Loss and recovery of *Arabidopsis*-type telomere repeat sequences 5'-(TTTAGGG)_n-3' in the evolution of a major radiation of flowering plants

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Fluorescent *in situ* hybridization and Southern blotting were used for showing the predominant absence of the *Arabidopsis*-type telomere repeat sequence (TRS) 5'-(TTTAGGG)_n-3' (the 'typical' telomere) in a monocot clade which comprises up to 6300 species within Asparagales. Initially, two apparently disparate genera that lacked the typical telomere were identified. Here, we used the new angiosperm phylogenetic classification for predicting in which other related families such telomeres might have been lost. Our data revealed that 16 species in 12 families of Asparagales lacked typical telomeres. Phylogenetically, these were clustered in a derived clade, thereby enabling us to predict that the typical telomere was lost, probably as a single evolutionary event, following the divergence of Doryanthaceae ca. 80–90 million years ago. This result illustrates the predictive value of the new phylogeny, as the pattern of species lacking the typical telomere would be considered randomly placed against many previous angiosperm taxonomies. Possible mechanisms by which chromosome end maintenance could have evolved in this group of plants are discussed. Surprisingly, one genus, *Ornithogalum* (Hyacinthaceae), which is central to the group of plants that have lost the typical telomere, appears to have regained the sequences. The mechanism(s) by which such recovery may have occurred is unknown, but possibilities include horizontal gene transfer and sequence reamplification.

Keywords: phylogeny; telomere; chromosomes; monocots; *Ornithogalum*

1. INTRODUCTION

Telomeres are the physical ends of chromosomes in eukaryotes. They have a specialized and complex DNA–protein structure (see the review in Price 1999), thereby enabling them to function in several critical cell processes (see the review in Pryde *et al.* 1997). In particular, telomeres have a special role in overcoming the problem of end replication. Conventional template-dependent DNA polymerases do not replicate the ends of linear DNA molecules and so chromosome ends are gradually lost over successive cell generations. Telomere sequences, however, are typically restored by telomerase, a ribonucleoprotein that synthesizes DNA sequences onto chromosome ends using an internal RNA template (Greider & Blackburn 1985; Blackburn *et al.* 1989). Understanding the role and functioning of telomeres and their associated proteins has also proved to be extremely important in assessing the potential of cloned animals such as 'Dolly' to live their normal life spans, the immortalization of cell lines and tumorigenesis (Price 1999). This has led to telomere length being used in the assessment of ageing and apoptosis (Harley & Villeponteau 1995; Karlseder *et al.* 1999).

As telomeres have so many vital functions, it is perhaps not surprising that their nucleotide sequence is highly conserved across a broad phylogenetic spectrum. For example, there is only a single base pair difference

between the telomeric sequence of all investigated vertebrates, the fungus *Neurospora* and the protozoan *Trypanosoma*, which share (TTAGGG)_n and nearly all plants previously studied, which have the sequence (TTTAGGG)_n. Most arthropod telomeres have (TTAGG)_n repeats, whereas all ciliate protista studied are either (TTGGGG)_n or (TTTTGGGG)_n (Murray 1990). These 'typical' telomeres are thought to be stabilized by an invasion of the 3'-single-stranded telomeric overhang into its proximal duplex telomeric repeat array, which forms the so-called T-loop (Griffith *et al.* 1999).

The typical telomere sequence in plants was first characterized in *Arabidopsis thaliana* (Richards & Ausubel 1988). This *Arabidopsis*-type telomere sequence has since been found in many plant species investigated, including bryophytes (*Pellia*), lycopods (*Selaginella*), gymnosperms (*Pinus* and *Quercus*) and most angiosperms studied (Cox *et al.* 1993; Fuchs *et al.* 1995). The typical plant telomere sequence was thought to be ubiquitous until *Allium* and two other genera in Alliaceae (*Nothoscordum* and *Tulbaghia*) were reported to lack these sequences (Fuchs *et al.* 1995; Pich *et al.* 1996a,b). Recently, we showed that it was also lacking in *Aloe* (Asphodelaceae) (Adams *et al.* 2000). This result led us to look at the new angiosperm classification (Chase *et al.* 2000; Fay *et al.* 2000), which places both *Allium* and *Aloe* in Asparagales, a large group of 28 monocot families containing ca. 28 000 species (ca. 11% of all angiosperms). We used the phylogenetic data of Fay *et al.* (2000) for predicting and selecting representative species from Asparagales families in order to evaluate the hypothesis that the loss of typical telomere sequences was

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a unique event occurring early in the evolution of Asparagales (estimated to be nearly 90 million years ago) (Bremer 2000). In order to test for the presence/absence of *Arabidopsis*-type telomere sequences, we used fluorescent *in situ* hybridization (FISH) and dot-blot approaches using established methods for demonstrating the absence of these sequences in *Aloe* (Adams *et al.* 2000).

2. MATERIAL AND METHODS

(a) Plant material

Plant materials and their sources are listed in Appendix A.

(b) *In situ* hybridization and Southern slot blotting

The methods for FISH followed Leitch *et al.* (1994) and Adams *et al.* (2000), with minor modifications. Briefly, digoxigenin-11-dUTP-labelled (Roche Diagnostics Ltd, Lewis, UK) or biotin-11-dUTP-labelled (Sigma-Aldrich Company Ltd, Gillingham, UK) DNA probes were hybridized to root-tip metaphase spreads and sites of probe hybridization detected using anti-digoxigenin-fluorescein isothiocyanate and avidin-Cy3 (Vector Laboratories, Burlingame, CA, USA), respectively. In order to detect the *Arabidopsis*-type telomeric DNA, the telomere repeat sequence (TRS) was prepared by polymerase chain reaction (PCR) concatenation using TTTAGGG primers and methods described in Cox *et al.* (1993). As a positive control for the FISH technique, either digoxigenin-labelled pTa71 (9 kb *Eco*RI fragment of the wheat 18–25S ribosomal DNA (rDNA) unit) (Gerlach & Bedbrook 1979) or digoxigenin-labelled pTZ19-R (120 bp fragment of the *Nicotiana rustica* 5S rDNA unit) (Venkateswarlu *et al.* 1991) were used.

The methods for Southern slot blotting followed Adams *et al.* (2000). Briefly, 1 µg of total genomic DNA was loaded into the slot blot and probed first with the TRS probe and then reprobed with pTa71 for rDNA in order to control for the possibility of poor DNA adhesion during Southern transfer. The Gene Images® random prime labelling and detection modules (Amersham Pharmacia Biotech, Little Chalfont, UK) were used for probe labelling and detection following the manufacturer's instructions.

Plant species from families known or expected to have *Arabidopsis*-type telomeres were used as positive controls for the TRS probe in both FISH and Southern slot blots (see the 'outgroups' in Appendix A).

3. RESULTS

Recently, Fay *et al.* (2000) produced a well-supported phylogeny of the 28 Asparagales families (figure 1). This places Alliaceae and Asphodelaceae, which both contain species shown to lack the *Arabidopsis*-type telomeres, in more derived positions than Orchidaceae, the only other Asparagales family studied and shown to contain a species (*Paphiopedilum insigne*) with the typical telomere sequences (Cox *et al.* 1993). We selected 27 representative taxa from 16 Asparagales families (see Appendix A) using the angiosperm phylogeny in a predictive manner in order to evaluate the hypothesis that loss of typical telomere sequences was a unique event occurring early in the evolution of Asparagales.

The presence of *Arabidopsis*-type telomeres can be detected using FISH and the probe TRS which typically appear as paired fluorescent dots at or near the termini of

Figure 1. Phylogenetic tree for Asparagales and related outgroups (taken from Fay *et al.* 2000) showing which families contained the typical telomere sequences and the predicted node at which the typical telomeric sequences were lost during the evolution of Asparagales. The plus and minus signs indicate the presence and absence of typical telomere sequences in the families investigated, respectively.

the chromosomes (figure 2). No species investigated had a TRS signal at an interstitial or centromeric location. All species labelled appropriately with 5S and/or 18–26S rDNA probes as a positive control (as well as those in the outgroups, including *Tradescantia*, Commelinaceae) (see figure 2 and Appendix A). Our results showed that only 11 of the Asparagales taxa examined generated a FISH signal using TRS probes. The remaining 16 species lacked a TRS *in situ* hybridization signal and by inference the typical telomeric sequence. Southern slot blot analysis corroborated these data (see figure 3a and Appendix A). Proof of appropriate DNA transfer to the membrane was confirmed by reprobing the membrane with the 18–26S rDNA probe. The uneven intensity of signal using the telomere and rDNA probes reflects different copy numbers of the sequence rather than uneven loading of the membrane with genomic DNA.

4. DISCUSSION

The results strongly suggest that many species in Asparagales lack the *Arabidopsis*-type telomere sequence. We discuss how reliable a negative result is likely to be and then describe the phylogenetic distribution of species that appear to lack the sequence. We discuss mechanisms that might replace the *Arabidopsis*-type telomere sequence and explain how some species appear to have secondarily regained these sequences.

(a) Demonstrating an absence of *Arabidopsis*-type telomeres

Our results showed that only 11 of the 27 Asparagales taxa examined contained the typical telomeric sequences. The remaining 16 taxa appeared to lack this sequence.

Figure 2. FISH of root-tip metaphase chromosome preparations: (a) *Tradescantia purpurea*, (Commelinaceae), (b) *Kniphophia uvaria* (Asphodelaceae) and (c) *Ornithogalum umbellatum* (Hyacinthaceae). Double labelling was performed in each experiment using a TRS probe (red signal) and either 5S rDNA (cyan signal) (arrows in a,b) or 18–26S rDNA (cyan signal) (arrows in c) as a positive control. Note that the typical telomeric signal is only seen in (a) *Tradescantia* and (c) *Ornithogalum*. Scale bar = 10 µm.

However, demonstrating an absence of a sequence beyond all doubt is difficult. Based on the following discussion, we estimate that our methods are sensitive enough to detect low numbers of the (TTTAGGG)-type telomere in the genome and, thus, those species that did not generate a signal with the TRS probe may indeed lack functional *Arabidopsis*-type telomeric sequences.

- (i) In a previous study by Adams *et al.* (2000) we carried out asymmetric PCR on *Aloe* DNA. Like *Allium*, genomic DNA from *Aloe* failed to generate a DNA product using single-direction (TTTAGGG)₇ primers, clearly indicating that these telomeric sequences were either absent or present in low numbers. In positive controls using *Nicotiana sylvestris* DNA (a species known to possess typical telomeres) as the template, single-direction (TTTAGGG)₇ primers successfully generated the expected product.
- (ii) It has previously been shown in Southern blotting that telomeric probes will hybridize to non-identical but related sequences under high stringency (Allshire *et al.* 1988). We therefore conclude that those species for which DNA failed to hybridize to the TRS probe in Southern slot blots not only lack the *Arabidopsis*-type telomere but also related telomeric sequences.
- (iii) In previously reported Southern blotting experiments (Adams *et al.* 2000), we serially diluted *N. sylvestris* DNA (which contains typical telomeric sequences) with *Allium cepa* DNA (which lacks typical telomeric sequences) keeping the same total DNA concentration in each mixture. These dilution mixtures contained ever fewer typical telomeric repeats as the amount of *Allium* DNA increased. These mixtures were bound to a nylon membrane. We then probed the blots with TRS and observed that we could still generate a hybridization signal when *N. sylvestris* DNA was diluted 10 000-fold with *Allium* DNA. Assuming that each chromosome arm of *N. sylvestris* ($2n=24$) has 0.2–1.3 kb of telomeric repeats (Fajkus *et al.* 1996), a 10 000-fold dilution of *N. sylvestris* DNA would be predicted to reduce the

Figure 3. (a) Southern slot blot probed with TRS and (b) stripped and reprobed with 18–26S rDNA in order to ensure that genomic DNA transfer to the membrane had occurred. One microgram of the following genomic DNAs were loaded onto the membrane: (a) *Aspidistra lurida*, (b) *Hosta* hybrid, (c) *Hemerocallis* hybrid, (d) *Albuca altissima*, (e) *Hippeastrum* hybrid, (f) *Zephyranthes candida*, (g) *Iris tectorum*, (h) *Chlorophytum tetraphyllum*, (i) *Tecophilaea violiflora*, (j) *Blandfordia nobilis*, (k) *Scilla cooperi*, (l) *Hypoxis occidentalis*, (m) *Kabeya hostifolium*, (n) *Milligania densiflora*, (o) *Aloe tenuior*, (p) *Allium cepa* and (q) *Strelitzia reginae*.

abundance of telomeric repeats to less than one copy per chromosome arm, yet we could still detect TRS probe hybridization at this dilution. Despite this sensitivity, we recognize that the TRS probe will not hybridize efficiently to short lengths of a (TTTAGGG)-type repeat, and so very low numbers of the repeat might still be present but undetected.

- (iv) Theoretically, even if a few copies of the telomeric repeat are present at the chromosome ends, the repeats are unlikely to be maintained because they would fail to serve as efficient primers for telomerase. Furthermore, end degradation of the lagging strand at each round of DNA replication causes tens of 5'-(CCCTAAA)-3' units to be lost (50–200 bp). Short stretches of the TTTAGGG repeat would require immediate telomerase-generated synthesis of the leading strand and then lagging strand replication to replace the loss. If this failed to happen at each cell cycle, then the telomere sequence would immediately erode.
- (v) *Allium cepa* is the only member of Asparagales in which DNA sequencing of the chromosome ends has

been conducted. Here, end cloning failed to reveal (TTTAGGG) or similar DNA sequences (Pich & Schubert 1998).

(b) Phylogenetic distribution of the *Arabidopsis*-type telomere

All but one of the 11 Asparagales species with *Arabidopsis*-type telomeres were clustered in families occupying the first three nodes of the Asparagales tree (figure 1). The remaining 16 species lacking typical telomere sequences all belong to families clustered in a derived clade that diverged after the separation of Doryanthaceae (figure 1), which is estimated to have occurred perhaps 80 million years ago. The most parsimonious interpretation of these results is that there was a single evolutionary event when the *Arabidopsis*-type telomere sequence was lost in an early progenitor of most families of Asparagales, such that now up to 6300 species (ca. 2.5% of angiosperms) are predicted to lack typical telomeres. Interestingly, none of the species investigated that lacked *Arabidopsis*-type telomeres had interstitial telomeric signals that could be a relic from ancient chromosome fusion/rearrangement events. Such signals are known in a number of plant species (e.g. *Vicia*, *Petunia*, *Pinus* and *Gibasis*) (Fuchs *et al.* 1995). Loss of typical telomere sequences has also been observed in some arthropods (including dipterans and an arachnid) (Sahara *et al.* 1999).

(c) Regain of the *Arabidopsis*-type telomere in Hyacinthaceae

Although *Scilla cooperi* and *Albuca altissima* lacked the *Arabidopsis*-type telomeres as predicted, four species in *Ornithogalum* (*Ornithogalum umbellatum*, *Ornithogalum virens*, *Ornithogalum montana* and *Ornithogalum arabicum*) and probably *Scilla siberica* (D. Schweizer, personal communication) were shown to possess the typical telomere (all species in Hyacinthaceae) (e.g. figure 2c). Hyacinthaceae has a central position within the group of Asparagales that we predict should lack typical telomeres (figure 1). The most parsimonious explanation is that, after the loss of the typical telomeres in Asparagales, *Ornithogalum* recovered them secondarily. However, this recovery is clearly very restricted since J. Manning, M. F. Fay and M. W. Chase (personal communication) and Pfosser & Speta (1999) have shown that, phylogenetically, *Albuca* and *Ornithogalum* are interdigitated and *Albuca* has been shown to lack typical telomeres.

Secondary acquisition could have occurred through one of several mechanisms, any of which would be novel for telomeres.

- (i) Horizontal gene transfer. The typical plant telomere sequence and/or enzyme machinery for its synthesis may have been reintroduced by horizontal gene transfer from another species, as has been implicated in *Nicotiana tabacum* for the acquisition of *rep* from geminiviruses and *rolC* from *Agrobacterium* (Bejarano *et al.* 1996).
- (ii) Sequence inactivation. A mutation (e.g. point or frame shift) or epigenetic change (e.g. DNA methylation or histone acetylation) in typical telomere-negative species could have inactivated telomerase or

associated enzymes essential for *Arabidopsis*-type telomere synthesis, but in some species this change has reverted. Such reversions may be possible over time-frames of 0.6–6 million years (Marshall *et al.* 1994).

- (iii) Sequence reamplification. The methods reported here which fail to detect typical telomere sequences in the majority of Asparagales investigated indicate a loss of these sequences in these species. However, it is possible that very low numbers of the telomeric sequence may still be present in at least some of the species. If true, this opens up the possibility that, under certain conditions, telomeric sequences could be reamplified and once again form typical, functional telomeres. If this is the explanation then it seems likely that other species with typical telomeres will also be discovered, occurring sporadically throughout the Asparagales clade that predominantly lacks the typical telomeres.

(d) Alternative mechanisms for resynthesizing telomeres

It is clearly of major importance to discover what DNA sequences have replaced the typical telomeric DNA in the majority of Asparagales families and whether all taxa lacking the sequence have the same replacement mechanism. The typical telomeric sequences in *Drosophila* have been replaced by retroelements (*HeT-A* and *TART*), which balance terminal loss of DNA (Danilevskaya *et al.* 1994; Mason & Biessmann 1995; Biessmann & Mason 1997). In *A. cepa* (onion) different chromosome ends may be terminated by different DNA sequences such as those related to En/Spm-transposable elements or rDNA (Pich *et al.* 1996a,b; Pich & Schubert 1998). Perhaps some Asparagales families have similarly replaced the typical telomere with such elements. If so, the elements were probably already present in the ancestral Asparagales genome and able, perhaps by competition, to replace the typical telomere at the point of loss.

Alternatively, it has been shown that chromosome ends can be synthesized *de novo* by a mechanism involving gene conversion (Mikhailovsky *et al.* 1999; Kass-Eisler & Greider 2000) and there is growing recognition that this is an important mechanism in eukaryote telomere maintenance (Blackburn 2000). Asparagales include many species with large genomes (Bennett, M. D., Cox, A. V. and Leitch, I. J. 1998 Angiosperm DNA C-values database. <http://www.rbgekew.org.uk/cval/database1.html/>). Perhaps chromosome elongation by gene conversion is operating both to increase genome size and to synthesize and stabilize chromosome ends. If gene conversion or (retro)transposition are involved we would expect the DNA sequence at the chromosome ends to reflect the 'flavour' of that species' chromatin and the sequences would not necessarily be restricted to chromosome termini. Alternatively, it may be that the species shares a common sequence that differs from the *Arabidopsis*-consensus sequence at the ends of their chromosomes and this sequence carries out the essential functions of the telomere.

Whichever model for *Arabidopsis*-type telomere replacement is correct, most families within Asparagales have evolved a novel way of stabilizing their chromosome ends. It will be important to discover the nature of these alternative mechanism(s) and the DNA sequences involved.

Table A1 Occurrence of *Arabidopsis*-type telomere sequence in Asparagales and related monocots

		TRS detection		positive rDNA control
		Southern	FISH	
Asparagales				
<i>Hosta</i> hybrid (1)	Agavaceae	absence	absence	5S/18S–26S
<i>Allium cepa</i> (2)	Alliaceae	absence	—	18S–26S
<i>Hippeastrum</i> hybrid (3)	Amaryllidaceae	absence	—	18S–26S
<i>Zephyranthes candida</i> (1)	Amaryllidaceae	absence	—	18S–26S
<i>Chlorophytum comosum</i> (3)	Anthericaceae	—	absence	5S/18S–26S
<i>C. tetraphyllum</i> (3)	Anthericaceae	absence	—	18S–26S
<i>Asparagus sprengeri</i> (1)	Asparagaceae	—	absence	18S–26S
<i>Aloe</i> various (2)	Asphodelaceae	absence	absence	5S
<i>Kniphofia uvaria</i> (3)	Asphodelaceae	absence	—	5S
<i>Milligania densiflora</i> (2)	Asteliaceae	presence	—	18S–26S
<i>Blandfordia nobilis</i> (2)	Blandfordiaceae	presence	—	18S–26S
<i>Aspidistra lurida</i> (2)	Convallariaceae	absence	absence	18S–26S
<i>Doryanthes excelsa</i> (4)	Doryanthaceae	—	presence	18S–26S
<i>Hemerocallis</i> hybrid (1)	Hemerocallidaceae	absence	absence	18S–26S
<i>Albuca altissima</i> (2)	Hyacinthaceae	absence	—	18S–26S
<i>Ornithogalum montana</i> (4)	Hyacinthaceae	—	presence	18S–26S
<i>O. arabicum</i> (4)	Hyacinthaceae	—	presence	18S–26S
<i>O. virens</i> (4)	Hyacinthaceae	—	presence	18S–26S
<i>O. umbellatum</i> (4)	Hyacinthaceae	—	presence	18S–26S
<i>Scilla cooperi</i> (2)	Hyacinthaceae	absence	absence	18S–26S
<i>Hypoxis occidentalis</i> (2)	Hypoxidaceae	presence	—	18S–26S
<i>Iris tectorum</i> (3)	Iridaceae	absence	absence	5S/18S–26S
<i>I. winkleri</i> (3)	Iridaceae	—	absence	18S–26S
<i>Cordylina australis</i> (1)	Laxmanniaceae	—	absence	18S–26S
<i>Kabeya hostifolium</i> (2)	Tecophilaeaceae	presence	—	18S–26S
<i>Tecophilaea cyanocrocus</i> (5)	Tecophilaeaceae	—	presence	18S–26S
<i>T. violiflora</i> (2)	Tecophilaeaceae	presence	—	18S–26S
outgroup: commelinoids/liliales				
<i>Tradescantia purpurea</i> (3)	Commelinaceae	—	presence	5S
<i>Rhoeo discolor</i> (3)	Commelinaceae	—	presence	18S–26S
<i>Strelitzia reginae</i> (1)	Strelitziaceae	presence	—	18S–26S
<i>Lilium</i> hybrid (1)	Liliaceae	—	presence	18S–26S
outgroup: dicots				
<i>Tanacetum parthenium</i> (1)	Asteraceae	—	presence	18S–26S
<i>T. vulgare</i> (1)	Asteraceae	—	presence	18S–26S
<i>Nicotiana sylvestris</i> (3)	Solanaceae	presence	presence	5S/18S–26S
<i>Nicotiana</i> various (3)	Solanaceae	presence	presence	5S/18S–26S
<i>Rosa canina</i> (3)	Rosaceae	—	presence	18S–26S
<i>Podophyllum hexandrum</i> (4)	Berberidaceae	—	presence	18S–26S

The source of plant material is indicated after species designation: (1) Bardill's Garden Centre, Nottingham, (2) Royal Botanic Gardens, Kew, (3) Queen Mary, University of London, (4) Chelsea Physic Gardens, London, and (5) Royal Horticultural Society, London.

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APPENDIX A

Table A1 shows the taxa investigated, listed alphabetically by family and then by species. The results in the Southern slot blotting and FISH columns show either the absence or presence of typical TRS (TTTAGGG) as detected with a TRS probe. Double labelling with the TRS probe and either 5S or 18S–5.8S–26S rDNA was performed for each experiment as a control. A positive control species was used for each run of experiments in order to ensure the TRS probe was hybridizing and detectable.

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