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The telomeric sequence is directly attached to the HRS60 subtelomeric tandem repeat in tobacco chromosomes

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Abstract PCR and primers derived from the telomeric repeat $(CCCTAAA)_n$ and from the tobacco subtelomeric tandemly repetitive sequence HRS60 (EMBL X12489) were used to amplify the region linking the two loci. A 131 bp PCR product was obtained both from total tobacco DNA and from the DNA fraction enriched for telomeres. Its sequence only consists of the telomeric primer and the attached region of the HRS60 repetitive unit up to the end of the sequence complementary to the HRS60 primer. The site of direct continuity between the two sequences is formed by a $(dA)_7$ tract.

Key words: DNA sequence; Telomere; Subtelomeric repeat; *Nicotiana tabacum*; Chromatin; DNA curvature

1. Introduction

The ends of eukaryotic chromosomes consist of many tandemly repeated copies of a DNA sequence $(T/A)_{1-4}G_{1-8}$ [1]. In plants, telomeric sequences were first cloned and sequenced in *Arabidopsis thaliana* [2]. Their terminal heptanucleotide repeat $(TTTAGGG)_n$ has been shown to be present at the chromosome ends of many plant species, including *Nicotiana tabacum* [3].

The long-range organization of chromosome ends has been investigated by means of in situ hybridization and pulsed field gel electrophoresis (PFGE). In tomato, for example, the telomeric repeats are associated with the TGRI satellite repeat at 20 of the 24 chromosome ends, and the distance between the two repeated sequences ranges from a few bp to 150 kb of spacer DNA [1]. Our recent PFGE study on tobacco chromosomes [4] has shown a similar long-range organization of their termini. A 184 bp satellite DNA repeat, HRS60 [5], which was previously detected in terminal regions of 30 out of the 48 total tobacco chromosomes [3], forms tandems that are >100 kb in length. This satellite DNA is in close proximity to the telomeric repeat that have an average length of 90–130 kb; thus, tobacco telomeres are the longest described in plants so far. Furthermore, we have observed differences between the chromatin structure of the telomeric $(TTTAGGG)_n$ and the HRS60 repeat, especially in the spacing and micrococcal nuclease sensitivity of their nucleosomes [4]. The PFGE data provided only a general estimate of the distance between the telomeric and subtelomeric repeat clusters in tomato and tobacco [3,4], which is based on the average cutting frequency of restriction enzymes.

Here we were interested in properties of the region connecting the HRS60 with the telomeric repeat.

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The nucleotide sequence data reported will appear in the GenBank, EMBL and DDJB Nucleotide Sequence Databases under the Accession Number L37044.

Abbreviations: PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis.

2. Materials and methods

Since the orientation of HRS60 with respect to telomeres was not known, we tested primers complementary to both HRS60 strands: the HRS-FOR (5′-GATCCATCCGGGCCCAAGG-3′) and HRS-REV primer (5′-CGTCGTGGAATCGCCTAATAT-3′). As a telomeric primer, PLTELC $(5'\text{-CCCTAAA-}3')_6$ was used to direct this primer extension towards the HRS60 repeat (taking into account that the telomeric G-rich strand extends past the C-rich strand as a 3′ overhang [6]). Both combinations of the primers (HRS-FOR + PLTELC, HRS-REV + PLTELC) were tested and conditions were chosen under which neither of the primers present alone in control reactions gave any detectable PCR products: initial denaturation (3 min/95°C), hot start at 72°C, 30 three-step cycles of (i) 1 min/95°C, (ii) 1 min/60°C, (iii) 1 min/72°C, and final extension (6 min/72°C). Two units of Taq polymerase (AmpliTaq, Perkin Elmer), Taq polymerase buffer (Amersham), 0.5 μM primers (BioVendor, MWG Biotech), 0.5 mM dNTPs (Boehringer) and 2.5 mM $MgCl_2$ were used in a 100 μl reaction.

3. Results and discussion

While HRS-FOR gave only a smeared electrophoretic pattern of PCR products, HRS-REV produced specific products with PLTELC: a ladder of bands (Fig. 1, lane 1) hybridizing with both HRS60 and PLTELC probes (not shown), in which the shortest fragment of 131 bp was followed by others with 184 bp increments as determined by sequencing (see below). These fragments were accompanied by less prominent products 7 bp longer (the length of the telomeric repeating unit). Increasing the extension time to 3 or 5 min promoted the production of longer fragments (315 bp, 496 bp) but did not change the overall pattern. Preliminary sequencing of the 131 bp PCR fragment, using the HRS-REV primer and a CircumVent Sequencing Kit (NEB), showed that it consisted of 42 bp of telomeric sequence directly connected to the 89 bp part of the HRS60 sequence (positions 96–184 in the EMBL X12489 sequence).

To avoid a possibility that our PCR products originate from intrachromosomal telomere-like sequences, which occur for example in the *Arabidopsis thaliana* genome [7], the whole procedure was repeated using a high molecular weight fraction of *Mbo*I or *Hae*III digested DNA as a template for PCR because this fraction is enriched in chromosomal termini and poor in intrachromosomal sequences (Fig. 2A). Both *Mbo*I and *Hae*III

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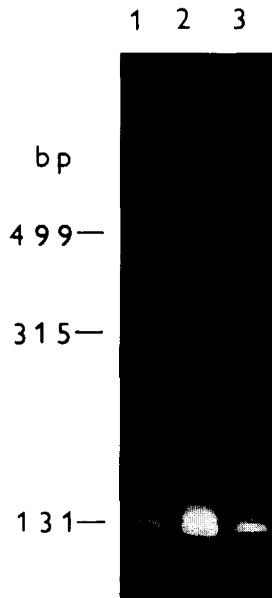


Fig. 1. Amplification of the boundary region between the telomere and the HRS60 subtelomeric repeat. PCR products are shown which were obtained using total tobacco genomic DNA (lane 1) and the high molecular weight fraction of *Hae*III or *Mbo*I-digested tobacco DNA (lanes 2 and 3, respectively) as template. PCR fragments were separated on a 8% polyacrylamide gel and stained with ethidium bromide.

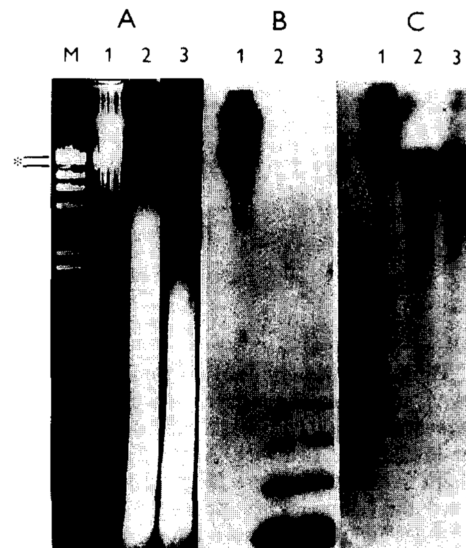
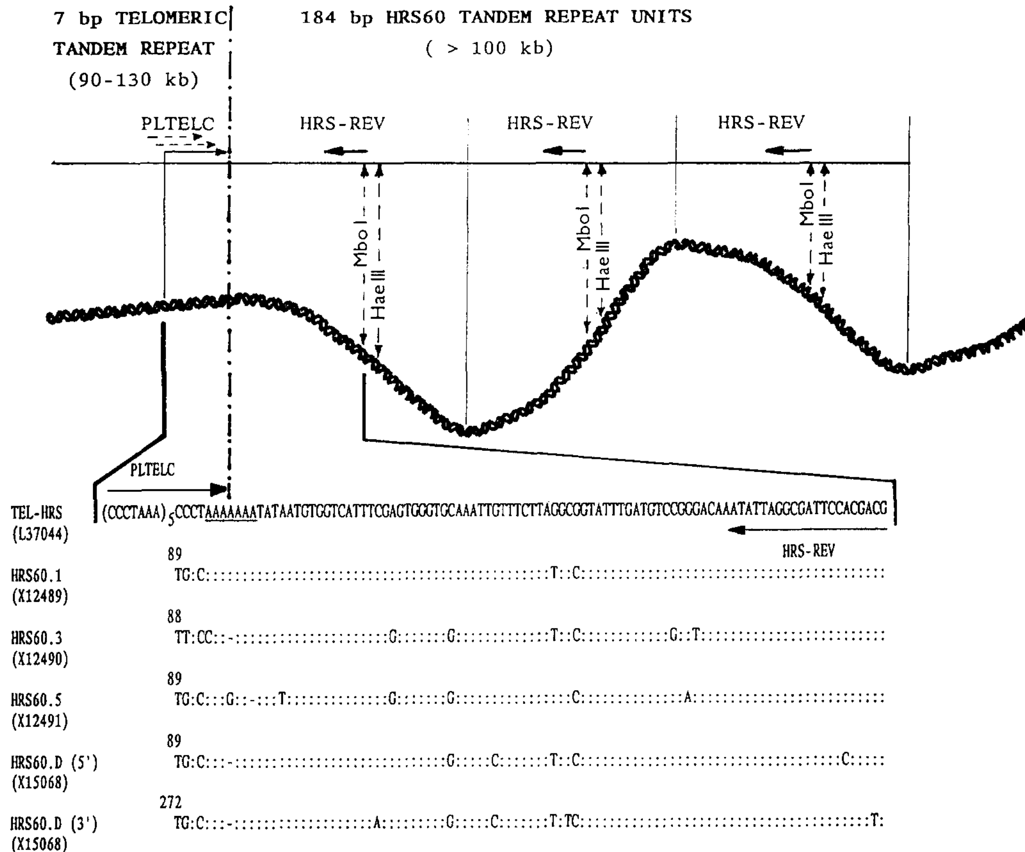


Fig. 2. Detection of telomeric and subtelomeric repeats in *Hae*III and *Mbo*I-digested tobacco DNA. A 1% agarose gel was stained with ethidium bromide (panel A), alkali-blotted onto Hybond N+ and hybridized with end-labeled HRS-REV (B) and PLTELC (C) oligonucleotide probes. Patterns of tobacco DNA without digestion (lane 1) and after *Hae*III (2) or *Mbo*I digestion (3) are shown. Pieces of the agarose gel (their boundaries are indicated next to the lane M) containing the high molecular weight DNA fractions of the *Hae*III or *Mbo*I digests of tobacco DNA were excised and the extracted DNAs were used as template DNAs for PCR (Fig. 1). *Hin*dIII-digested lambda DNA was used as a marker (Panel A, lane M).



cleave HRS60 once per repeating unit, at positions 1 and 12, respectively. Both cleavage sites are located in the part of HRS60 beyond the first HRS-REV site in the centromeric direction, and consequently, outside the 131 bp boundary fragment (Fig. 3). Each digest was separated on a 1% agarose gel, blotted and hybridized with the HRS-REV and PLTELC probes. As assumed, HRS60-specific signal (Fig. 2B) was detected as a 184 bp ladder of short oligomers (from monomers to tetramers), while all the telomere-specific signal remained in fragments longer than 20 kb in both *Hae*III- and *Mbo*I-digested samples (Fig. 2C). These telomere-enriched DNA fractions were excised from the gel (Fig. 2A) and the extracted DNAs were used as templates for PCR, providing the results shown in Fig. 1, lanes 2 and 3. As expected, 131 bp products (and a lesser amount of 138 bp products) were obtained, while no 315 and 499 bp products were observed because the remainder was removed of the HRS60 tandem repeat extending the *Hae*III or *Mbo*I site closest to the telomere (Fig. 3). The 131 bp PCR product was blunt-end ligated into the *Sma*I site of pBluescript SK-(Stratagene). Ligation was carried out at 25°C using T4 DNA ligase in the presence of *Sma*I restriction endonuclease to prevent the empty vector from religating. The cloned boundary fragment was sequenced again, and the results confirmed direct sequencing of the PCR product (Fig. 3).

The boundary sequence, TEL-HRS (L37044), extended by the addition of adjacent units of both telomeric and subtelomeric repeats, was processed by the CURVATURE computer program [8] to predict a sequence - directed curvature of the boundary DNA (Fig. 3). It is worth noting that the site of direct contact between HRS60 and telomere sequences occurs within an oligo(dA) tract which participates in forming a locus of intrinsic HRS60 curvature. In the HRS60 chromatin in vivo, this locus is occupied by a histone octamer [9]. A telomeric tandem repeat itself displays no DNA helix axis deformation. The boundary locus 5′-TAAAAAAATA-3′ is homologous to the consensus sequence found in Matrix Attachment Regions (MARs) and potential replication-initiation regions [10]. Alignment with five sequenced members of the HRS60 family shows (Fig. 3) that a deletion of one dA within the oligo (dA) tract occurs in four of them. The deleted dA probably has not been included in the process of the HRS60 tandem amplification. The same length of the oligo(dA) tract in both units of the HRS60 dimer (HRS60.D) agrees with this notion.

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Fig. 3. Schematic view of the boundary region between the telomeric $(GGGATTT)_n$ and the subtelomeric HRS60 tandem repeat. The intrinsic DNA curvature of a locus consisting of 17 units of telomeric heptanucleotide tandem repeat and 3.5 repetitive 184 bp units of HRS60 was predicted using the CURVATURE software [8]. Positions of *Hae*III and *Mbo*I restriction sites within HRS60 are shown with respect to sites recognized by primer HRS-REV. The sequence of the 131 bp boundary fragment, given below in the 5′-3′ direction, is aligned with the corresponding part of five sequenced members of the HRS60 family. Accession numbers are given below the name of each sequence. The positions of PLTELC and HRS-REV primers are marked with arrows. The boundary oligo(dA) tract is underlined.