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Asparagales telomerases which synthesize the human type of telomeres

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Abstract

The order of monocotyledonous plants Asparagales is attractive for studies of telomere evolution as it includes three phylogenetically distinct groups with telomeres composed of TTTAGGG (*Arabidopsis*-type), TTAGGG (human-type) and unknown alternative sequences, respectively. To analyze the molecular causes of these switches in telomere sequence (synthesis), genes coding for the catalytic telomerase subunit (TERT) of representative species in the first two groups have been cloned. Multiple alignments of the sequences, together with other TERT sequences in databases, suggested candidate amino acid substitutions grouped in the Asparagales TERT synthesizing the human-type repeat that could have contributed to the changed telomere sequence. Among these, mutations in the C motif are of special interest due to its functional importance in TERT. Furthermore, two different modes of initial elongation of the substrate primer were observed in Asparagales telomerases producing human-like repeats, which could be attributed to interactions between the telomerase RNA subunit (TR) and the substrate.

Introduction

The ends of eukaryotic chromosomes are capped by a special structure called the telomere whose DNA component is formed by long arrays of tandemly repeated short minisatellite sequences, e.g. TTAGGG in vertebrates (first described in humans, (Cheng *et al.*, 1989)) and TTTAGGG in model plants (*Arabidopsis*, (Richards and Ausubel, 1988)). The maintenance of telomeres is provided by special enzymes, the telomerases. These consist of the protein catalytic subunit (telomerase reverse transcriptase, TERT) and the RNA subunit (TR) which provides the template for telomere synthesis (Greider and Blackburn, 1987). The telomerase RNA subunit was first cloned and sequenced in *Tetrahymena thermophila* (Greider and Blackburn, 1989) and the first TERT was cloned later in another ciliate species, *Euplotes aediculatus* (Lingner *et al.*, 1997). Nowadays, TERTs and TRs from many model organisms, including yeasts, ciliates, humans and other vertebrates are characterised (see e.g. (Krupp and Parwaresch, 2002) for a review). The knowledge of both telomerase subunits has enabled researchers to study the function and characteristics of telomerases *in vivo* and *in vitro*. Such studies also initiated efforts to use telomerase as a target of anticancer therapies.

Among the known TERTs, two have been characterised in model plants, *Arabidopsis thaliana* and *Oryza sativa* (Fitzgerald *et al.*, 1999; Oguchi *et al.*, 1999; Heller-Uszynska *et al.*, 2002; Oguchi

et al., 2004). On the other hand, no RNA subunit has been described in any plant. Our interest in cloning of telomerases was driven by an extensive study leading to the identification of a large group of plants from the monocotyledonous plant order Asparagales with telomerase maintaining the human-type telomeric repeat (Adams *et al.* 2001; Sykorova *et al.*, 2003b). The occurrence of a human type of telomere repeat is associated with the evolution of Asparagales (see Figure 1). Asparagales also contain families with 'ancient' telomeres formed by the *Arabidopsis*-type of repeat, and another group of plants which have lost the human type of telomeric minisatellites and telomerase activity (genus *Allium*, (Pich *et al.*, 1996; Sykorova *et al.*, 2005 submitted). In those Asparagales possessing human-type telomeres, analysis of the TRAP products revealed error-prone synthesis of the human type of repeat by telomerases (Sykorova *et al.*, 2003b). It was shown recently in Hyacinthaceae (one of families in this group) that variant telomeric motifs (of the *Arabidopsis* and *Tetrahymena* type) are present in telomeres in mixed arrays, and proteins that are able to bind specifically to the *Arabidopsis* and/or the human type of telomeric repeats have been detected (Rotkova *et al.*, 2004). Novel telomeres and telomerases in Asparagales point to mutation and adaptation events which have affected telomerase and the other telomere-associated proteins involved in telomere maintenance.

The question arises about the nature of the evolutionary changes leading to a telomerase synthesizing the human type of telomeric repeat, and there are two probable explanations. The first is that a point mutation occurred in the template region of the RNA subunit that was subsequently stabilized by adaptation of the protein machinery, an explanation supported by the high tolerance to such mutations observed in many *in vitro* studies of TR subunits e.g., (Ware *et al.*, 2000). However, an incorrect telomeric repeat caused the loss of telomere cap integrity *in vivo* and several mitotic defects in yeasts, *Tetrahymena* and human (Petcherskaia *et al.*, 2003; Lin *et al.*, 2004; reviewed in Blackburn, 2005). A second explanation is that a structural change of the catalytic subunit resulted in altered template usage, similarly to the error-prone telomerase of *Paramecium tetraurelia* or the telomerases of *Oxytricha nova* and *Euplotes crassus* (Melek *et al.*, 1994; McCormick-Graham and Romero, 1996; Ye *et al.*, 1999).

Because Asparagales consists of plants synthesizing different types of telomeric sequences by telomerase, they provide a unique opportunity to ask how telomere synthesis mechanisms can evolve. This study enables determination of any significant similarities/differences in telomerase action or sequence that could be connected to changed telomerase action. The first step to efficiently address questions about plant telomerases synthesizing the human type of telomeric sequences is to identify and analyse telomerase genes in their evolutionary context.

Materials and methods

Plant material sources

Protein extracts for telomerase assays were prepared from root tips of *Ornithogalum virens* (Hyacinthaceae; Chelsea Physic Gardens, London, UK), *Scilla peruviana*, *Muscari armeniacum* (both Hyacinthaceae; Taylor Bulbs, London, UK), *Dracaena marginata* (Ruscaceae, commercial cultivar) or seedlings of *Asparagus officinalis* (Asparagaceae; commercial cultivar), *Agapanthus umbelatus* (Agapanthaceae; Royal Botanic Gardens Kew (RBGK)), *Hosta rectifolia* (Agavaceae; RBGK 1979-5048), *Gennaria chaplinii* (Amaryllidaceae; RGBK 1986-5342), *Galtonia candicans* (Hyacinthaceae; RBGK 1969-19589), *Bulbine glauca* (Asphodelaceae; RBGK 1998-4063), *Phormium cookianum* (Hemerocallidaceae; RBGK 1996-434) and *Iris pseudacorus* (Iridaceae; RBGK).

RNAs were isolated from root tips of *Scilla peruviana* (Hyacinthaceae; Taylor Bulbs, London, UK), *Ornithogalum virens* (Hyacinthaceae; Chelsea Physic Gardens, London, UK), *Doryanthes excelsa* (Doryanthaceae; Ginkgo Nursery, Ravenscourt Park, London, UK), *Iris tectorum* (Iridaceae; Queen Mary University of London, UK), *Phragmipedium longifolium* (Orchidaceae; RBGK 2002-3341, cultivated origin) or seedlings of *Hosta rectiflora* (Agavaceae; RBGK 1979-5048), *Hordeum vulgare* cv. Reni (Poaceae; Central Institute for Supervising and Testing in Agriculture, Brno, Czech Republic) and *Zea mays* ssp. *mays* (Poaceae; Maize Genetics Stock Centre Illinois, Urbana USA).

RT-PCR and cloning

RNAs (DNA free) were isolated using Absolutely RNA RT-PCR Miniprep kit (Stratagene). The Superscript III One Step RT-PCR kit with Platinum Taq polymerase (Invitrogen) and designed primer pairs were used to directly amplify parts of TERT genes from total RNA. Products of the expected size were amplified using a combination of a degenerate primer NOmotF (QFP motif) and a specific primer Os4R (A motif) against cDNA templates of *Scilla peruviana*, *Doryanthes excelsa* and *Hordeum vulgare*. When the direct RT-PCR reaction did not generate suitable products, a second semi-nested PCR reaction (50 μl) was performed using 2 μl of 50$\times$ diluted product from the primary RT-PCR as a template with one primer being the same as that used in the first PCR and a second primer designed to be specific for the different species' TERT. For semi-nested secondary PCR we used specific primers (SpFw3, DoFw1, DoFw2, SpRev2, DoRev1) which covered the T motif region in combination with the NOmotF or Os4R primer. These were designed originally for cloning the 5′/3′ ends of cDNA of *Scilla* or *Doryanthes* TERT (see below). After sequencing, further species-specific gene primers were designed and used for amplification of the 5′ and 3′ cDNA ends of TERT using a SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) in a few steps according to the manufacturer's instructions. Due to the high GC content, the RT-PCR SMART reactions for the distal 5′ end of *Iris* TERT had to be performed using a 10% DMSO supplement in the reaction mixture. To confirm the sequence of full length cDNAs, the specific primers covering the initiation ATG and stop codons of *Zea*, *Doryanthes*, *Scilla* or *Iris* TERT gene were used for a PCR reaction on cDNA made by PowerScript™ Reverse Transcriptase (BD Biosciences, Clontech) or M-MLV Reverse Transcriptase (Invitrogen). To create full length products, gradient PCR (range 60–65 °C) with the BD Advantage™ 2 Polymerase (BD Biosciences, Clontech) or Phusion DNA Polymerase (Finnzymes) was used according to the manufacturer's instructions (the latter used the HF or GC buffer recommended for higher GC content sequences). The PCR products were inserted into pCR(II)TOPO® or pCR Blunt II TOPO® vector (Invitrogen) and transformed into TOP10 *E. coli* cells. Sequence data were submitted to the DDBJ/EMBL/GenBank databases under accessions AY363163, AY506856-AY506865, AY790901–AY790923, AY805119–AY805123, AY818181–AY818188, AY818158–AY818173, AY850566–AY850610 and DQ057996–DQ058006, including products that are not TERT sequences.

Analysis in silico *and primer design*

Primer design, protein characteristics and alignments of DNA sequences were made using WINSTAR software (DNAStar Inc.); the protein alignments were made manually with the help of partial alignments from ClustalW and Dot-plot methods and published alignments in cited papers (see Results). BLAST search for putative TERT sequences included the searching of all nucleotide GenBank databases including GSS and EST sequences. Putative phosphorylation sites and nuclear localization-like signals were predicted by Prosite [http://www.expasy.org/prosite/] and PSORT [http://psort.ims.u-tokyo.ac.jp/].

The conserved regions of TERT for the first primer design were identified by comparison of the mRNA sequences of *Oryza sativa* (Genbank AF288216, AF494453), *Arabidopsis thaliana* (Genbank AF135454, AF172097) and partially of *Homo sapiens* (GenBank NM_003219). The specific primers for further cloning were designed subsequently as exactly matching sequences for each species.

The specific primers for cloning of partial *Zea mays* TERT sequences were designed using EST and GSS sequences from GenBank identified by BLAST searching (see Supplementary Figure 1). After sequencing, further specific primers were designed for cloning of the 5′ end. Using the newly cloned partial sequences for BLAST search, other GSS sequences were identified and primers for full length mRNA sequence were designed.

For primer sequences used in this work, see Supplementary Table 1.

TRAP assay

Peparation of protein extracts and TRAP assays were performed as described in (Sykorova *et al*., 2003b). Briefly, a two step TRAP protocol was applied using 1 μl of 10 μM substrate primer mixed with telomerase extract containing

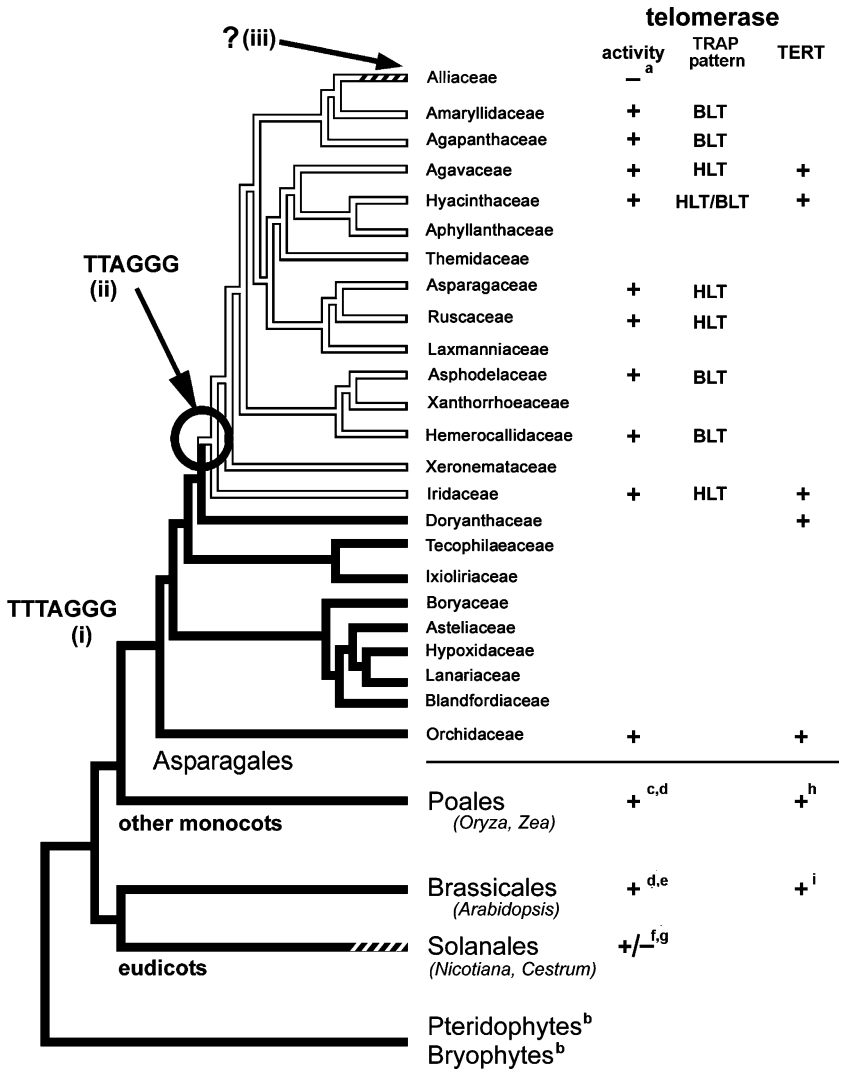

Figure 1. Overview of knowledge of telomeres and telomerases in plants. The phylogenetic scheme (on the left) and data on telomerase activity in Asparagales (left column) are adapted from (Sykorova *et al.*, 2003b) unless otherwise stated. The pattern of TRAP products (BLT or HLT) typical for telomerases synthesizing human-type telomeres and the presence of a sequenced TERT are indicated if a representative species of the family have been studied. Asparagales are split into group (i) with the *Arabidopsis* type of telomeres (black line), group (ii) with human-type telomeres (white line) and group (iii) with uncharacterised telomeres (hatched line, a, *Allium cepa* also negative for telomerase activity). For comparison, the liverwort, fern [b, (Suzuki *et al.*, 2001)] and some model plants are shown with relevant references. Note that the Solanales also include genera with unknown types of telomeres [hatched line, (Sykorova *et al.*, 2003a)]. References for telomerase activity are: c (Heller *et al.*, 1996); d, (Fitzgerald *et al.*, 1996); e, (Fitzgerald *et al.*, 2001); f, tobaco (+) (Fajkus *et al.*, 1996); g, *Cestrum* (−) (Sykorova, unpublished results, 2003) and for TERT subunits: i, *Oryza sativa* (Heller-Uszynska *et al.*, 2002; Oguchi *et al.*, 2004) and *Zea mays* (this work); h, *Arabidopsis thaliana* (Fitzgerald *et al.*, 1999; Oguchi *et al.*, 1999).

100–500 ng of total protein. Primer elongation proceeded in reaction buffer at 26 °C for 45 min. After extension, the samples were incubated at 95 °C for 10 min and cooled to 80 °C. The PCR step started by adding a mix containing 1 μl of 10 μM reverse primer and 2 units of Dynazyme II Taq DNA polymerase (Finnzymes) and the reaction mixture was subjected to 35 cycles of 95 °C/30 s, 65 °C/30 s, 72 °C/30 s followed by final extension at 72 °C/5 min. TRAP products were run on 12.5% PAGE in 0.5×TBE and stained with SybrGreen I (Molecular Probes). The gels were

scanned by a STORM 860 Phosphofluoroimager (Amersham Biosciences) in blue fluorescence mode.

The following primers were used in TRAP assays:

CAMV	5′-CGTCTTCAAAGCAAGTGGATT-3′
CAMVGG	5′-ATTCGTCTTCAAAGCAAGTGG -3′
TS21	5′-GACAATCCGTCGAGCAGAGTT -3′
HUTPR	5′-CCGAATTCAACCCTAACCCTAAC CCTAACCC -3′

Results

The first telomeric switch point occurred after the divergence of Doryanthes (with the *Arabidopsis*-type telomere) in the ancestor to all species of Asparagales with a human motif (Figure 1). Therefore, a comparison of Doryanthes with Asparagales carrying human-type telomeres is likely to be most informative. To cover a wider spectrum of Asparagales, more species were analysed from both groups (see below). Another focus of cloning TERT sequences was concentrated on Hyacinthaceae, the telomeres of which were previously analysed in more detail (Adams *et al*., 2001; Sykorova *et al*., 2003a, b; Rotkova *et al*., 2004; Weiss-Schneeweiss *et al*., 2004).

Cloning of Asparagales TERT subunits

For convenience, the TERT sequence was split into the N-terminal part which covers the less conserved regions of TERT and the C-terminal part which comprises well-conserved regions including the T-motif and all RT motifs suggested as important for catalytic function. Several sets of degenerate and non-degenerate primers were designed in regions of the highest similarity of nucleotide and/or protein sequences of *Oryza sativa*, *Arabidopsis thaliana* and partially of *Homo sapiens* (favoring the *O. sativa* sequence) and used in one-step RT-PCR (not shown). The best results were with a combination of a degenerate primer NOmotF (QFP motif) and a specific primer Os4R (A motif) giving products of ca. 875 bp from cDNA of *Scilla peruviana* (Hyacinthaceae, AY506857-60) and *Doryanthes excelsa* (Doryanthaceae, AY506861-64) and ca. 785 bp from barley (*Hordeum vulgare*, Poaceae; AY506865, AY363163). The products were partial sequences from the putative exon 9 coding for the T motif region (numbering of the exons corresponds to that of 12 exons of the AtTERT/OsTERT gene). The direct one-step RT-PCR reaction with NOmotF/Os4R primers was not successful for *Hosta rectifolia* (Agavaceae), *Ornithogalum virens* (Hyacinthaceae), *Phragmipedium longifolium* (Orchidaceae) and *Iris tectorum* (Iridaceae) but the semi-nested secondary PCR reaction using primers NOmotF or Os4R combined with primers specific for *Scilla* or *Doryanthes* sequences (see Materials and methods and Supplementary Table I) was successful for all listed species.

The two-step RT-PCR protocol and RACE cloning were used for the 5′ and 3′ end cloning of cDNA using SMART^{TM} technology (Clontech). Three genes, from *S. peruviana, D. excelsa, I. tectorum*, were contiguously cloned including the 5′ and 3′ untranslated regions (UTR). Partial sequences covering the C-terminal part of the genes and the T-motif were obtained from the other three plants (*P. longifolium, H. rectifolia*, *O. virens*) of the Asparagales species with a different type of synthesized telomeric repeat (see Figure 1). Only the cloning of the 5′ end of *Iris tectorum* TERT was difficult due to high GC content and a DMSO supplement had to be used in both RT-PCR steps. Finally, we designed specific primers to clone the full length TERTs from *Doryanthes excelsa* (AY790922-23), *Scilla peruviana* (AY818171) and *Iris tectorum* (DQ057996-97) to confirm contiguous TERT sequences from partial clones. In cloning the full length TERT sequences from *Doryanthes*, *Scilla* and *Zea* (see below), the expected products were generated in gradient PCR using various *Taq* DNA polymerases (not shown). Since these PCR conditions were not able to amplify the full length sequence from *Iris tectorum*, the number of PCR cycles was increased and the reaction buffer was altered (see Material and methods). A wide spectrum of products was formed, namely due to alternatively spliced variants. (GenBank DQ057998-DQ058005).

Cloning of Zea TERT

A different strategy was used for the cloning of *Zea mays* (Poaceae) TERT sequence. The partial TERT sequence covering the region from the putative exon 5 to the 3′ end was obtained using

primers designed against sequences identified by BLAST search using *Oryza* TERT as a query (BH879628, CC342251, BZ639144). This 2616 bp long clone (AY506856) interconnected the above partial sequences from database and confirmed that they were TERT sequences. Later, we were able to identify more *Zea* TERT sequences in the GSS database (CG227905, CG277726) due to sequence identity to new clones from 5′ end-cloning which contained the 5′UTR region of the gene. 5′ end-cloning enabled us to design primers for cloning the full length TERT sequence from *Zea mays* (AY818187-88) which was necessary to confirm the coding region of TERT since the consensus sequence of *Zea mays* from partial clones lacked the T2 motif. This motif was present in all full-length clones of *Zea mays* TERT, so the partial mRNA sequence with deletion in the T2 motif region (missing putative exon 3) represented an alternatively spliced mRNA variant.

Comparison of TERT sequences

The size of cloned TERTs ranged from 1227 to 1323 amino acids (aa) and they differed in length of the linker region in the less conserved N-terminal part of the protein and by the presence of another linker sequence between the B′ and C motifs in Asparagales TERTs (Figure 2, Supplementary Figure 2). When comparing TERT sequences from Asparagales, special attention was given to the C-terminal part of the gene that is generally more conserved and considered as substantial for catalytic activity. Even outside the motif regions, mutations of this part of the gene influence telomerase activity and other enzyme properties (see Kelleher *et al*., 2002 for review).

To better determine the conserved regions of plant telomerases, we performed BLAST searches in GenBank databases using AtTERT, OsTERT and all Asparagales cloned sequences as queries. The EST and/or the GSS sequences from *Brassica oleracea*, *Glycine max*, *Manihot esculenta*, *Medicago truncatula*, *Nuphar advena*, *Malus*×*domestica*, *Saccharum officinarum*, *Sorghum bicolor* and many *Oryza* species were identified as putative TERT sequences (see Supplementary Figure 1). The alignment of available plant sequences showed clearly the regions of high similarity among groups like all plant, monocot, eudicot or Asparagales-specific sequences (see Supplementary Figure 1) and their relation to the other known TERTs for further analysis (Figure 2). We were especially interested in conserved changes specific to TERTs from Asparagales synthesizing human-type telomeric repeats in comparison to any TERTs synthesizing *Arabidopsis*-type repeats (from Asparagales and/or other plants, see Figure 2 and Supplementary Figure 2).

Telomerases from both groups shared all conserved motifs with known telomerases like reverse transcriptase motifs [1, 2, A–E, (Nakamura *et al*., 1997)] responsible for catalytic activity and two telomerase-specific motifs [T and T2, (Malik *et al*., 2000; Xia *et al*., 2000)]. Other regions of similarity (QFP, CP motif, C-extension region) were also identified (Figure 2 and Supplementary Figure 1). Putative changes that might influence enzyme properties were found in the C-terminal part of TERT and are described in detail below.

N-terminal part of the TERT subunit

One of the possible explanations of the altered catalytic function of plant telomerases synthesizing human-type repeats is that mutations in TERT subunit lead to changes of catalytic properties and/or changed template usage. A similar situation has been described in *Paramecium tetraurelia* TERT. Two amino acid positions in the N-terminal part of TERT have been suggested to be responsible, the first in the ciliate-specific motif (CP) and the other in the T2 motif due to its proposed function in positioning of RNA subunits and template definition (Ye *et al*., 1999). There is a low similarity in the CP motif region among the ciliates and Asparagales TERT sequences and the possible influence of this region cannot be deduced for Asparagales telomerases. Comparison of TERT subunits from Asparagales and other organisms (Figure 2) revealed that the Asparagales catalytic subunits share conserved features in the T2 motif with other telomerases, independent of the type of telomeric repeat synthesized but do not share the amino acid substitution proposed as responsible for the altered action of *Paramecium tetraurelia* TERT.

Besides the T2 motif, the similarities in the N-terminal part of the gene determined a small conserved region in exon 1 which was previously suggested as a subject of putative Ser

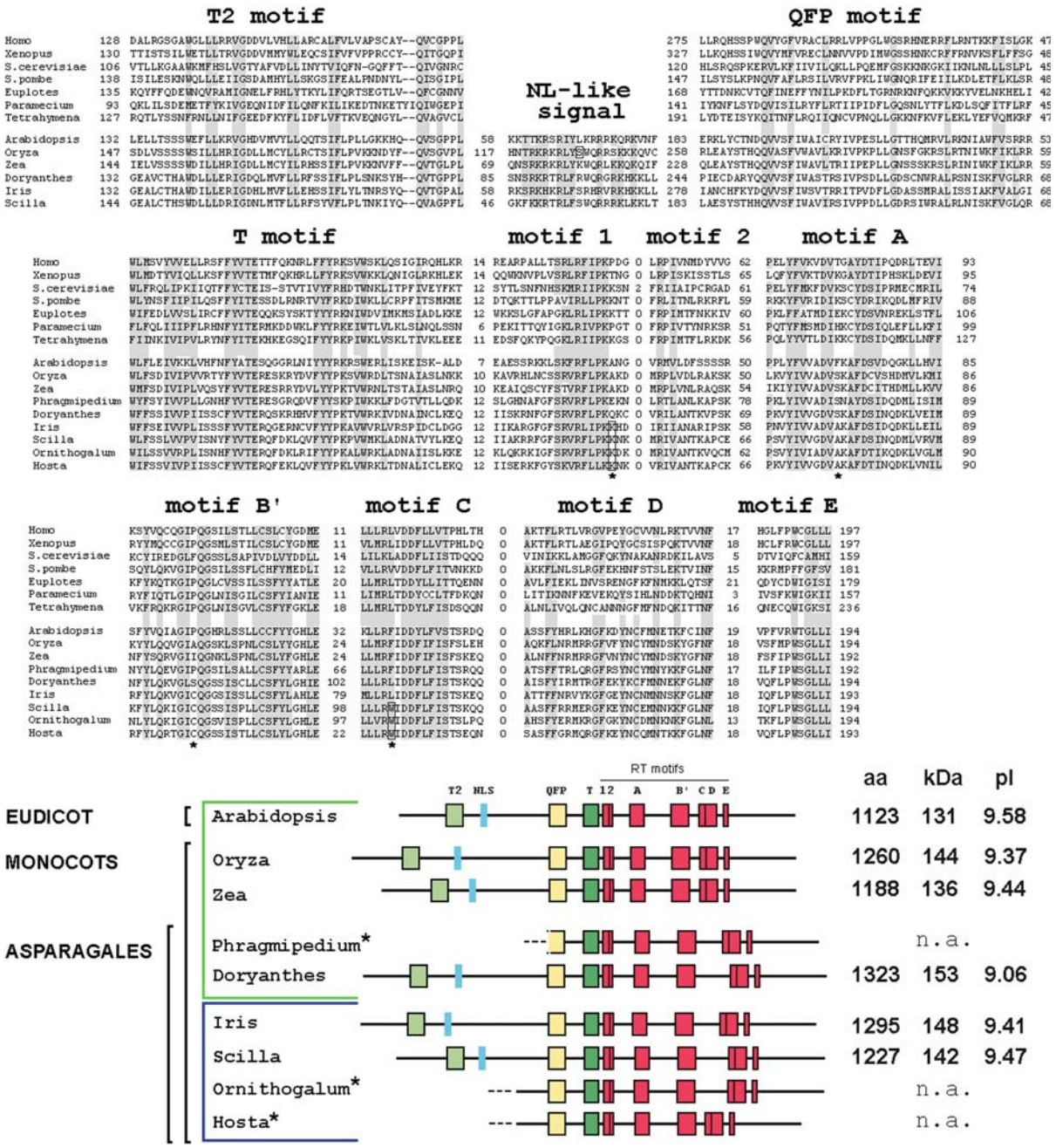

Figure 2. Alignment of conserved telomerase motifs (above) and scheme of organization (below) of plant TERTs. The conserved motifs are as described previously (Nakamura *et al*., 1997; Malik *et al*., 2000; Xia *et al*., 2000), except for the nuclear localization-like signal (NL-like signal) predicted by Prosite and PSORT. The distance (number of amino acids) from the two termini and between the motifs are indicated, shaded amino acids are either identical or represent conservative substitutions for homologs included in this alignment. Conserved amino acid changes in motifs are indicated by asterisks, those discussed in detail are framed (including the position of Ser in the NL-like signal, see Results). The scheme below compares major features of telomerases such as motifs, linker regions, the length (aa), size (kDa) or isoelectric point (pI) as predicted by Winstar (n.a. - not analysed). The partially sequenced TERTs are marked by asterisks, the taxonomy of species is shown on the left with the plant telomerases synthesizing *Arabidopsis*-type telomeres grouped in green bracket and telomerases synthesizing human-type telomeres in blue bracket.

phosphorylation of OsTERT (see Supplementary Figure 1) with a possible regulatory function (Oguchi *et al.*, 2004). However, the presence of the serine residue in this region is specific for *Oryza sativa* sequence, and it is missing in the close relative *Zea* and the other plants investigated. The second suggested Ser phosphorylation site is in a conserved basic region (see Figure 2 and Supplementary Figure 1) which is predicted by Prosite or PSORT to be a nuclear targeting sequence in all plant and some vertebrate TERTs including human. A greater similarity among plant TERT sequences has also been observed in the QFP motif with eight neighboring amino acid residues forming a highly conserved cluster.

C-terminal part of the TERT subunit

The Asparagales TERTs share conserved T motifs and reverse transcriptase motifs with the other known telomerases, including the conserved position of aspartic acids of the catalytic triad in motifs A and C. However, comparison of the C-terminal part of plant TERT genes identified eight amino acid changes inside and 14 outside of conserved reverse transcriptase motifs (Figure 2 and Supplementary Figure 2). Among these, the change in the C motif is of special interest. The telomerases usually possess a Leu (L), Val (V) or Phe (F) residue in the position -2 upstream of the catalytic DD dublet [except for retroviral reverse transcriptases which have Tyr (Y)]. Mutations in this position strongly affect telomerase action (Bryan *et al.*, 2000a; Peng *et al.*, 2001). All telomerases synthesizing the human type of telomeric repeat (except *Iris* TERT) have phenylalanine or leucine substituted by tryptophan (W). The *Iris* TERT shares a leucine (L) residue with *Doryanthes* TERT, while the orchid TERT and other plants have phenylalanine (F); thus an evolution-driven process of adaptation connected to telomerase function could be suggested. Other substitutions in conserved RT motifs were also found but their impact cannot be evaluated due to the lack of data from other systems for comparative analysis. Some changes could be noted in the regions adjacent to RT motifs or in the conserved C terminal extension part (Figure 2 and Supplementary Figure 2). For example, all Asparagales TERTs synthesizing human-type repeats share a Lys residue near the conserved motif 1 with retroviral reverse transcriptases, instead of Ala which is conserved in representatives of Poaceae and Brassicaceae. This lysine could also be found in ciliates and yeasts, but mutation in this less conserved residue in *Tetrahymena* TERT did not influence telomerase activity or RNA binding (Bryan *et al.*, 2000b). A potential impact of this residue on telomerase action cannot be excluded because motif 1 is part of the putative 'finger' domain possibly involved in contacting and positioning of the nucleotide substrate (Xiong and Eickbush, 1990; Peng *et al.*, 2001). The C-terminal part of TERTs contains a few more conserved changes in the C-extension region, as indicated in Supplementary Figure 2. This part of the protein subunit is more conserved in a group-specific manner (e.g. vertebrates, ciliates, plants) and, therefore, comparison between phylogeneticaly distant groups could be misleading. However, a potential functional importance of any of these substitutions cannot be excluded without further experiments. The C-terminal part of Asparagales TERT genes revealed also the presence of a linker region between the B′ and C motifs (Figure 2) which is ca. 40–70 amino acids longer than the corresponding region of other plant TERTs. This is not unique for species with telomerases synthesizing human-type repeats since *Hosta* TERT lacks this extended linker as confirmed by RT-PCR (not shown).

Template region of RNA subunits analysed by TRAP assays

The RNA component of telomerase contains the template for synthesis of telomeric DNA which determines directly the sequence of the telomeric repeat synthesized. The synthesis of variant types of repeats could be caused by a single mutation in the template region or by changed template usage caused either by properties of the TERT subunit (McCormick-Graham *et al.*, 1997) or by its structure outside the template region (Roy *et al.*, 1998; Miller and Collins, 2002). No telomerase RNA subunit has been identified from plants. Analysis of telomerase products *in vitro* showed that model plant telomerases naturally differ in accuracy (mainly T/G-slippage) and in preferences for substrate oligonucleotides (Fitzgerald *et al.*, 1996, 2001). The sequencing of the TRAP products from *Bulbine* and *Ornithogalum* revealed error-prone

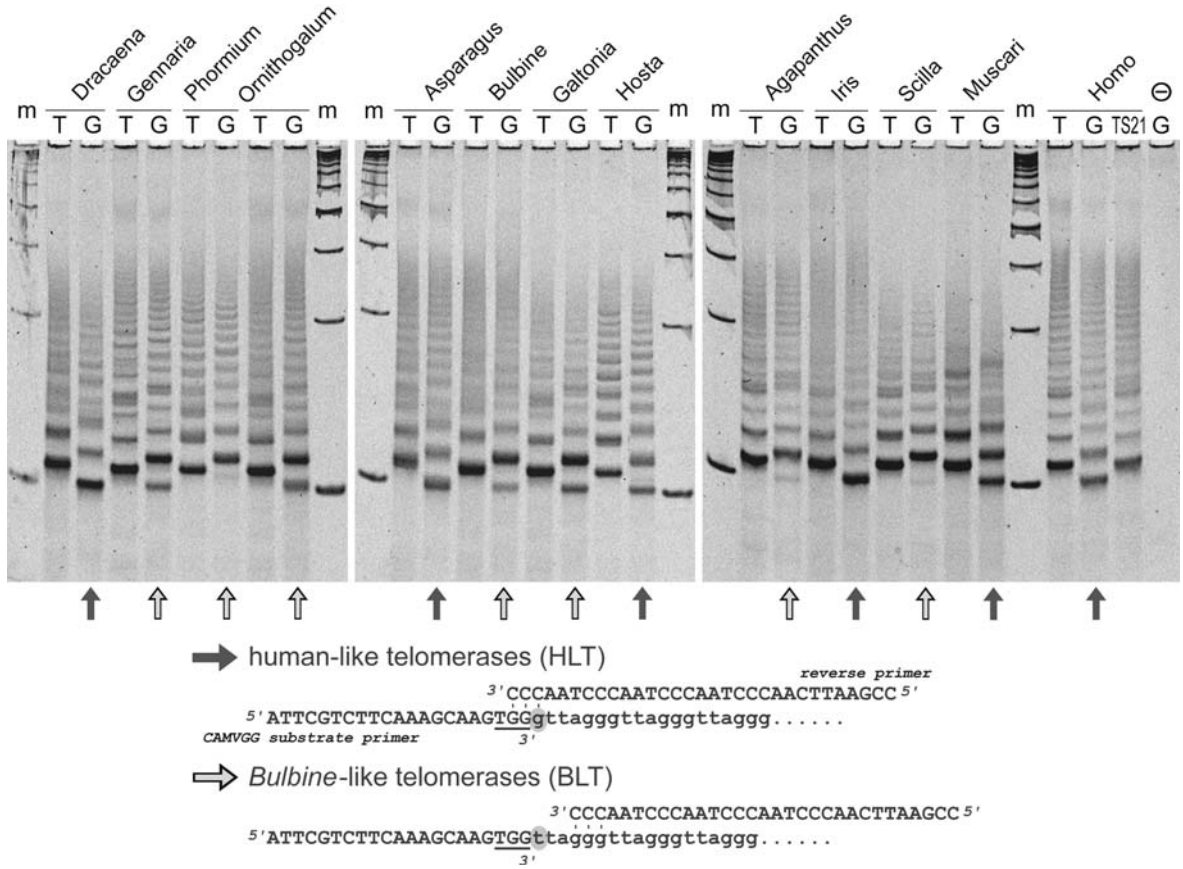

Figure 3. TRAP assays on Asparagales TERT synthesizing human-type telomeric sequence using CAMV (T) or CAMVGG (G) substrate primers. Representative species (indicated by generic names) were studied and compared to human telomerase (*Homo*). Result using TS21 substrate primer in human telomerase is also shown as a control. Negative control (−) contained no protein extract. Lanes m show 50 bp ladder (MBI Fermentas). Two groups of telomerases, the HLT group (black arrows) and the BLT group (grey arrows), differ in addition of the first nucleotide and finally in the pattern of TRAP products generated with the CAMVGG primer. In the scheme below, the primer sequences are shown in upper case letters, telomerase-generated sequences are shown in lower case, and the first nucleotide added by telomerases of each group is shadowed. The position of annealing of the reverse primer (HUTPR) is shown. Note that the CAMV primer (lanes T) provides the same product independently of the group.

telomerases synthesizing the human type of repeat (Sykorova *et al*., 2003b).

Here we tested telomerases synthesizing human-type telomeric repeats from twelve species covering nine families of Asparagales using different oligonucleotides as a substrate primer in TRAP assays (Figure 3). Using the CAMV substrate primer, no differences in TRAP products were observed between Asparagales synthesizing human-type repeats and human telomerase used as a positive control. Surprisingly, two distinct types of TRAP patterns were observed when the CAMVGG substrate primer was used. One type of telomerases (human-like telomerases, HLT) pooling *Iris*, *Hosta*, *Asparagus*, *Dracaena* and *Muscari* TERTs behaved differently to the group termed *Bulbine*-like telomerases (BLT) which includes *Bulbine*, *Phormium*, *Agapanthus*, *Galtonia*, *Scilla*, *Ornithogalum* and *Gennaria* (Figure 3). The basic (shortest) TRAP product of the HLT group was shorter than that of the BLT group, suggesting strongly a different assembly of the template-substrate complex among these telomerases. The lengths of the basic TRAP products depend on the elongation step of the substrate primer by telomerase, but are finally established in the PCR step of the assay which is influenced by the reverse primer sequence (Weinrich *et al*., 1997). The difference between BLT- and HLT-like patterns of TRAP products made by Asparagales telomerases

synthesizing human type of telomeric repeats has to be concealed in the sequence of the first repeat added by telomerase (Figure 3). The CAMVGG primer contains only two guanines at its 3′ end and the first nucleotide added by telomerases generating HLT-like pattern is a third guanine as also occurs for human telomerase (see also Figure 4). The reverse primer HUTPR has three cytosines at the 3′ end that pair to the first synthesized GGG triplet in the elongated substrate, thus generating the shortest TRAP product. In the group of telomerases generating BLT-like pattern, the substrate primer is not initially extended by G and, consequently, the amplification of the basic extension product occurs in a shifted position as soon as the first complete GGG triplet has been added by telomerase.

In some telomerases of the BLT group (*Bulbine*, *Galtonia*, *Gennaria* and *Ornithogalum*) a shorter by-product (probably resulting from G-slippage in the first repeat synthesized) can also be seen. However, in the other BLT telomerases (*Phormium*, *Scilla*, *Agapanthus*) the preference for substrate-template annealing is so strong that this by-product is barely detected. Interestingly, the affiliation of telomerases to either the HLT or BLT group is independent of the phylogenetic position. Moreover, both kinds of telomerases have been detected in a single family of Hyacinthaceae (Figure 1).

It has been demonstrated that telomerase reactions and also their template usage could depend on reaction conditions *in vitro*, namely the concentration of dGTP (Hammond and Cech, 1997; Sun *et al.*, 1999). The properties of Asparagales telomerases were therefore tested in TRAP assays using concentrations of dGTP ranging from 5 to 500 μM, but no switch between the BLT- and HLT-like patterns of TRAP products was observed (not shown). These results support the notion that the BLT/HLT affiliation bears on differences in the structure of the telomerase template region and/or in the assembly of its complex with the substrate (see Discussion).

Discussion

The telomerase catalytic subunit TERT

Previous studies of the catalytic subunit of telomerase (TERT) in a number of eukaryotes showed that in spite of a high degree of complexity and sequence diversity, certain functional domains are conserved and essential to maintain the functional characteristics of the enzyme. These regions involve the reverse transcriptase motifs, the telomerase-specific motifs (T and T2), and some taxon-specific motifs like CP and CP2 in ciliates. The effects of mutations in individual TERTs on telomerase activity, processivity, RNA binding, protein-protein interactions, and telomerase recruitment to telomeres have been tested extensively *in vitro* and *in vivo* [see (Kelleher *et al.*, 2002) for a review]. In some mutants, reduced telomerase function was not lethal and telomerase activity was restored by a second mutation (Friedman and Cech, 1999; Peng *et al.*, 2001; Hossain *et al.*, 2002). These findings demonstrate the enzyme's adaptability to various changes, except at certain essential positions.

In this study we took advantage of the sequence heterogeneity of Asparagales telomeres which enabled studies of related telomerases producing human- or the *Arabidopsis*-type of telomeres. Contrary to the more common approach in which site-specific or random mutations are introduced into either of the telomerase subunit, here we studied the effects of natural mutation of telomerase during evolution which are associated with long-term survival. Besides the Asparagales plants, a similar variability in telomeric sequence has been found only in unicellular eukaryotes (ciliates and yeasts).

Comparison of Asparagales TERTs with others has pointed to mutations which could have played a role in the establishment of changed telomeric sequences (Figure 2 and Supplementary Figure 2). The conservation of the C-terminal part makes it possible to speculate on the impact of changes in, e.g., the C-motif, in motif 1 (a putative part of the finger domain) or in the C-extension region (downstream of the E motif).

Mutations in the highly conserved C motif can affect telomerase processivity [e.g., the *Tetrahymena* mutant L813Y showed increased processivity (Bryan *et al.*, 2000a), while double mutation LA658YT of *S. cerevisiae* TERT had the opposite effect (Peng *et al.*, 2001)].

Asparagales TERTs synthesising human-type telomeres, with the only exception of *Iris*, share W (in contrast to the otherwise present F or L) in the C motif, 2 amino acids upstream of the catalytic DD doublet. A putative TERT also shares W in

the corresponding position in the telomerase sequence of the intracellular protozoan parasite *Theileria annulata* released recently (Apicomplexa/Piroplasmida, GenBank CR940352) and in our database search, the same holds for another protozoan parasite, *Cryptosporidium hominis* (Apicomplexa/Coccidia, GenBank AY034376). *Plasmodium falciparum*, which belongs to the Apicomplexa/Haemosporidia group, possesses F in this position [GenBank AX112155, (Figueiredo *et al*., 2005)]. According to genomic data, these organisms possess different telomeric repeats [TTTTAGGG in *Theileria* (Sohanpal *et al*., 1995), TTTAGG in *Cryptosporidium* (Liu *et al*., 1998) and TT(T/C)AGGG with prevailing TTTAGGG (Bottius *et al*., 1998) in *Plasmodium*]. Thus, in analogy to the situation in Asparagales, single-base changes of the telomeric repeat with respect to the TTTAGGG motif can be seen in this group. Unfortunately, as in plants, the RNA subunits of these telomerases are not known.

Mutations in the catalytic telomerase subunit, especially those in the C-terminal part of the sequence, could contribute to evolutionary change towards imprecise synthesis of the human-type telomeric repeats. The only known example in which synthesis of a variant telomere sequences is TERT-directed is *Paramecium tetraurelia* whose telomeres contain a high proportion (ca. 30%) of the variant repeat TTTGGG, while TTGGGG is coded by the TR template region. The major repeat is identical to that coded by the TR template, but TERT affects fidelity of synthesis perhaps by affecting the conformation of the template region in the TR-TERT complex. Similarly, the telomerases of *Bulbine* and *Ornithogalum* which produce TTAGGG as a major repeat also show a high error rate (ca. 25%) resulting namely in TTTAGGG. Studies of chromosome healing in wheat (Tsujimoto *et al*., 1997, 1999) reveal a similar situation to that observed here in that telomerase, which normally synthesizes TTTAGGG repeats, produces roughly the same proportion of errors, but this time mostly resulting in TTAGGG repeats.

The template region of the telomerase RNA subunit

Two explanations for the change in the synthesized telomeric motif from TTTAGGG to TTAGGG can be formulated in relation to the template region of the RNA subunit:

1. A deletion inside the template region.
2. A shift in template usage. This may have occurred either due to a mutation in ribonucleotides adjacent to the template region which participate in template definition (e.g., via changed secondary RNA structure), or due to changes in the catalytic subunit (TERT-driven template usage, as detailed above).

The first explanation is straightforward, and is supported by experiments on yeast mutants synthesizing human telomere repeats (Brevet *et al*., 2003). In *Paramecium* telomerase, the template region defines the major type of synthesized repeats, but variant repeats can be produced by the mechanism suggested in point 2 (McCormick-Graham and Romero, 1996). Thus, both explanations need not be mutually exclusive.

The second explanation can be illustrated by the example of parasitic protozoa synthesizing TTAGGG repeats and whose template region is probably different from that of human TR [Figure 4, (Cano *et al*., 1999)]. In *Euplotes* the template usage *in vitro* depends on dGTP concentration (Hammond and Cech, 1997), the effect of which is probably absent in Asparagales. A different part of the template region is apparently used by *Oxytricha* and *Euplotes* telomerases, although the telomere sequence synthesized remains the same in this case (Melek *et al*., 1994). Explanations for the telomere sequence switch in Asparagales await complete characterisation of Asparagales TR and determination of factors involved in template definition. It is however predictable that the current template region of the TR corresponds to the major TTAGGG repeat produced, no matter what the mechanism of the change was.

Asparagales telomerases synthesizing human-type telomeres can be grouped into HLT and BLT classes

Classification of Asparagales telomerases into BLT/HLT classes (according to the initial substrate elongation products) is apparently not connected to Asparagales phylogenetic divergence. The differences between HLT and BLT telomerase classes may be due to:

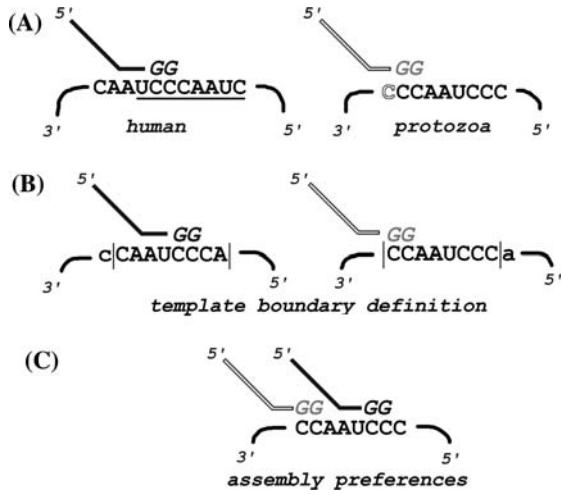

Figure 4. Template regions. (A) human and protozoan template regions and possible template usage of the CAMVGG substrate primer by the HLT (black) or BLT (grey) group of Asparagales telomerases synthesizing human-type telomeres. The region conserved throughout all vertebrates is underlined; (B) the difference could be caused also by changed template boundary definition in otherwise similar template region, or (C) by different assembly preferences on the same template.

1. different template regions (Figure 4A).
2. similar template sequence, but different template boundary definition (Figure 4B).
3. the same template region but different preference in annealing substrate to the alternative positions of the template site (Figure 4C).

When evaluating our results, it should be recalled that they were obtained *in vitro* and using the TRAP assay. Nevertheless, a reproducible qualitative difference between BLT and HLT is obvious enough to define such classification.

The first explanation presumes that, as in the human TR, the template region in the HLT group is distinguished by the central position of the CCC triplet. Consequently, these telomerases require annealing to a substrate primer ending with GG. The BLT group might show a shift of the CCC to the 5′ end of the template region similarly to protozoa and, correspondingly, two possibilities (with a different preference) of positioning the substrate primer. The likelihood of the first explanation is decreased by the facts that (i) BLT and HLT occur independently of Asparagales phylogeny, and ii) in sequenced vertebrate TRs, the conserved part of the template region is shared by all these TRs (Chen *et al.*, 2000). The occurrence of quite different template regions even in the same Asparagales family (Hyacinthaceae) would be surprising, and thus the third explanation seems more probable. On the other hand, the lengths of the template region differ across vertebrates (Figure 4A), providing a potential for differential template boundary definition, thus also favouring the second explanation (Figure 4B). In conclusion, BLT/HLT affiliation is likely to be dependent on the secondary structure of the telomerase complex which determines template usage or assembly of the complex of telomerase with substrate, rather than being caused by mutation in the TR template region, a model which also corresponds with the observations in *Euplotes* and *Oxytricha* cited above.

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