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Nicotiana chloroplast genome

6. Deletion and hot spot – a proposed origin of the inverted repeats

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Summary. A physical map containing six restriction sites of the *Nicotiana tabacum* chloroplast genome, together with the BamHI maps of *N. tabacum, N. otophora* and *N. knightiana,* and the SmaI maps of *N. acuminata, N. plumbaginifolia, N. langsdorffii, N. otophora, N. tabacum, N. tomentosiformis* and *N. knightiana* was constructed. In *Nicotiana* chloroplast genomes, the most frequently observed variations are point mutations. Deletions are also detected. Most of the observed changes are confined to one area of the large single copy region, which is designated as the "hot spot". Based on the evidence obtained from *Nicotiana* chloroplast genomes, an origin of the inverted repeats in this genus is proposed. We suggest that the inverted repeats represent a vestige of what were once two identical, complete chloroplast genomes joined together in a head-to-head and tail-to-tail fashion, and that deletions generated the current chloroplast genome organization.

Key words: *Nicotiana* – Chloroplast DNA – Deletion – Restriction – Hot spot – Inverted repeats

Introduction

In *Nicotiana* it appears that both nuclear and chloroplast genomes share a similar mechanism for structural alterations. *Nicotiana* chloroplast genomes exhibit a high degree of variability. EcoRI restriction fragment patterns, for example, are species specific (Kung et al. 1982). The most frequently observed variations in the *Nicotiana* chloroplast genome are point mutations and deletions or insertions (Kung et al. 1982; Shen et al. 1982). Many of the detected changes of restriction sites are probably the result of point mutations. This is indicated by the observations that there are frequent gains and losses of restriction sites (Kung et al. 1982). Deletions or insertions cause more drastic changes in structure and genome size than do point mutations. All of the observed mutations, whether point mutations, insertions, or deletions are not widely scattered along the circular molecule of the *Nicotiana* chloroplast genome, but rather are confined in a few regions.

A small deletion, which was localized in the same region where an extra segment was discovered in *N. acuminata* (Shen et al. 1982), was discovered in *N. knightiana* and *N. paniculata.* This, together with the comparative studies on the restriction map of many *Nicotiana* species, demonstrates that alterations occur mostly in the large single-copy region, particularly near the right-hand border of an inverted repeat. This region has been designated as the "hot spot" in the *Nicotiana* chloroplast genome (Kung et al. 1982). In this communication strong supportive evidence for such a designation is provided. The biological implication of these changes in view of molecular evolution is discussed and, from this, the origin of the inverted repeats is proposed.

Materials and methods

Plant material

Seeds from *N. tabacum* (V. Maryland 609), *N. tomentosiformis, N. knightiana,* and *N. paniculata* were grown in soil in a greenhouse. Seedlings having 4–6 leaves were transplanted into 5-inch soil pots and allowed to grow for 4 to 8 weeks after transplanting, depending on the season. Before harvest of leaf tissue, the plants were placed in the dark for 48 h to deplete them of starch.

Isolation of chloroplast DNA

Chloroplasts were purified by the procedure previously described by Rhodes and Kung (1981). Freshly harvested leaves were homogenized in a metal Waring blender with liquid nitrogen. Purified chloroplasts were lysed in 2% sarkosyl and DNA was isolated according to the method of Kolodner and Tewari (1975).

Restriction endonuclease digestion: agarose gel electrophoresis: physical map of chloroplast DNA

Chloroplast DNA was digested with the endonucleases BglI, PvuII, SmaI, SalI, XhoI, and BamHI as directed by the supplier, New England Biolabs. Two procedures for double digestion with SalI/PvuII and SalI/XhoI were utilized. In the first method both enzymes were used simultaneously in the buffer for the enzyme which tolerated the lowest salt concentration. The second method involved sequential digestion; the second enzyme was added without additional buffer. 0.8% and 1.2% horizontal agarose slab gels were used to separate the restriction fragments according to the procedure of Helling et al. (1974). The EcoRI and HindIII single or double digest fragments of lambda DNA were used as molecular weight markers. Ethidium bromide-stained bands were visualized with a model C-62 Chromatovue and transilluminator (Ultra-Violet Product, Inc.). The physical map of *N. tabacum* constructed by Seyer et al. (1981) with SalI, BglI, XhoI, and PvuII was used in the construction of the map of *N. knightiana.* Also, the physical map for *N. otophora* constructed by Zhu et al. (1982) with SmaI and BamHI was used in the construction of the maps of *N. tabacum* and *N. knightiana.*

Nick translation and hybridization

Some ct-DNA fragments were recovered from the gels according to Dretzen et al. (1981) and labelled with [2d-^{32}P] dATP by the nick-translation reaction of Maniatis et al. (1975). These were used as probes to hybridize BamHI and SmaI fragments of *N. tabacum, N. otophora* and *N. knightiana* ct-DNA blotted on nitrocellulose paper according to Smith and Summers (1980).

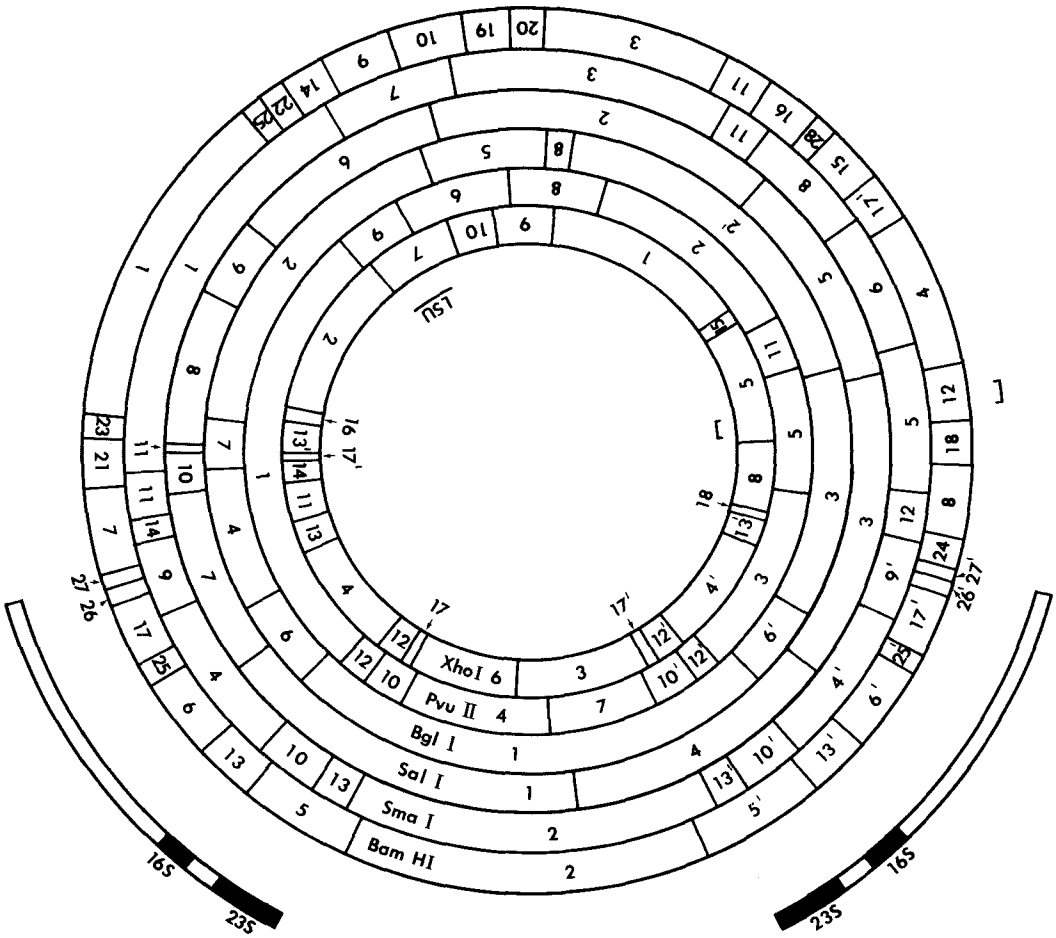

Fig. 1. The restriction map of the *N. tabacum* chloroplast genome constructed with BamHI, SmaI, BglI, PvuII and XhoI. The large and small single-copy regions are separated by the inverted repeats as marked. The position of the genes for 23S and 16S and the large subunit (*LSU*) of RuBPCase are marked. The narrow, bracketed region designates the location of the deletion detected in *N. knightiana* (Fig. 2)

Results

The construction of XhoI, PvuII, BglI, SalI, SmaI and BamHI restriction map of N. tabacum ct-DNA

Physical maps of the *N. tabacum* chloroplast genome using selected restriction enzymes were recently constructed by various workers. By combining the physical maps of *N. tabacum* (Seyer et al. 1981; Jurgenson and Bourque 1981) and *N. otophora* (Zhu et al. 1982), a map consisting of the restriction sites of six restriction enzymes was constructed for *N. tabacum* (Fig. 1). The physical map of Seyer et al. (1981) contained the restriction sites of XhoI, PvuII, BglI and SalI, while that of Jurgenson and Bourque (1981), extensively modified according to our results, included that of SalI and SmaI. The *N. otophora* map was made with SmaI and BamHI enzymes (Zhu et al. 1982). Because *N. tabacum* and *N. otophora* ct-DNA share all but one SmaI site (Zhu et al. 1982), it was possible to incorporate the *N. tabacum* map of Seyer et al. (1981) and the *N. otophora* map of Zhu et al. (1982) into one map for *N. tabacum* via the *N. tabacum* map of Jurgenson and Bourque (1981).

Figure 1 illustrates some unique features of the *Nicotiana* chloroplast genome. One is that the distribution of various restriction sites is not entirely at random. There are regions where fewer restriction sites exist than others. The most obvious region is the small single-copy region, where only one or two restriction sites are found for all six enzymes used. Another feature is that in the inverted repeats complete symmetry of restriction sites can be generated only by BamHI, SmaI and XhoI, which cleave at more restriction sites than SalI, PvuII and BglI.

The location of the gene of the large subunit (LS) of RuBPCase is indicated in Fig. 1. Among the six restriction enzymes used, only BamHI cut this gene into two unequal fragments. This was recently verified by the DNA sequence data of Shinozaki and Sugiura (1982).

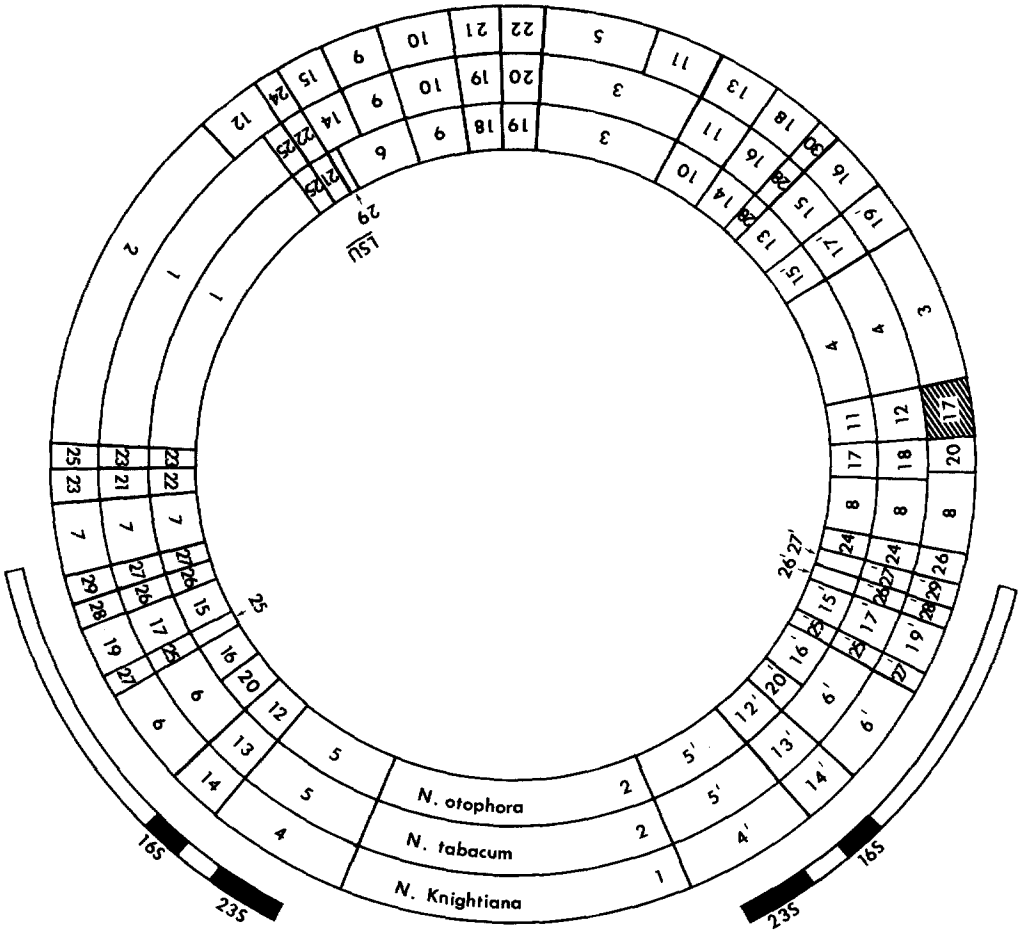

Fig. 2. Comparison of physical maps of BamHI restriction sites in *N. knightiana, N. tabacum* and *N. otophora* chloroplast genomes. The positions of the genes for ribosomal RNA, the large subunit (*LSU*) of RuBPCase and the regions of inverted repeats are marked. Fragment No. 17 of *N. knightiana* (*shaded*) was identified as the site of a small deletion

A similar case was also found in *N. otophora* (Zhu et al. 1982).

Comparison of the BamHI restriction maps of N. tabacum, N. otophora, and N. knightiana and the detection of a deletion

The BamHI comparison map of *N. otophora, N. tabacum* and *N. knightiana* is shown in Fig. 2. The physical BamHI map of *N. otophora* presented here is different from the one originally constructed by Zhu et al. (1982) due mainly to the change of numbering systems. In the original map fragment 10 is a doublet, which was designated as 10 and 10′. In this study 10 and 10′ were separated on a gel and therefore are numbered as 10 and 11. All the fragments numbered after 10 in the original map are re-numbered here and are one integer higher. In addition, there are also some slight alterations as dictated by some new evidence obtained from this study. In one case the original order of 20, 25, and 16 in the inverted repeat was re-arranged to 20, 16, and 25. In another case, the sequence of 17 and 11 was reversed to 11 and 17 because fragment 17 corresponds to XhoI fragment 8 (Fig. 1). Fragment 29 was deleted from the original map but is included here.

There are three regions in the BamHI map where differences between *N. tabacum* and *N. otophora* are evident (Fig. 2). Two of the three regions are located in each of the inverted repeats in which an additional site was observed in *N. otophora.* These sites divide the *N. tabacum* BamHI fragments 6 (5.1 kb) and 6′ (5.1 kb) into *N. otophora* BamHI fragments 20 (2.2 kb) and 16 (2.8 kb), and 20′ (2.2 kb) and 16′ (2.8 kb), respectively (Fig. 2). The third region is in the large single-copy region near the LS gene. Apparently the BamHI restriction sites between fragments 9 (4.5 kb) and 14 (3.1 kb), and 14 and 22 (2.05 kb) of *N. tabacum* are rearranged in *N. otophora,* generation BamHI fragments 6 (6.6 kb), 29 (1.12 kb), and 21 (2.16 kb). This rearrangement of restriction sites has no effect on genome size of the two species. When a similar comparison was made between the BamHI maps of *N. tabacum* and *N. knightiana* three different regions containing variable BamHI sites were apparent (Fig. 2). In contrast to those of *N. otophora,* all three regions in *N. knightiana* are located in the large single-copy region. One in-

Table 1. Fragment sizes (Kb) and stoichiometries** of *N. tomentosiformis,* and *N. knightiana* generated by SmaI

Fragment no.	*N. tomentosiformis*	*N. knightiana*
1	28.0	28.0
2	25.8	25.8
3	17.4	17.4
4	11.2**	11.2**
5	10.7	10.7
6	9.5	8.9
7	8.6	8.6
8	8.5	8.5
9	7.9	7.9
10	6.5	6.5
11	4.0**	4.0**
12	3.3	3.3
13	1.95**	1.95**
Total	160.5	160.0

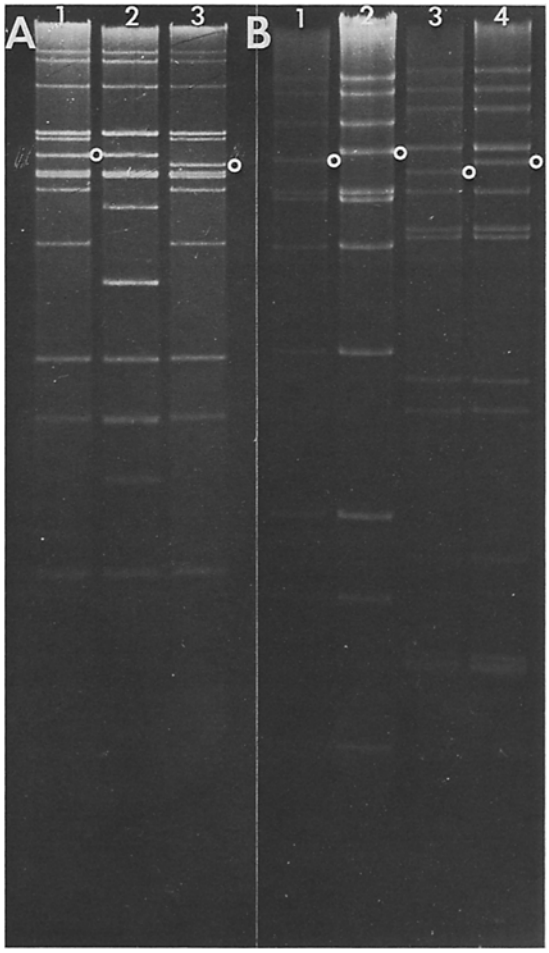

Fig. 3. A Restriction fragment patterns of *Nicotiana* ct-DNA generated by SmaI; *1 N. tomentosiformis, 2 N. tabacum* and *3 N. knightiana. N. tomentosiformis* and *N. knightiana* ct-DNA share identical SmaI restriction fragment patterns except for fragment No. 6 (⊙). In *N. knightiana* this fragment is smaller than in *N. tomentosiformis* (see Fig. 6 for detailed SmaI map of all these three species). **B** Restriction fragment patterns of Nicotiana ct-DNA generated by PvuII (*1* and *2*) and XhoI (*3* and *4*); from *N. knightiana* (*1* and *3*) and *N. tabacum* (*2* and *4*). The size of fragment No. 5 in both PuvII and XhoI digests of *N. knightiana* ct-DNA (*1* and *3*) is notably smaller than that of *N. tabacum* (*2* and *4*). The remaining fragments are identical

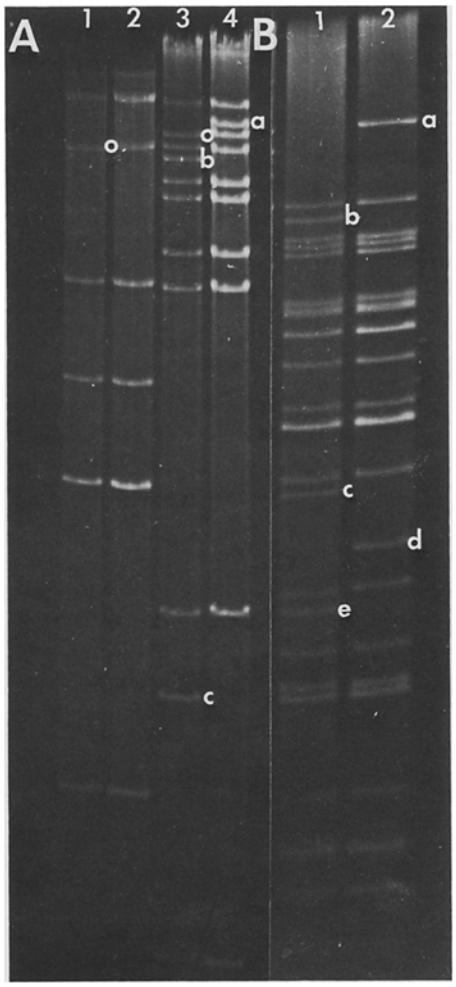

Fig. 4. **A** BglI (*1* and *2*) and SalI (*3* and *4*) restriction fragment patterns of *N. knightiana* (*1* and *3*) and *N. tabacum* (*2* and *4*) ct-DNA. *N. knightiana* and *N. tabacum* ct-DNA share identical BglI restriction fragment patterns except that the size of fragment No. 3 of *N. knightiana* is reduced (*o*). In the case of SalI treatment (*3* and *4*), a slight size reduction in fragment No. 2 *N. knightiana* (*o*) is detected upon comparison with the corresponding *N. tabacum* fragment (*o*). The single additional SalI site present in *N. knightiana* ct-DNA cleaves fragment (*a*) of *N. tabacum* into fragments (*b*) and (*c*) of *N. knightiana.* **B** XhoI/SalI double digestion of *N. knightiana* (*1*) and *N. tabacum* (*2*) ct-DNA. The extra SalI site (see **A** (*3*)) in *N. knightiana* cleaves the largest fragment (*a*) of *N. tabacum* ct-DNA into fragments (*b*) and (*c*) of *N. knightiana* ct-DNA. The size reduction of *N. knightiana* ct-DNA resulted in the shift of fragment (*d*) of *N. tabacum* ct-DNA to fragment (*e*) of *N. knightiana* ct-DNA

cludes the LS gene where BamHI fragments 22 (2.03 kb), 25 (1.32 kb) and 1 (21.5 kb) of *N. tabacum* are replaced by *N. knightiana* BamHI fragments 24 (2.03 kb), 12 (3.40 kb) and 2 (19.2 kb). In the second region a gain of a single BamHI site simply divides fragment 3 (10.4 kb) of *N. tabacum* into fragments 11 (6.3 kb) and 5 (3.90 kb) of *N. knightiana.* In every case there is no actual change of the sub-total of fragment size except for experimental errors in estimation.

The most interesting change is observed in the third region, where a deletion occurred in *N. knightiana.* Consequently, BamHI fragment 12 (3.45 kb) of *N. tabacum* was reduced in size and became BamHI fragment 17 (2.94 kb) in *N. knightiana.* A segment of 0.51 kb was deleted. This deletion was also detected in the SmaI restriction patterns of *N. knightiana* and *N. tomentosiformis,* which share identical fragment patterns except for fragment 6 (Fig. 3 A and Table 1). The SmaI fragment 6 of *N. knightiana* is smaller than SmaI fragment 6 of *N. tomentosiformis* by approximately 0.5 kb. It is evident that a segment of this size has been deleted from *N. knightiana.* Various other restriction enzyme digests performed to verify the existence of this deletion yielded confirmatory results (Figs. 3–4).

Location and demonstration of the deletion

Figure 2 illustrates the location of the deletion in the BamHI map of *N. knightiana.* The shaded BamHI fragment 17, where a 0.5 kb segment was deleted in *N. knightiana,* corresponds to BamHI fragment 12 of *N. tabacum* and is contained in *N. tabacum* ct-DNA fragments SmaI-5, SalI-3, BglI-3, PuvII-5, and XhoI-5. These fragments are all located in the same region, as marked in Fig. 1 (□). By comparing the PuvII and XhoI fragment patterns between *N. tabacum* and *N. knightiana,* it is clear that a segment is deleted from *N. knightiana* fragments PvuII-5 and XhoI-5 (Fig. 3 B as marked). Since the sizes of *N. knightiana* fragments PvuII-5 and XhoI-5 are 12.1 and 11.75 kb, respectively, a reduction of 0.5 kb or 4% can be easily detected in the shift of fragment mobility. In the case of BglI and SalI digests, a 0.5 kb reduction from 19.7 kb (BglI-3) and 22.2 kb (SalI-2) of *N. knightiana* is only barely detectable even when an extra-long gel is used (Fig. 4 A as marked). Nevertheless, the location of this small deletion on the *N. knightiana* chloroplast genome is confirmed by using 6 different restriction enzymes in single or double digestions.

Figure 4 B is the double digestion (SalI/XhoI) pattern of *N. tabacum* and *N. knightiana* ct-DNA in which 5 different fragments between these two species are marked (a–e). All of the different fragments were generated either by an extra SalI site (Fig. 4A) or by a deletion in *N. knightiana.* This extra SalI site is responsible for the cleavage of fragment (a) in *N. tabacum* into fragments (b) and (c) in *N. knightiana* (Fig. 4 B). The reduction in size of fragment (d) in *N. tabacum* to fragment (e) in *N. knightiana* is due to a deletion. In all cases, the size of the deleted segment is

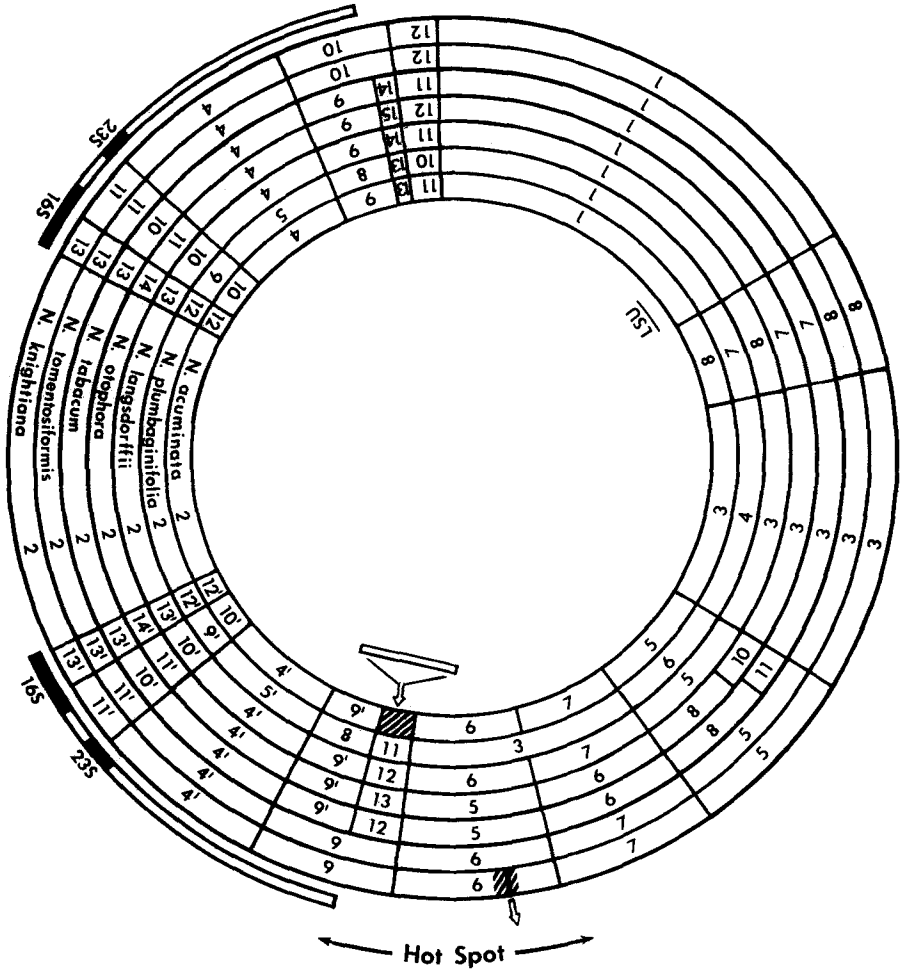

Fig. 5. The SmaI restriction map of ct-DNA from seven *Nicotiana* species. The variations of SmaI sites are clustered in one area of the large single-copy region in which the deletions (0.5 kb) in *N. knightiana* (*shaded*) and extra segment (11 kb) in *N. acuminata* (*shaded*) are identified. This region is designated as the "hot spot"

estimated to be 0.5 kb and contains no restriction sites for BamHI, SmaI, SalI, BglI, PuvII or XhoI, since the total number of cleavage sites for all enzymes remains unchanged. A similar deletion was also detected in *N. paniculata,* a species closely related to *N. knightiana* (Goodspeed 1954). Reciprocal molecular hybridization experiments using Southern blotting proved that the diminished size of SmaI-6 was due to deletion and not to translocation or any other form of re-arrangement.

Identification of hot spots

When the SmaI sites were used to construct the physical map for seven *Nicotiana* species, as shown in Fig. 5, regions of variations were observed. The variations consist mainly of gains or losses of SmaI sites and are located exclusively in the large single-copy region. Two of the four variations are on the end of this region adjacent to the inverted repeats. Furthermore, three of the four variations are confined in the same area where deletions are detected. There is no variation and no SmaI site in the small single-copy region. As depicted in the diagrammatic representation of Fig. 6, it is very clear that a deletion of approximately 11 kb from the genome of 171 kb of *N. acuminata* generated the 160.5 kb genome of *N. tabacum.* A second deletion of 0.5 kb from *N. acuminata* or from *N. tabacum* gave rise to the 160.0 kb genome of *N. knightiana.* The two deletions occurred in locations which are within Sal-3, and Bgl-3 of *N. tabacum* and are 5–6 kb apart. These locations are now identified as a "hot spot" as previously suggested.

Proposal: origin of the inverted repeats

Since *N. acuminata* is an ancient species (Goodspeed 1954), it is tempting to speculate that the *N. acuminata* genome size may represent an ancient molecular dimension and that the sizes of *N. tabacum* and of *N. knightiana* were enclosed by successive deletions. Deletion may constitute a major force in *Nicotiana* chloroplast genome evolution. Based on this, the origin

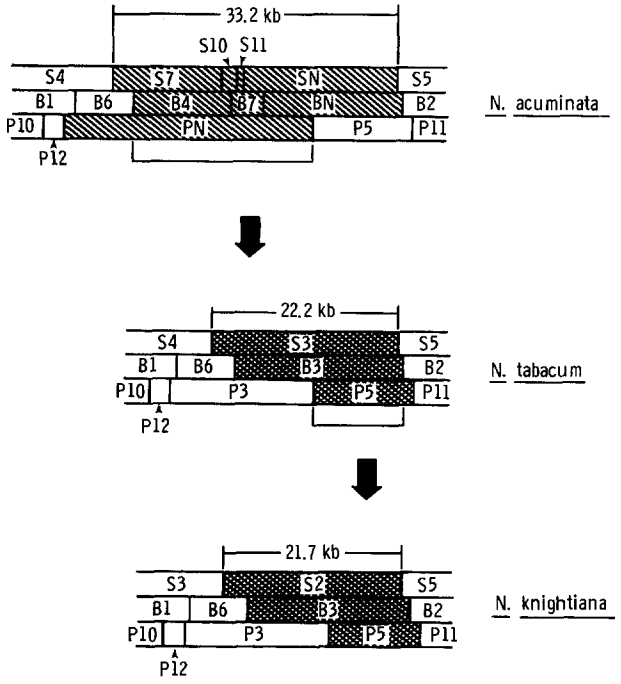

Fig. 6. Diagrammatic representation of the hot spot illustrating a scheme of possible consecutive deletions in *Nicotiana* chloroplast genomes. *N. acuminata* represents the largest *Nicotiana* chloroplast genome. A deletion of 11 kb from the shaded SalI region of 32.2 kb reduces SalI fragments S7, S10, S11, and SN to a single S3 fragment in *N. tabacum*. The first deletion is localized within the marked area and contains three SalI sites. A further deletion of 0.5 kb in S3 fragment of *N. tabacum* generates the genome of *N. knightiana*. The second deletion is identified within the fragments of S3, B3 and P5 as marked. The sites of these two deletions are estimated to be within 5–6 kb. *P* = PuvII; *S* = SalI; *B* = BglI

of the inverted repeats is proposed. The genome size of *N. acuminata* is 7% larger than all other *Nicotiana* chloroplast genomes so far examined (Shen et al. 1982). The fragment responsible for this increase in size is located in the large single-copy region near the right-hand border of one inverted repeat, distant from the LS gene. The presence of this segment near one inverted repeat results in an enlargement of the segment which extends the symmetry of the inverted repeats in *N. acuminata* (Shen et al. 1982). This led to the proposal that the present chloroplast genomes may be evolved from a much larger one that consisted of two identical copies of chloroplast genomes joined together in a head-to-head and tail-to-tail fashion – a total-genome inverted repeat. During the course of evolution, the size of the chloroplast genome may have been reduced by the occurance of repeated deletions in some defined regions. One such region could be the hot spot. This would give rise to the current chloroplast genome organized in the fashion in which there are large and small single-copy regions separated by the inverted repeats. These inverted repeats may be a vestige of what once was two full chloroplast genomes. The extra segment in *N. acuminata* and the portion of the inverted repeat adjacent to it may be relics of one copy of the two originals that remained after a series of deletions in that copy. A complete deletion of one of the two original copies of a chloroplast genome would generate a much smaller genome size with no inverted repeat. If this proposal is correct, then one would expect to find a correlation between the size of the inverted repeats and total genome size. Indeed, there is

Table 2. The chloroplast DNA genome size (Kb) of higher plants and the corresponding size of inverted repeat

Species	Genome size	Inverted repeat		Reference
		Size	% of total	
Spirodela oligorrhiza (duckweed)	184	25.7	14	Van Ee et al. (1980)
Zea mays (corn)	136.1	22.5	16	Kolodner and Tewari (1979)
Triticum eastivum (wheat)	135	21.0	16	Bowman et al. (1981)
Hordeum vulgare (barley)	133	20.9	16	Poulsen (1983)
Oenothera	153	22.7	15	Seyer et al. (1981)
Spinacia oleracea (spinach)	153.5	29.4	16	Kolodner and Tewari (1979)
Lactuca sativa (lettuce)	156.5	24.4	16	Kolodner and Tewari (1979)
Sinapis alba L. (mustard)	158	23.7	15	Link et al. (1981)
Vigna radiata (mung bean)	150	23	15	Palmer and Thompson (1981)
Petunia hybrida (petunia)	150	> 15	> 10	Bovenberg et al. (1981)
Glycine max (soy bean)	151	23.9	16	Chu and Tewari (1982)
Nicotiana tabacum (tabacco)	160	> 20.4	13	Seyer et al. (1981)
	160	24.0	15	Fluhr and Edelman (1981)
Nicotiana acuminata	171	28.0	16	Shen et al. (1982)
Pisum sativum (pea)	120	–		Palmer and Thompson (1981)
Vicia faba (broad bean)	121	–		Koller and Delius (1980)
Cicer arietinum (chick pea)	119	–		Chu and Tewari (1982)

evidence to support this. Kolodner and Tewari (1979) observed that the inverted repeats represent a constant portion of the entire chloroplast genome. Regardless of the variation of genome size, the inverted repeat always constituted approximately 16% of the native length of the chloroplast genome (Table 2). The clear examples are spinach and corn chloroplast genomes which were measured by EM as 153.5 and 136.1 kb, respectively. In each case, 16% of the total length (24.4 and 22.5 kb) is an inverted repeat. In *N. acuminata,* as well as in duckweed, the sizes of the inverted repeats and their corresponding chloroplast genomes are proportionally larger. Likewise, when one of the inverted repeats is absent from a species, the genome size is reduced by approximately the size of one copy in an inverted repeat (20–25 kb) to about 120 kb. There are chloroplast genomes such as pea, chickpea and broad bean which do not possess such structures and have a genome size of about 120 kb (Chu and Tewari 1982; Palmer and Thompson 1981; Koller and Delius 1980).

Discussion

Nicotiana species, as well as most other higher plant species, contain large (approximately 160 kb) circular molecules of ct-DNA organized into four distinct regions. The inverted repeats are separated by a small and a large single-copy region. The small single-copy region in *Nicotiana* species is highly conserved – no variations in either restriction sites or size have been detected (Figs. 1, 2 and 5). Similar results were also obtained from other chloroplast genomes (Table 2). The inverted repeats contain only infrequent alterations. Some limited restriction site shifts, but no structural changes have been detected in this region in *Nicotiana,* though in *Oenothera,* (Gordon et al. 1982) structural changes have been detected in the inverted repeat region. Many of the differences found in *Nicotiana* chloroplast DNA are located in the large single-copy region of the molecule. This seems to be generally true for chloroplast genomes from many other plants (Herrmann et al. 1980).

The distribution of differences in restriction sites vary, depending on which enzyme is used. For example, differences in BamHI restriction sites among *N. otophora, N. tabacum* and *N. knightiana* chloroplast genomes are scattered throughout the molecule-exclusive of the small single-copy region. On the other hand, 3 out of 4 differences in SmaI sites are located in a section of the large single-copy region near the right-hand border of an inverted repeat, distant from the LS gene. It is in this section that the deletion identified in *N. knightiana* and *N. paniculata,* as well as the extra segment reported in *N. acuminata* (Shen et al. 1982) were located. This region has been designated as the hot spot. It is not known whether this region contains A+T rich stretches that were shown to exist in yeast mitochondrial DNA or inverted repeats similar to those associated with transposable elements (Calos et al. 1980).

Our results also support the suggestion that a molecular mechanism exists to ensure sequence homology for both copies of the inverted repeats of the *N. otophora* chloroplast genome is a clear example. Whether there is a causal relationship between the degree of stability and the presence of inverted repeats is still unknown. Palmer and Thompson (1981) suggested that the presence of inverted repeats stabilizes certain genomes against extensive rearrangements. Kolodner and Tewari (1979) implicate the inverted repeats in intermolecular recombination. The actual function of the inverted repeat should be further elucidated. It is hoped that the hypothesis presented here will stimulate further research on this subject.

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