TAS49—a dispersed repetitive sequence isolated from subtelomeric regions of *Nicotiana tomentosiformis* chromosomes

Mirka Horáková and Jiří Fajkus

Abstract: We have isolated and characterized a new repetitive sequence, *TAS49*, from terminal restriction fragments of *Nicotiana tomentosiformis* genomic DNA by means of a modified vectorette approach. The *TAS49* was found directly attached to telomeres of *N. tabacum* and one of its ancestors, *N. tomentosiformis*, and also at inner chromosome locations. No association with telomeres was detected neither in *N. otophora* nor in the second tobacco ancestor, *N. sylvestris*. PCR and Southern hybridization reveal similarities in the arrangement of *TAS49* on the chromosomes of 9 species of the genus *Nicotiana*, implying its occurrence as a subunit of a conserved complex DNA repeat. *TAS49* belongs to the family of dispersed repetitive sequences without features of transposons. The copy number of *TAS49* varies widely in the genomes of 8 species analyzed being lowest in *N. sylvestris*, with 3300 copies per diploid genome. In *N. tomentosiformis*, *TAS49* forms about 0.56% of the diploid genome, corresponding to 17 400 copies. *TAS49* units are about 460 bp long and show about 90% of mutual homology, but no significant homology to DNA sequences deposited in GenBank and EMBL. Although genomic clones of *TAS49* contain an open reading frame encoding a proline-rich protein similar to plant extensins, no mRNA transcript was detected. *TAS49* is extensively methylated at *CpG* and *CpNpG* sites and its chromatin forms nucleosomes phased with a 170 ± 8 bp periodicity.

Key words: repetitive DNA sequence, subtelomere, plant, *Nicotiana*.

Résumé : Les auteurs ont isolé et caractérisé une nouvelle séquence répétée, *TAS49*, à partir des fragments de restriction terminaux du génome du *Nicotiana tomentosiformis* à l'aide d'une approche « vectorette » modifiée. La séquence *TAS49* était liée aux télomères du *N. tabacum* et d'un de ses ancêtres, le *N. tomentosiformis*, tout en étant localisée également au niveau de certains sites chromosomiques internes. Aucune association avec les séquences télomériques n'a été détectée ni chez le *N. otophora* ni chez le second ancêtre du tabac, le *N. sylvestris*. Des analyses PCR et des hybridations Southern ont révélé des similitudes quant à l'agencement des séquences *TAS49* sur les chromosomes de neuf espèces du genre *Nicotiana*, ce qui implique sa présence comme sous-unité d'un complexe conservé d'ADN répété. *TAS49* appartient à une famille de séquences répétées dispersées ne présentant pas de caractéristiques d'un transposon. Le nombre de copies de *TAS49* varie grandement parmi les génomes des huit espèces analysées et il était le plus faible chez le *N. sylvestris* avec 3,300 copies par génome diploïde. Chez le *N. tomentosiformis*, *TAS49* compte pour environ 0,56 % du génome diploïde, ce qui correspond à 17,400 copies. Les monomères de *TAS49* mesurent environ 460 pb et montrent environ 90 % d'homologie les uns avec les autres mais aucune homologie significative avec aucune autre séquence disponible dans GenBank ou EMBL. Bien que des clones génomiques de *TAS49* comprennent un cadre de lecture pouvant coder pour une protéine riche en proline semblable aux extensines végétales, aucun transcrit n'a été détecté. *TAS49* subit une importante méthylation au niveau des sites *CpG* et *CpNpG* et sa chromatine forme des nucléosomes avec une périodicité de 170 ± 8 pb.

Mots clés : séquence d'ADN répétitif, subtélomérique, plante, *Nicotiana*.

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Introduction

Telomeres are nucleoprotein structures forming the very ends of linear eukaryotic chromosomes. Their DNA component usually consists of short oligonucleotide sequences repeated in tandems of various lengths. Plant telomeres are mostly assembled from $(TTTAGGG)_n$ sequences (Richards and Ausubel 1988) that are synthesized by a specialized reverse transcriptase, telomerase (Fajkus et al. 1996; Fitzgerald et al. 1996), similarly to other eukaryotic cells (Greider and Blackburn 1985). Telomeres are followed by subtelomeric regions. In most cases these are composed of repetitive sequences that form a border between distally positioned structural genes and telomeres, which possibly func-

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M. Horáková. Masaryk University, Department of Analysis of Biologically Important Molecular Complexes, Brno, Czech Republic.
J. Fajkus.[1] Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, CZ-612 65 Brno, Czech Republic.

[1]Author to whom all correspondence should be addressed (e-mail: fajkus@ibp.cz).

tion as a buffer zone, protecting coding sequences from eventual damage resulting from incomplete replication.

In a number of plant species, tandemly repetitive sequences have been shown to form a major component of subtelomeres, e.g., *TGR1* in tomato (Ganal et al. 1988), *HRS60* in tobacco (Koukalova et al. 1989), *HvRT* and *HvT01* in barley (Belostotsky and Ananiev 1990; Brandes et al. 1995). Some of these sequences were found in a direct association with telomeres e.g., *HvRT* (Kilian and Kleinhofs 1992) and *HRS60* (Fajkus et al. 1995b), and, consequently, were termed telomere-associated sequences. It has been shown recently that the telomere–subtelomere border is a site of transition between telomeric chromatin, characterized by a short nucleosome spacing (about 155 bp) and multiple translational positioning without rotational phasing of nucleosomes (Makarov et al. 1993; Tommerup et al. 1994; Fajkus et al. 1995*a*; Rossetti et al. 1998) and the subtelomeric chromatin with preferred rotational and translational positioning of nucleosomes, which show identical or similar spacing to that in other genome regions (about 180 bp, Fajkus et al. 1992; Fajkus et al. 1995*a*; Vershinin and Heslop-Harrison 1998). These findings support a hypothesis that coding for chromatin structure is one of the possible roles of subtelomeres (Vogt 1990).

Several other functions have been suggested for subtelomeres. Specific recombination "hot-spots" are probably present at subtelomeric positions (Hofker et al. 1990; Ganal et al. 1992) resulting in exchanges of DNA tracts between chromosome ends. Thus, sister chromatid exchange (Louis et al. 1994), homologous recombination (Wada and Nakamura 1996), or gene conversion (Zou et al. 1996; Pluta and Zakian 1989) take place within. Homogenization of heterologous chromosome ends through recombination between subtelomeric tandem arrays has been reported (Dover 1982; Flavell 1986; Zhong et al. 1998). Using fluorescence in situ hybridization (FISH), very clear aggregation of subtelomeric arrays near the nuclear matrix in the interphase nucleus was observed before the first meiotic division (Spence et al. 1998). A putative nuclear matrix-attachment region was found in a boundary between telomere and associated *HRS60* subtelomeric sequences (Fajkus et al. 1995*b*). Subtelomeric sequences participate in initiation of chromosome pairing (Loidl 1990; Cooke et al. 1985) and in synapsis (Eckert et al. 1997). Chromosome-specific local differences in subtelomeric structure may contribute to the correct recognition of homologous chromosomes in this process, especially in the case of composite genomes (Feldman et al. 1997).

In tomato, a reversed order of telomeric and subtelomeric (*TGR1*) sequences has been observed at native chromosome ends, suggesting a backfolding telomeric structure with *TGR1* occupying the extremity of the chromosomes (Zhong et al. 1998). Further, Amarger et al. (1998) proposed a general subtelomeric origin of minisatellite structures represented by tandem repeats in the kilobase size range. Together, these observations suggest that subtelomeric sequences contribute to the structural architecture of the nucleus.

Another possible function of subtelomeres has arisen from observations in *Allium* species, whose chromosome ends lack the common plant telomeric repeats. Instead, highly repetitive satellite and (or) rDNA sequences were found to form the very ends of chromosomes (Pich et al. 1996). Correspondingly, Alliaceae species were negative for telomerase activity in dividing cells (Pich and Schubert 1998). It was suggested that the original TTTAGGG telomeric sequences became lost from the subtelomeres of Alliaceae chromosomes and were replaced by highly repetitive sequences. Replication-mediated shortening of chromosome ends might be overcome by frequent recombination and (or) conversion events involving these sequences. A similar situation has been described previously in the dipteran *Chironomus pallidivittatus*, in which 340-bp satellite sequences have apparently replaced the original telomeric sequences (Saiga and Edstrom 1985; Zhang et al. 1994). Thus, subtelomeric repetitive sequences may play the role of a compensatory telomerase-independent mechanism of chromosome-end stabilization.

In order to gain better insight into telomeric and subtelomeric structure and function within an amphidiploid tobacco genome, we have previously characterized DNA sequences of telomere–subtelomere junctions in *Nicotiana tabacum* chromosomes from the S-component of the tobacco genome (originating from one of tobacco's probable ancestors, *N. sylvestris*). A tandem repeat of *HRS60* units was found in direct connection with telomeres (Fajkus et al. 1995*b*), and differences in chromatin structure of telomeres and subtelomeres were described. To enrich the picture of tobacco subtelomeres by data from the T-component of the tobacco genome (originating most probably from *N. tomentosiformis* or, with a lower probability, from *N. otophora*) (Narayan 1987), we have cloned and characterized a telomere-associated sequence, *TAS49*, from terminal restriction fragments of *N. tomentosiformis* genomic DNA.

Materials and methods

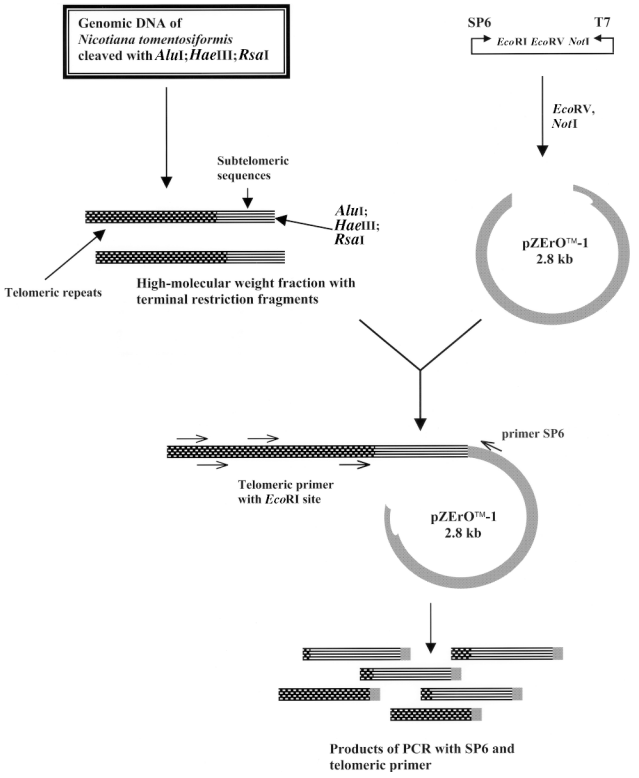
Isolation of genomic DNA, nucleosomal DNA, and plasmid DNA

Cell nuclei were prepared from leaves of different species of the genus *Nicotiana* according to protocols previously described (Espinás and Carballo 1993). About half of the material was digested to mono- and oligonucleosomes (Fajkus et al. 1995*a*). The rest of the nuclei were lysed in lysis solution (0.5 M EDTA pH 9, 2 M NaCl, 1% laurylsarcosine, 0.5 mg/mL Proteinase K) at 50ºC for 24 h and DNA was extracted twice with phenol–chloroform and once with chlorophorm. After ethanol precipitation, genomic DNA was dissolved in TE (10 mM Tris–HCl, 1 mM EDTA pH 8.0). Plasmid DNA was prepared using alkaline lysis of bacterial cells (Birnboim and Doly 1979).

Cloning of telomere-associated sequences

The modified vectorette approach (Riley et al. 1990; Arnold and Hodgson 1991; Kilian and Kleinhofs 1992) was used for cloning telomere-associated sequences (Fig. 1). High-molecular-weight terminal restriction fragments containing the telomeric tandem array and associated subtelomeric sequences were prepared by digestion of *N. tomentosiformis* genomic DNA with restriction enzymes creating blunt ends (*Hae*III, *Alu*I, or *Rsa*I), separated on 0.8% agarose from short restriction fragments, and isolated from agarose by a QIAquick Gel Extraction Kit (Qiagen, U.S.A.). Vector pZErO-1 (Invitrogen, U.S.A.) was double-digested in the polylinker with

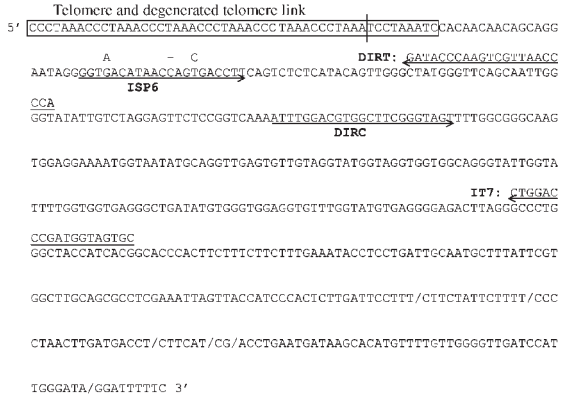
Fig. 1. The procedure used for amplification of telomere-associated sequences of *Nicotiana tomentosiformis* chromosomes. Terminal restriction fragments were ligated into the vector pZErO and amplified by PCR with a telomeric primer and vector primer SP6. Multipositional annealing of the telomeric primer resulted in variable lengths of the telomeric part of the products, and eventually in products containing only telomeric parts.



*Eco*RV creating blunt ends, and *Not*I producing cohesive ends. Subsequently, the terminal restriction fragments were ligated to the digested vector pZErO-1, and PCR (polymerase chain reaction) was performed with the vector primer SP6 (5′-GATTTAGGTGACACTATAG-3′) and the telomeric primer C containing an *Eco*RI restriction site (5′-CC*GAATTC*AACCTAAACCCTAAACCCTAAACCC-3′) using Taq DNA polymerase (Promega, U.S.A.) under conditions: 2 min at 95°C, 35× (30s at 94°C, 30s at 45.5°C, 90s at 70°C), 3 min at 72°C.

Products of the first round of PCR were then amplified under the same conditions, and the resulting products were separated by 6% non-denaturing polyacrylamide gel electrophoresis (PAGE). Products isolated from polyacrylamide gel (Sambrook et al. 1989) were cleaved with *Eco*RI and ligated into vector pZErO-1 cleaved with the same restriction enzyme. Ligation mixtures were used for transformation of Epicurian Coli XL1-Blue Subcloning-Grade Competent Cells (Stratagene, U.S.A.) and clones positive for telomeric sequences were selected by colony hybridization. Inserts of selected plasmid clones were sequenced using a Sequenase v. 2.0 DNA Sequencing Kit (USB, U.S.A.). This procedure enabled us to obtain the sequence of *TAS49*. The authenticity of the sequence obtained was verified by PCR using telomeric primer C 5′-$(CCCTAAA)_6$-3′, telomeric primer G 5′-$(TTTAGGG)_6$-3′, and *TAS49*-specific primers DIRT, DIRC, IT7, and ISP6 (Fig. 2; Table 1) on genomic DNA of *N. tomentosiformis*, *N. tabacum*, and *N. sylvestris* in various combinations. PCR products were cloned in pZErO-1 vector and sequenced.

Fig. 2. The complete *TAS49* unit and its association with the telomere via a linker of degenerate telomeric sequence. Sequences of primers ISP6, DIRT, DIRC, and IT7 are underlined and their 3′ ends are marked with arrows. The sequence of primer ISP6 (originally designated according to the sequence of clone p49) differs from the underlined consensus sequence by three nucleotides marked above.



Polymerase chain reactions

PCR conditions used for amplification of genomic DNAs of *Nicotiana* species using Taq polymerase and primers IT7, ISP6, and telomeric primer C or G were: 2 min at 95°C, 35× (30s at 94°C, 30s at 48°C, 90s at 70°C), 3 min at 72°C. The Expand High Fidelity PCR System (Boehringer Mannheim, Switzerland) was used for the amplification of *N. tabacum*, *N. tomentosiformis*, *N. otophora*, and *N. sylvestris* genomic DNA using primers DIRT, DIRC, and telomeric primer C or G to enable amplification over a wide range of distances. PCR with primers DIRT and DIRC, and primer DIRT with telomeric primer C or G was performed under optimal conditions: 2 min at at 94°C, 10× (15s at 94°C, 30s at 60°C, 4 min at 68°C), 15× (15s at 94°C, 30s at 56°C, 4 min 20s each cycle at 68°C), 7 min at 72°C. For amplification with primer DIRC and telomeric primer C or G, the conditions were: 2 min at 94°C, 10× (15s at 94°C, 30s at 60°C, 4 min at 68°C), 15× (15s at 94°C, 30s at 60°C, 4 min 20s each cycle at 68°C), 7 min at 72°C. Concentrations of all PCR components were adjusted according to the manufacturer's recommendations. All PCR components used were shown to be non-contaminated.

Isolation of protoplasts and pulsed-field gel electrophoresis (PFGE)

Protoplasts from *N. tomentosiformis* and *N. tabacum* cells were prepared and embedded in agarose plugs according to the protocol of Wu et al. (1992) with minor modifications described previously (Fajkus et al. 1995*a*). After restriction digestion of DNA in agarose plugs, PFGE was carried out in a CHEF-DR II apparatus (BioRad, U.S.A.) under the following conditions: 1% Fast Lane Agarose (FMC, U.S.A.), 0.5× TBE, 14°C, 190 V, 20 h; switch interval ramped from 2–25 s.

Copy number of *TAS49* in genomic DNA

The contents of sequence *TAS49* in genomic DNAs of different *Nicotiana* species were determined by a slot-blot procedure using Hybond N^+ membrane (Amersham, Sweden) and a BIO-DOT SF apparatus (BioRad, U.S.A.) according to the manufacturer's instructions. Genomic DNA was loaded in 100-ng and 10-ng amounts. A calibration curve was prepared from serial dilutions of the *TAS49* sequence (300 pg, 200 pg, 100 pg, 50 pg, 10 pg, 5 pg, 0.5 pg) which was subsequently used as a probe. As a negative

Table 1*a*. Results of PCRs using various combinations of primers DIRT, DIRC, IT7, ISP6, telomeric primer C and telomeric primer G.

	DIRC/DIRT	DIRT/tel C	DIRT/tel G	DIRC/tel C	DIRC/tel G	IT7/tel C	ISP6/tel G	IT7/ISP6
N. tabacum	y	y	n	n	n	y	n	y
N. tomentosiformis	y	y	n	n	n	y	n	y
N. sylvestris	y	y	n	n	n	n	n	y
N. otophora	y	n*	n	n	n	–	–	y
N. bigelovii, glutinosa, kawakamii, rustica, solanifolia, suaveolens	–	–	–	–	–	–	–	y

Table 1*b*. Results of PCRs using single primers DIRT, DIRC, IT7, ISP6, telomeric primer C and telomeric primer G.

	DIRT	DIRC	IT7	ISP6	tel C	tel G
N. tabacum	y	n	n	n	n	n
N. tomentosiformis	n	n	n	n	n	n
N. sylvestris	n	n	n	n	n	n
N. otophora	y	n	n	n	n	n
N. bigelovii, glutinosa, kawakamii, rustica, solanifolia, suaveolens	–	–	n	n	n	n

Note: –, PCR not performed.
[y]Positive PCR.
[n]Negative PCR.
*PCR products correspond to the products of single-primed reaction of DIRT.

control, 100 ng of bacteriophage λ was used. Intensities of hybridization signals were evaluated using a STORM 860 Phosphofluoroimager and IMAGEQUANT software (Molecular Dynamics, U.S.A.).

DNA sequencing and DNA–DNA or DNA–RNA hybridization

DNA fragments were sequenced using a Sequenase Version 2.0 DNA Sequencing Kit and sequencing reactions were separated on a LKB 2010 Macrophor Electrophoresis Unit. Probes were labelled with Ready-to-Go [α-^{32}P]dCTP-labelling beads (Pharmacia, Sweden) or, in the case of oligonucleotide probes, using T4-polynucleotide kinase (NEB) and [γ-^{32}P]dATP (Amersham) according to Sambrook et al. (1989). Hybridizations and washings of Southern blots, Northern blots, and colony-lifts were performed in bottles in a hybridization incubator using a hybridization solution containing 0.25 M $NaHPO_4$ pH 7.5, 7% SDS, and 100 ug/mL BSA. Temperatures of hybridization and washing were 65°C for the *TAS49* probe, and 55°C for plant telomeric oligonucleotides. Hybridization signals on Hybond N^+ membranes were visualized using autoradiography on X-ray film or, for quantitative evaluation, using a Storm860 Phosphofluoroimager and IMAGEQUANT software.

Results

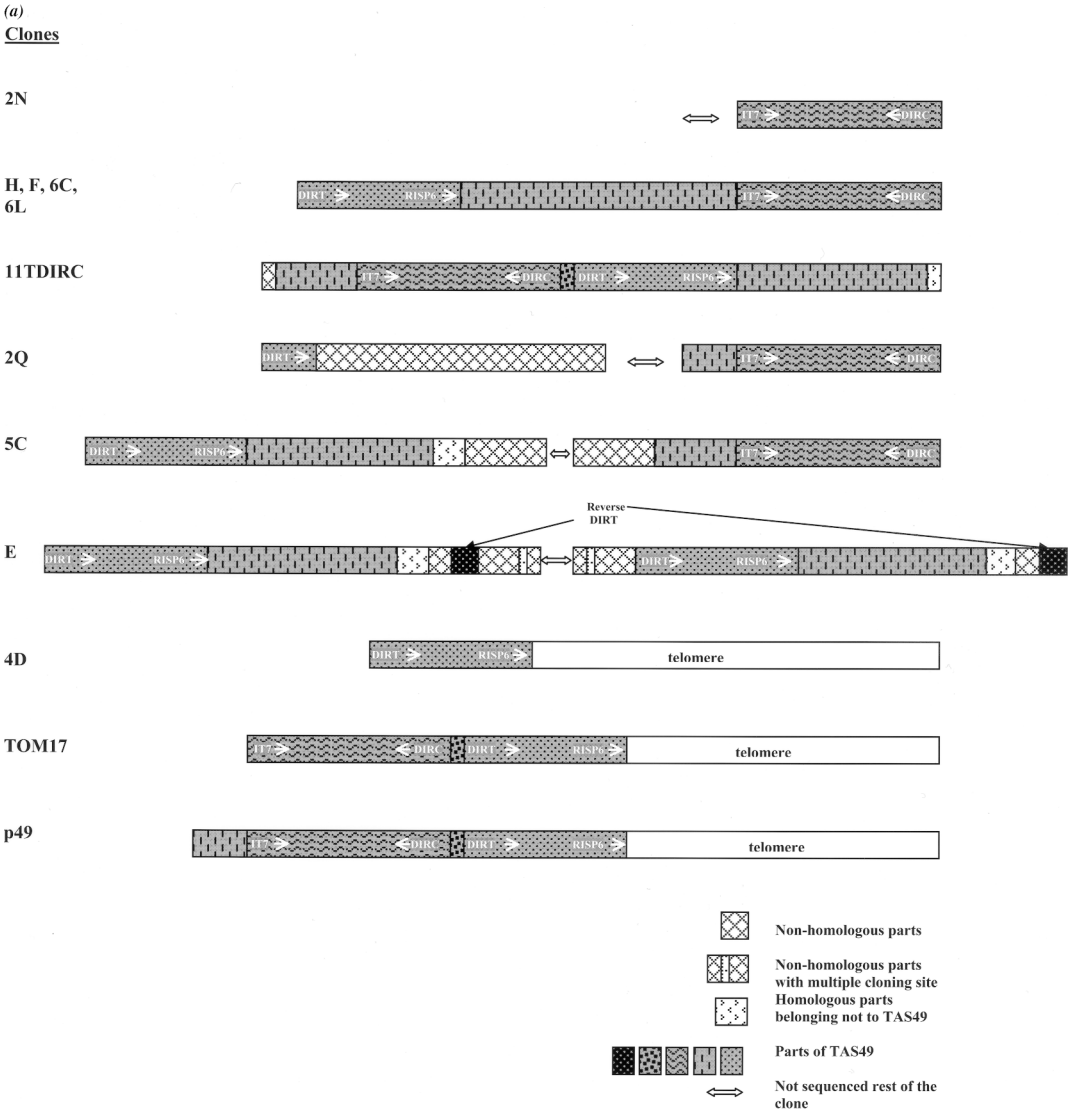
Cloning and sequencing of telomere-associated sequences

Based on colony hybridization of 90 colonies obtained by a modified vectorette approach, five clones hybridizing with plant telomeres were chosen for sequencing. While four of these clones contained only arrays of telomeric repeats, the insert of the fifth clone (p49, GenBank Acc. No. AF146059) containing the product of PCR on *Hae*III-terminal restriction fragments revealed the presence of four telomeric units CCCTAAA linked by degenerate telomeric repeat TCCTAAATC to a 390-bp region of telomere-associated sequence, which was designated as *TAS49*. To obtain *TAS49* directly from genomic DNA, PCR was performed on genomic DNA of *N. tomentosiformis*, *N. sylvestris*, and *N. tabacum* with the IT7 primer and telomeric primer C. Only *N. tomentosiformis* and *N. tabacum* gave PCR products; no products were obtained with any of these DNAs using primer ISP6 with telomeric primer G or telomeric primers G or C alone (Table 1). Based on these results, the orientation of *TAS49* with respect to the telomere was deduced, with the IT7 primer directed towards the telomere (Fig. 2). Direct association with the telomere was absent in *N. sylvestris*.

PCR products obtained with primer IT7 and telomeric primer C on *N. tomentosiformis* DNA were cloned and the resulting colonies were screened for homology to *TAS49*. One of the selected clones, tom17 (GenBank Acc. No. AF146062), was sequenced. The tom17 insert shows 92.9% homology to the insert of p49 clone (Fig. 3*b*) and, like *TAS49*, contains a 128-bp sequence with a markedly asymmetrical distribution of GC between the strands: similarly to telomeric DNA, individual strands in this region are almost completely devoid of GC. Interestingly, the C-rich telomeric strand becomes G-rich in the adjacent telomere-associated sequence and vice versa. The tom17 sequence was then used to design another pair of specific primers, DIRT and DIRC (Fig. 2).

To further analyze the complete sequence unit of *TAS49* and its variants associated with telomeres, plasmid DNAs of 700 clones originating from transformations by PCR products of primer combinations DIRT–DIRC and DIRT–telomeric primer C were isolated, and after restriction and (or) PCR analysis and hybridization with a *TAS49* probe, ten clones were chosen for sequencing (Fig. 3*a*). Clone 4D (GenBank Acc. No. AF146063) contains tobacco DNA and consists of eight telomeric units CCCTAAA, a degenerate telomere–subtelomere linker TCCTAAATC, and an associated *TAS49* showing 90% homology with p49 and tom17 clones (Fig. 3*b*). The *TAS49* unit is attached at an identical

Fig. 3*a*. An overview of the analyzed clones containing different permutations of homologous blocks of *TAS49*.

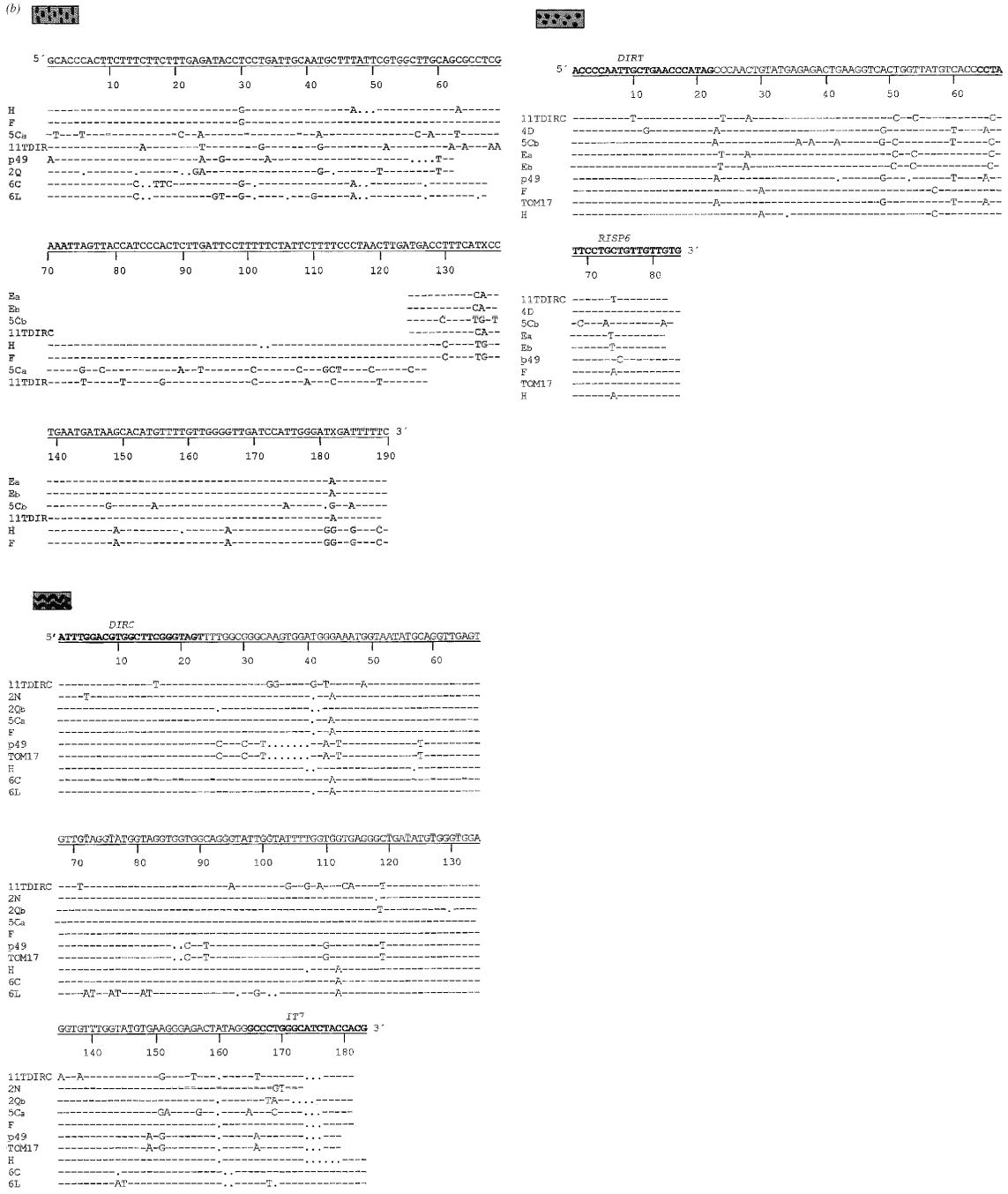


point to the telomere in all three telomeric clones analyzed (p49, tom17, and 4D), starting from the sequence CACAACAACA. Two *N. tomentosiformis* clones (2N, GenBank Acc. No. AF146060; H, Acc. No. AF146073), one *N. tabacum* clone (F, Acc. No. AF146072), and two *N. sylvestris* clones (6C, Acc. No. AF146071; 6L, Acc. No. AF146070) contain different overlapping parts of the *TAS49* unit, showing about 90% of mutual homology between corresponding regions (Fig. 3*b*). One *N. tomentosiformis* clone (5C, Acc. Nos. AF146067, AF146066) and two *N. tabacum* clones (11TDIRC, Acc. No. AF146061; E, Acc. Nos. AF146064, AF146065) are assembled from parts of *TAS49*, immediately followed by 65 bp of mutually homologous sequence distinct from *TAS49*, indicating the occurrence of *TAS49* in a complex DNA repeat. The arrangement of the connection between *TAS49* and the 65-bp sequence is identical in the above clones (it occurs at the same position of *TAS49* and the 65-bp sequence is surrounded by mutually non-homologous sequences). Clone 2Q (Acc. Nos. AF146069, AF146068) isolated from *N. tomentosiformis*, contains *TAS49* surrounded by sequences showing no significant homology to the others analyzed. Moreover, clone E contains an inverted sequence of primer DIRT, which enabled us to explain the amplification of genomic DNA of *N. tabacum* by primer DIRT alone, even at a highly stringent annealing temperature. Since we were not able to clone a DNA fragment containing at least a dimer of *TAS49* units, which may reflect a dispersed character of *TAS49* and (or) its location in a conserved complex repeat, the consensus sequence of the *TAS49* repetitive unit was deduced from overlapping homologous *TAS49* regions found in single clones. The conserved site of attachment of *TAS49* to the telomere is considered as the start and (or) end point of *TAS49*. The length of the consensus *TAS49* unit was determined as 469 bp (Fig. 2).

PCR screening of *TAS49* arrangement in different *Nicotiana* species

PCR products of identical length (280 bp) were obtained by amplifications of genomic DNA of *N. rustica*, *N. suaveolens*, *N. solanifolia*, *N. glutinosa*, *N. bigelovii*, *N. kawakamii*, *N. otophora*, *N. tomentosiformis*, *N. sylvestris*, and *N. tabacum*

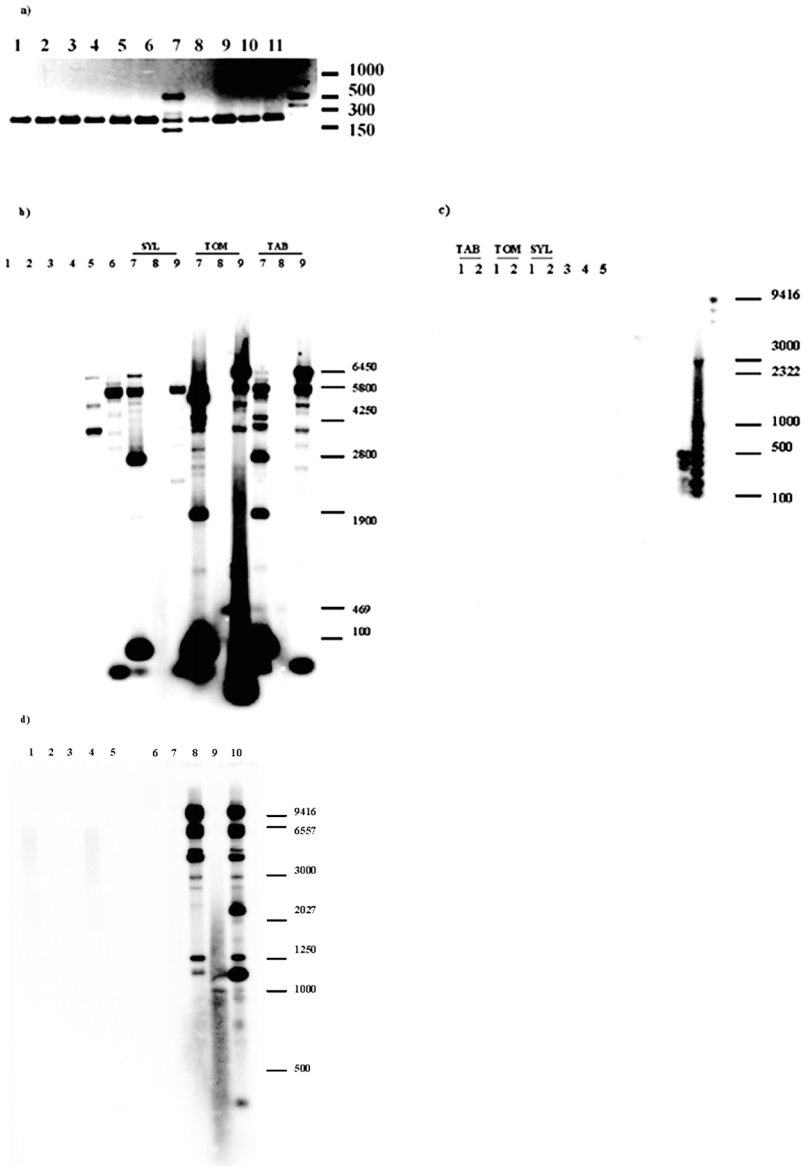
Fig. 3*b*. Alignment of *TAS49* homologous blocks from the clones analyzed (H, F, 5C, 11TDIRC, E, p49, TOM17, 4D, 6C, 6L, 2N, and 2Q). Individual blocks are marked by shaded boxes at the upper left corner, and the sequences of clones 5C, 2Q, and E are presented as parts *a* and *b* corresponding to Fig. 3*a*. The nucleotide differences are indicated. Identical nucleotides are shown by dashes, and deletions by dots.



with primers IT7 and ISP6. The only exception was *N. tomentosa*, where additional products of 500 bp, 300 bp, and 150 bp were formed (Fig. 4*a*). PCR amplification of *N. tometosiformis*, *N. sylvestris*, *N. tabacum*, and *N. otophora* genomic DNA were performed with various combinations of primers DIRT, DIRC, and telomeric primers C or G (Table 1). PCR products were separated on an agarose gel (Fig. 4*c*), transferred onto Hybond N^+ membrane by Southern blotting and hybridized with the *TAS49* probe (Fig. 4*b* and 4*d*). The absence of a regular ladder typical for tandemly repetitive sequences implies the dispersed character of the *TAS49* sequence.

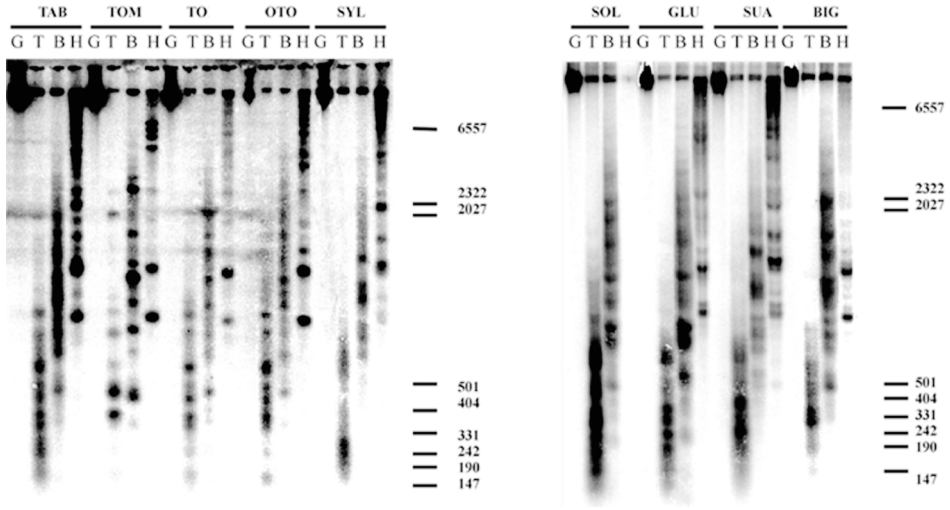
Sequence analysis of clone E revealed the presence of inverted DIRT repeats in *N. tabacum* genomic DNA, which was confirmed by PCR with DIRT primer alone. It is interesting that similar inverted repeats of *TAS49* were not revealed in *N. tomentosiformis* or *N. sylvestris* chromosomes, indicating the occurrence of DNA rearrangements in the course of amphidiploidization. PCR using DIRT and telomeric primer C on *N. tabacum* and *N. tomentosiformis*

Fig. 4. (*a*) PCR products resulting from amplification of genomic DNA of *Nicotiana rustica* (1), *N. suaveolens* (2), *N. solanifolia* (3), *N. glutinosa* (4), *N. bigelovii* (5), *N. kawakamii* (6), *N. tomentosa* (7), *N. otophora* (8), *N. tomentosiformis* (9), *N. sylvestris* (10), and *N. tabacum* (11) with primers IT7 and ISP6, stained with ethidium bromide in an agarose gel. (*b*) PCR products resulting from amplification of *N. sylvestris* (SYL), *N. tomentosiformis* (TOM), and *N. tabacum* (TAB) genomic DNA with primers DIRC and DIRT (7), DIRT and telomeric primer G (8), DIRT and telomeric primer C (9), or by single-primed reactions with primer DIRT on *N. sylvestris* (1) and *N. tomentosiformis* (2). Genomic DNA of *N. tabacum* was amplified with telomeric primer G (3), telomeric primer C (4), DIRC (5), and DIRT (6). PCR products were transferred from agarose to a nitrocellulose membrane and hybridized with the *TAS49* probe. (*c*) PCR products resulting from amplification of *N. sylvestris* (SYL), *N. tomentosiformis* (TOM) and *N. tabacum* (TAB) genomic DNA with primer DIRC and telomeric primer G (1), DIRC and telomeric primer C (2) or by single-primed reactions with primer DIRC (3), telomeric primer C (4), and telomeric primer G (5) on *N. tabacum* genomic DNA. PCR products were separated in an agarose gel and stained with ethidium bromide. (*d*) PCR products resulting from amplification of genomic DNA of *N. otophora* with telomeric primer G (1), telomeric primer C (2), DIRC (3), DIRC and telomeric primer G (4), DIRC and telomeric primer C (5) using an annealing temperature of 60°C, and with telomeric primer G (6), telomeric primer C (7), DIRT (8), DIRT and telomeric primer G (9), DIRT and telomeric primer C (10) using an annealing temperature of 56°C. PCR products were transferred from agarose to a nitrocellulose membrane and hybridized with the *TAS49* probe. The lengths of DNA fragments are in bp.



genomic DNA provided corresponding results, showing a similar arrangement of *TAS49* in subtelomeres. PCR with primer DIRT and telomeric primer C on *N. sylvestris* revealed only the presence of DIRT sequences remote from telomeres (5700, 5000, 4100, 3500, 2400 bp) and no telomere-associated fragments, in contrast to *N. tomentosiformis*. No PCR products were obtained using DIRT with telomeric primer G.

Fig. 5. Southern blot hybridization of genomic DNA (G) of *Nicotiana tabacum* (TAB), *N. tomentosiformis* (TOM), *N. tomentosa* (TO), *N. otophora* (OTO), *N. sylvestris* (SYL), *N. solanifolia* (SOL), *N. glutinosa* (GLU), *N. suaveolens* (SUA), and *N. bigelovii* (BIG) cleaved with *Taq*I (T), *Bst*NI (B), or *Hin*dIII (H) restriction enzymes. The probe was *TAS49*. The lengths of DNA fragments are in bp.



Addition of the PCR patterns originating from amplification of *N. tomentosiformis* and *N. sylvestris* with primers DIRC and DIRT coincides with the amplification products of *N. tabacum*. This additive character may reflect the conserved arrangement of DNA sequences in the neighbourhood of *TAS49*, as well as evolutionary relations between these species. In *N. otophora*, the pattern of PCR products from primers DIRT and telomeric primer C is hardly distinguishable from the pattern of products primed by DIRT alone. Although some weak bands of short fragments were detectable upon hybridization using the former primer combination, their reproducible absence in ethidium bromide - stained gel argues against their specificity.

No specific products were obtained by amplification of *N. tomentosiformis*, *N. sylvestris*, *N. otophora*, and *N. tabacum* genomic DNAs with primer DIRC and telomeric primers G or, using the same PCR conditions, with DIRT, DIRC, or telomeric primers alone.

Together, these results confirm the unique arrangement of *TAS49* at telomeres, where *TAS49* is positioned as shown in Fig. 2. Assuming the absence of direct association of *TAS49* with telomeres of *N. sylvestris* and *N. otophora*, we can regard the chromosome ends of *N. tabacum* with *TAS49* directly attached to telomeres as components of the T-genome originating exclusively from *N. tomentosiformis* chromosomes.

Restriction analysis of genomic DNA

Genomic DNA of nine species of the genus *Nicotiana* (*N. tabacum*, *N. tomentosiformis*, *N. tomentosa*, *N. otophora*, *N. sylvestris*, *N. solanifolia*, *N. glutinosa*, *N. suaveolens*, and *N. bigelovii*) was cleaved with *Taq*I, *Bst*NI or *Hin*dIII restriction enzymes. The restriction fragments were separated in a 0.8% agarose gel, transferred onto a Hybond N^+ membrane, and hybridized with labelled *TAS49* sequence (Fig. 5). *TAS49* contains restriction sites for *Taq*I and *Bst*NI, and therefore the hybridization patterns of genomic DNAs cleaved with these enzymes reveal both *TAS49* and associated sequences. Since *TAS49* revealed no *Hin*dIII restriction sites, hybridization patterns of genomic DNA cleaved with *Hin*dIII characterize the surroundings of *TAS49*. The high homology among the hybridization patterns (Fig. 5) implies a similar arrangement of *TAS49* and its surroundings in all the species tested, regardless of their exact evolutionary relations. Addition of the *N. sylvestris* and *N. tomentosiformis* patterns coincides with the pattern of *N. tabacum*. The lack of periodicity of the hybridization bands shows the absence of large tandems of sequence units, and suggests a dispersed character of *TAS49*, in accordance with the PCR results above.

To characterize the methylation state of *TAS49*, genomic DNA of *N. tabacum* was cleaved with three pairs of isoschizomers; *Mbo*I and *Sau*3AI, *Bst*NI and *Eco*RII, and *Msp*I and *Hpa*II. *Mbo*I is insensitive to the presence of methylcytosine in the target sequence GATC, whereas *Sau*3AI cleavage is inhibited by cytosine methylation. Similarly, *Bst*NI and *Eco*RII represent isoschizomers insensitive and sensitive, respectively, to methylation of the inner cytosine of the target sequence CC(A/T)GG. *Hpa*II cleaves the site CCGG, provided no methylation is present in either cytosine, while *Msp*I cleavage is inhibited only by methylation of the outer cytosine. Digested DNA was separated in a 1% agarose gel, transferred onto Hybond N^+ membrane, and hybridized with labelled *TAS49* containing part of the telomere. The telomeric part of the probe produces a hybridization signal of high-molecular-weight terminal restriction fragments. As seen in Fig. 6, methylation-sensitive enzymes did not cleave the *TAS49* sequence (*Eco*RII, *Msp*I, and *Hpa*II) or cleaved the *TAS49* sequence only partially (*Sau*3AI), indicating a high level of cytosine methylation in the *CpG* and *CpNpG* sites analyzed.

A long-range analysis of the distribution of *TAS49*

High-molecular-weight genomic DNAs of *N. tomentosiformis* and *N. tabacum* were cut with *Hin*dIII, which does not cleave inside the *TAS49* unit. The products were separated by PFGE in a 1% Fast Lane (FMC) agarose gel and DNA fragments were transferred onto Hybond N^+ mem-

Fig. 6. Southern blot hybridization of genomic DNA of *Nicotiana tabacum* cleaved with *Alu*I (1), *Taq*I (2), *Hae*III (3), *Hpa*II (4), *Msp*I (5), *Eco*RII (6), *Bst*NI (7), *Sau*3AI (8), or *Mbo*I (9). Isoschizomers are underlined. The insert of the p49 clone was used as probe, containing *TAS49* associated with telomeric sequences. The lengths of DNA fragments are in bp.

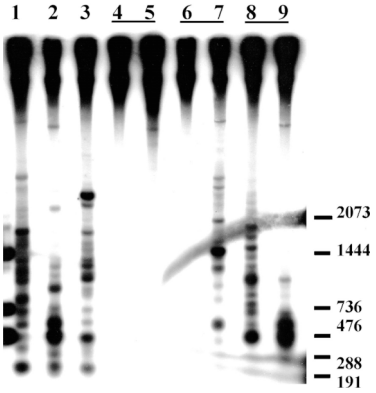
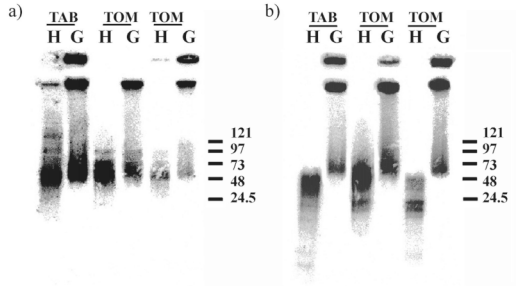


Fig. 7. Southern blot hybridization of genomic DNA (G) of *Nicotiana tabacum* (TAB) and *N. tomentosiformis* (TOM) cleaved with *Hin*dIII (H) restriction enzyme and separated by PFGE. The probes were telomeric primers C $(CCCTAAA)_6$ and G $(TTTAGGG)_6$ (*a*) or *TAS49* (b). The lengths of DNA fragments are in kb.



brane, and hybridized with labelled *TAS49*. After detection of the *TAS49* signal, the membrane was stripped and reprobed with an end-labelled telomeric oligonucleotide (Fig. 7). Co-hybridizing DNA fragments containing telomere and the *TAS49* repeats occur in the range of 25–130 kb. DNA fragments showing the presence of *TAS49* but lacking telomere-specific signals range from 17–22 kb. Together, the PFGE data show that *TAS49* is not restricted to telomere-associated locations. This result is supported by the absence of discrete foci of *TAS49* upon performing in situ detection of *TAS49* on interphase or metaphase chromosomes (not shown).

Copy number of *TAS49* in eight species of the genus *Nicotiana*

The number of copies of *TAS49* in the genomes of eight plant species of the genus *Nicotiana* (*N. tabacum*, *N. tomentosiformis*, *N. tomentosa*, *N. sylvestris*, *N. solanifolia*, *N. suaveolens*, *N. otophora*, and *N. glutinosa*) was derived from a calibration curve based on the intensities of hybridization signals from different amounts of *TAS49*. As shown in Fig. 8, the highest number of copies occurs in *N. tabacum* genomic DNA (22 600) and the lowest number in *N. sylvestris* DNA (3300). The total number of copies of *TAS49* in *N. tomentosiformis* and *N. sylvestris* genomes (about 20 700) is nearly equivalent to the number of copies in tobacco genomic DNA.

Fig. 8. Copy numbers of *TAS49* (black squares) in genomic DNAs of various *Nicotiana* species compared with their 2C nuclear DNA content (columns).

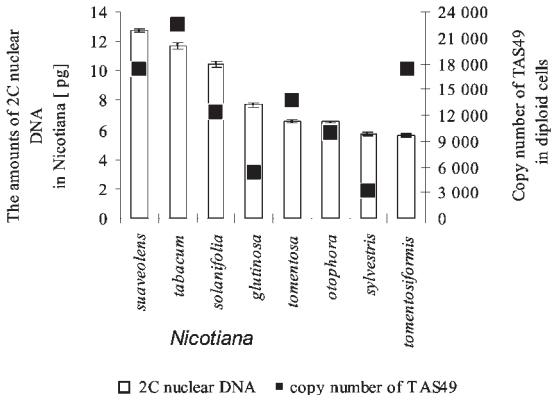
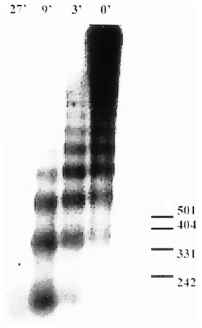


Fig. 9. Southern blot hybridization of nucleosome ladders generated by cleavage of *Nicotiana tabacum* nuclei by micrococcal nuclease (150 U/mL) and exonuclease III (100 U/mL) for 0, 3, 9, and 27 min. *TAS49* was used as probe.



Chromatin structure of *TAS49*

Oligonucleosomes were separated on a 2% agarose gel, transferred onto Hybond N^+ membrane and hybridized with labelled *TAS49* sequence (Fig. 9). A periodical nucleosome pattern was observed with a mononucleosome unit size of 170 ± 8 bp, as assessed using a pUC18/*Msp*I molecular weight marker. The size of the internucleosomal linker DNA in *TAS49* chromatin is 24 ± 8bp. These parameters correspond to the common phasing of nucleosomes in tobacco non-telomeric chromatin, as shown by ethidium bromide - stained gels. They are also similar to nucleosome periodicities, which were determined previously in the tandemly repetitive tobacco sequences *HRS60* (Fajkus et al. 1992), *GRS* (Gazdová et al. 1995), and *NTRS* (Matyášek et al. 1997).

Computational analysis of *TAS49*

The *TAS49* sequence shows no significant homology to sequences deposited in GenBank and EMBL, using Advanced BLAST 2.0.6. Protein-coding capabilities of *TAS49* were analyzed by the ORF finder and by BLASTP 2.0.8. at NCBI. About 50% homology was found between *TAS49* and

various proline-rich plant proteins, extensins, or extensin precursors using the matrix BLOSUM 62 as default. For instance, clone 11TDIRC contains *TAS49* arranged in a 459-bp open reading frame, translating into 152 amino acids showing 55% homology (E-value 0.54) to the precursor of tobacco extensin, a cell wall hydroxyproline-rich glycoprotein (Acc. No. P13983). However, the largest part of this homology is formed only by prolines, which reduces the information value of such results. Also, when a Northern blot of total tobacco callus RNA was hybridized with the *TAS49* probe, no signal was detected (not shown) indicating that *TAS49* DNA is probably not transcribed in vivo. Further, *TAS49* contains no promoter-like region and shows no features of transposons.

Discussion

TAS49 is a newly described dispersed repetitive sequence of 469-bp length. Although isolated from terminal restriction fragments of *N. tomentosiformis* chromosomes, it is not restricted to subtelomeric locations. Results of PCR and Southern hybridization suggest that *TAS49* is a part of a complex DNA repeat, the arrangement of which appears to be conserved in genomic DNA of nine species of the genus *Nicotiana*. It differs from the previously characterized tobacco telomere-associated sequence *HRS60*, which is directly associated with telomeres of the S-genome chromosomes forming >100-kb-long tandem arrays (Fajkus et al. 1995*a*).

Plant genomes are rich in highly repetitive DNA sequences interspersed with other highly repeated or low-copy sequences (Flavell 1980; Smyth 1991). Generally, species with relatively large genomes such as maize, rye, and wheat exhibit a short period interspersion pattern characterized by the presence of short (5–2000 bp) repetitive sequences interspersed with short (200–4000 bp) single-copy DNA (Flavell 1980; Gupta et al. 1984; Hake and Walbot 1980). On the other hand, species with small genomes show a long period interspersion pattern (reviewed in Lapitan 1992). The structure and organization of such DNA regions are much more variable and complex than those of tandemly repeated sequences (Lapitan 1992). In our experiments on cloning of DNA sequence units, we usually obtained various permutations of *TAS49* and (or) associated sequences. Remnants of significant open reading frames and the conserved structure of both *TAS49* and its environs among the *Nicotiana* species tested suggest that *TAS49* might have arisen from a part of an unknown gene that has been amplified together with its neighbouring sequences. In contrast to the families of highly transcribed subtelomeric repetitive sequences described by Wu et al. (1994) in rice (*Os48* and *Osc-567* in long tandem arrays accompanied with interspersed *OsG3-340*), *TAS49* is not transcribed. The present function of the *TAS49* sequence is probably restricted to the chromatin structure-coding level.

Polyploidization has played a major role in the evolution of many plant species like tobacco, cotton (*Gossypium barbadense* L., AD genome), and wheat. However, little is known regarding the subsequent evolution of DNA sequences that become newly introduced to a common nucleus. Zhao et al. (1998) used the spread of some dispersed repetitive sequences specific for Old World (A) and New World (D) diploid ancestors for the analysis of such subsequent processes in tetraploid cotton.

The important feature of *TAS49* is its direct association with telomeres in the case of amphidiploid *N. tabacum* and its progenitor *N. tomentosiformis* and, conversely, the absence of such an association in *N. sylvestris* and *N. otophora*. Thus, we can distinguish between chromosome ends belonging to the T-genome originating from *N. tomentosiformis* and the S-genome ends originating from *N. sylvestris*. Our results further minimize the probability that the *N. otophora* genome participates in forming the ends of tobacco chromosomes. PCR experiments, as well as hybridization patterns of restriction nuclease digestions, showed an additive character of *TAS49*-specific fragments in *N. tomentosiformis* and *N. sylvestris*, forming together the pattern of *TAS49* observed in *N. tabacum*.

The important conceivable function of subtelomeric sequences is their potential to substitute for short-oligonucleotide telomeres, as has probably occurred in the case of terminal satellite sequences in chromosomes of Alliaceae or *Chironomus*, mentioned in the introduction. The dispersed *TAS49* sequence is probably not in the same case, being a poorer substrate for eventual maintenance by recombination-based telomerase-independent mechanisms than long tandem arrays. However, the observed telomere-like G/C asymmetry described in *TAS49* and the frequent occurrence of GGT and GTT trinucleotides in this region, which are efficient substrates for both human and plant telomerase at the 3′-end of TS(18), TS(21), and other oligonucleotide substrates used for TRAP assays (Kim et al. 1994; Fitzgerald et al. 1996; Heller et al. 1996; Ríha et al. 1998) makes the *TAS49* sequence a suitable substrate for telomere synthesis de novo in the process of healing of broken chromosome ends (Tsujimoto 1993).

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