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Extensive homologous chloroplast DNA recombination in the pt14 *Nicotiana* somatic hybrid

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Summary. In a previous study, six recombination sites have been confirmed in the chloroplast DNA (cpDNA) of pt14, a somatic hybrid of *Nicotiana tabacum* and *Nicotiana plumbaginifolia*. In the present study, physical mapping revealed six recombination sites in the 11.4-kb SalI fragment alone, only one of which has been previously identified. This fragment is located in the large unique region. We assume, therefore, that the pt14 cpDNA is a fine mosaic of the parental genomes with a recombination site about every 2 kb. A 748-bp region that comprised the intergenic region between ORF73 and ORF74B, and 460 bp of the petD intron have been sequenced. Parent-specific sequences in the pt14 DNA defined the regions within which recombination took place. The exact site of recombination events could not be determined because the parental sequences were identical between the polymorphic markers, and these sequences have been preserved in the pt14 line.

Key words: Chloroplast DNA – Homologous recombination – *Nicotiana*

Introduction

Evidence for recombination of chloroplast genomes of different genetic lines is extensive in the unicellular alga, *Chlamydomonas* (Gillham 1978; Lemieux et al. 1981, 1988). However, most evidence for chloroplast recombination in higher plants is indirect. Intragenomic rearrangements or intergenomic recombination events among the multiple cpDNA copies have contributed to cpDNA evolution, yet are relatively infrequent (Palmer 1985). Heterogeneity maintained in the cpDNA population suggests relatively frequent cpDNA rearrangements. CpDNA multimers observed by electron microscopy (Kolodner and Tewari 1979) and after pulse-field electrophoresis (Deng et al. 1989) were interpreted to be products of intermolecular recombination events.

The presence of two types of chloroplast genomes in a single plant that differ in their relative orientation of the single-copy regions was attributed to intramolecular recombination by interconversion of the circular genome (flip-flop recombination) through the inverted repeats (Palmer 1983; Mubumbila et al. 1983). Intramolecular recombination during microsporogenesis is the likely mechanism by which deleted forms of cpDNA are generated to yield albino wheat and barley plants (Day and Ellis 1984, 1985).

Evidence for recombination of chloroplast genomes of different genetic lines of higher plants was recently reported (Medgyesy et al. 1985; Thanh and Medgyesy 1989). The cpDNA of the pt14 line, a somatic hybrid of *Nicotiana tabacum* and *Nicotiana plumbaginifolia* was shown to be a product of interspecific cpDNA recombination. Based on physical mapping, six recombination sites have been deduced in the pt14 cpDNA (Medgyesy et al. 1985). In this paper we present evidence that the recombination is much more extensive than has been previously assumed. We also show, by sequencing two regions, that the recombination process does not result in any DNA sequence alterations.

Materials and methods

SalI fragments of *N. tabacum* SR1, *N. plumbaginifolia* and line pt14 were cloned into pBR322 (fragments S4-S11) or pUC18

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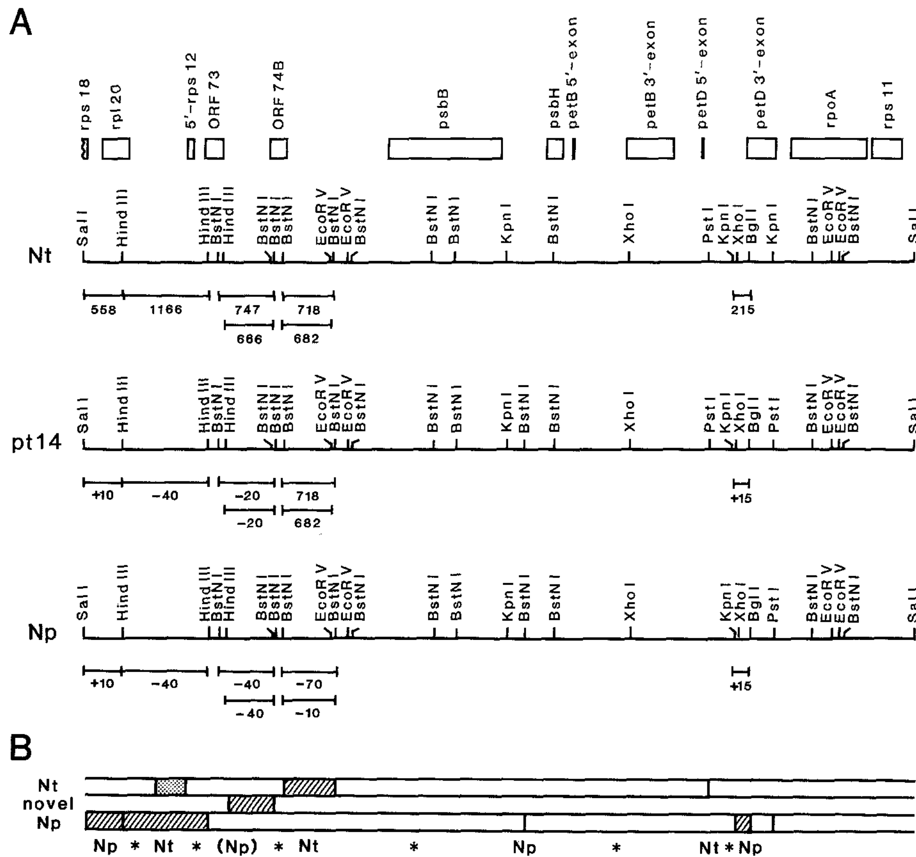

Fig. 1 A and B. Identification of recombination sites in fragment S8. **A** Restriction maps for *N. tabacum* (Nt), line pt14 (pt14) and *N. plumbaginifolia* (Np). Position of genes indicated as in Shinozaki et al. (1986). Length polymorphism is shown below the maps. Map distances are given in bp for *N. tabacum* and are based on the cpDNA sequence. Length polymorphism in *N. plumbaginifolia* and pt14 is given relative to *N. tabacum* (+ or − bp), estimated from relative fragment mobility. **B** Origin of polymorphic regions (*bars*) and restriction sites (*vertical lines*) in the pt14 line. Relative position of parental regions (Np, Nt) is marked. The inferred Np-derived region is bracketed. *Asterisks* indicate regions involved in recombination.

(fragment S2), as described in Medgyesy et al. (1985). Physical mapping of cpDNA was carried out by standard techniques (Maniatis et al. 1982). For sequencing, cpDNA was cloned in M13mp18, M13mp19 (Yanish-Perron et al. 1985) or in pUC118 and pUC119 (Vieira and Messing 1987) vectors. The sequence of both strands has been determined using the dideoxynucleotide chain termination method (Sanger et al. 1977).

Map distances were determined by comparing fragment mobility in gels with appropriate molecular weight standards. When the sequence of the *N. tabacum* cpDNA became available (Shinozaki et al. 1986), absolute map distances calculated from fragment mobility were corrected according to the sequence information for the *N. tabacum* parent. If the mobility of a *N. plumbaginifolia* or of a pt14 fragment was identical with that of a *N. tabacum* fragment, the size established for *N. tabacum* is given. If fragment mobilities were different, the estimated deviation (+ or −) from the *N. tabacum* value is given in base pairs.

Results

Recombination sites in the large unique region

The 11.4-kb SalI fragment (S8 fragment) is in the large unique region of the cpDNA. Recombination events in the pt14 line have been deduced from the physical map of the cloned fragments (Fig. 1 A). Regions in which recombination took place are bordered by markers from a different parent. Three parent-specific restriction sites were found in the pt14 fragment: one BstNI site from *N. plumbaginifolia* and two PstI sites, one from *N. tabacum* and one from *N. plumbaginifolia* (Fig. 1 B). In addition, identification of regions containing recombination sites has been facilitated by restriction length polymorphisms (RFLPs). RFLPs in cpDNA are generated by small deletions or insertions and result in altered distances between conserved sites (reviewed in Palmer 1985). RFLP markers in the pt14 fragment are shown as bars in Fig. 1 B. Striped bars mark data based on Fig. 1 A. In addition, the map position of the 414-bp *N. tabacum*-specific HpaII fragment is shown as a speckled bar. The map position of this fragment is known from the published *N. tabacum* cpDNA sequence (Shinozaki et al. 1986). This fragment was isolated from the pt14 line and shown to hybridize to the 1,166-bp HindIII fragments (data not shown).

From data shown in Fig. 1 B, six recombination events have been deduced. Four regions containing recombination sites (asterisks in Fig. 1 B) are defined by alternating parent-specific polymorphic regions or restriction sites, one (the first) on the left and three on the right of Fig. 1 B. Two additional recombination sites (second and third asterisks on left) were inferred between the two neighboring *N. tabacum*-specific polymorphic regions (Fig. 1 B). There is a novel fragment between these RFLP markers. RFLP markers from the same parent flanking a novel fragment can only be obtained by the formation of an even number of recombination sites enclosing regions from the other (*N. plumbaginifolia*)

parental line. Indeed, one *N. plumbaginifolia*-specific site and a recombination event has been confirmed by DNA sequence data in the novel BstNI fragment (Fig. 3A).

Idenfication of recombination sites in the inverted repeat

A 7.4-kb region of the inverted repeat that includes a portion of the rRNA operon was also tested for recombination (Fig. 2). The region was cloned as four fragments (a 2.37-kb KpnI-BamHI, and 3.25-, 1.22- and 0.48-kb BamHI fragments) and tested for polymorphisms with 19 restriction enzymes (AluI, AvaII, BstNI, DdeI, FnuDII, Fnu4HI, FokI, HaeIII, HhaI, HgiAI, HincII, HinfI, HpaII, NciI, RsaI, Sau3AI, Sau96I, ScrFI and TaqI). By comparing restriction digests of the parental species, two RFLPs were identified. One was localized within an ApaI-DdeI fragment, the other in an HgiAI fragment, both of them within the intron of $tRNA^{Ile}$, about 400 bp away. In the pt14 line, both polymorphic regions derive from *N. plumbaginifolia* (Fig. 2). Streptomycin resistance in the SR1-A15 line is due to a base change (C to A) in the 16S rRNA gene (Etzold et al. 1987). As the pt14 line is resistant to streptomycin, we assume that this mutation derives from the *N. tabacum* SR1-A15 line. The two polymorphic sites from *N. plumbaginifolia* and streptomycin resistance from *N. tabacum* confirm one recombination event in this region.

DNA sequence analysis of regions containing recombination sites

Two regions were selected for sequencing based on information presented in Fig. 1. One of them was a BstNI fragment that showed an intermediate fragment mobility in the pt14 line as compared to the fragments from the parental species. This fragment is 747 bp in *N. tabacum,* and the corresponding fragments, based on mobility in gels, were estimated to be 707 bp (747 bp−40 bp) and 727 bp (747 bp−20 bp) in *N. plumbaginifolia* and the pt14 line, respectively. This fragment comprises the region between ORF73 and ORF74B and the proximal end of the open reading frames (Figs. 1 and 3A). The borders of the other sequenced region are defined by PstI and EcoRI sites, and this region is entirely within the intron of the petD gene (Figs. 1 and 3B). The sequence of strand B (Shinozaki et al. 1986) of the sequenced regions is shown in Fig. 3.

Within the BstNI fragment, the parental species differed by three insertion/deletion mutations (Fig. 3A). One length mutation and two point mutations were found in the region delineated by the PstI/EcoRI sites (Fig. 3B). Since the DNA sequences in the pt14 and parental lines were identical between the polymorphic sites, it is only possible to define the regions within which recombination took place.

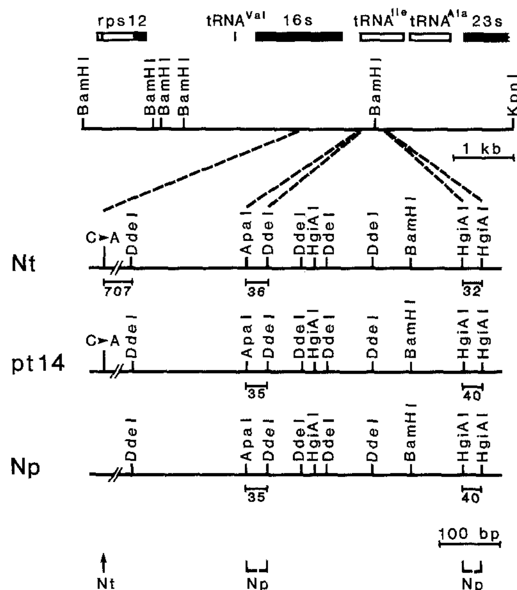

Fig. 2. Identification of recombination sites in the 7.4-kb region from the inverted repeat. Restriction maps for the parental species (Nt, Np) and the recombinant (pt14) are shown together with the position of genes coded in the region. Map distances in the polymorphic regions are given in bp. The site of the C to A base change conferring streptomycin resistance is marked

Discussion

Seven parental restriction sites, three from *N. tabacum* and four from *N. plumbaginifolia,* in an alternating order, have previously been found in the pt14 cpDNA. This suggested the presence of six recombination sites scattered within the 155.8-kb chloroplast genome (Medgyesy et al. 1985). We have presented here physical mapping data for the 11.4-kb S8 fragment localized in the large unique region and have identified six recombination sites (Fig. 1), only one of which has been previously described. Assuming that recombination is as frequent throughout the cpDNA as it is in fragment S8, there would potentially be a recombination site formed every 2 kb, i.e. there would be approximately 80 recombination sites in the 155.8-kb cpDNA. We believe that the pt14 cpDNA is indeed a fine mosaic of the parental genomes, since we have found recombination sites in the nine SalI fragment tested that represent 70% of the pt14 cpDNA (Maliga and Fejes 1985 and unpublished results; shaded area on Fig. 4). Restriction sites specific to *N. tabacum* and to *N. plumbaginifolia* alternate randomly in fragment S8 of line pt14 (Figs. 1 and 4), indicating that recombination sites are formed more frequently than 2-kb intervals. Thus, the suggested number of 80 recombination sites in the pt14 cpDNA may be an underestimate.

A 7.4-kb region of the repeated region was also searched for evidence of recombination events. In this region, however, only a single recombination site could be inferred (Fig. 2). Lack of cpDNA sequence polymor-

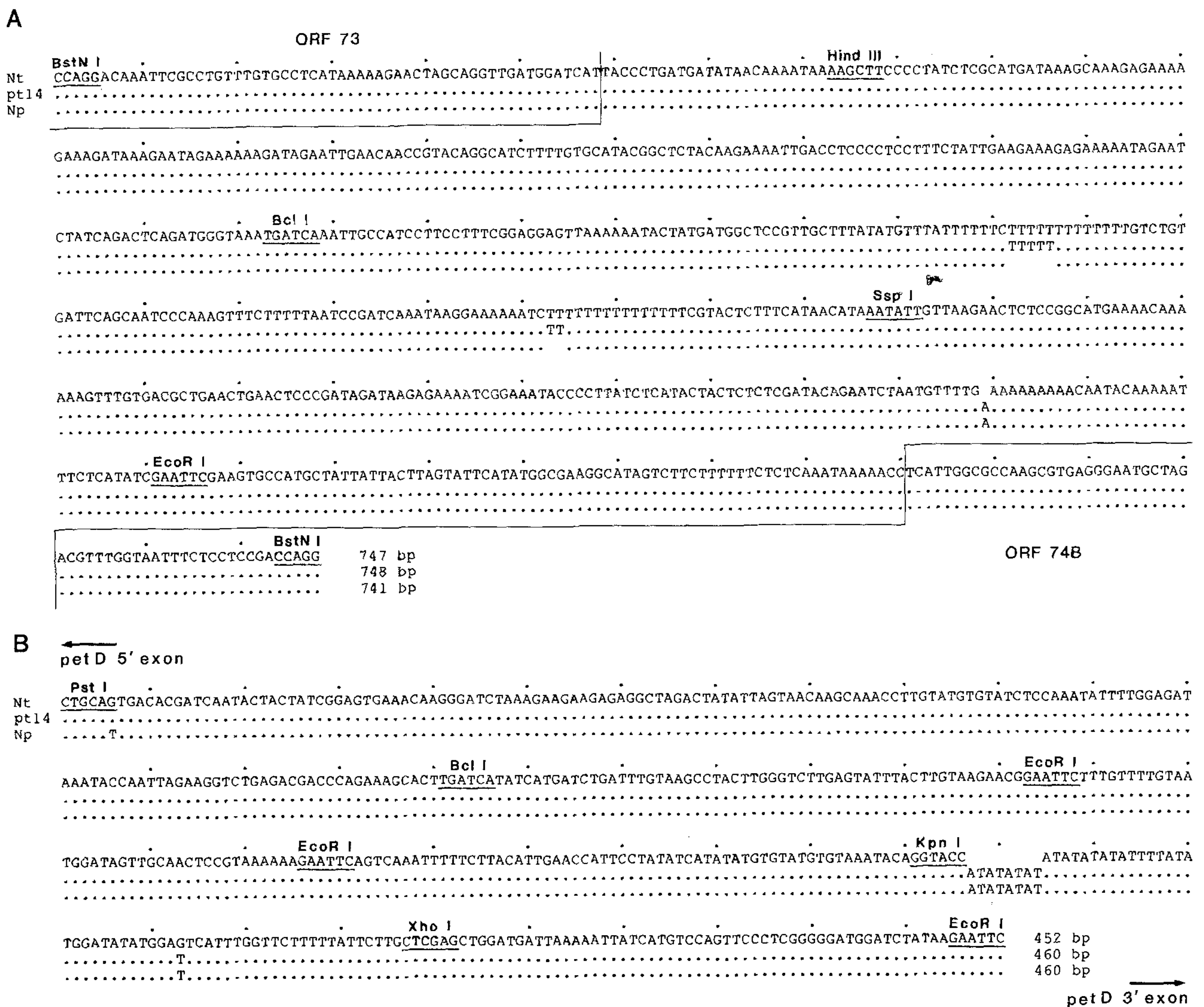

Fig. 3 A and B. Sequence of regions containing recombination sites in pt14 cpDNA and of corresponding regions in parental species, *N. tabacum* (Nt) and *N. plumbaginifolia* (Np). **A** Intergenic region between ORF73 and ORF74B. **B** Part of the petD intron. *N. plumbaginifolia* and pt14 sequence is given only if different from the *N. tabacum* sequence. Deletions are indicated by *blank space*. Open reading frames are *boxed*. Note that the size of polymorphic fragments determined by mobility (Figs. 1 and 2), and of their true length, as determined by sequencing, is different

phism between the parental species limits estimation of the true number of recombination sites in this region. Sequence conservation between *N. tabacum* and *N. plumbaginifolia* is not surprising, since the cpDNA of these two species is the most closely related within the genus (Salts et al. 1984).

Data in this paper suggest that cpDNA recombination occurs readily. This conclusion contrasts with the lack of chloroplast recombinants in sexual crosses of higher plants (Chiu and Sears 1985) and the rarity of the event in fused heteroplastidic somatic cells (Maliga et al. 1987, 1989). We assume that the rate-limiting step of interspecific cpDNA recombination is the physical proximity of the different chloroplast genomes in chloroplasts, i.e. the formation of plastids carrying a mixed cpDNA population. Ultrastructural studies suggest that organelle fusion is the mechanism by which heterogenomic plastids form (Wellburn and Wellburn 1979). We propose, therefore, that cpDNA recombination is rare because plastid fusion is rare.

Recombination in the pt14 cpDNA has been confirmed by sequencing two regions within which recombination took place (Fig. 3). Due to a high degree of sequence conservation between the parental species and lack of footprints of the recombination process, only the endpoints of regions within which recombination took place could be identified; the exact location of breakpoints could not be pinpointed. The results described in this paper indicate homologous recombination between chloroplast genomes.

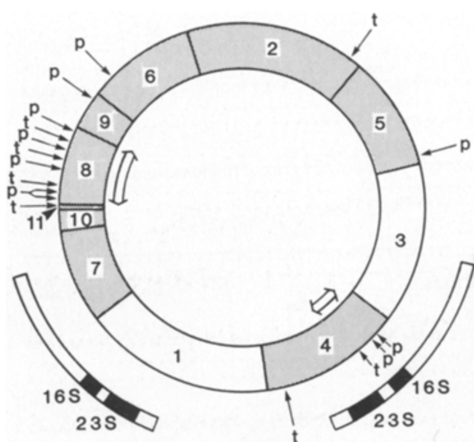

Fig. 4. SalI restriction map of the pt14 chloroplast genome. The inverted repeats are denoted by concentric *bars*, showing the location of the 16S and 23S rRNA genes. SalI fragments tested for recombination sites are *shaded*. *Arrows* point to sites or polymorphic regions, the origin of which has been determined (*N. tabacum*, t; *N. plumbaginifolia*, p). Regions mapped in this study are marked by *arrows* within the map, and are based on data in Figs. 1 and 2. Data published earlier (Medgyesy et al. 1985; Maliga and Fejes 1985) have also been included

Recombination in the plastid genome of the pt14 line is very extensive and the pt14 ptDNA appears to be a mosaic of the two parental genomes (see above). In contrast, the plastid genome derived from the recombination of fused *N. tabacum* and *Solanum tuberosum* parents carries the large and small unique regions from *N. tabacum* and the repeats from *S. tuberosum* (Thanh and Medgyesy 1989). Recombination in this second case seems to have involved only the junctions of the unique regions and the repeated regions. The two lines studied so far in higher plants appear to represent extremes and are not comparable since, in the second case, nucleoplastidic incompatibility may have played a role in selection of a specific plastid recombinant type. The rules of ptDNA recombination in higher plants will be established by comparing a larger number of plastid recombinants and from studies on the integration of transforming DNA.

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