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Developmental Control of Telomere Lengths and Telomerase Activity in Plants

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Telomere lengths and telomerase activity were studied during the development of a model dioecious plant, *Melandrium album* (syn *Silene latifolia*). Telomeric DNA consisted of Arabidopsis-type TTTAGGG tandem repeats. The terminal positions of these repeats were confirmed by both Bal31 exonuclease degradation and in situ hybridization. Analysis of terminal restriction fragments in different tissues and ontogenetic stages showed that telomere lengths are stabilized precisely and do not change during plant growth and development. Telomerase activity tested by using a semiquantitative telomerase repeat amplification protocol correlated with cell proliferation in the tissues analyzed. Highest activity was found in germinating seedlings and root tips, whereas we observed a 100-fold decrease in telomerase activity in leaves and no activity in quiescent seeds. Telomerase also was found in mature pollen grains. Telomerase activity in tissues containing dividing cells and telomere length stability during development suggest their precise control during plant ontogenesis; however, the telomere length regulation mechanism could be unbalanced during in vitro dedifferentiation.

INTRODUCTION

Telomeres are essential elements of all eukaryotic chromosomes, protecting them from exonuclease degradation and end-to-end chromosomal fusions. Telomeres are associated with the nuclear matrix and are involved in the organization of chromosomes in interphase nuclei (reviewed in Dernburg et al., 1995). It also has been shown that telomeres are essential for proper separation of chromosomes in anaphase (Kirk et al., 1997). Telomeres of most eukaryotes are composed of short, G-rich, tandemly repeated sequences. The G-rich strand is extended over the C-rich strand and forms a single-stranded overhang. A telomere is replicated by a specialized reverse transcriptase by using its own RNA subunit as the template. This enzyme, called telomerase, compensates for the inability of DNA polymerase to replicate the 5′ ends of linear DNA molecules (reviewed in Blackburn, 1991; Zakian, 1995).

In humans, telomeres shorten during cell differentiation and aging (Harley et al., 1990). Normal human somatic cells cultured in vitro are only able to pass through a limited number of cell divisions, after which their growth is arrested and cells undergo senescence. As cultured cells divide, their telomeres gradually shorten (Harley et al., 1990; Allsopp et al., 1995). After telomere lengths decrease to a critical level, a higher frequency of chromosomal rearrangements has been observed (Counter et al., 1992). This indicates that sufficiently short telomeres may provide a signal for replicative senescence (Allsopp et al., 1992). Tumor- or virus-transformed cell lines possess an unlimited proliferation capacity. In most of these cell lines, high telomerase activity and stabilization of telomere lengths have been detected. Similarly, telomerase is expressed in cells that are predicted to be immortal in vivo, such as germ line cells and cells in 90% of malignant tumors (Kim et al., 1994). On the other hand, telomerase activity has not been detected in most human somatic tissues. These observations led to the telomere hypothesis, which suggests that telomeres may serve as a mitotic clock by which cells count their divisions (Allsopp et al., 1992; Autexier and Greider, 1996).

As in other eukaryotes, plant telomeres are maintained with telomerase. Telomerase activity has been detected in plants by using a direct method (Fajkus et al., 1996) or a polymerase chain reaction (PCR)–based telomerase repeat amplification protocol (TRAP) assay (Fitzgerald et al., 1996; Heller et al., 1996). Telomerase seems to be developmentally regulated in plants, which is similar to the way it is regulated in humans. Fitzgerald et al. (1996) were able to detect telomerase activity in root tips of soybean but not in leaves or the shoot apex. Heller et al. (1996) also found telomerase in barley embryo, anther, and carpel extracts. These data indicate that telomerase activity is connected with cell proliferation, but little is known about the role of telomerase in telomere length regulation during plant development. Kilian et al. (1995) observed that barley telomeres shorten during growth and aging, which could be explained by developmental regulation of telomerase activity. On the other hand, no changes in terminal restriction fragment (TRF) lengths

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were observed during aging of tomato plants (Broun et al., 1992). However, in both barley and tomato, telomeres reach up to hundreds of kilobases; therefore, it is difficult to precisely determine relatively small changes in telomere lengths that could occur during plant ontogenesis.

In this study, we determined telomere lengths and telomerase activity in various tissues of the dioecious plant *Melandrium album* (syn *Silene latifolia*; white campion), which possesses relatively short telomeres. By comparing TRF lengths in pollen grains, seedlings, and leaves, we show that telomere lengths do not change during plant ontogenesis. The data presented also suggest that stable maintenance of telomere lengths is connected with telomerase activation in dividing cells.

RESULTS

Telomeres of *M. album* Are Composed of TTTAGGG Telomeric Repeats

Telomeres of most higher plant species are composed of the repeated sequence $(TTTAGGG)_n$. To investigate whether this also holds for *M. album*, we performed hybridization analyses. DNA from leaves of male and female plants were cut with MspI, BstNI, or TaqI restriction endonucleases, separated by agarose gel electrophoresis, blotted onto a membrane, and hybridized with a γ–^{32}P-ATP end-labeled $(CCCTAAA)_3$ probe (Figure 1). BstNI digestion gave a strong smeared signal from 3 to 8 kb. The cleavage with TaqI resulted in a smear from 2.5 to 4.5 kb and a ladder signal composed of five bands from 0.5 to 1.6 kb. This result showed that the TRFs in *M. album* are relatively short and that the telomeres are 4.5 kb long at maximum. MspI, a methylation-sensitive enzyme, yielded high molecular weight signals. Because the cleavage site of MspI is a tetranucleotide sequence (CCGG), long TRFs generated with MspI must be caused by heavy methylation of the outer cytosine in telomere-associated sequences. No apparent differences in the restriction patterns between male and female samples, which could arise from the presence of the male Y chromosome, were observed (Figure 1).

Telomere motifs often are localized not only at chromosome ends but also intrachromosomally. To distinguish between the terminal and intrachromosomal telomeric positions, we used a test for telomere sensitivity to exonuclease degradation. High molecular weight DNA from leaves (ranging from 100 to 150 kb) was treated with Bal31 exonuclease and then cut with TaqI. DNA fragments were analyzed by DNA gel blot hybridization using the $(CCCTAAA)_3$ oligonucleotide as a probe, and the membrane was washed at reduced stringency (Figure 2A). Time-course exonuclease analysis revealed the insensitivity of the ladder signal to Bal31: after 2 hr of exonuclease degradation, all of the bands from 0.5 to 1.6 kb remained stable. On the other hand, as the reaction progressed, the smear gradually moved to lower molecular weight values until it completely disappeared after 45 min of Bal31 treatment. This experiment showed that the ladder signal obviously arose from some intrachromosomally localized homologous sequences, whereas the smear from 2.5 to 4.5 kb represented the TRFs. Moreover, when this membrane was washed at a higher stringency (Figure 2B), the bands insensitive to exonuclease degradation were removed completely. This suggests weaker homology of these intrachromosomal sequences to the TTTAGGG telomeric motif.

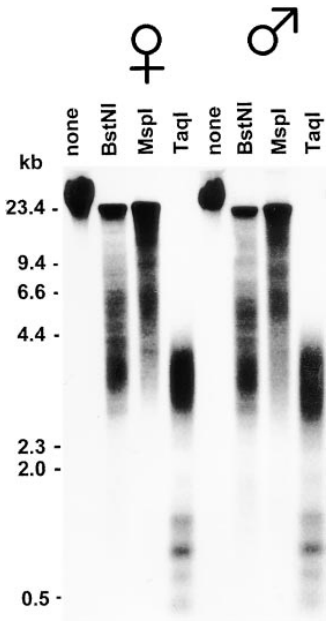

Figure 1. DNA Gel Blot Analysis of TRFs.

DNA samples from female (left) and male (right) plants were cleaved with restriction endonucleases as indicated, and TRFs were detected by hybridization with the radioactively labeled $(CCCTAAA)_3$ probe. Nondigested samples were included as controls (none; left lanes). Numbers at left indicate the lengths of DNA fragments in kilobases.

The terminal localization of the TTTAGGG telomeric repeats was verified further by in situ hybridization (Figure 3). Analysis of 30 individual male metaphases with a special emphasis on the sex chromosomes (X and Y, indicated in Figure 3) confirmed the presence of the telomeric motif at both ends of each chromosome.

Telomeres Do Not Shorten during *M. album* Development, but They Increase Their Lengths in Callus Culture

Because the TRFs generated with TaqI ranged from 2.5 to 4.5 kb, it was possible to analyze telomere lengths by using

conventional agarose gel electrophoresis. If *M. album* telomeres shortened during cell differentiation, substantial differences in TRF size should be expected between gametes in pollen grains and somatic tissues, such as leaves from adult plants. To test this hypothesis, we determined the lengths of TRFs generated by TaqI in mature pollen grains, young seedlings, and leaves (Figure 4). In all of these tissues, the TRF lengths varied from 2.5 to 4.5 kb, and no significant differences were observed.

To study the telomere length in a rapidly growing in vitro culture, we derived calli from leaf explants on a synthetic medium supplemented with auxin. Three months after callus culture initiation, DNA was extracted, and TRF sizes were estimated and compared with those in plants from which the calli were derived (Figure 5). Two callus lines, each initiated from a different plant, were investigated. In these callus cultures, telomere lengths were extended to 5.5 or 6.5 kb. Thus, compared with leaves, the telomeres increased in length by 1 or 2 kb during in vitro growth and dedifferentiation.

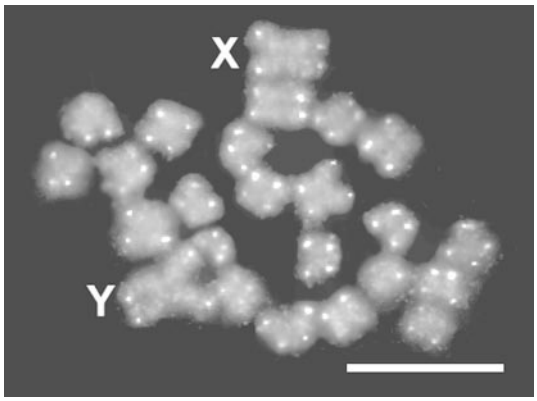

Figure 3. In Situ Localization of TTTAGGG Telomeric Motifs on *M. album* Chromosomes.

The biotin-labeled telomeric probe was hybridized with *M. album* male metaphase chromosomes and counterstained with propidium iodide. Sex chromosomes X and Y are indicated. Bar = 10 μm.

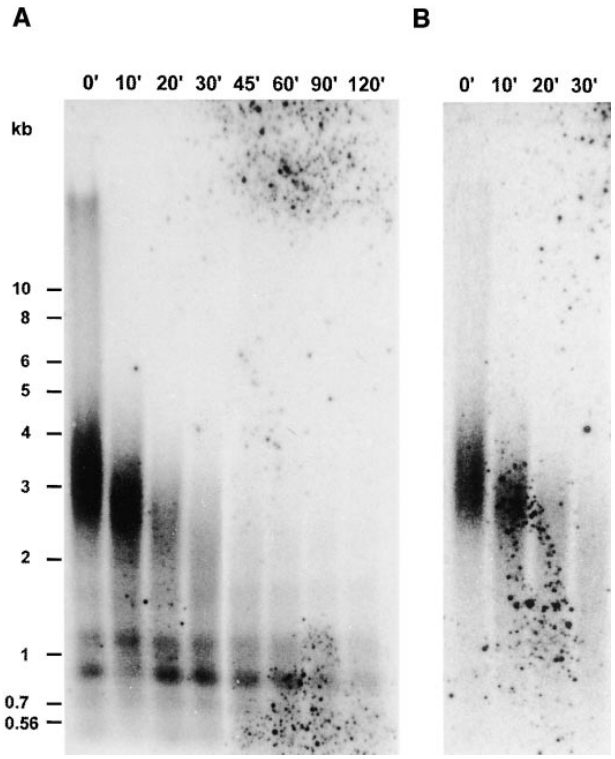

Figure 2. Bal31 Exonuclease Degradation of Telomeric Sequences in *M. album* DNA.

DNA from leaves was treated with Bal31 for 0, 10, 20, 30, 45, 60, 90, and 120 min and then cleaved with TaqI. DNA blots were hybridized with the $(CCCTAAA)_3$ probe. The lengths of DNA fragments in kilobases are indicated at left.

(A) Blot washed at low stringency.

(B) Blot washed at high stringency.

Telomerase Activity at Different Stages of Plant Development

The stability of telomere lengths during plant ontogenesis indicates that telomerase should be expressed in all dividing cells. For detection of telomerase, we used a modified PCR-based TRAP assay according to Fitzgerald et al. (1996). Protein precipitation with polyethylene glycol was included during extraction to reduce the amount of telomerase inhibitors. Use of the 23-mer oligonucleotide 47F as a forward primer (see Methods) permitted an increase in the annealing temperature to 65°C during the PCR step, which completely eliminated the creation of PCR artifacts. Moreover, we performed a set of control reactions in which telomerase extracts or forward primers were omitted in the telomerase step (Figure 6B, lanes 1 and 2) or in which telomerase was inactivated by heat denaturation or RNase A treatment (Figure 6B, lanes 3 and 4). The absence of any products in these reactions confirmed that the ladder signal was a result of telomerase activity.

To map telomerase expression, we determined telomerase activity in various plant organs and tissues at different stages of development. To compare relative amounts of telomerase in various samples, we performed titration experiments in which a series of 10-fold dilutions of each extract was tested for telomerase activity (Figure 6A). The highest activities were detected in organs containing rapidly proliferating meristems, such as germinating seedlings and root tips. In these samples, strong signals were generated in reactions containing 1 ng of protein (Figure 6A, lanes 3 and 13), and the typical ladder was apparent even in reactions with 0.1 ng of protein (Figure 6A, lanes 4 and 14). Slightly lower telomerase activity was detected in callus cultures, immature floral buds, and developing axillar shoots. Approximately

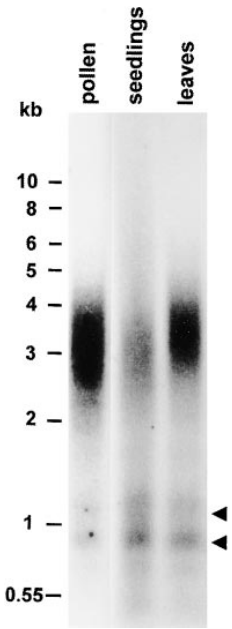

Figure 4. Telomere Lengths at Different *M. album* Developmental Stages.

The TRFs generated by TaqI digestion of DNA isolated from pollen grains, young seedlings, and leaves were visualized by DNA gel blot hybridization with the telomeric probe. Arrowheads indicate signals derived from intrachromosomal sequences. Numbers at left indicate the lengths of DNA fragments in kilobases.

100-fold lower activity (compared with the seedlings) was found in leaves (cf. lanes 4 and 7 of Figure 6A). Telomerase also was assayed in mature pollen grains and seeds, which both represent quiescent stages of plant development. Although no telomerase was detected in seeds, relatively high activity was found in pollen grains. Telomerase present in 1 ng of pollen total protein was still sufficient to generate detectable signals (Figure 6A, lane 38). To exclude the possibility that the lack or decrease of telomerase activity in some samples was due to putative tissue-specific inhibitors, we conducted TRAP reactions in which 10 ng of root proteins was combined with 100 ng of their respective samples, but no inhibition was observed in any case (data not shown).

DISCUSSION

A plant telomere sequence was first cloned from Arabidopsis by Richards and Ausubel (1988), and the Arabidopsis telomeric repeat motif TTTAGGG is conserved in a majority of higher plant species, as shown by in situ hybridization (reviewed in Fuchs et al., 1995) and DNA gel blot analysis (reviewed in Richards, 1995). In this study, we provide evidence, obtained by both in situ hybridization and exonuclease analysis, that *M. album* telomeres are composed of the Arabidopsis-like telomeric motif and that their lengths range from 2.5 to 4.5 kb.

Telomeres shorten during human cell differentiation. This phenomenon also has been observed in barley (Kilian et al., 1995): telomere lengths decreased from 80 kb in young embryos to 30 kb in mature leaves, and telomere shortening also was found during development of barley inflorescences. On the other hand, Broun et al. (1992) observed stable telomere lengths in tomato leaves when comparing 4-week-old and 6-month-old plants. An average decrease in telomere size in humans was determined to be ~50 bp per cell division (Counter et al., 1992; Allsopp et al., 1995). At least 50 cell divisions are required for mature leaf formation from the zygote, as calculated on the basis of DNA content (Burr et al., 1992). Thus, if telomeres in *M. album* shortened at each round of cell division by ~50 bp, as occurs in humans, the difference in telomere length between gametes and leaves would be at least 2.5 kb. However, we did not detect any changes in *M. album* telomere lengths in pollen grains and leaves.

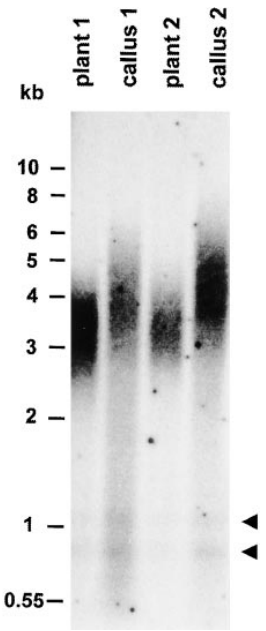

Figure 5. Comparison of Telomere Lengths between *M. album* Leaves and Leaf-Derived Callus Cultures.

Shown are the results of DNA gel blot hybridization of the TRFs obtained by TaqI cleavage of DNA samples isolated from two different plants and the corresponding calli cultured for 3 months on synthetic medium. Arrowheads indicate signals derived from intrachromosomal sequences. Numbers at left indicate the lengths of DNA fragments in kilobases.

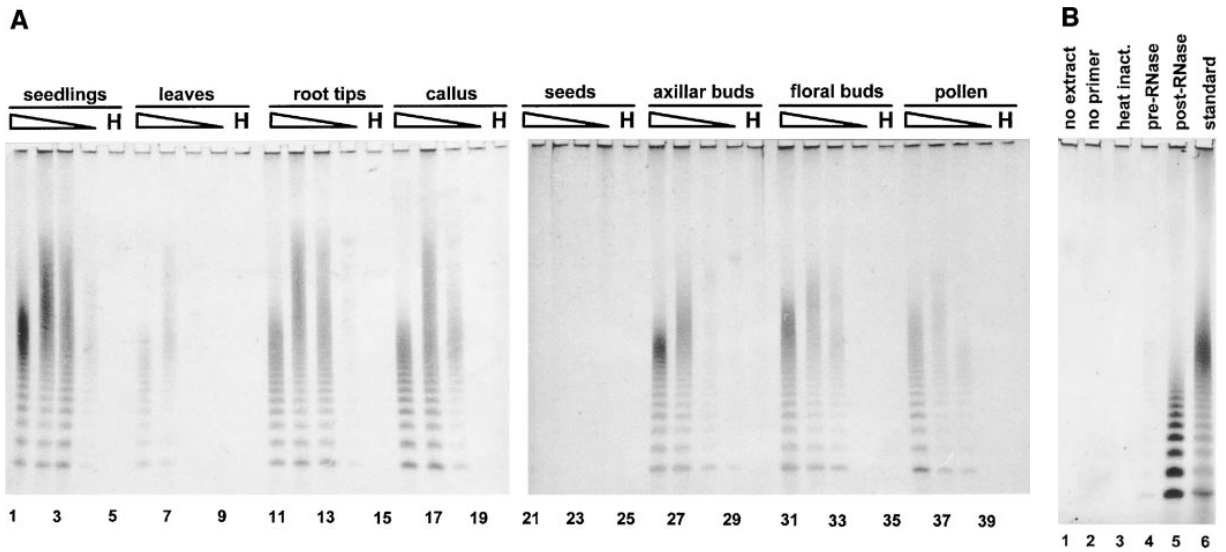

Figure 6. Developmental Regulation of Telomerase Expression in *M. album.*

(A) Telomerase activities in various tissues and different stages of plant development were assayed by TRAP, using 47F as the forward primer. Reactions were performed with decreasing amounts of total protein (100, 10, 1, or 0.1 ng from left to right in each sample as indicated by the triangles). As a negative control, 100 ng of heat-inactivated proteins from each sample was tested for telomerase activity (lanes marked by H).
(B) TRAP assays were performed either without telomerase extract (lane 1) or with 100 ng of protein from root tips: the forward primer was omitted from the telomerase step and added before the PCR step (lane 2); the protein extract was boiled for 10 min before the telomerase step (lane 3); the protein extract was treated for 10 min with 100 ng of RNase A before the telomerase step (lane 4), or 100 ng of RNase A was added before the PCR step (lane 5); standard TRAP assay (lane 6). inact., inactivated.

In plants, no true germ line is set aside in early embryogenesis, and both vegetative and generative parts are derived from meristems during growth and differentiation. This implies 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. Our finding that telomeres are stably maintained during *M. album* ontogenesis fits the pattern of plant body formation well and suggests strict control of telomere lengths during development. If such a control mechanism did not operate and the telomeres shortened during multiple cell divisions, resulting in the formation of gametes, this change would finally, after repeated sexual generations, lead to the complete loss of telomeres.

Whereas telomere lengths are maintained stably during plant development, a substantial increase in TRF sizes was observed during cell dedifferentiation and growth in callus cultures. This supports the results of Kilian et al. (1995), who reported an increase of up to threefold in the number of telomere repeats in long-term barley callus cultures. Telomere elongation also was observed in some immortalized human cell lines in vitro (Bryan et al., 1995). Interestingly, no telomerase activity was detected in these human cell lines, and TRF elongation probably was caused by a telomerase-independent mechanism. However, high telomerase activity in *M. album* callus cultures indicates that TRF elongation was due to nonregulated synthesis of TTTAGGG repeats by telomerase rather than being a consequence of structural rearrangements in telomere-linked regions, which may have been induced by in vitro culture conditions.

As we show in this study, telomeres do not shorten during successive cell divisions during *M. album* development. This suggests that telomerase is required for complete 5′ end chromosome replication in dividing plant cells. Our results suggest a tight correlation of telomerase with cell division in plants. We found highest telomerase activity in germinating seedlings and root tips containing a high proportion of actively dividing meristematic cells. Because developing axillar buds and immature floral buds are more heterogeneous organs, we suppose that their lower telomerase activities reflect a lower content of proliferating cells rather than a general decrease in telomerase expression. We also detected low telomerase activity in leaves. This activity could have originated from marginal or plate meristems, which are responsible for growth of lamina during leaf development and in some cases can retain their mitotic activity until the leaf has reached one-half or three-fourths of its final length (Steeves and Sussex, 1994). Similar results were obtained by Fitzgerald et al. (1996) and Heller et al. (1996), who detected telomerase activity in tissues containing actively proliferating cells. Moreover, low telomerase activity also was detected in cauliflower leaves and stems that may have

been due to a residual amount of telomerase in cells recently derived from the vascular cambium (Fitzgerald et al., 1996). The presence of telomerase activity in such differentiated cells supports our data on the stable maintenance of telomere lengths in plants. Telomerase also was detected in suspension cultures of tobacco (Fajkus et al., 1996; Heller et al., 1996), carrot, soybean, Arabidopsis, and rice (Fitzgerald et al., 1996). These results as well as our data on the telomerase activity in *M. album* calli indicate that telomerase (re)activation is essential for plant cell proliferation.

Surprisingly, we also found low telomerase activity in mature pollen grains. Because sperm cells in pollen grains are haploid, they often are used to induce mutants by irradiation. In *M. album*, such mutant plants have been isolated with an aberrant Y chromosome possessing a terminal deletion (Donnison et al., 1996), which was obviously healed by a telomere to maintain its mitotic stability. It is possible that the telomerase activity in pollen grains could be involved in DNA repair processes, or it may reflect residual activity after the second pollen mitosis. A low level of telomerase activity also has been detected in extracts prepared from mature sperm cells of Xenopus (Mantell and Greider, 1994).

The stability of TRF sizes during *M. album* development suggests the presence of a precise mechanism controlling telomere length. Telomerase is required for telomere synthesis, but telomerase activity per se probably does not control telomere length. Recent studies indicate that telomerase activity is regulated by double-strand telomere binding proteins. The human telomere binding protein TRF1 acts as a negative regulator of human telomerase (van Steensel and de Lange, 1997). A protein-counting mechanism for telomere length regulation was described for yeast in which the Rap1p telomere binding protein plays a key role (Marcand et al., 1997). We observed comparable telomerase activities in callus cultures and young seedlings, which are both characterized by the presence of rapidly dividing cells. However, whereas in seedlings telomeres are stable, telomere length regulation in calli seems to be unbalanced. Genetic analysis of different inbred lines of maize revealed that telomere lengths are under strict genetic control (Burr et al., 1992). Moreover, proteins binding to telomeric repeats also have been found in plants (Regad et al., 1994; Zentgraf, 1995). Taken together, these data suggest that mechanisms similar to those recently described for humans and yeasts could participate in telomere length regulation in plants.

METHODS

Plant Material

Plants (*Melandrium album* [syn *Silene latifolia*]; Caryophyllaceae) were from a large greenhouse population cultured and crossed for 5 years at the Institute of Biophysics (Brno, Czech Republic). *M. album* pollen grains, young seedlings, immature male flower buds, leaves, root tips, young axillar shoots, seeds, and callus cultures were used to study telomere lengths and telomerase activity at different stages of plant development. Root tips were isolated from hydroponically cultured 3-week-old seedlings. Pollen grains were collected from mature male flowers and stored at 4°C until used. We prepared seedlings at the earliest stages of postembryonic development by sterilizing, washing, and then germinating them for 36 hr in water. Seedlings (3 to 5 mm long) were extirpated from the rest of the seeds and immediately used for extraction. Male buds, 3 mm long, corresponding to flower developmental stage 11, according to Grant et al. (1994), were bulked. Callus cultures were derived from leaf explants cultured on medium BMS-30 (Ye et al., 1990) supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/L).

DNA Isolation

DNA for restriction analysis was isolated using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). One hundred milligrams of plant tissues (pollen, young seedlings, and leaves) or 40 mg of lyophilized callus was ground using a mortar and pestle under liquid nitrogen, and DNA was extracted according to the manufacturer's instructions. Nuclear high molecular weight DNA for Bal31 exonuclease degradation experiments was isolated according to Jofuku and Goldberg (1988).

DNA Gel Blot Analysis and Bal31 Exonuclease Degradation

For terminal restriction fragment (TRF) length analyses, we cleaved DNA samples from various tissues with TaqI, BstNI, or MspI, electrophoretically separated them in a 1% agarose gel, and blotted them onto a membrane. Hybridizations were performed in 7% SDS, 0.25 M phosphate buffer, and 100 μg/mL BSA at 55°C. The blots were washed twice for 30 min at 55°C either in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate; low stringency) or in 0.2 × SSC (higher stringency). Radioactively 5′ end-labeled $(CCCTAAA)_3$ was used as a probe. The probe was labeled with T4 polynucleotide kinase and γ–^{32}P-ATP (3000 Ci/mmol; Amersham). Signals were detected by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For the Bal31 nuclease time-course analysis, 120 μg of DNA was incubated in 800 μL of buffer (0.6 M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris-Cl, pH 8.0, and 0.2 mM EDTA) at 30°C in the presence of 5 units of Bal31 (Amersham). One hundred–microliter aliquots (containing 15 μg of DNA) were taken at 0, 10, 20, 30, 45, 60, 90, and 120 min, and Bal31 was inactivated by adding EGTA to a final concentration of 20 mM and heating the mixture at 65°C for 10 min. DNA samples were precipitated with ethanol, digested with TaqI, and analyzed by DNA gel blot hybridization as described above.

Chromosome Preparation and in Situ Hybridization

Root tip meristems of germinating seeds were used as a source of metaphase chromosomes according to the method described by Buzek et al. (1997). As a telomere-specific DNA probe, the oligonucleotide $(CTAAACC)_5$ in which each thymidine was biotinylated was used. After hybridization, slides were washed under nonstringent conditions (1 × SSC, twice for 10 min at 42°C; Meyne and Moyzis, 1994), biotin-labeled DNA duplexes were detected with fluorescein isothiocyanate–avidin D (Vector, Burlingame, CA), and signals were

amplified using biotinylated anti-avidin antibody (Vector) and a second layer of fluorescein isothiocyanate–avidin D. Mitoses were observed on an Olympus AX70 fluorescence microscope (Tokyo, Japan) and captured with a CCD camera by using FISH-imaging software (ISIS; MetaSystems, Sandhausen, Germany).

Telomerase Assay

Telomerase was detected using a modified version of the telomere repeat amplification protocol (TRAP) as described by Fitzgerald et al. (1996), except that staining of TRAP assay products with SYBR Green I (Molecular Probes, Eugene, OR) was used rather than radioactive labeling. Oligonucleotides 47F (5′-CGCGGTAGTGATGTGGTTGTGTT-3′) and PTelC$_3$ (5′-CCCTAAACCCTAAACCCTAAA-3′) were used as forward and reverse primers, respectively. Plant extracts containing telomerase were prepared exactly according to Fitzgerald et al. (1996). The concentration of total protein in the extracts was determined according to Bradford (1976). TRAP assays were performed in 49 μL of reaction mixture consisting of 50 mM Tris-acetate, pH 8.3, 50 mM potassium glutamate, 0.1% Triton X-100, 1 mM spermidine, 1 mM DTT, 50 μM each deoxyribonucleotide triphosphate, 5 mM $MgCl_2$, 10 mM EGTA, 100 ng/μL BSA, 500 nM T4 gene 32 protein (U.S. Biochemical), 2 units of Taq polymerase (Promega), and 10 pmol of 47F forward primer. After the addition of the protein extract, the telomerase reaction was allowed to proceed at room temperature for 45 min. The reaction mixtures were covered with 50 μL of paraffin oil and warmed to 94°C; then 10 pmol of PTelC3 reverse primer was added. Polymerase chain reaction (PCR) amplification followed immediately using 35 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 90 sec. Samples were precipitated with ethanol, dissolved in water, and separated in 10% nondenaturing polyacrylamide gels. Gels were stained for 45 min in 0.01% SYBR Green I nucleic acid gel stain and scanned on a PhosphorImager.

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