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Comparison of the mitochondrial genome of *Nicotiana tabacum* with its progenitor species

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Summary. Mitochondrial DNAs from *Nicotiana tabacum*, an amphiploid, and its putative progenitor species, *N. sylvestris* and *N. tomentosiformis* were compared in structure and organization. By using DNA transfer techniques and cloned fragments of known genes from maize and *N. sylvestris* as labeled probes, the positions of homologous sequences in restriction digests of the *Nicotiana* species were analyzed. Results indicate that the mitochondrial DNA of *N. tabacum* was inherited from *N. sylvestris*. Conservation in organization and sequence homology between mtDNAs of *N. tabacum* and the maternal progenitor, *N. sylvestris*, provide evidence that the mitochondrial genome in these species is evolutionarily stable. Approximately one-third of the probed restriction fragments of *N. tomentosiformis* mtDNA showed conservation of position with the other two species. Pattern variations indicate that extensive rearrangement of mtDNA has occurred in the evolution of these *Nicotiana* species.

Key words: *Nicotiana* – Progenitor species – mtDNA – Restriction endonuclease – Cloned fragments

Introduction

Nicotiana tabacum, as most polyploid species (Grant 1981), arose as the result of interspecific hybridization between two diploid progenitor species, followed by chromosome doubling to allow for fertility of the hybrid. The origin and evolution of this commercially important species has been the focal point of much interest and research. The relationship of *N. tabacum* with its putative parental species, *N. sylvestris* and *N. tomentosiformis*, has been analyzed by classical methods including cytogenetics, artificial hybridization and comparisons of flower morphology (Gerstel 1960, 1976). At the molecular level, comparisons of isozyme patterns (Sheen 1972), polypeptide composition of ribulose-1,5-bisphosphate carboxylase (Gray et al. 1974) and tentoxin sensitivities (Burk and Durbin 1978) support the view that *N. sylvestris* and *N. tomentosiformis*, or ancestral types similar to these species (Smith 1979), are the progenitors of tobacco.

Speciation and evolution in higher plants involve the compartmentalized nuclear, mitochondrial and chloroplast genomes. As in most angiosperms, cytoplasmic organelles in *Nicotiana* show strict maternal inheritance (Sears 1980). Uniparental inheritance primarily excludes the sources of evolutionary variation available in sexual cycles.

Little is known about the structure and organization of plant mitochondrial DNA (mtDNA). Unlike animal mitochondrial genomes which are typically 15 to 18 kb in length, plant mitochondria contain unusually large and complex genomes, varying in size from 250 to 2,500 kb (Levings 1983). The variation in size among plant mitochondrial DNAs is not understood. The tobacco mitochondrial genome is estimated to be approximately 250 kb (Dale et al. 1983) and, in cultured cells, is composed of heterogeneous classes of circular molecules (Sparks and Dale 1980; Dale et al. 1981, 1983).

To provide insight into the origin and organization of the *N. tabacum* mitochondrial genome, we have used cloned fragments of known genes from maize and unidentified genes from *N. sylvestris* mitochondria as labeled probes to detect specific sequence homologies on Southern transfers of *N. tabacum* and progenitor species mtDNAs. The results provide definitive evidence of the maternal parentage of *N. tabacum* and the stability of the mitochondrial genome.

Materials and methods

Plant materials

N. tabacum L. cv. 'Hicks' is an inbred line maintained in our nursery by self pollination. Seeds for *N. sylvestris* (UCBG

49-G-87) and *N. tomentosiformis* (UCBG 59-G-54) were originally obtained from the University of California Botanical Garden. They have been maintained by self pollination as well, and are highly inbred. A single accession of each species was examined.

Isolation of mtDNA

Leaves (250–350 g) from 8–12 week-old greenhouse plants or from field grown plants were harvested, de-ribbed, washed in dilute chlorox, rinsed extensively, cut into small pieces, and held overnight at 4 °C. All subsequent operations were conducted at 4 °C unless otherwise specified. Leaves were homogenized for 10 to 15 s in a Waring blender in homogenizing medium (0.5 M sucrose, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, 1% bovine serum albumin, 10 mM β-mercaptoethanol) at a 1:3 (w/v) ratio. The extract was filtered through four layers of cheesecloth and two layers of miracloth and centrifuged at 2,500 rpm (Sorval GSA rotor) for 10 min. The supernatant was recentrifuged at 3,500 rpm (Sorval GSA rotor) for 10 min, and the resulting supernatant was then centrifuged at 10,500 rpm (GSA rotor) for 20 min.

The crude mitochondrial pellet was resuspended in a buffer containing 0.4 M sucrose, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 50 µg/ml DNase I (Sigma) and incubated at room temperature for 15 min. The suspension was centrifuged at 12,000 rpm (Sorval SS-34 rotor) for 20 min at 4 °C. The pellet was resuspended in homogenizing medium and layered on a step gradient consisting of 1.6 M and 1.2 M sucrose solutions containing 10 mM EDTA. The gradient was placed in a SW-627 rotor and centrifuged at 20,000 rpm for 80 min. Purified mitochondria were removed from the 1.6 M–1.2 M interphase, diluted with 2 volumes of 10 mM EDTA, pH 7.5 and centrifuged at 12,000 rpm (Sorval SS-34 rotor) for 20 min. The pellet was gently resuspended in 10 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2% sarkosyl [w/v]) and immediately extracted twice with tris-saturated phenol:chloroform (Maniatis et al. 1982). The aqueous phase was then ether extracted. The mtDNA was precipitated overnight in 2 volumes of 95% ethanol at –20 °C and recovered by centrifugation at 12,000 rpm (SS-34 rotor) for 20 min.

RNA isolation

N. sylvestris mitochondria were isolated as above except that the crude mitochondrial pellet was not incubated with DNase I prior to purification on sucrose step gradients. Mitochondrial nucleic acids were purified as described above. The nucleic acids were resuspended and the mtDNA was removed by treatment with DNase for 30 min on ice. The RNA was phenol:chloroform extracted and re-precipitated with ethanol.

Chloroplast DNA isolation

Chloroplasts were isolated from leaves of greenhouse grown *N. tabacum* as described by Palmer (1982). Chloroplast DNA was purified by repeated phenol:chloroform extractions and ethanol precipitated.

Recombinant DNA clones

Recombinant DNA plasmids containing the ribosomal genes from normal maize mitochondria were isolated from a BamHI clone library in pBR322 (Spruill et al. 1980). Clone 542 contains the genes for the 5S and 18S ribosomal RNA and a partial DNA sequence has been published (Chao et al. 1983). Maize clone 625-2 has a 14.4 kb insert known to encode the 26S rRNA gene. DNA of the maize EcoRI clone for cytochrome *c* oxidase subunit II (COII) was a gracious gift from Dr. Thomas Fox (Fox and Leaver 1981). The XbaI clone containing the sequence for the maize mitochondrial ATPase complex subunit 9 was isolated from a normal maize library in our laboratory (Dewey et al. 1984). *N. sylvestris* PstI clone 9A4 and PstI–HindIII clone 1B6 were prepared in the plasmid vector pUC 9 (Vieira and Messing 1982). These *N. sylvestris* clones were found to be transcribed as determined by hybridization with end-labeled total mtRNA from *N. sylvestris*.

Enzymes and gel electrophoresis.

BamHI, HindIII and PstI restriction endonucleases (Bethesda Research Labs or New England Biolabs) were used as recommended by the manufacturer except that incubations were carried out for 5–6 h. The fragments were separated by electrophoresis on 1% agarose gels (Seakem) at 45 V for 16–17 h in a horizontal apparatus. Gels were stained for 30 min in ethidium bromide, destained for 1 h in deionized water and photographed with Polaroid type 55 film under UV light.

Blotting and hybridizations

After photographing, the restriction fragments were transferred from the gels to nitrocellulose or Gene Screen (New England Nuclear) as described by Maniatis et al. (1982). To achieve better transfer of large fragments, partial depurination was performed as described by Wahl et al. (1979). Filters were pre-hybridized in 5X SSC/25X Denhardt's reagent/50 mM sodium phosphate, pH 6.5/1% glycine/50% formamide/denatured salmon sperm DNA at 250 µg/ml.

Recombinant plasmid DNAs were labeled by nick translation (Maniatis et al. 1982) using (α-^{32}P) dATP (New England Nuclear, 3,200 Ci/m mole). Labeled DNA was separated from unincorporated nucleotides by chromatography on a Sephadex G-50 column. Labeled probe was boiled for 5 min and added to the hybridization mix (5X SSC/20 mM sodium phosphate, pH 6.5/1X Denhardt's reagent/10% dextran sulfate/50% formamide) containing denatured salmon sperm DNA at 100 µg/ml. The filters were hybridized in this solution for 20–24 h at 37 °C, washed and autoradiographed as previously described (Sederoff et al. 1981). *N. sylvestris* mitochondrial RNA was 5′ end-labeled with (γ-^{32}P) ATP (Maxam and Gilbert 1980) and hybridized to Southern blots of *N. sylvestris* clones IB6 and 9A4 as described above.

Results

Restriction endonuclease digests of mtDNA from *N. tabacum* and the progenitor species show a complex pattern of approximately 40 bands using PstI, to over 50 bands using HindIII (Fig. 1). The BamHI and PstI restriction patterns of *N. tabacum* and *N. sylvestris* appear nearly identical, while the HindIII pattern shows additional bands in *N. sylvestris* of about 6.7 and 3.0 kb and the absence of a 3.4 kb fragment in *N. sylvestris*. Restriction patterns of *N. tabacum* and *N. sylvestris* mtDNA using XhoI and SalI endonucleases (Galun et al. 1982) were shown to be indistinguishable. Nagy et al. (1983) obtained similar results using BamHI and SalI digests of mtDNA isolated from *N. tabacum* and *N. sylvestris* regenerated from tissue culture. Re-

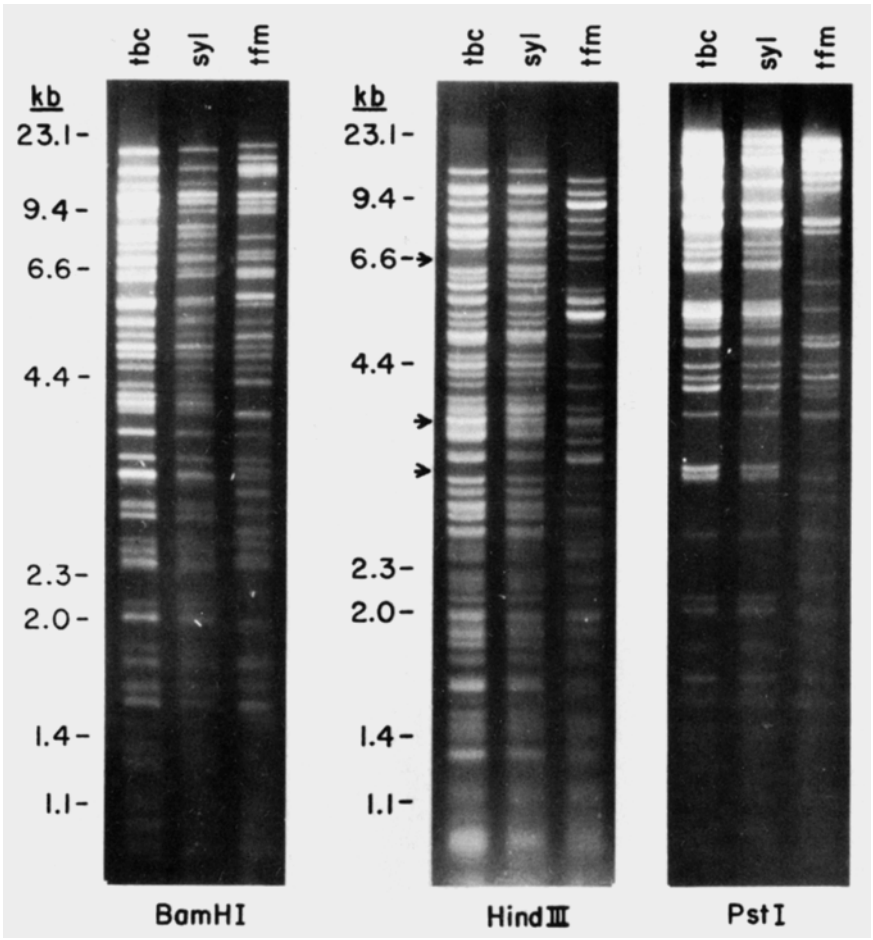

Fig. 1. Restriction fragment patterns of mtDNA from *N. tabacum* (tbc), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) generated by digestion with *BamHI*, *HindIII* and *PstI*. *Arrows* indicate differences between *N. tabacum* and *N. sylvestris* patterns

striction patterns of *N. tomentosiformis* mtDNA obtained with BamHI, HindIII and PstI digestions show a complex array of fragments that bears little similarity in migration with those of the other two species (Fig. 1).

Mitochondrial genomes of *Nicotiana* species are large and sufficiently complex that it is difficult to follow organizational changes by analyzing the positions of visible restriction fragments. The use of hybridization to detect homologous sequences offers a more precise method of studying mtDNA variation in these species. Accordingly, six clones of mtDNA containing either defined genes or regions known to be transcribed were hybridized with restriction endonuclease fragments of mtDNA from the three *Nicotiana* species.

The maize BamHI clone 542, containing the 5S and 18S ribosomal RNA genes, hybridizes strongly to a 3.4 kb HindIII fragment in all three *Nicotiana* species (Fig. 2). Other HindIII and PstI fragments probed are conserved between *N. tabacum* and *N. sylvestris* but differ in *N. tomentosiformis*. Subfragments of the 542 clone containing only the 5S or the 18S rDNAs probed to the *Nicotiana* digests, hybridize to the same fragment (not shown) indicating these genes are closely linked in

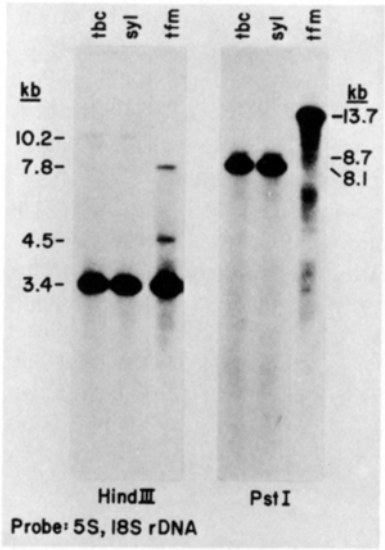

Fig. 2. Autoradiographs of Southern blots of *Nicotiana* mtDNAs after hybridization to a radioactive probe of the maize clone containing the genes for the 5S and 18S rRNAs. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) were digested with *HindIII* or *PstI*. The sizes of restriction fragments showing homology are indicated

Nicotiana species as has been shown for other plant mitochondrial genomes (Huh and Gray 1982).

Hybridization of the maize clone 625-2, which contains the 26S rRNA gene, to *Nicotiana* HindIII, PstI and BamHI digests (Fig. 3) gives an identical pattern in *N. tabacum* and *N. sylvestris*. The *N. tomentosiformis* gene is found in a larger BamHI fragment than in the other two species and the PstI digest shows two large PstI fragments indicating an additional PstI site in the *N. tomentosiformis* gene. In the HindIII pattern, two bands are conserved among the three species. There appears to be a change in one HindIII site in *N. tomentosiformis*, however, as evidenced by hybridization to an 11.1 kb fragment in *N. tomentosiformis* and a corresponding 1.3 kb fragment in the other two species.

The cytochrome oxidase subunit II hybridization pattern is again conserved in *N. tabacum* and *N. sylvestris* (Fig. 4). In the BamHI digest, fragments of 1.6 kb and 0.6 kb are conserved in all three species as is a 2.3 kb HindIII fragment. In *N. tomentosiformis* a 18.2 kb BamHI fragment is reduced to 7.4 kb.

The 2.2 kb maize fragment containing the gene for subunit 9 of the ATPase complex hybridizes to only one BamHI fragment of about 770 bp that is shared by the three *Nicotiana* species (Fig. 5). The maize sequences flanking the 210 bp subunit 9 gene thus appear to have no homology with the *Nicotiana* mitochondrial genome. Hybridization to PstI digests gives identical *N. tabacum* and *N. sylvestris* patterns, while the subunit 9 gene hybridizes to a larger *N. tomentosiformis* fragment.

The *N. sylvestris* HindIII–PstI clone IB6 has a 3.3 kb insert which is transcribed. The clone hybridizes strongly to the 4.0 kb HindIII and 8.9 kb PstI bands of its origin (Fig. 6) but hybridizes in addition to conserved fragments of 2.1 kb in HindIII digests and 18 kb in PstI digests of all three *Nicotiana* species. The hybridization of clone IB6 to the additional fragments indicates partial homology to sequences elsewhere in the *Nicotiana* mitochondrial genome. To test the possibility that the unusual hybridization pattern was due to chloroplast DNA contamination of mitochondrial preparations, hybridization to restriction digests of *N. tabacum* chloroplast DNA was performed. Surprisingly, clone IB6 hybridizes weakly to chloroplast DNA, but the fragment sizes (0.85 kb in the HindIII digest, and two bands of about 19 kb and 26 kb in the PstI digest) do not correspond to the hybridization pattern in mtDNA (data not shown).

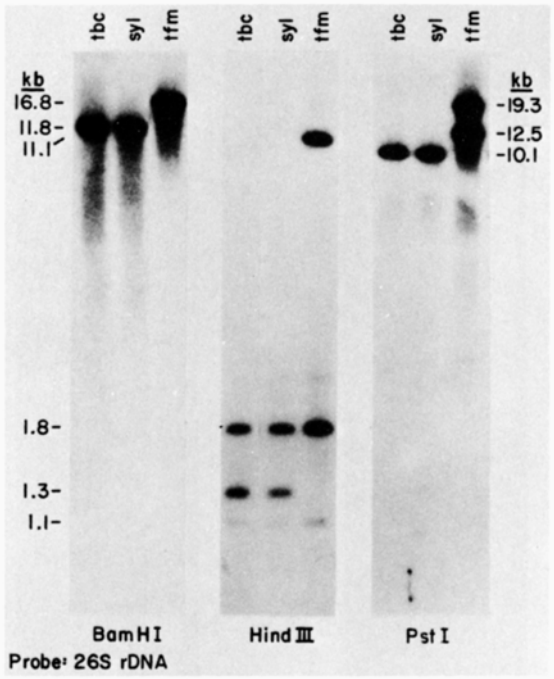

Fig. 3. Autoradiographs of Southern blots of *Nicotiana* mtDNAs after hybridization to a radioactive probe of the maize clone containing the 26S rRNA gene. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) were digested with *BamHI*, *HindIII* or *PstI*. The sizes of restriction fragments showing homology are indicated

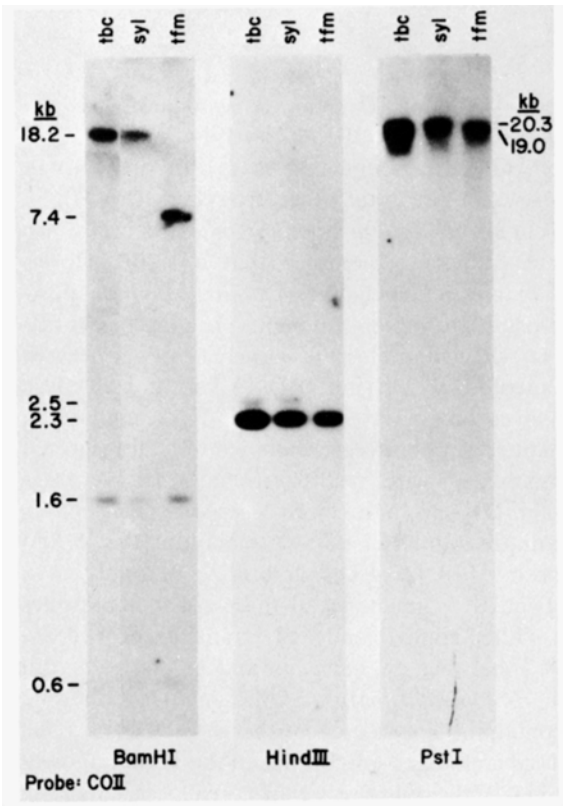

Fig. 4. Hybridization of nick-translated maize clone containing the gene for cytochrome oxidase subunit II to Southern blots of *Nicotiana* mtDNAs. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) were digested with *BamHI*, *HindIII* or *PstI*. The sizes of restriction fragments showing homology are indicated

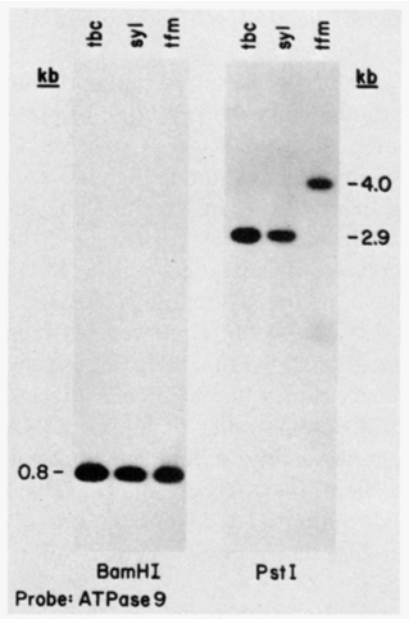

Fig. 5. Hybridization of nick-translated maize clone containing the gene for *ATPase* subunit 9 to Southern blots of *Nicotiana* mtDNA. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*), and *N. tomentosiformis* (*tfm*) were digested with *BamHI* or *PstI*. The sizes of restriction fragments showing homology are indicated

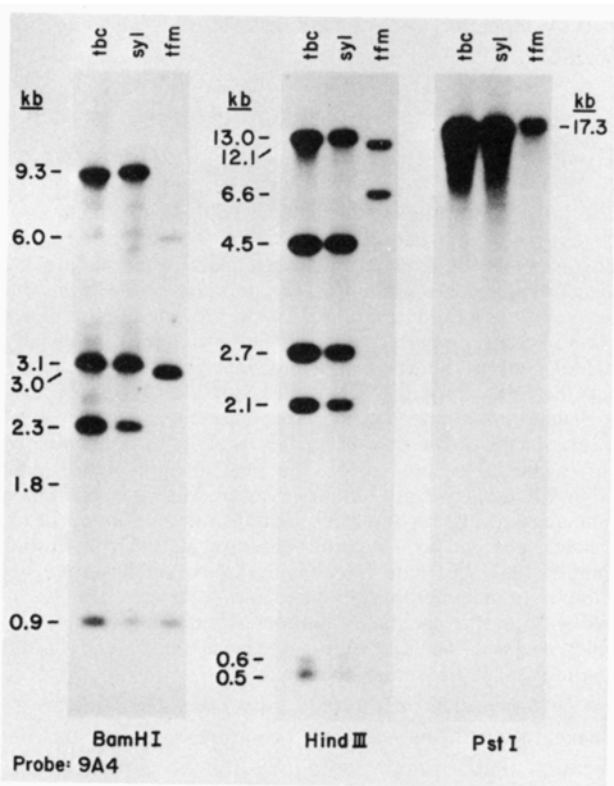

Fig. 7. Hybridization of ^{32}P labeled *N. sylvestris Pst* clone 9A4 to Southern blots of *Nicotiana* mtDNA. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) were digested with *BamHI*, *HindIII* or *PstI*. The sizes of restriction fragments showing homology are indicated

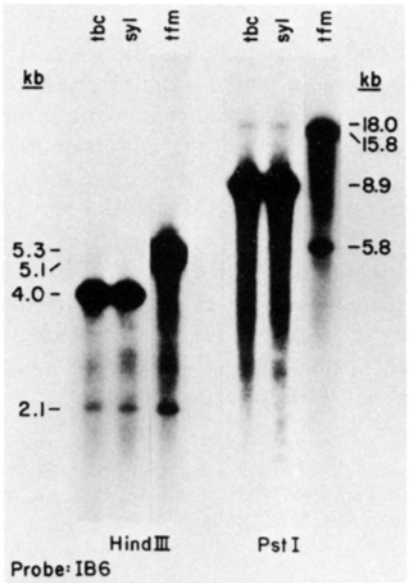

Fig. 6. Hybridization of ^{32}P labeled *N. sylvestris HindIII-PstI* clone *IB6* to Southern blots of *Nicotiana* mtDNA. MtDNA from *N. tabacum* (*tbc*), *N. sylvestris* (*syl*) and *N. tomentosiformis* (*tfm*) were digested with *HindIII* or *PstI*. The sizes of restriction fragments showing homology are indicated

N. sylvestris PstI clone 9A4 contains a 17.3 kb insert that is transcribed. This clone hybridizes with a complex pattern of HindIII and BamHI fragments which are identical in *N. tabacum* and *N. sylvestris* (Fig. 7). Hybridization of clone 9A4 to *N. tomentosiformis* seems less intense than with the other species and the clone hybridizes to fewer restriction fragments indicating that a portion of the 9A4 sequence may be absent in *N. tomentosiformis*. *N. tomentosiformis* shares homology of only the 0.5 kb HindIII fragment and the 0.9 kb BamHI fragment with the other two species. A summation of the *N. tomentosiformis* BamHI fragments hybridizing to the clone accounts for only 11.7 kb versus the cloned insert size of 17.3 kb. It appears that a minimum of 5.6 kb could be missing from the *N. tomentosiformis* mitochondrial genome.

An estimate of the genetic divergence between the species can be made by comparing the number of conserved fragments to the total number of probed fragments. The calculation of divergence between *N. sylvestris* and *N. tabacum* is zero because all fragments were common to both species. *N. tomentosiformis* shared 11 of the 33 fragments probed in *N. sylvestris*

and *N. tabacum* and has an estimated divergence of 66.7%.

Discussion

The large and variable mitochondrial DNAs of plants may contain an abundance of dispersed non-coding regions. The discovery of a 794 bp intron in the maize gene coding for cytochrome oxidase subunit II (Fox and Leaver 1981) was the first report of an intron in a mitochondrial gene from a higher plant. Recent reports of maize mitochondrial sequences homologous to the chloroplast inverted repeat region coding for the 16S ribosomal RNA and two tRNAs (Stern and Lonsdale 1982) and to the large subunit of ribulose-1,5-bisphosphate carboxylase (Lonsdale et al. 1983) are evidence for organelle recombination. The phenomenon of mtDNA–ctDNA homology is not unique to maize. Mitochondrial DNA homology to ctDNA has been demonstrated in mung bean, spinach, pea and four cucurbits (Stern et al. 1983; Stern and Palmer 1984). To date no biological function has been attributed to the chloroplast-homologous sequences. The *N. sylvestris* clone IB6 contains a chloroplast sequence. Preliminary analysis maps the area of homology to the 5′ end of the chloroplast 23S ribosomal RNA gene.

N. tomentosiformis and *N. tabacum* show 66% divergence in positions of restriction digest fragments, indicating that a major reorganization of sequences has occurred during the evolution of *Nicotiana* mtDNAs. Single restriction site changes partially explains the differing hybridization patterns between *N. tabacum* and *N. tomentosiformis.* Possible examples of gain or loss of restriction sites are observed in the HindIII digest of Fig. 3 where the 1.3 kb band in *N. tabacum* is replaced by an 11.1 kb band in *N. tomentosiformis* and in the BamHI digest of Fig. 4 where the 18.2 kb band in *N. tabacum* corresponds to a 7.4 kb band in *N. tomentosiformis.* The hybridization pattern of PstI clone 9A4, however, indicates that there are DNA sequences maintained in *N. sylvestris* and *N. tabacum* mitochondria that are absent from *N. tomentosiformis.* One explanation is that the mitochondria of *N. sylvestris,* or an ancestral species, acquired the additional sequence through a recombinational event with a foreign genome. Protoplast fusion studies of *Nicotiana* species provide evidence for mitochondrial recombination. Rearrangements in the DNAs of plants derived from heteroplasmic fusions have been interpreted as recombinations between parental mtDNAs (Belliard et al. 1979) or as reshuffling and recombination between populations of parental mitochondrial chromosomes (Nagy et al. 1981, 1983). An alternative explanation is that *N. tabacum* and *N. sylvestris* contain duplications in the region of 9A4. Genome expansion by duplication and by increases in the number of gene copies provides variation required for evolution of new gene functions. The variation in intensity of hybridization of 9A4 to the three species indicates that *N. tomentosiformis* may contain fewer copies of the 9A4 sequences than the other species.

Although rearrangements and recombinational events play an important role in the evolution of plant mitochondrial genomes, the evidence also suggests an inherent stability and conservation among the mtDNAs. For instance, all plant mitochondria examined, including the *Nicotiana* species in this paper, show the same novel arrangement of ribosomal genes, the 5S and 18S genes closely linked and the 26S remotely located.

Conservation of DNA coding sequences between plant mtDNAs is evidenced by the relatively strong hybridization of maize clones to *Nicotiana* digests. Molecular cross-hybridization studies of total mtDNA from plant species representing different taxonomic ranks suggest that there is a group of conserved sequences between distantly related species, and sequence homologies follow classic phylogenetic relationships (Stern et al. 1983).

Evidence for the stability of the plant mitochondrial genome is demonstrated by the near-identical molecular hybridization patterns for *N. tabacum* and its maternal progenitor, *N. sylvestris.* Conservation between these species of the regions of clone 9A4 not present in *N. tomentosiformis,* and the chloroplast-homologous sequences of clone IB6 indicate that even non-essential sequences are maintained by slowly evolving plant mitochondria. In maize mitochondria the sequence homologous to the chloroplast ribulose-1,5-bisphosphate carboxylase large subunit gene is sufficiently conserved to allow in vitro transcription-translation of the mtDNA clone and immunoprecipitation of the resulting truncated polypeptide (Lonsdale et al. 1983).

It is clear from our results that the mitochondrial DNA of *N. tabacum* was inherited from *N. sylvestris* with no apparent heteroplasmic contribution from the paternal species. The stability of the mitochondrial genome is evident by the near identical restriction and molecular hybridization patterns of *N. tabacum* and its maternal progenitor. Moreover, non-essential DNAs acquired through recombinational events during the evolution of the genus are also maintained in the mitochondrial genome. Analysis of the "acquired" DNAs may provide further insight into phylogenetic relationships within the *Nicotiana* genus.

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