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Isolation and characterization of two middle repetitive DNA sequences of nuclear tobacco genome

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Summary. Two DNA sequences, R8.1 and R8.3, representing two distinct classes of tobacco genomic repeated DNA, were cloned and characterized by Southern blot analysis. Both R8.1 and R8.3 were found to be homologous to the *Nicotiana tomentosiformis* component of the allotetraploid *Nicotiana tabacum* genome, and each of them represents about 0.3% of nuclear DNA. The R8.1 and R8.3 differ in the mode of distribution in chromosomes, as revealed by in situ DNA/DNA hybridization.

Key words: Repeated DNA – *Nicotiana tabacum* – *Nicotiana tomentosiformis* – Hybridization in situ

Introduction

The interspersion of repetitive and single-copy DNA sequences is a common property of eukaryotic genomes. In plants, repetitive sequences often represent a prevailing part of the genome (Walbot and Goldberg 1979). Although the role of repetitive sequences is still a subject of discussion, it is commonly accepted that they contribute to genomic instability.

One of our main interests is the study of the structure and instability of genomes of some model plants. Recently we described a new family of highly repetitive DNA sequences of the tobacco nuclear genome, denoted as HRS60-family (Koukalová et al. 1989; Matyášek et al. 1989). DNA/DNA hybridization experiments revealed that HRS60 was present in the *N. sylvestris* component of the allotetraploid genome of *N. tabacum* (Koukalová et al. 1990).

In this paper we characterize two other DNA sequences belonging to the class of middle repetitive sequences isolated from the *N. tabacum* genomic library. In contrast to the HRS60-family, which is present mainly in the *N. sylvestris* genome, R8.1 and R8.3 sequences originate from the *N. tomentosiformis* component of the tobacco genome.

Materials and methods

Plant material and DNA isolation

Tobacco nuclear DNA was extracted from fresh leaves of *N. tabacum*, according to Koukalová et al. (1989). Plasmid DNA was isolated as reported by Birnboim and Doly (1979).

Restriction endonuclease digestion, gel electrophoresis, DNA/DNA hybridization at high stringency conditions (0.2 × SSC, 65 °C), and ^{32}P-labelling of DNA probes were performed as described previously (Koukalová et al. 1989).

Construction of genomic library

A 15- to 20-kb DNA fraction of the partial BamHI digest of the tobacco nuclear DNA was ligated to the BamHI arms of the EMBL3 phage vector (Frischauf et al. 1983).

Screening of the phages carrying repetitive sequences

Recombinant phages were plaque-hybridized with ^{32}P-labelled tobacco nuclear DNA, and the plaques providing distinct signals were identified and purified.

Subcloning of phage inserts and screening of secondary clones for the presence of repetitive DNA sequences

Plaques showing a positive signal with labelled tobacco nuclear DNA were further characterized. Plant DNA inserts were cleaved by restriction endonuclease BamHI and subcloned into the plasmid pUC19 (Yanisch-Perron et al. 1985). The DNA of recombinant plasmids was hybridized with labelled tobacco nuclear DNA. Plasmids showing positive hybridization signals were selected. Those with signals significantly weaker than the signal for an HRS60 DNA repeat as a control were selected. Two such clones were further analyzed. The respective nonhomologous inserts originating from one contiguous genomic lambda clone were designated R8.1 and R8.3.

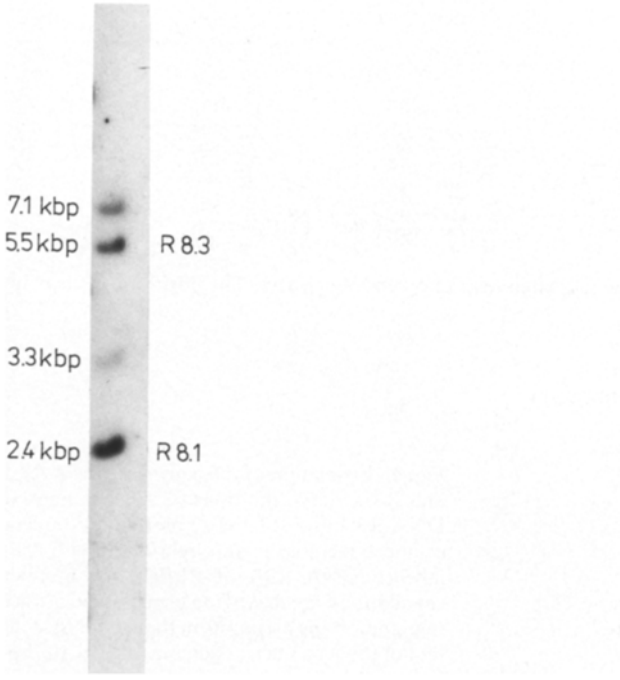

Fig. 1. Hybridization of the recombinant phage No8 with nuclear tobacco DNA. DNA of recombinant phage clone with 18.3-kbp nuclear tobacco DNA insert was cleaved with BamHI endonuclease (5 units per microgram of DNA). After electrophoretic separation and hybridization with ^{32}P-labelled nuclear tobacco DNA (at high stringency conditions – 0.2 × SSC, 65 °C), four internal fragments with a positive hybridization signal were obtained. Two of them, designated R8.1 and R8.3 were further analyzed

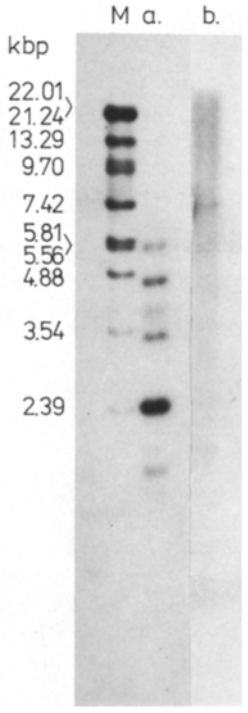

Fig. 2. Hybridization patterns of DNA R8.1 and R8.3. Total DNA isolated from leaves of *N. tabacum* was digested with BamHI endonuclease. After agarose gel electrophoretic separation of the fragments and their transfer onto nitrocellulose membrane, they were hybridized with ^{32}P-labelled probes R8.1 (*lane a*) and R8.3 (*lane b*). *Lane M* contained molecular markers

Hybridization in situ

To localize the DNA repeats on chromosomes, DNA/DNA hybridizations were performed on cytological preparations. Root tips from axenic *N. tabacum* plants macerated with cellulolytic enzymes were squashed and treated with DNase-free RNase. After alkaline denaturation of chromosomal DNA, the nick-translated ^{3}H-DNA probes (specific activity of at least 1×10^8 dpm per microgram of DNA) were applied on slides in the hybridization cocktail after Ambros et al. (1986), and the hybridization was run at 37 °C for 16 h. The posthybridization treatment consisted of two washings in 2 × SSC and in 50% formamide in 2 × SSC (2 × 5 min, 37 °C). The slides, covered with Ilford L4 autoradiographic emulsion, were developed after about 1 week's exposure and stained with acetoorcein.

Determination of the copy number of the subcloned repeated DNA sequences

Serial dilutions of tobacco nuclear DNA and those of R8.1 or R8.3 were blotted onto nitrocellulose membranes using a Schleicher and Schuell Minifold II apparatus. After hybridization with ^{32}P-labelled probes (R8.1 and R8.3), autoradiograms were scanned on a densitometer. Calculations of the copy numbers of R8.1- and R8.3-like repeats were based on the estimate of 1.5×10^9 bp for the DNA content of the *N. tabacum* haploid genome (Walbot and Goldberg 1979).

Results and discussion

The screening of the *N. tabacum* genomic library in the phage vector EMBL3 (1.2×10^4 clones) with ^{32}P-labelled nuclear DNA gave phage clones that produced weaker hybridization signals when compared with the already well-characterized family of highly repetitive sequences HRS60 (Koukalová et al. 1989). We suspected that these phage clones could contain DNA representing some kind of middle repetitive sequence.

One phage clone was chosen, the insert was cleaved using BamHI restriction endonuclease, and individual mutual nonhybridizing subfragments were cloned into the BamHI site of pUC19. Two of them were designated as R8.1 and R8.3. Their lengths were 2.4 kb and 5.5 kb, respectively (Fig. 1).

The Southern blots of the digests of *N. tabacum* DNA hybridized with the R8.1 and R8.3 probes revealed characteristic differences: probe R8.1 produced multiple distinct bands, whereas probe R8.3 produced smears (Fig. 2). This particular result suggested that the sequence R8.3 was extensively dispersed within the genome.

The physical maps of R8.1 and R8.3 for different restriction endonucleases are shown in Fig. 3. There are

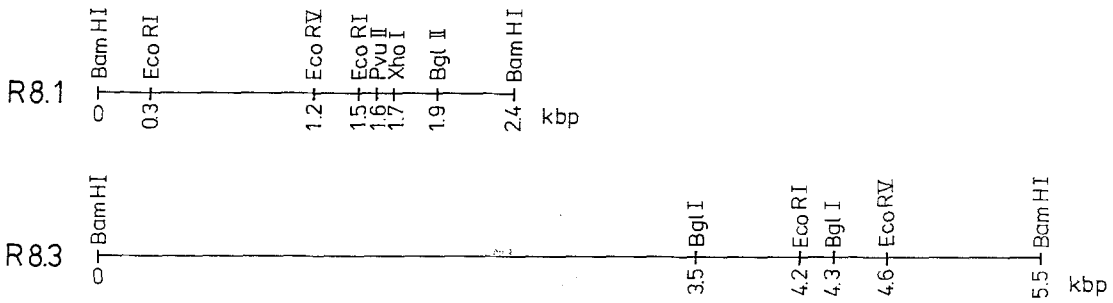

Fig. 3. Physical maps of DNA R8.1 and R8.3. DNA was cut with the indicated restriction endonucleases. The positions of cleavage sites are shown

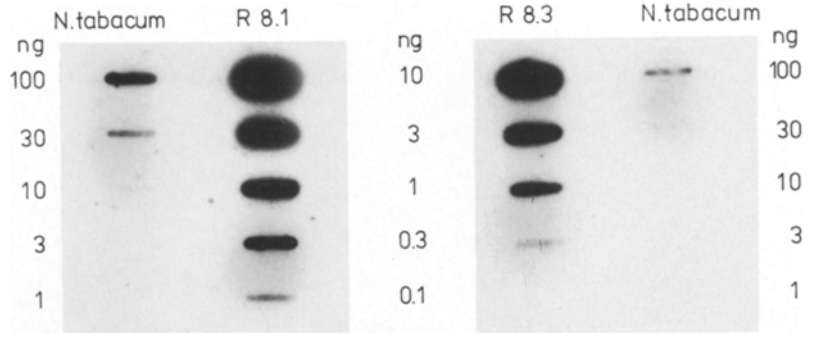

Fig. 4. Estimation of the copy number of R8.1 and R8.3. Serial dilutions of tobacco nuclear DNA R8.1 and R8.3 were blotted onto nitrocellulose membrane and hybridized with ^{32}P-labelled DNA R8.1 or R8.3. Copy number calculations are based on densitometer tracings and on the assumption that 1c DNA content of tobacco nuclear genome is 1.5×10^9 bp. Stringent hybridization conditions ($0.2 \times$ SSC, 65 °C) were used

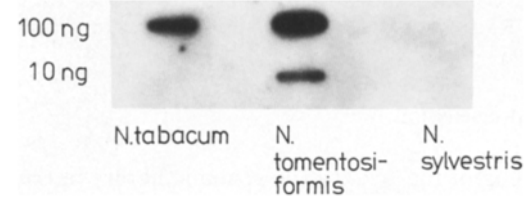

Fig. 5. Species-specificity of the DNA sequence R8.1. DNAs of *Nicotiana tabacum* and its progenitors – *N. tomentosiformis* and *N. sylvestris* – were blotted onto nitrocellulose membrane (100 and 10 ng per slot) and probed with ^{32}P-labelled R8.1 at high stringency conditions

no restriction sites for SalI, XbaI, PstI, HindIII, MluI, BclI, or BglI in the fragment R8.1, and no restriction sites for SalI, XbaI, PstI, HindIII, MluI, BclI, PvuII, or BglII in the fragment R8.3.

The fraction of the haploid tobacco genome corresponding to sequences R8.1 and R8.3 was estimated as ca. 0.3% for both of them (Fig. 4). Based on the lengths of R8.1 (2.4 kb) and R8.3 (5.5 kb), the copy numbers per haploid tobacco genome are 1.6×10^3 and 1.0×10^3, respectively. For comparison, the copy number of a monomer unit of HRS60 is 1.6×10^5 per haploid genome.

Since *N. tabacum* is an allotetraploid species whose genome consists of two genetic components, *N. sylvestris* and *N. tomentosiformis*, we tested the relatedness of sequences R8.1 and R8.3 to both progenitor genomes. Two concentrations (100 and 10 ng) of DNAs originating from *N. tabacum*, *N. tomentosiformis*, and *N. sylvestris* were fixed onto a nitrocellulose membrane and hybridized with ^{32}P-labelled R8.1 or R8.3 DNA probes at high stringency. The result obtained with the R8.1 probe is shown in Fig. 5. Positive hybridization signals were obtained for DNAs from *N. tabacum* and *N. tomentosiformis* and no signal was detected for *N. sylvestris*. There were also no hybridization signals with DNAs originating from some other species of the family Solanaceae: *Lycopersicon esculentum, Capsicum annuum, Solanum tuberosum* (data not shown). The same results were obtained for probe R8.3. From these results we infer that both of these repeated DNA sequences are most probably species specific, and that in the *N. tabacum* genome they label the genetic components originating from *N. tomentosiformis*.

To further characterize R8.1 and R8.3 sequences, these were used as probes for in situ DNA/DNA hybridizations with metaphase *N. tabacum* chromosomes and interphase nuclei. Autoradiograms (Fig. 6) revealed that both of the above-mentioned DNA repeats were dispersed in the majority of chromosomes. This implies that R8.1 and R8.3 spread throughout the tobacco genome from the *N. tomentosiformis* progenitor after the formation of the *N. tabacum* species. Whereas the R8.1 sequence seems to be homogeneously dispersed in the genome, the R8.3 tends to form dense clusters (Fig. 6). Both of them were contained within one genomic clone

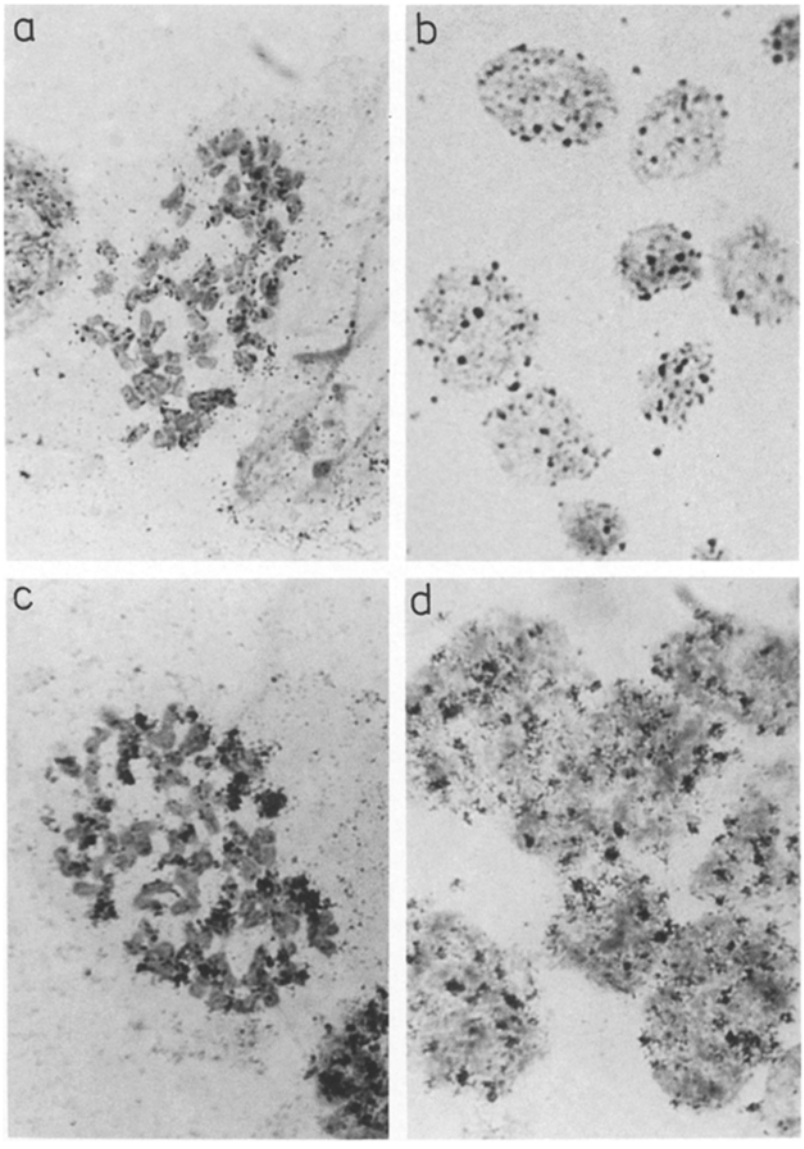

Fig. 6a–d. In situ DNA/DNA hybridization of DNA R8.1 and R8.3 with *N. tabacum*. **a** Metaphase chromosomes, and **b** interphase nuclei probed with R8.1 showed a homogeneous distribution of the repeat in the genome. The slides hybridized with R8.3 DNA displayed a similar dispersion in the genome, but a stronger tendency to form dense clusters was seen both on chromosomes (**c**) and interphase chromatin (**d**)

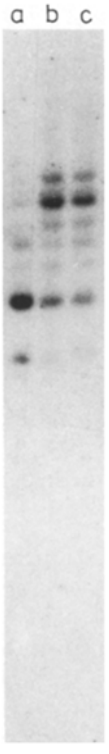

Fig. 7. Use of R8.1 for the screening of somaclonal variation. Total DNA isolated from the original *N. tabacum* plant and two selected plants regenerated from protoplasts were digested with BamHI restriction endonuclease (25 units per microgram DNA, 6 h at 37 °C). After electrophoresis and Southern blotting, hybridization with ^{32}P-labelled probe R8.1 was carried out. Different intensities of individual bands among the plants tested can be seen on the autoradiogram. The control of total cutting was made using chloroplast DNA as an inner standard. *Lanes a* and *b* – DNA of regenerated protoplasts; *lane c* – DNA of the original plant

used for the analysis. These distinct patterns of sequence arrangements agree with silver grain distributions over interphase nuclei.

A typical example of plant genome plasticity is that of somaclonal variation and genome plasticity caused by external stress factors (Karp and Bright 1985). Our preliminary experiments with R8.1 as a probe proved that this DNA sequence could also be a valuable tool for detecting structural changes in the tobacco genome in populations of plants regenerated from leaf mesophyll protoplasts (Fig. 7 and paper in preparation).

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