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How do Alliaceae stabilize their chromosome ends in the absence of TTTAGGG sequences?

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The *Arabidopsis*-type telomeric repeats (5′-TTTAGGG-3′) are highly conserved. In most families of different plant phyla they represent the basic sequence of telomeres that stabilize and protect the chromosome termini. The results presented here show that Alliaceae and some related liliaceous species have no tandemly repeated TTTAGGG sequences. Instead, their chromosomes reveal highly repetitive satellite and/or rDNA sequences at the very ends. These apparently substitute the original plant telomeric sequences in Alliaceae. Both sequence types are very active in homologous recombination and may contribute to the stabilization of chromosome termini via compensation of replication-mediated shortening.

Key words: Alliaceae, rDNA, satellite DNA, telomeric sequences

Introduction

Telomeres protect the termini of eukaryotic chromosomes from degradation by nucleases, illegitimate fusion and progressive shortening as a result of incomplete replication of linear DNA molecules at their 5′-ends.

Short repetitive sequences of similar base composition form the telomeres of chromosomes of most groups of protozoan, avertebrate and vertebrate animals as well as of fungi, algae and higher plants (for review see Fuchs *et al.* 1995).

Although telomeric sequences appear to be highly conserved during evolution of eukaryotes, some groups, e.g. Diptera (Okazaki *et al.* 1993), have apparently lost and replaced these sequences with others (Saiga & Edström 1985, Biessmann & Mason 1992, Zhang *et al.* 1994, for review see Mason & Biessmann 1995). The *Arabidopsis*-type telomeric repeats (5′-TTTAGGG-3′, Richards & Ausubel 1988) characteristic of chromosome termini of all other plant families tested so far are not detectable by *in situ* hybridization on chromosomes of *Alliaceae* (Fuchs *et al.* 1995).

Here we report our efforts to search for *Arabidopsis*-type telomeric sequences in chromosomes of the genus *Allium* and related liliaceous species and to refine the localization of the chromosomal positions of an approximately 375-bp tandemly repetitive satellite sequence (Barnes *et al.* 1985, Irifune *et al.* 1995) and the 18/25S rDNA sequences (Schubert & Wobus 1985, Ricroch *et al.* 1992) using fluorescence *in situ* hybridization (FISH). Both sequence types may, separately or together, occupy the chromosome termini in some *Allium* species.

Materials and methods

Plant material

A. cepa, *A. fistulosum* and top onions [*Allium* × *proliferum* (Moench) Schrad., a hybrid species of *A. cepa* and *A. fistulosum*], *Nothoscordum inodorum*, *N. gracile*, *N. fragans* and *Tulbaghia violacea* were investigated. The karyotypes of the top onion strains *461*, *413*, *383* (formerly *437*, *124*, and *80*) as well as *228* from the Gatersleben collection were described previously (Schubert 1984, Schubert & Wobus 1985).

Root tips (approximately 2 cm) from bulbs were treated for 2–3 h in 0.5% colchicine, fixed in 3 : 1 ethanol–acetic acid for 1 h, washed in sodium phosphate buffer, pH 4.5, for 1 h and digested in 1% pectinase (Sigma) and 1% cellulase (Onuzuka R10) for 1 h at 37°C. Meristems were squashed in 45% acetic acid. Slides were stored in 96% ethanol at − 20°C.

Polymerase chain reaction and probe labelling

In order to find cryptic amounts of TTTAGGG or satellite sequences not detectable by FISH, PCR with sequence-specific primers and 50 ng of total genomic DNA or DNA of five microdissected chromosome arms (Pich *et al.* 1994) of *Allium cepa* as a template was used.

To detect *Arabidopsis*-type telomeric repeats, a single primer $(TTTAGGG)_7$ was used for asymmetric PCR. For labelling via PCR two heptameres of the *Arabidopsis*-type telomere repeat were used according to Ijdo *et al.* (1991). Primers specific for the *A. cepa* satellite sequences (5′-CCA CGT GAC GAA AAA ACG AAG GGT; 5′ CGG GAT CCC CGT GGC CGG TCT ATG) were designed according to the sequence data of Barnes *et al.* (1985) (ACSAT, EMBL accession number X02572). PCR reactions were performed as described previously (Pich & Schubert 1993a).

A satellite-specific probe of approximately 375 bp was produced and labelled for FISH via PCR using the same primers. For labelling, the final dTTP concentration in the reaction mix was reduced to 0.75 μM and 0.25 μM biotin-16-dUTP (Boeh-

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ringer) was added. The PCR products were characterized electrophoretically on 1.2% agarose gels.

A plasmid (VER17, Yakura & Tanifuji 1983) containing parts of the 18S, the 5.8S, most of the 25S rDNA sequences and the internal transcribed spacer cloned into pBR325 was used as nucleolus organizing region (NOR)-specific probe. It was labelled using a nick-translation kit (Amersham) and digoxigenin-11-dUTP (Boehringer) according to the manufacturer's instructions.

FISH

FISH was performed as described by Fuchs & Schubert (1995). About 50 ng of labelled probes per slide were used for single or double *in situ* hybridization. Biotin was detected using the FITC–streptavidin–antistreptavidin system (Vector Laboratories). Digoxigenin was detected by the rhodamine- or FITC-antidigoxigenin system (Boehringer). A Zeiss fluorescence microscope with an appropriate filter system was used for signal detection. Photographs were taken with an MC 100 camera system on Kodak Ektachrome film, ASA 400. In the case of biotinylated *Arabidopsis*-telomere sequences a cooled (charge-coupled device) CCD camera was also used.

Southern hybridization

For Southern hybridization, total genomic DNAs of *Allium cepa* and *Vicia faba* were isolated according to Pich & Schubert (1993b) and digested by restriction endonucleases *Sau*3AI and *Bam*HI (Boehringer) according to the manufacturer's instructions. Digested DNA was separated electrophoretically on 1% agarose gels and transferred to nylon membranes (Hybond N^+, Amersham) by alkaline transfer using a vacuum blotter (Appligene). An *Arabidopsis*-type telomere probe was synthesized from heptameres (49 bp) of the basic repeat (5′-TTTAGGG-3′) both in sense and antisense orientation and amplified by PCR (Ijdo *et al.* 1991). Labelling was done with a random hexanucleotide labelling kit (Boehringer) and [^{32}P]dCTP (Feinberg & Vogelstein 1983).

Hybridization was performed at high (65°C) and low (55°C) stringency in 0.5 M phosphate buffer (pH 7.2), 7% sodium dodecyl sulphate (SDS), 1% bovine serum albumin (BSA) overnight after 16 h of prehybridization including 0.01% (w/v) salmon sperm DNA. Filters were washed with increasing stringency up to 0.1 × SSC, 1% SDS at 50°C. Signals were detected on X-ray films (Kodak).

Results

TTTAGGG repeats

In repeated FISH experiments with biotinylated TTTAGGG sequence repeats, no hybridization signals could be observed (even using a CCD camera) on chromosomes of *Allium cepa*, *A. fistulosum*, *A. globosum*, *A. chevsuricum*, *A. saxatile* and *A. sativum* (Fuchs *et al.* 1995). The same results were obtained for species of related genera such as *Nothoscordum inodorum*, *N. gracile*, *N. fragans* and *Tulbaghia violacea*. In each experiment the same probe successfully hybridized to chromosomes of other plant species, and other probes, derived from rDNA or 5S RNA genes, yielded signals on chromosomes of *Allium* or other liliaceous species (data not shown).

To refine this observation, total genomic DNA of *A. cepa* was tested in Southern hybridization experiments with TTTAGGG sequences. This probe did not label total *A. cepa* DNA even under low-stringency conditions. With *V. faba* DNA as a positive control the same probe resulted in strong signals even under high stringency conditions (Figure 1).

Additionally, asymmetric PCR with only one primer specific for *Arabidopsis*-type telomeric sequences resulted usually in a smear of fragments when total DNA of *V. faba* but not when total DNA of *A. cepa* served as a template (Figure 2).

These results indicate the absence of *Arabidopsis*-type telomeric sequences from the chromosomes of the tested *Alliaceae*.

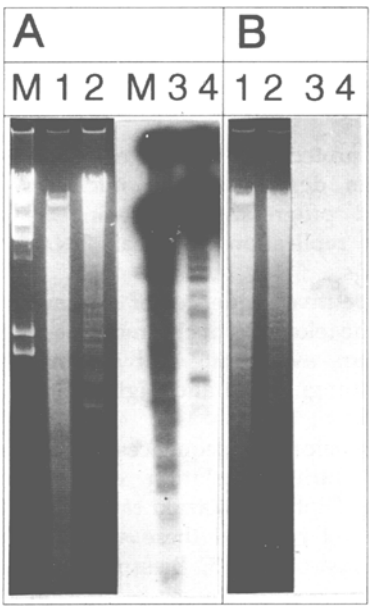

Figure 1. Southern hybridization with TTTAGGG telomeric repeats. *Vicia faba* (**A**) and *Allium cepa* DNA (**B**) was digested by restriction endonucleases *Sau*3AI (lanes 1) and *Bam*HI (lanes 2), separated electrophoretically and blotted on nylon membranes. After hybridization under high-stringency conditions (approximately 85–90% homology) with the telomere repeat-specific probe to the corresponding lanes on blots, strong signals were detected for *Vicia faba* (**A**, lanes 3 & 4); *Allium cepa* lanes remain without signals even under low stringency (approximately 65–70% homology) conditions (**B**, lanes 3 & 4). For comparison marker VI (Boehringer) was loaded on the same gel (M).

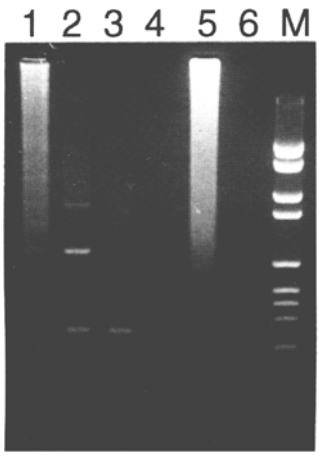

Figure 2. PCR-mediated search for cryptic satellite and TTTAGGG repeats. Amplification of satellite sequence repeats using total genomic *Allium cepa* DNA (1), microisolated terminal regions of *A. cepa* chromosomes with the exception of chromosome 6 (2) and microisolated terminal regions of the short arm of the NOR-bearing *A. cepa* chromosomes 6 (4) as target. Digestion of satellite-specific amplification products from *A. cepa* DNA with *Bam*HI resulted in a repeat specific-sized ladder starting with 375 bp and its multimers (3). Amplification of TTTAGGG repeats via asymmetric PCR using total genomic *Vicia faba* (5) and *Allium cepa* DNA (6) as target. For orientation marker VI (Boehringer) is shown (M).

Satellite and rDNA sequences

A. cepa, *A. fistulosum* and the top onion strains *461*, *413*, *383* and *228* were chosen as examples to study which sequences might form the chromosome termini in Alliaceae. FISH with the satellite-specific probe characteristically labelled the terminal heterochromatin of chromosomes of these species (Figures 3 & 4; for evolutionary conservation of the satellite sequences between various *Allium* species see Pich *et al.* 1996). Additional signals (variable bands) have occasionally been detected at centromeric and interstitial positions of various chromosomes of *A. fistulosum*, *A. cepa* and top onions (Figure 4). These hybridization signals corresponded in position and intensity to the Giemsa banding (Schubert *et al.* 1983) and late replication patterns (Fussel, 1975).

The only chromosome ends without signals were those of the NOR-bearing short arm of *A. cepa* chromosome 6 and the NOR-bearing *A. fistulosum* chromosome 8 in those top onions characterized by the irreversible loss of the satellite from this chromosome. These chromosome termini revealed instead signals after hybridization with the rDNA probe (Figure 3).

After simultaneous hybridization with rDNA and satellite sequences, the short arm ends of the smallest chromosome of *A. cepa* and all chromosome termini of top onions that revealed additional NORs (Schubert 1984, Schubert & Wobus 1985) were labelled by both probes. The short arm terminus of the smallest *A. cepa* chromosome in some lines revealed an active NOR (Sato 1981). One to three additional NORs were found in clonal strains *228*, *383*, *413*, *461*. Interestingly, both strains *228* and *383* – formerly strain *15* – revealed in previous observations (Schubert 1984) only one active NOR positioned on the satellite chromosome of *A. fistulosum*.

The relative position of both signals could not be determined exactly in each case. However, in top onion strains *383* (data not shown), *228* and *461* two-colour FISH clearly revealed chromosome termini with rDNA label at the outermost position, whereas in strain *413* the opposite, i.e. satellite sequences in the most distal position, was observed (Figure 3).

After PCR with primers designed according to an approximately 375-bp tandemly repetitive satellite sequence and total genomic *A. cepa* DNA several products of different size were observed. Digestion with *Bam*HI having a single recognition site in the satellite sequence yielded the basic repeat and a limited number of di- and trimers. The same-sized ladder was obtained when DNA of microdissected terminal regions of *A. cepa* chromosomes were used as a target for PCR with satellite sequence-specific primers (Figure 2).

Again the only exception was the short arm of *A. cepa* chromosome 6 with the NOR in terminal position or flanked distally by a small heterochromatic satellite (Schubert 1984), which seems to be composed of condensed rDNA sequences (Ricroch *et al.* 1992). DNA of the terminal region of this chromosome arm yielded no PCR products with satellite-specific primers (Figure 2).

Discussion

Our data confirm and extend the previous inference that *Arabidopsis*-type telomeric repeats are absent from chromosomes of the Alliaceae (Fuchs *et al.* 1995). The chromosomal location of the approximately 375-bp GC-rich satellite sequences at chromosome termini of *A. cepa* (Barnes *et al.* 1985) and *A. fistulosum* (Irifune *et al.* 1995) was confirmed by FISH. Additional loci were found in interstitial heterochromatic positions on chromosomes of both species and in top onions.

Furthermore, the rDNA composition of the *A. cepa* satellite (Ricroch *et al.* 1992) and the mobility of NORs between terminal positions of various chromosomes of some top onion strains (Schubert 1984, Schubert & Wobus 1985) were confirmed and refined by FISH. The previous observation that top onion strains with only one NOR may acquire additional terminal NORs as a result of mobile rDNA sequences (Schubert & Wobus 1985) was extended to further clones (*228* and *383*).

Either rDNA or satellite sequences may yield the most distal signal after double-colour FISH at chromosome termini where both occur together. The simplest explanation is that both sequences in fact may occupy outermost positions and substitute each other as well as TTTAGGG repeats.

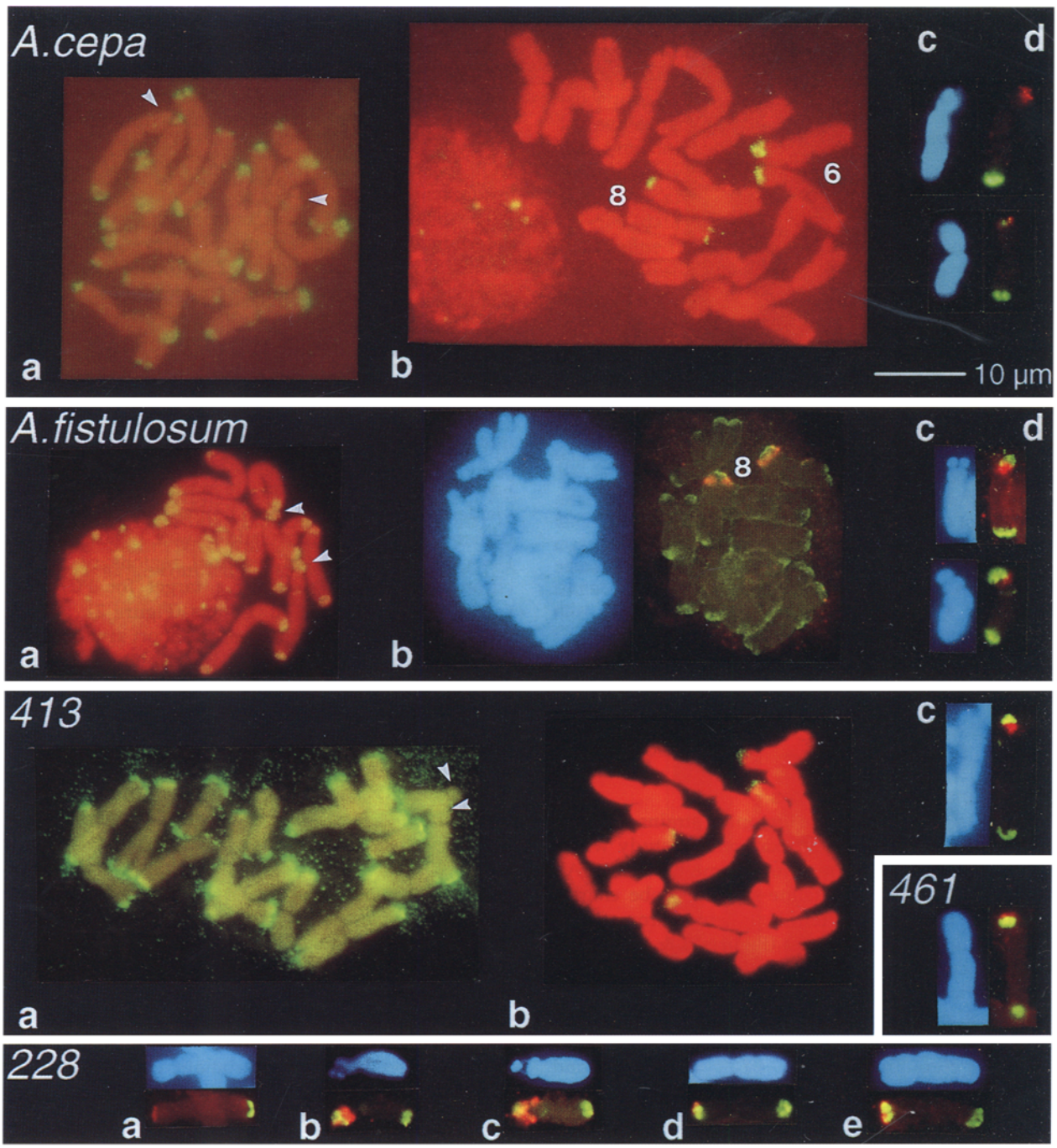

Figure 3. Compilation of *in situ* hybridization patterns obtained with satellite- and rDNA-specific probes on chromosomes of *A. cepa*, *A. fistulosum* and selected top onion strains (*413*, 461, *228*). *A. cepa*: **a** Satellite-specific signals at all chromosome ends except the short arm of chromosome 6 (arrows). **b** rDNA-specific signals on chromosomes 6 and 8. **c** DAPI-stained chromosomes 6 (above) and 8. **d** The same chromosomes after FISH with satellite- (green) and rDNA- (red) specific probes. *A. fistulosum*: **a** Partial metaphase with satellite-specific signals at all chromosome ends. Satellite chromosomes are indicated (arrows). **b** DAPI-stained metaphase and the same metaphase after FISH with satellite- (green) and rDNA- (red) specific probes, rDNA signals only on chromosome 8. **c** DAPI-stained chromosomes 8. **d** The same chromosomes after FISH with satellite (green) and rDNA- (red) specific probes. Strain *413*: **a** Satellite DNA-specific signals at metaphase chromosomes. The NOR-bearing chromosome 6 of *A. cepa* and the NOR-bearing chromosome 8 of *A. fistulosum*, which have lost their satellite remain without signals at their short arm ends (arrows). **b** rDNA-specific signals on four chromosome termini. **c** Signals obtained by satellite- (green) and rDNA-(red) specific probes at the end of an *A. cepa* chromosome with an additional NOR. Strain *461*: Position of rDNA (red) and satellite (green) sequences at the end of a chromosome with an additional NOR. rDNA sequences occupy the outermost position. Strain *228*: Selected chromosomes after DAPI-staining and after FISH with satellite (green) and rDNA (red) probes. **a** Chromosome 6 of *A. cepa* without satellite sequences on the short arm. **b & c** Satellite chromosomes of *A. fistulosum*. The satellite is labelled by satellite sequences. **d & e** Chromosomes with additonal NORs and rDNA sequences at the outermost position.

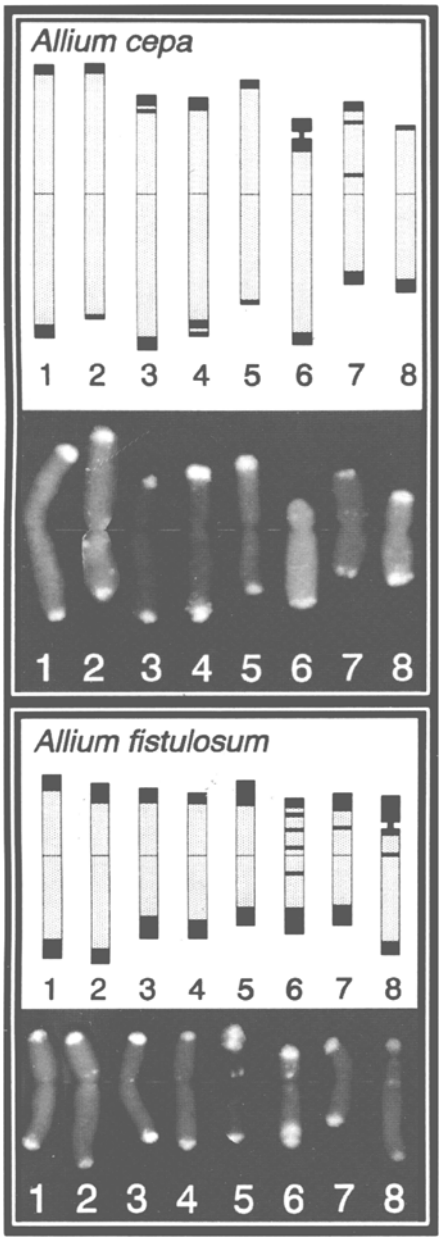

Figure 4. Karyotypes of *A. cepa* and *A. fistulosum*. Scheme of chromosome complements of both species after Giemsa banding with strong terminal and weaker variable bands at interstitial positions (top). The corresponding chromosome complements after FISH with the satellite-specific probe. Signals are mostly correlated to the Giemsa banding patterns, except for the NOR-bearing chromosome 6 of *A. cepa* (which has lost its satellite). The heterochromatin of the NOR is composed of condensed rDNA (bottom).

Taking all data into account we arrived at the following assumptions:

- The primary plant telomeric sequences probably became lost and the original TTTAGGG telomeric sequences were replaced by highly repetitive satellite- and rDNA sequences during evolution of *A. cepa* and *A. fistulosum*.
- The replication-mediated shortening at the chromosome ends might be overcome by frequent recombination and/or conversion events involving these sequences.
- The same mechanism might also be responsible for polymorphism of size and position of NORs in *Allium* species (Bougourd & Parker 1976, Schubert 1984, Schubert & Wobus 1985).
- Satellite and rDNA sequences might also fulfil other functions of telomeres in *A. cepa* and *A. fistulosum*.
- Possibly similar sequences also form telomeres in other related species that lack TTTAGGG repeats at their heterochromatic chromosome termini.

A similar situation has been described for diptera in which retroposons or satellite sequences have apparently substituted the original telomeric sequences (Young *et al.* 1983, Saiga & Edström 1985, Biessmann & Mason 1992, Zhang *et al.* 1994). In *Chironomus pallidivittatus* only the centric end of the telocentric chromosome 4 does not reveal the species-specific telomeric satellite sequence. Instead, a repetitive sequence occurring at all centromeres of this species was found at this chromosome end (Rovira *et al.* 1993). This is another example of substitution on one and the same species of the primary telomeric repeats by two different sequences that might also have other functions.

It remains an open question whether interstitial satellite DNA loci on *Allium* chromosomes are primary ones, similar to those of HetA-retroposons within the β-heterochromatin in *Drosophila* or whether their interstitial positions are acquired secondarily, e.g. by inversions. rDNA loci seem to occupy interstitial positions originally and become mobile when not flanked distally by heterochromatic structures (Schubert & Wobus 1985). In terminal position they 'stick' to each other and may transiently form bridges between NOR-bearing chromosomes as visualized by silver staining (Figure 5). These structures probably indicate recombination of terminal rDNA sequences.

It will be of interest to show the extent of substitution of TTTAGGG repeats by other sequences in further groups of Liliaceae. It will also be informative to test whether in other phyla NORs at chromosome ends may occupy true terminal positions or are flanked distally by original telomeric repeats.

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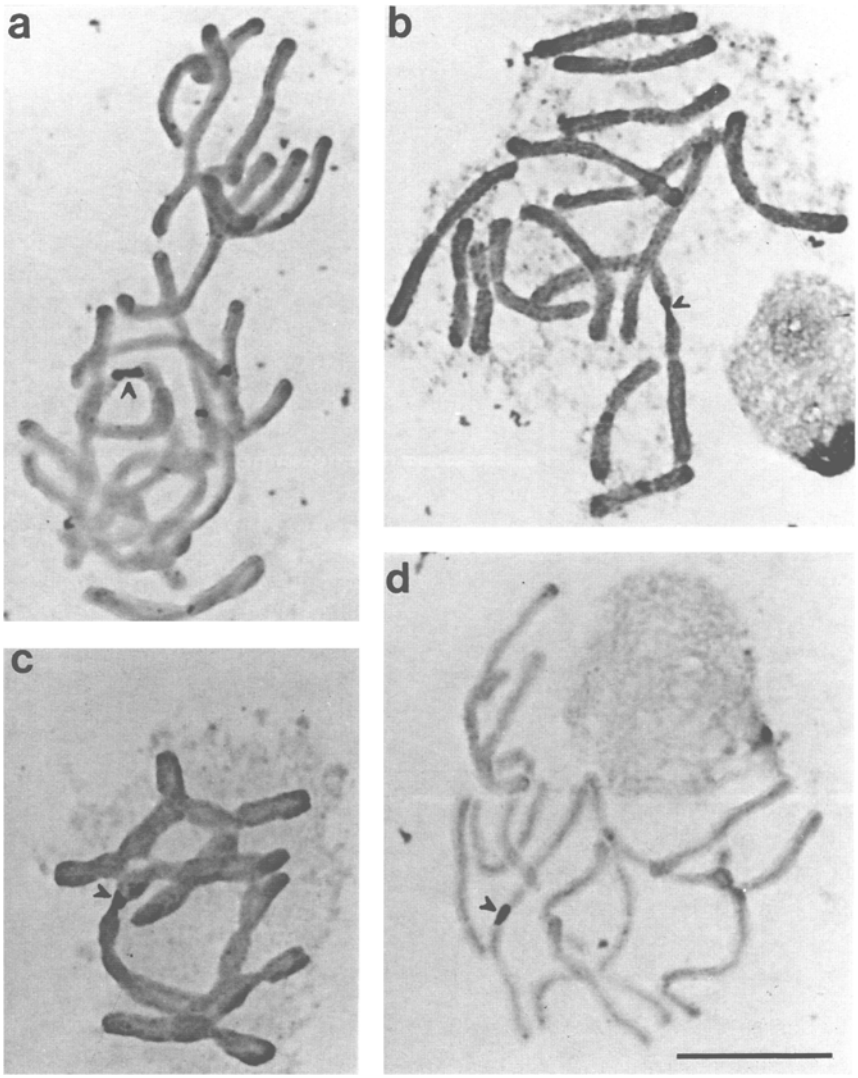

Figure 5 Complete (**a, b & d**) and partial (**c**) metaphases of *Allium cepa* after silver staining of NORs (Schubert *et al.* 1983). Arrowheads mark 'bridges' between NORs of the short arm ends of chromosomes 6. Scale bars = 5 μm.

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