Theor Appl Genet (1992) 83:515–527

THEORETICAL AND APPLIED GENETICS

Physical maps of *Nicotiana* chloroplast DNA constructed by an efficient procedure

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Received December 27, 1990; Accepted April 30, 1991
Communicated by Yu. Gleba

Summary. The restriction profiles of chloroplast DNA (cpDNA) from *Nicotiana tabacum*, *N. sylvestris*, *N. plumbaginifolia*, and *N. otophora* were obtained with respect to *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I. An efficient mapping method for the construction of cpDNA physical maps in *Nicotiana* was established via a computer-aided analysis of the complete cpDNA sequence of *N. tabacum* for probe selection. The efficiency of this approach is demonstrated by the determination of cpDNA maps from *N. sylvestris*, *N. plumbaginifolia*, and *N. otophora* with respect to all of the above restriction endonucleases. The size and basic structure of the cpDNA from the three species are almost identical, with an addition of approximately 80 bp in *N. plumbaginifolia*. The restriction patterns and hence the physical maps between *N. tabacum* and *N. sylvestris* cpDNA are identical and there is no difference in the *Pvu*II digests of cpDNA from all four species. Restriction site variations in cpDNA from different species probably result from point mutations, which create or eliminate a particular cutting site, and they were observed spanning the whole chloroplast molecule but highly concentrated in both ends of the large, single-copy region. The results presented here will be used for the forthcoming characterization of chloroplast genomes in the interspecies somatic hybrids of *Nicotiana*, and will be of great value in completing the exploration of the phylogenetic relationships within this already extensively studied genus.

Key words: *Nicotiana* – Chloroplast DNA – Restriction patterns – Physical mapping – Restriction site variations

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Introduction

The structural organization of chloroplast genomes has been studied extensively in the past decade (see Palmer 1985). The chloroplast DNA molecules (cpDNA) in most higher plants exist as a circular form with a length ranging from 120 to 160 kb (Palmer 1985). One of the most distinct features found in cpDNA is the existence of two inverted repeat segments (IR_A and IR_B) which are separated by a small and a large single-copy region (designated as SSC and LSC, respectively). This basic structure is highly conserved among most plant species ranging from algae to higher plants, with only a few exceptions. For instance, the cpDNAs in broad bean, pea, and chickpea lack the inverted repeats (Koller and Delius 1980; Palmer and Thompson 1981; Chu et al. 1981; Chu and Tewari 1982).

With respect to a variety of restriction enzymes, there have been many reports on the restriction patterns and the construction of physical maps of cpDNA from algae, *Chlamydomonas reinhardtii* (Rochaix 1978; Gauthier et al. 1988), to lower plants, *Marchantia polymorpha* (Ohyama et al. 1983), and higher plants, *Zea mays* (Bedbrook and Bogorad 1976; Larrinua et al. 1983), *Oryza sativa* (Hirai et al. 1985), *Pinus monticola* (White 1990), *Spinacia oleracea* (Driesel et al. 1979; Herrmann et al. 1980), *Glycine max* (Spielmann et al. 1983), *Dioscorea bulbifera* (Terauchi et al. 1989), *Triticum* (Bowman et al. 1981), *Cucumis* species (Perl-Treves and Galun 1985), and *Nicotiana* species (Seyer et al. 1981; Fluhr and Edelman 1981; Hildebrand et al. 1985). Of all plant species, the chloroplast genomes of tobacco have been studied the most, and the complete sequence of the cpDNA from *N. tabacum* was determined by Shinozaki et al. (1986a,b). The cpDNA of *N. tabacum* is a circular molecule (155,844 bp) that contains two copies of identical

inverted repeats (25,339 bp), which are separated by a large (86,684 bp) and a small (18,482 bp) single-copy region. In this molecule, the genes for four different rRNAs, 30 different tRNAs, 39 different proteins, and 11 predicted protein coding regions were located (Shinozaki et al. 1986a, b).

The restriction patterns of the cpDNA are similar but not identical among different *Nicotiana* species (Tassopulu and Kung 1984). This characteristic was used as the plastome marker in the studies of chloroplast segregation (Belliard et al. 1978) and for evaluating genetic distance between fused partners (Aviv et al. 1980). It has also been used for investigating the mechanism of streptomycin-resistant mutants that inherited maternally (To et al. 1989). Although the restriction profiles alone can provide phylogenetic and evolutionary indications, the availability of physical maps allows a much more detailed analysis of cpDNA rearrangement in the fused cells (Medgyesy et al. 1985; Fejes et al. 1990) and deletions or additions of particular sequences in different species (Shen et al. 1982; Tassopulu and Kung 1984). The physical maps, together with the evidence obtained from biochemical studies, were also utilized to build the phylogenetic tree along the course of evolution among the diverse species of the genus *Nicotiana* (Chen et al. 1976; Kung et al. 1982).

In this article, we demonstrate an efficient mapping procedure for cpDNA from the genus *Nicotiana*, and we present the restriction profiles and the maps obtained from *N. plumbaginifolia*, *N. otophora*, and *N. sylvestris* with respect to eight common restriction enzymes: *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I. Physical maps of *N. tabacum* are also included for comparison and the results are discussed.

Materials and methods

Plant materials

Four species of the *Nicotiana* genus were used in the present study. *N. plumbaginifolia*, *N. sylvestris*, and *N. otophora* were used extensively in the somatic genetic studies (Chen et al. 1985; To et al. 1989), and *N. tabacum* was included mainly for comparative analysis because the complete sequence of its cpDNA is determined. The plants were grown at 25° ± 2 °C with 12 h of illumination. Before the isolation of cpDNA, the plants were kept in the dark for 3–5 days to reduce the starch content in the leaves.

Chloroplast DNA extractions

The method for chloroplast DNA isolation was mainly based on the procedures described by Fluhr and Edelman (1981), with the modification that 30%–60% discontinuous sucrose density gradients were used.

Restriction endonuclease analysis of the cpDNA

Restriction endonucleases were obtained from Boehringer-Mannheim or Biolabs. Chloroplast DNA (5–10 μg) was completely digested in a 25-μl reaction mixture, as recommended by the suppliers, with 5–10 units of each enzyme at 37 °C for 2 h. Double digestions were carried out by adding the two enzymes simultaneously and extending the digestion time to 3 h. The electrophoresis analysis of the resulting DNA fragments was performed as described by Maniatis et al. (1982). Fragment sizes were calculated from the standard curve using *Hin*dIII digests of λ phage as size markers. In addition, theoretical values were generated from the published sequence (Shinozaki et al. 1986a, b) by a computer program.

Excision of DNA fragments from agarose gels and probe preparation

The restricted DNA fragments were isolated from the gels by the method of Vogelstein and Gillespie (1979) using a commercially available Geneclean kit (Bio 101 Inc., La Jolla/CA). The fragments were labeled by random-primed incorporation (Feinberg and Vogelstein 1983) of digoxigenin-labeled deoxyuridine-triphosphate, as described by the supplier (DNA Labeling and Detection Kit, Boehringer-Mannheim).

DNA transfer and hybridization

After electrophoresis, DNA fragments on gels were denatured, neutralized, and transferred onto Nylon 66 membrane (Hoeffer Instruments, San Francisco) according to Southern's method (1975). The hybridization procedure was carried out under normal stringency as described by Maniatis et al. (1982). The hybrids were detected by enzyme immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate) and subsequent enzyme-catalyzed color formation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (DNA Labeling and Detection Kit, Boehringer-Mannheim).

Results

In the present study, 30–60% sucrose step gradients were used for chloroplast isolation. The cpDNAs subsequently obtained were free from nuclear DNA contamination since background staining on gel was negligible, and they could be completely digested by restriction endonucleases without further purification. The average yields varied from 5 to 10 μg cpDNA per gram of fresh leaves, depending on the growth conditions and amounts of starting materials used. In a typical preparation, approx. 100–200 μg cpDNA was obtained from 20 g green leaves, which is enough for at least 20 gel electrophoresis analyses.

Figure 1 shows the restriction patterns generated by *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*II, *Sal*I, and *Xho*I digestion of cpDNA from the four *Nicotiana* species. The sizes of each fragment are listed in Table 1. They were determined to single base-pair values if corresponding fragments existed in the *N. tabacum*. Alternatively, the fragment sizes were calculated from a standard curve constructed with molecular-size markers. The restriction patterns highly resembled each other among all *Nicotiana* species studied, although obvious variations were detected. In all of the restriction enzymes used, identical restriction patterns were obtained from *N. tabacum* and

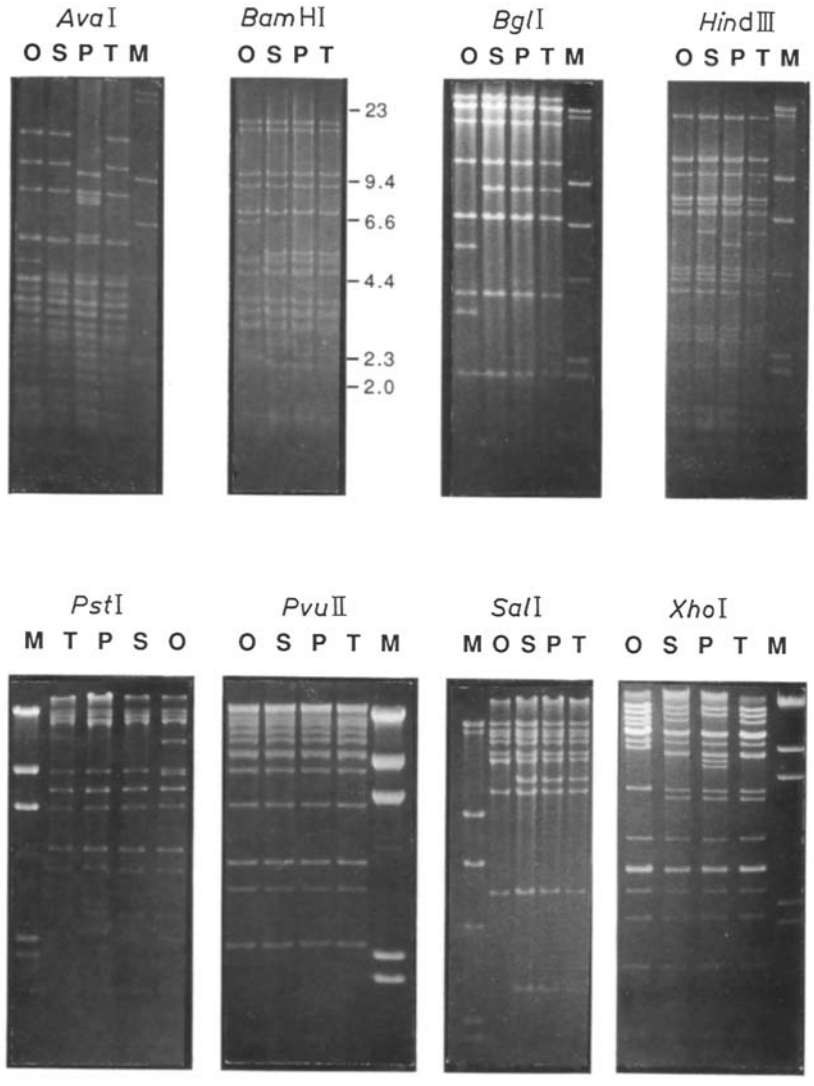

Fig. 1. Restriction patterns of cpDNA isolated from four *Nicotiana* species. Tobacco cpDNA was cut with eight restriction endonucleases and the fragments were separated on an 0.8% agarose gel. *M:* size markers, *Hin*dIII digests of λ phage DNA. *T*, *P*, *S*, and *O* represent *N. tabacum*, *N. plumbaginifolia*, *N. sylvestris*, and *N. otophora*, respectively. Fragment sizes are listed in Table 1. It is difficult to visualize bands smaller than 0.5 kb on these photographs, but the restriction fragment sizes can be obtained from computer analysis of the *N. tabacum* chloroplast genome

N. sylvestris. In addition, the restriction profiles of *N. plumbaginifolia* and *N. sylvestris* cpDNA generated by *Bam*HI, *Bgl*I, *Pvu*II, and *Sal*I were the same as those obtained from *N. tabacum*, while cpDNA from *N. otophora* exhibited the most different restriction patterns except when digested with *Pvu*II. In fact, no differences could be found in either the number of bands or their stoichiometries in *Pvu*I digests of all four species. These data indicate that the chloroplast genomes were very similar, if not identical, in size and overall structural organization. Therefore, the variations observed in the restriction patterns were most likely due to point mutation occurring in the cpDNAs. However, because of the resolution limit of agarose gel electrophoresis, small deletions or additions could not be characterized here.

In order to establish the physical maps of interest, the maps of *N. tabacum* cpDNA were first obtained from the previously published data and through sequence analysis with the aid of a computer program (Fluhr and Edelman 1981; Tassopulu and Kung 1984; Shinozaki et al. 1986a, b). For each physical map with respect to an individual restriction endonuclease, the invariable fragments were located and the inconsistent fragments (generated from varied restriction sites) were resolved by (1) analysis of restriction patterns from double digestions, or (2) cross-hybridization of probes prepared from specific restriction fragments of *N. tabacum* cpDNA to the digests of cpDNA from other *Nicotiana* species, or a combination of both. Suitable restriction enzymes and probes were chosen according to the physical maps of *N. tabacum* cpDNA. In the following descriptions, the designation of restriction fragments was the same as those in Table 1, unless otherwise specified. The maps are presented in linearized forms by cutting at the junction (J_{LA}) between IR_A and LSC. J_{LA} was designated as zero and nucleotides are numbered proceeding towards the LSC, according to

Table 1. Nomenclatures and sizes of the restricted cpDNA fragments form *Nicotiana tabacum*, *N. sylvestris*, *N. plumbaginifolia*, and *N. otophora*

Fragment designation[a]	Size[a] (bp)	*Nicotiana* species			
		NT	NS	NP	NO
*Ava*I					
A69	13,533	+	+	−	+
A68	10,303	+	+	−	+
				9.5 kb	
A67	8,179	+	+	+	+
				7.9 kb	
				7.5 kb	
				6.0 kb	
A66	5,704	+	+	−	+
A65	5,640	+	+	+	+
					4.8 kb
A64	4,385	+	+	+	−
A63	4,218	+	+	+	+
A62	4,180	+	+	+	+
					4.2 kb
A61	4,116	+	+	+	+
A60	3,908	+	+	+	+
A59	3,829	+	+	−	+
A58	3,776	+	+	+	+
A57	3,622	+	+	+	+
A56	3,561	+	+	+	−
A55	3,550	+	+	+	+
				3.3 kb	
A54	3,271	+	+	+	+
A53	3,261	+	+	+	+
A52	2,990	+	+	+	+
A51	2,790	+	+	+	+
A50	2,689	+	+	+	+
A49	2,638	+	+	−	+
A48	2,567	+	+	+	+
				2.4 kb	
A47	2,319	+	+	+	+
A46	2,128	+	+	+	+
A45	1,896	+	+	+	+
A44	1,876	+	+	+	+
A43 (×2)	1,597	+	+	+	+
A42	1,479	+	+	+	+
A41 (×2)	1,319	+	+	+	+
A40 (×2)	1,283	+	+	+	+
A39	1,212	+	+	+	+
A38 (×2)	1,137	+	+	+	+
A37 (×2)	1,060	+	+	+	+
A36	929	+	+	+	+
A35	771	+	+	+	+
A34 (×2)	757	+	+	+	+
A33	699	+	+	+	+
A32 (×2)	696	+	+	−	+
A31	690	+	+	+	+
A30	613	+	+	+	−
A29	606	+	+	+	+
A28	597	+	+	+	+
A27	582	+	+	+	+
A26	514	+	+	+	+
A25	509	+	+	+	+
A24	506	+	+	+	+
A23 (×2)	498	+	+	+	+
A22	458	+	+	+	+
A21 (×2)	413	+	+	+	+
A20	407	+	+	+	−

Fragment designation[a]	Size[a] (bp)	*Nicotiana* species			
		NT	NS	NP	NO
*Bam*HI					
B40	19,706	+	+	+	+
B39	17,481	+	+	+	+
B38	10,173	+	+	+	+
B37	9,007	+	+	+	+
B36 (×2)	7,067	+	+	+	+
					6.6 kb
B35 (×2)	5,164	+	+	+	−
B34	4,971	+	+	+	+
B33	4,790	+	+	+	+
B32	4,537	+	+	+	−
B31	4,465	+	+	+	+
B30	3,629	+	+	+	+
B29	3,619	+	+	+	+
B28 (×2)	3,269	+	+	+	+
B27	3,251	+	+	+	+
B26	3,181	+	+	+	+
B25	3,005	+	+	+	+
					3.0 kb
B24 (×2)	2,949	+	+	+	+
B23	2,868	+	+	+	+
B22	2,506	+	+	+	+
B21	2,322	+	+	+	+
					2.2 kb
B20	2,126	+	+	+	+
B19	2,098	+	+	+	−
B18	1,349	+	+	+	+
B17	1,238	+	+	+	+
B16 (×2)	1,226	+	+	+	+
B15 (×2)	1,190	+	+	+	+
B14	1,189	+	+	+	+
B13 (×2)	1,112	+	+	+	+
B12	1,063	+	+	+	+
B11	449	+	+	+	+
*Bgl*I	sizes in kb				
Bg9	39.0	+	+	+	+
Bg8	29.0	+	+	+	+
Bg7	27.0	+	+	+	+
Bg6	23.0	+	+	+	+
Bg5	12.5	+	+	+	+
Bg4	8.3	+	+	+	−
Bg3 (×2)	7.2	+	+	+	+
					5.2 kb
Bg2	3.8	+	+	+	+
					3.1 kb
Bg1	2.0	+	+	+	+

Table 1. (continued)

Fragment designation[a]	Size[a] (bp)	*Nicotiana* species			
		NT	NS	NP	NO
*Hin*dIII					
H33	20,994	+	+	+	+
H32	11,487	+	+	+	+
H31 (×2)	11,302	+	+	+	+
H30	9,758	+	+	+	+
H29	7,803	+	+	+	+
H28	7,667	+	+	+	+
H27	7,476	+	+	+	+
H26	7,010	+	+	+	+
H25	6,836	+	+	+	+
H24	5,904	+	+	−	−
				5.4 kb	
H23	4,498	+	+	+	+
H22	4,305	+	+	+	+
H21	4,146	+	+	+	+
					3.8 kb
H20	3,774	+	+	+	+
H19	2,854	+	+	+	+
H18	2,731	+	+	+	+
H17	2,575	+	+	+	+
H16	2,081	+	+	+	+
					1.6 kb
H15 (×2)	1,313	+	+	+	+
H14	1,166	+	+	+	+
H13	1,119	+	+	+	+
H12 (×2)	1,098	+	+	+	+
H11 (×2)	1,043	+	+	+	+
H10 (×2)	846	+	+	+	+
H9	688	+	+	+	+
H8	544	+	+	+	+
H7	525	+	+	+	+
				0.5 kb	0.5 kb
*Pst*I					
P14	23,689	+	+	+	+
P13	21,492	+	+	+	−
					21,0 kb
P12	21,012	+	+	−	−
P11	18,949	+	+	+	+
P10	18,869	+	+	+	+
				17.9 kb	12.8 kb
P9	9,047	+	+	+	+
					8.7 kb
P8 (×2)	7,607	+	+	+	+
P7	6,505	+	+	+	+
P6 (×2)	4,635	+	+	+	+
				4.0 kb	4.0 kb
P5	3,966	+	+	−	−
				3.1 kb	
P4	2,876	+	+	+	+
				2.5 kb	
P3	2,395	+	+	−	+
P2	1,573	+	+	+	+
P1	987	+	+	+	+

Fragment designation[a]	Size[a] (bp)	*Nicotiana* species			
		NT	NS	NP	NO
*Pvu*II					
Pv12	41,704	+	+	+	+
Pv11	20,124	+	+	+	+
Pv10	17,350	+	+	+	+
Pv9	14,042	+	+	+	+
Pv8	12,129	+	+	+	+
Pv7	10,103	+	+	+	+
Pv6	9,876	+	+	+	+
Pv5	8,225	+	+	+	+
Pv4	6,043	+	+	+	+
Pv3 (×2)	3,979	+	+	+	+
Pv2	3,430	+	+	+	+
Pv1 (×2)	2,430	+	+	+	+
*Sal*I					
S11	26,757	+	+	+	+
S10	23,121	+	+	+	+
S9	21,671	+	+	+	+
S8	19,467	+	+	+	+
S7	16,511	+	+	+	+
S6	15,469	+	+	+	+
					15.4 kb
S5	12,518	+	+	+	−
S4	11,344	+	+	+	+
S3	5,471	+	+	+	+
S2	2,880	+	+	+	−
S1	653	+	+	+	+
*Xho*I					
X24	20,998	+	+	+	+
X23	17,751	+	+	+	+
X22	15,260	+	+	−	+
					14.0 kb
X21 (×2)	12,059	+	+	+	+
X20	11,429	+	+	+	+
X19	9,944	+	+	+	+
					9.1 kb
X18	8,721	+	+	+	−
X17	8,492	+	+	+	−
				8.0 kb	
				7.2 kb	
X16	5,640	+	+	+	+
X15	5,241	+	+	+	−
X14	3,728	+	+	+	+
X13 (×2)	2,990	+	+	+	+
X12 (×2)	2,978	+	+	+	+
X11	2,954	+	+	+	+
X10	2,567	+	+	+	+
X9	2,128	+	+	+	+
X8	1,479	+	+	+	+
X7	825	+	+	+	+
X6 (×2)	795	+	+	+	+
X5	639	+	+	+	−

[a] Designations and sizes of the restriction fragments are obtained by computer-aided analysis of the cpDNA sequence. Fragments smaller than 0.4 kb are not detected on gels, therefore they are not included in this table

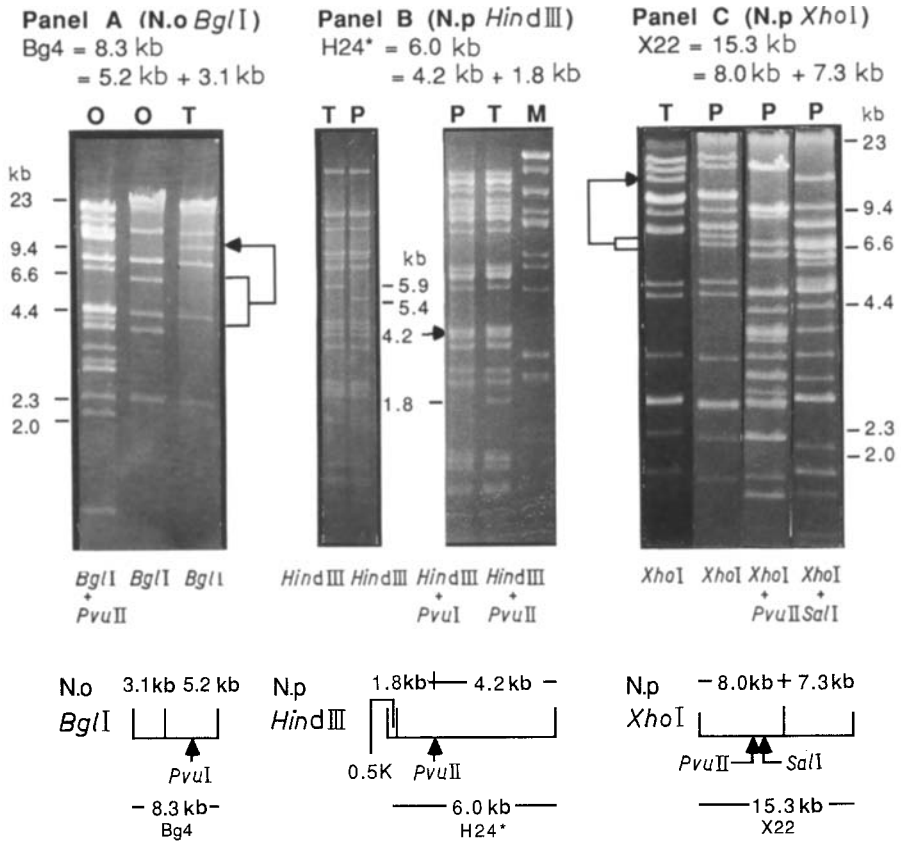

Fig. 2A–C. Determination of subfragment orders by double digestions. cpDNA of *N. plumbaginifolia* and *N. otophora* cpDNA were double digested with restriction enzymes as indicated. Fragments were separated by electrophoresis for 10–15 h in 1.0% agarose gels. Panels **A**, **B**, and **C** indicate the three double digestions by which subfragment order was determined (N.o. *Bgl*I, N.p. *Xho*I, and N.p. *Hin*dIII). Results are summarized at the bottom of the figure. In Panel **B**, the H24* in *N. plumbaginifolia* is homologous to H24 (5,904 bp) in *N. tabacum*, but with a size addition of approximately 80 bp. Therefore, its size is estimated to be about 6.0 kb

Shinozaki et al. (1986a). In the following, descriptions concerning the left and right side of the specific fragment are in accordance with those mentioned above. For instance, the left and right side of the subfragments in *N. otophora* corresponding to B4 in *N. tabacum* are designated as B4L and B4R, respectively.

Figure 2 demonstrates the determination of the subfragment orders by analyzing the restriction patterns obtained by double digestions. The regions concerned are in the *Bgl*I maps of *N. otophora*, as well as the *Xho*I and *Hin*dIII maps in *N. plumbaginifolia*. All of the regions involve an additional restriction site when compared to those obtained in *N. tabacum*. In the *Bgl*I digest of *N. otophora* cpDNA, a fragment corresponding to the B4 band in *N. tabacum* did not exist, while two other smaller fragments were generated instead. Because the sum of their sizes (3.1 and 5.2 kb) is equal to B4 (8.3 kb), the most likely explanation is that an additional restriction site appears in *N. otophora*. It is already known that a *Pvu*II site exists on the right side of B4; therefore, the same cutting site should also exist in one of the subfragments in *N. otophora*. The cpDNA from *N. otophora* was then double digested with *Bgl*I and *Pvu*II, and the resulting fragments were resolved using the single digest of *Bgl*I as a reference (Fig. 2, panel A). The results show that the 3.1-kb fragment remained (indicated by the arrows), while the 5.2-kb fragment disappeared, indicating that the *Pvu*II site was located in the 5.2-kb fragment in *N. otophora*. This leads us to conclude that B4L is 3.1 kb while B4R is 5.2 kb. Likewise, the subfragment orders of H25L (1.8 kb) and H25R (4.2 kb), as well as X22L (8.0 kb) and X22R (7.3 kb) in *N. plumbaginifolia*, are determined (Fig. 2, panels B and C). It should be pointed out that the recognition site of *Xho*I (C/TCGAG) and *Ava*I (C/PyCGPuG) overlap, with *Xho*I being more specific; therefore, all alternations recognized by *Xho*I will be reflected in *Ava*I patterns. Accordingly, an extra *Ava*I site, which split the A68 into a 7.9 and a 2.4-kb fragment in *N. plumbaginifolia* is correspondingly determined.

The other type of mutation involved restriction site eliminations in *N. plumbaginifolia* and *N. otophora* when compared to those in *N. tabacum*. For instance, in comparing the *Xho*I and *Ava*I restriction profiles, larger fragments were found in *N. plumbaginifolia* and *N. otophora*, while other smaller fragments in *N. tabacum* no longer existed. Size calculations indicate that the addition of the smaller fragments eliminated in *N. tabacum* is always equal to those of the larger ones found in *N. plumbaginifolia* and *N. otophora*, suggesting the eliminations of re-

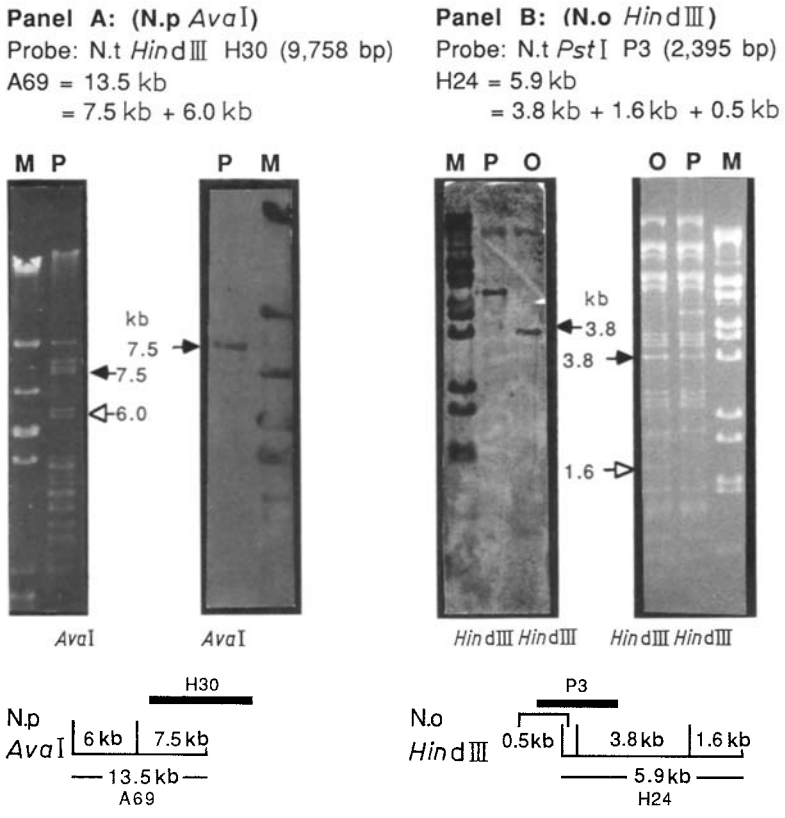

Fig. 3 A, B. Determination of subfragment orders by probe hybridizations. Panels **A** and **B** show the hybridization of probes prepared from *N. tabacum* cpDNA fragments to *Hin*dIII and *Ava*I digests of *N. otophora* and *N. plumbaginifolia* cpDNA, respectively. *Solid arrows* indicate the fragments that could be hybridized by the probes. For experimental details of nonradioactive probe labeling, see 'Materials and methods'

Table 2. Various *Ava*I (C/PyCGGpuG) and *Xho*I (C/TCGAG) restriction site eliminations found in *N. plumbaginifolia* and *N. otophora* compared to those in *N. tabacum*

Nicotiana species	Restriction enzymes	Original fragments found in *N. tabacum* (in bp)	Corresponding fragments generated in *N. plumbaginifolia* and *N. otophora*
N. plumbaginifolia	*Ava*I	A66 (5,704) + A59 (3,829)	9,533
	*Ava*I	A49 (2,638) + A32 (696)	3,334
N. otophora	*Ava*I	A64 (4,385) + A20 (407)	4,792
	*Xho*I	X18 (8,721) + X15 (5,241)	13,962
	*Ava*I	A56 (3,561) + A30 (613)	4,174
	*Xho*I	X17 (8,492) + X5 (639)	9,131

striction sites. This is summarized in Table 2. Due to the overlapping recognition sites of *Ava*I and *Xho*I mentioned above, two pairs of such "common restriction site elimination" are found in *N. otophora* (Table 2). In addition, a *Sal*I site elimination has also been detected in *N. otophora*, which occurs between S2 (2,880 bp) and S5 (12,518 bp) corresponding to the *N. tabacum* map.

In addition to the alternations in *Ava*I and *Xho*I sites listed in Table 2, an additional *Ava*I site that is not recognized by *Xho*I is detected in *N. plumbaginifolia*. This *N. plumbaginifolia Ava*I site is within A69 (13.5 kb) corresponding to the *N. tabacum* map. Due to the complexity of the *Ava*I pattern in *N. plumbaginifolia*, this *Ava*I site cannot be located by fragment size analysis. Therefore, H31 (9,758 bp, overlapping with the right side of A69) was used as a probe to determine the subfragment order within A69 in *N. plumbaginifolia*. The data show that H31 hybridizes with a 7.5-kb *Ava*I fragment in *N. plumbaginifolia*; therefore, the 7.5-kb fragment is identified as A69R (Fig. 3, panel A). The same method was used to construct a *Hin*dIII map of *N. otophora* cpDNA, in which a 3.8-kb *Hin*dIII fragment could be hybridized by the probe P3 (2,395 bp) and is identified as the H25L (Fig. 3, panel B).

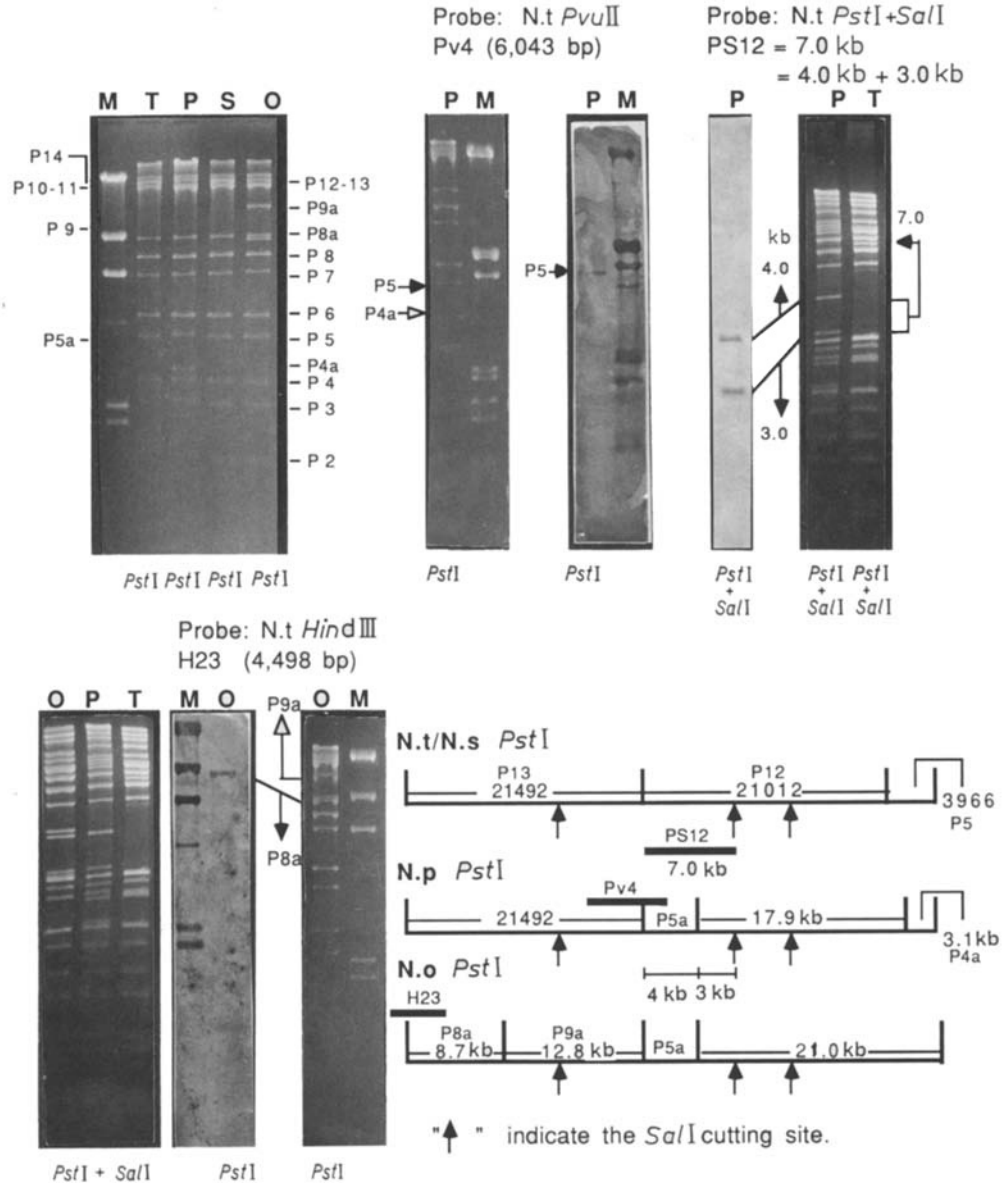

Fig. 4. Construction of *Pst*I maps of *N. plumbaginifolia* and *N. otophora* cpDNA corresponding to the P13-P12-P5 region of *N. tabacum*. Both double-digestion analysis and probe hybridization data are required to resolve these maps. PS12, Pv4, and H23 are the probes used and the results are summarized at the *lower right*. See text for details

The construction of *Pst*I maps is more complicated. The restriction profiles generated by this restriction enzyme are not only different in number but also in staining intensity of bands migrating to the same positions among the *Nicotiana* species examined (Figs. 1 and 4). From the sequence data, it is known that the P12–13 band in *N. tabacum* consists of two fragments, P12 (21,102 bp) and P13 (21,492 bp), which are adjacent to one another on the physical map. Examination of the overall *Pst*I restriction patterns revealed that the P12–13 intensity in *N. tabacum* was twice as high as those in *N. plumbaginifolia* and *N. otophora*; thus, additional *Pst*I restriction sites should be located either P12 or P13 in *N. plumbaginifolia* and *N. otophora*. After double digestion with *Pst*I and *Pvu*II, it became clear that an additional *Pst*I site is located in P12 in *N. plumbaginifolia* (data not shown).

Subsequently, Pv4 (6,043 bp, overlapping with the right side of P13 and the left side of P12) was used as a probe to hybridize with the *Pst*I digests of *N. plumbaginifolia*, and it could recognize the P12–13 band and a fragment with a size of about 4.0 kb, which migrated to the same position as P5 (3,066 bp) on agarose gels. Since Pv4 and P5 are distant from one another, the 4.0-kb fragment is designated P5a and assigned to the leftside of

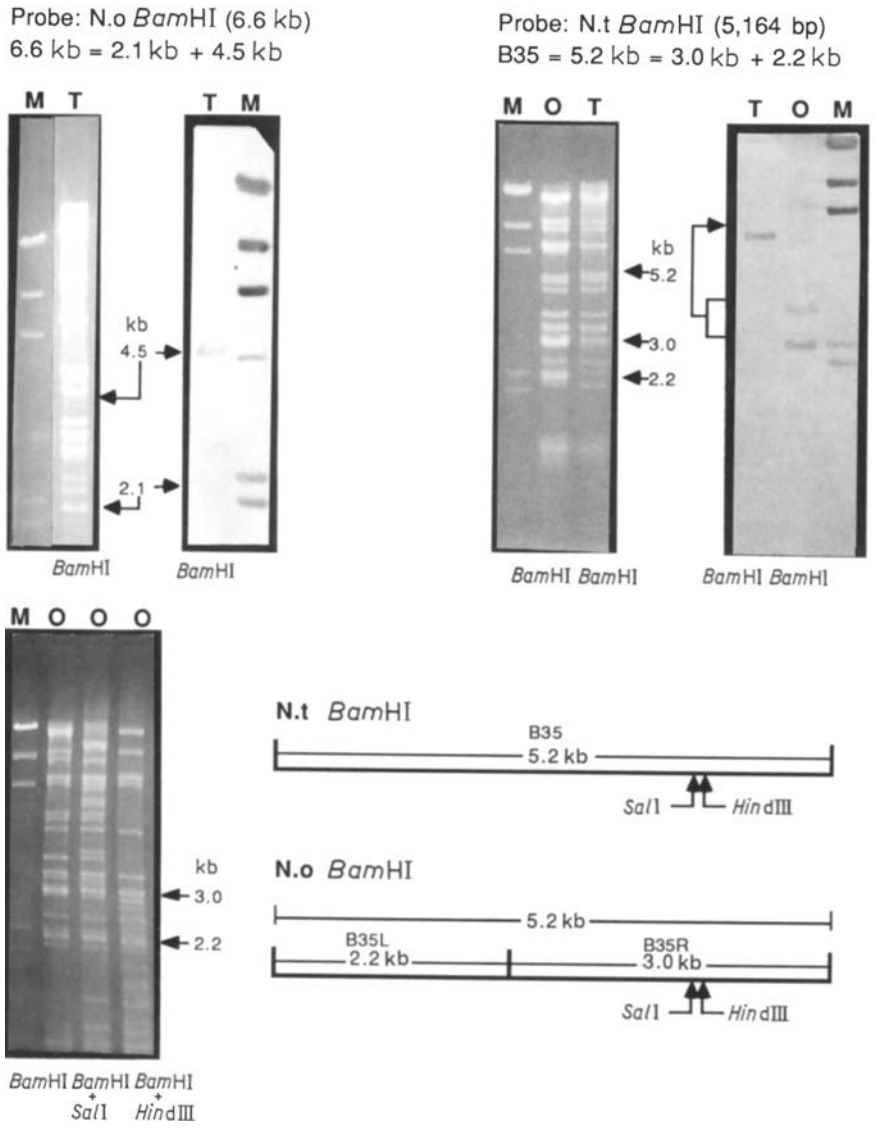

Fig. 5. Double digestion and reciprocal hybridization are combined for the construction of the *Bam*HI map of cpDNA from *N. otophora*. The probes used were the specific fragment of 6.6 kb found in *N. otophora* cpDNA and B35 (5,164 bp) of *N. tabacum* cpDNA

the P12. Another line of evidence to confirm the identity of P5a came from the *Pst*I + *Sal*I double digestion followed by hybridization experiments: a fragment of approx. 7.0 kb (as indicated by an arrow in Fig. 4 and designated as PS12) originally from the left side of P12 in *N. tabacum* was separated into two smaller fragments with lengths of 4.0 kb and 3.0 kb in *N. plumbaginifolia*. Obviously there is a *N. plumbaginifolia*-specific *Pst*I site in the PS12 region and the 4.0-kb fragment is P5a. As also shown in Fig. 4, a 3.2-kb fragment exists in *N. plumbaginifolia* but not in *N. tabacum*. This fragment migrates to a position between P4 (2,876 bp) and P5, (3,966 bp) and was therefore labeled P4a. It was eluted and used as a probe to hybridize with several enzyme digests of *N. tabacum* cpDNA (data not shown), and was found to be derived from P5 in *N. tabacum* with a size reduction of approx. 0.8 kb (3.2 kb versus 3,966 bp). Because a fragment of 0.8 kb was not observed in any of the *Pst*I digests of *N. plumbaginifolia* cpDNA, the most likely explanation is that a new *Pst*I site appears 3.2 kb away from the left end of P5, and the original *Pst*I site between the P12 and P5 in *N. tabacum* was simultaneously erased.

In the case of *Pst*I mapping in *N. otophora*, different features were involved. As mentioned above, at least one of the fragments in P12–13 is cleaved. Moreover, there are two additional bands, which were designated P8a and P9a, with sizes of approx. 8.7 and 12.8 kb, respectively. By the same strategy used above, a double digestion with *Pst*I + *Pvu*II was carried out (data not shown) and it showed that P13 should contain a *N. otophora*-specific *Pst*I site, which splits P13 into P8a and P9a. This result was further substantiated by size summation. The order of these two fragments was obtained by hybridization analysis using H23 (4,498 bp, overlapping with the left-side of P13) as a probe, which was found to recognize the P8a fragment. In addition to a new *Pst*I site within the

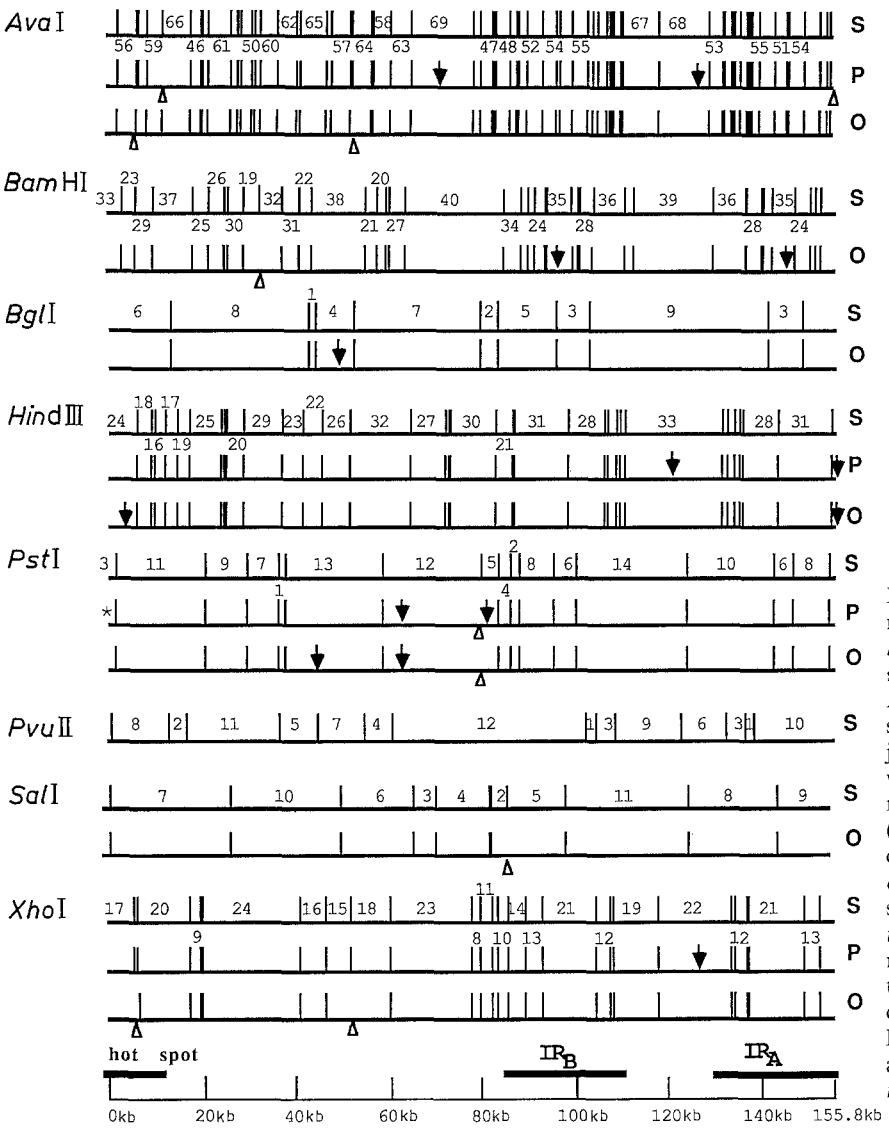

Fig. 6. Restriction maps of chloroplast genome from *N. tabacum*, *N. plumbaginifolia*, *N. sylvestris*, and *N. otophora* with respect to *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I. The maps are presented in linearized form by cutting at the junction (J_{LA}) between IR_A and LSC. J_{LA} was designated as zero and nucleotides are numbered proceeding towards the LSC (Shinozaki et al. 1986a). Only those physical maps of *N. plumbaginifolia* and *N. otophora* that bear site variations with respect to *N. tabacum* (and hence *N. sylvestris*) are listed. *Solid* and *open arrows* mark, respectively, the addition and the elimination of cleavage sites detected in the cpDNA molecules. The *asterisk* indicates P3 in *N. plumbaginifolia* cpDNA, which is approximately 80 pb larger than that in *N. tabacum*

P13 region of *N. otophora*, the *Pst*I + *Sal*I double digestion of the cpDNA showed the 3.0 kb and a 4.0 kb bands identical to those found in *N. plumbaginifolia*. These results indicated that P5a (therefore as the *Pst*I site on the left end of P5a) also exists in *N. otophora* and the new site should split the P12 into a 4.0-kb (P5a) plus a 17.0-kb fragment. However, no fragment with a size of 17.0 kb was detected, while a band of 21.0 kb still existed at P12–12. This result can be explained by the elimination of the *Pst*I site between P12 and P5, resulting in another fragment of 21.0 kb in *N. otophora*, which is partially overlapped by the P12 in *N. tabacum*. The region corresponding to the *N. tabacum* P13-P12-P5 section of the *Pst*I map in *N. plumbaginifolia* and *N. otophora* is summarized as a schematic drawing in Fig. 4.

During the course of the present study, some previously published mapping data were found to be inconsistent with the physical maps derived from the cpDNA sequence. For instance, inconsistency in the large, single-copy region was found in the *Bam*HI map of *N. otophora* described by Kung et al. (1982). The *N. otophora*-specific fragment with a size of 6.6 kb was then labeled and used as a probe to hybridize with the *Bam*HI digest of cpDNA from *N. tabacum*. The results show that this fragment derived from the cutting site elimination between B32 (4,537 bp) and B19 (2,098 bp) (Fig. 5). It was also found that the segment corresponding to B35 (5,164 bp) in *N. tabacum* cpDNA no longer existed in *N. otophora* and appeared as two fragments with sizes of 3.0 and 2.2 kb. Again, the subfragment order was determined by double

Table 3. Physical maps of cpDNA from *Nicotiana* species completed in the present study and in previously published studies

Nicotiana species	*Ava*I	*Bam*HI	*Bgl*I	*Hin*dIII	*Sal*I	*Sma*I	*Pst*I	*Pvu*II	*Xho*I
N. accuminata	−	−	+[a]	−	+[a]	+[c]	−	+[a]	−
N. knightiana	−	+[c]	−	−	−	+[c]	−	−	−
N. longsdorffii	−	−	−	−	−	+[c]	−	−	−
N. otophora	+	+[b]	+	+	+	+[b]	+	+	+
N. plumbaginifolia	+	+	+	+	+	+[c]	+	+	+
N. sylvestris	+	+	+	+	+	+	+	+	+
N. tabacum[d]	+	+	+	+	+	+	+	+	+
N. tomentosiformis	−	−	−	−	−	+[c]	−	−	−

[a] Shen et al. (1982)
[b] Zhu et al. (1982)
[c] Tassopulu and Kung (1984)
[d] The complete sequence of cpDNA was determined by Shinozaki et al. (1986a, b) and some of the maps are contructed by computer-aided sequence analysis

digestion and the results indicated that the 2.2-kb fragment is B35L and the 3.0-kb fragment is B35R (Fig. 5). In addition, the locations of P6 and P9 in the *Pvu*II maps of *N. tabacum* presented by Fluhr and Edelman (1981) were found to be assigned in reverse.

According to the evidence presented above and other published data, physical maps of *N. sylvestris* (same as *N. tabacum*), *N. plumbaginifolia*, and *N. otophora* cpDNA with respect to *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I are summarized in the form of linear schematic drawings (Fig. 6). In these maps, those of *N. sylvestris* are deemed to be identical to those of *N. tabacum*, because the restriction profiles of these two species are identical with respect to all the enzymes studied. Likewise, the *Pvu*II maps of all species are deemed to be the same. The *Bam*HI and *Sal*I maps were obtained by Kung and coworkers (Zhu et al. 1982; Tassopulu and Kung 1984) are corrected and included for comparative analysis.

Discussion

An efficient procedure to construct the physical maps of *Nicotiana* cpDNA has been established, using the sequence data of *N. tabacum* cpDNA as a guide. Its efficiency was demonstrated by the construction of the cpDNA maps of *N. sylvestris*, *N. plumbaginifolia*, and *N. otophora* with respect to eight restriction endonucleases; *Ava*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I. Although many cpDNA physical maps in *Nicotiana* are already availabe, as indicated in Table 3, the data were mainly concentrated in *N. tabacum* (Fluhr and Edelman 1981; Seyer et al. 1981; Hildebrand et al. 1985). For other species in same genus, the data were rather incomplete and scattered (Kung et al. 1982; Zhu et al. 1982; Tassopulu and Kung 1984).

In general, the construction of physical maps of cpDNA was accomplished by analyzing the restriction patterns generated by multiple digestion of the cpDNA molecules with two or more restriction endonuclease, followed by reciprocal hybridization between the restriction fragments obtained (Palmer 1986). There, in order to acquire a specific map, individual fragments generated by two or more restriction enzymes must be isolated, cloned, and made into radioactive probes. The procedure is both arduous and time-consuming. Moreover, if several fragments with similar sizes migrated as a single band on the agarose gels, erroneous assignments would result. Since the cpDNA sequence of *N. tabacum* is now available and physical maps with respect to any restriction enzyme can be obtained by computer analysis, it is possible to locate the varied restriction sites by comparing the restriction profile of one species with that of *N. tabacum*. Subsequently, relevant physical maps can be constructed by choosing appropriate fragments from *N. tabacum* as probes to derive the altered restriction sites with accuracy and efficiency. Although maps of only three species were presented, this approach should be applicable to the construction of cpDNA maps of all other *Nicotiana* species or to other genera in which one of the cpDNA sequence is determined, such as rice (Hiratsuka et al. 1989) and liverwort (Ohyama et al. 1986).

Restriction pattern is an excellent molecular marker for the identification of cpDNA in somatic hybrids of plants, including *Nicotiana* (Belliard et al. 1978). However, it is arduous to screen a large amount of somatic hybrids by purifying the chloroplasts from each hybrid lines and subsequently extracting their DNAs for restriction analysis. The task will become much easier if physical maps with respect to several restriction enzymes are available. In our particular case, comparative analysis of the physical maps revealed that certain cpDNA restriction fragments from *N. tabacum* could be used as a probe

to distinguish cpDNA from *N. tabacum* (or *N. sylvestris*), *N. plumbaginifolia*, and *N. sylvestris* without purification of their chloroplasts and cpDNA. For example, chloroplast genomes of these three species demonstrate distinct site (and thus fragment size) alterations over the regions of H24 in the *Hin*dIII map, while P3, one of the bands after *Pst*I digestion which is located at the position corresponding to H24 (see Fig. 5), could be cloned and labeled for subsequent hybridization to restriction endonuclease-treated total cellular DNA. By eliminiating the chloroplast purification step, labor as well as time is saved. Furthermore, only very little starting material is needed for this purpose. Nevertheless, this method would provide unambiguous identification of cpDNA in these species or somatic hybrids derived from them.

Aside from greatly assisting in the construction of phylogenetic trees, restriction maps of cpDNA could also help to localize the taxonomic position of specific species during the evolutional process (Kung et al. 1982; Palmer et al. 1983; Perl-Treves and Galun 1985). Limited by the small number of species involved in this study, only a little of such information was obtained. However, the wealth of restriction site variation in *N. otophora* cpDNA compared to *N. plumbaginifolia* and *N. sylvestris* (same as *N. tabacum*) suggests that *N. otophora* is distantly related to the other three species, while *N. sylvestris* and *N. tabacum* show the same restriction patterns in all restriction enzymes examined, indicating these two species are closely related. This observation is in accordance with the hypothesis that *N. tabacum* is the progeny of a cross between *N. sylvestris* and *N. tomentosiformis* (Kung et al. 1982). Moreover, it was shown that *Sma*I patterns of cpDNA from *N. tabacum* and *N. tomentosiformis* are different (Tassopulu and Kung 1984). Therefore, the data attest that *N. sylvestris* is the maternal and *N. tomentosiformis* is the paternal parent of *N. tabacum*. This argument is also supported by other cytogenetic (Goodspeed 1954) and biochemical (Gray et al. 1974; Chen et al. 1976) studies.

Our data and previous studies (Zhu et al. 1982; Tassopulu and Kung 1984) show that restriction site variations in *Nicotiana* cpDNA are generally derived from base-pair mutations which create or eliminate a cutting site. The distribution of differences in restriction sites varies quite a bit, depending on the enzymes used. In the present study, all site alterations were found beyond the inverted repeats but site variation in this region (a *N. otophora*-specific *Bam*HI site) was reported (Tassopulu and Kung 1984). On the other hand, we detected a *N. plumbaginifolia*-specific *Ava*I site in the small, single-copy region which is considered to be highly conserved (Tassopulu and Kung 1984). Therefore restriction site variation is observed over the span of the cpDNA molecule, only with different frequency over each localized region. For example, at least seven variations are located in the LSC (86,7 kb) within 13 kb to J_{LA}. This region was designated as the "hot spot" (Kung et al. 1982; Tassopulu and Kung 1984). However, at least five variations are detected on the other side of the LSC (within 14 kb to J_{LB}). In comparison with the IRs (25.3 kb each) and the SSC region (18.5 kb), where only one site change was detected in each, the region close to the junction between IR_B and LSC could possibly be classified as a "second hot spot." It is noteworthy that the 80-bp addition in *N. plumbaginifolia* was also situated in the "hot spot;" thus, it would be of interest to determine whether such regions contain structures or other specific sequences that could favor rearrangement.

Acknowledgements. We thank Ms. K. L. Chan for her excellent technical assistance. This work was supported by grants from the National Science Council, Republic of China, to Y. K. L. (NSC78-0203-B007-09 and NSC79-0203-B007-02).

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