B Telomerase activity in plant tissues. **A** TRAP assay amplification products, Lanes 1–5, immature barley embryo extract with 6 μ g (lane 1), 600 ng (2), 60 ng (3) and 6 ng (4) total protein (lane 5 is the same as (2), but RNase treated); lanes 6–9, barley anther extract with 6 μ g (6), 600 ng (7) and 60 ng (8) total protein; lane 9, same as (7), but RNase pretreated; lanes 10–13, barley carpel extract with 6 μ g (10), 600 ng (11) and 60 ng (12) protein. Lane 13, same as (11), but RNase pretreated; lanes 14–17, barley leaf extract with 6 μ g (14), 600 ng (15) and 60 ng (16) total protein; lane 17, same as (15), but RNase pretreated; lane 18, lysis buffer; lane 19, *Arabidopsis* immature seed extract (600 ng protein); lane 20, same as (19), but RNase pretreated. **B** PCR amplification of plasmid templates (see Materials and methods) pretreated with the same extracts as in **A**. Amplified products are ca. 100, 200 and 300 bp in size

length differences between meristematic (embryo) and differentiated (leaf) tissues of barley lead us to hypothesize that barley telomerase activity may be repressed during differentiation (Kilian et al. 1995). This hypothesis is supported by the telomerase activity data for those two tissues reported here. It is also possible that telomerase activity is associated with meristematic activities in tissue samples tested in this report. Additional enzyme activity analyses are needed to address these possibilities. With the functional and sensitive telomerase assay reported here we are now in a position to study tissue and developmental patterns of plant telomerase activity.

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References

- Bednarek A, Budunova I, Slaga TJ, Aldaz CM (1995) Increased telomerase activity in mouse skin premalignant progression. *Cancer Res* 55:4566–4569
- Blackburn EH (1992) Telomerases. *Annu Rev Biochem* 61: 113–129
- Fuchs I, Brandes A, Schubert I (1995) Telomere sequence localization and karyotype evolution in higher plants. *Plant Systematics Evolution* 196: 227–241
- Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extract. *Cell* 43:405–413
- Kilian A, Kleinhofs A (1992) Cloning and mapping of telomere-associated sequences from *Hordeum vulgare* L. *Mol Gen Genet* 235:152–156
- Kilian A, Stiff C, Kleinhofs A (1995) Barley telomeres shorten during differentiation but grow in callus culture. *Proc Natl Acad Sci USA* 92:9555–9559
- Kim N, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266:2011–2015
- Kipling D (1995) *The Telomere*. Oxford University Press, Oxford
- Lin JJ, Zakian VA (1995) An in vitro assay for *Saccharomyces* telomerase requires EST1. *Cell* 8:1127–1135
- Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, Shay JW (1995) Detection of telomerase activity in human cells and tumors by telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* 17: 1–15
- Prowse KR, Greider CW (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl Acad Sci USA* 92:4818–4822
- Richards EJ, Ausubel FM (1988) Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* 53:127–136
- Wright WE, Shay JW, Piatyszek MA (1995) Modifications of telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res* 23: 3794–3795