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Genetics

Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid

(protoplast fusion/chloroplast DNA/physical mapping)

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ABSTRACT **Genetic recombination between chloroplasts of two flowering plant species, *Nicotiana tabacum* and *Nicotiana plumbaginifolia*, after somatic cell fusion is described. The parental lines differed in three cytoplasmic genetic markers. The *N. tabacum* mutant SR1-A15 was streptomycin-resistant, defective in chloroplast greening, and lincomycin-sensitive. The *N. plumbaginifolia* mutant LR400 was streptomycin-sensitive, normal green, and lincomycin-resistant. Streptomycin-resistant clones in cell culture are identified by their ability to form a green callus on a selective medium. Streptomycin resistance in the SR1-A15 mutant could not be expressed due to defective chloroplasts. Protoplasts of the two species were fused, and calli grown from the fused population were screened for the expression of streptomycin resistance from the SR1-A15 line as the result of interspecific chloroplast recombination. A somatic hybrid, pt14, expressed a new combination of the cytoplasmic genetic markers. In the pt14 chloroplast genome three *N. tabacum* and four *N. plumbaginifolia* parent specific restriction sites have been identified, indicating that the pt14 chloroplast genome contains at least six recombination sites.**

Isolation and characterization of chloroplast genes is progressing quickly (1) and their modification by genetic engineering techniques is becoming a possibility. Chloroplast genetics, however, is relatively unexplored. In the unicellular alga *Chlamydomonas*, genetic studies have been limited to segregation and recombination of genetic markers (2); physical evidence for chloroplast recombination is recent (3, 4). In flowering plants chloroplast segregation has been extensively studied in species in which chloroplasts are biparentally inherited (2). Attempts to recover chloroplast recombinants after crossing independent pigment mutants have failed (5).

Protoplast fusion techniques have made it feasible to produce cells with mixed chloroplast populations in species in which chloroplasts are maternally inherited (6). This group includes all of the important crop species (2). It has been shown that mixed chloroplast populations are not maintained in the fused somatic cells, and segregation quickly results in progeny with one or the other parental chloroplast type (6, 7). During the transient mixing there is a chance for genetic recombination between the organelles. Genetic recombination has been described between mitochondria (8, 9, 10). In this paper we report on genetic recombination between different chloroplast genomes in flowering plants.

The isolation of a clone with recombinant chloroplasts was facilitated by direct selection for a new combination of cytoplasmic markers, including a streptomycin resistance mutation in a *Nicotiana tabacum* line, SR1 (11). Streptomycin resistance is expressed in cell culture as the ability to form green colonies on a medium containing streptomycin (11, 12). In a derivative of the SR1 mutant, SR1-A15, expression of streptomycin resistance—i.e., greening—is prevented by a second cytoplasmic mutation (unpublished data). Cells with mixed chloroplasts were obtained by fusing protoplasts prepared from line SR1-A15 (*N. tabacum*) and line LR400, a streptomycin-sensitive, normal green, and lincomycin-resistant derivative of *Nicotiana plumbaginifolia* (13). Identification of lines with recombinant chloroplasts was expected on the basis of expression of streptomycin resistance from the SR1-A15 chloroplast genome as the result of chloroplast recombination. Since the SR1-A15 and LR400 lines are from different species, confirmation of chloroplast recombination was planned by physical mapping.

MATERIALS AND METHODS

Plant Mutants. The cytoplasmic lincomycin-resistant mutant LR400 of *N. plumbaginifolia* has been described (13). The LR400 plants are normal green and are sensitive to streptomycin. The SR1-A15 line is a derivative of the SR1 cytoplasmic streptomycin-resistant mutant of *N. tabacum* (11). The SR1 plants are normal green. The SR1-A15 line was isolated in SR1 callus culture as a white shoot (Fig. 1). Pigment deficiency was inherited maternally in sexual crosses (unpublished data). Evidence that the chloroplast genome is the carrier of the cytoplasmic streptomycin and lincomycin resistance traits has been discussed (14).

Cell Culture Procedures. The LR400 and SR1-A15 plants were maintained on MS inorganic medium (15) containing 3% sucrose (MS medium). Protoplast isolation, fusion, and culture were carried out as described (16). Polyethylene glycol-induced fusion was carried out without any pretreatment to modify the contribution of the nuclear or chloroplast genomes to the somatic hybrid. Streptomycin-resistant colonies were selected in RMOP medium (17) containing 1.0 mg of streptomycin sulfate per ml following the protocol of Medgyesy *et al.* (12). Resistance was defined as the ability to form a green callus on the streptomycin medium that prevents the greening of sensitive calli. Lines were scored as resistant after an additional subculture on selective streptomycin medium. Plants were obtained from the resistant cell lines by rooting the shoots formed on antibiotic-free RMOP medium (17). The resistance phenotype of the regenerated plants was determined by placing leaf sections on RMOP medium containing streptomycin sulfate (1.0 mg/ml) or lincomycin hydrochloride (1.0 mg/ml). Callus formed on the leaf sections was green or white, indicating resistance or sensitivity, respectively (12, 13). The cell culture procedures cited above have been summarized in a technical paper (18).

Isolation and Mapping of Chloroplast DNA (cpDNA). cpDNA was prepared by the method of Kolodner and Tewari (19) with a few modifications. Leaves were collected from plants grown in sterile culture. The DNase treatment of the chloroplast preparation was omitted. Cloning of *Sal* I

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Abbreviations: cpDNA, chloroplast DNA; kb, kilobase(s).

chloroplast fragments into the *Sal* I site of the plasmid pBR322 was carried out according to Maniatis *et al.* (20) with *Escherichia coli* strain HB101 as recipient for transformation. Digestion with restriction endonucleases was carried out according to the manufacturers' specifications. Agarose and polyacrylamide gel electrophoresis was carried out according to Maniatis *et al.* (20).

Southern Blot Hybridization to Identify Chloroplast Sequences in Total Cellular DNA. Callus was grown on RMO medium (18). DNA from callus was isolated as described by Chilton *et al.* (21), digested by restriction enzymes according to the manufacturers' specifications, electrophoresed in horizontal agarose gels, blotted onto nitrocellulose, and probed with cpDNA cloned into pBR322 (20).

RESULTS

Isolation of Cell Lines Expressing Streptomycin Resistance. SR1-A15 and LR400 leaf mesophyll protoplasts were mixed (1:1) and fused with polyethylene glycol. Small calli obtained from the protoplasts were plated on a selective streptomycin medium, and the resistant green calli were isolated. On the streptomycin medium the parental SR1-A15 colonies, though they carried streptomycin resistance, did not form a green callus due to the cytoplasmic pigment mutation. Parental LR400 calli were sensitive to streptomycin and therefore also formed white colonies. [Bleaching is not lethal in cell culture due to the presence of sucrose in the culture medium. Growth of the sensitive calli, however, is slower (11).] Among 1.9×10^5 calli derived from fusion, 23 streptomycin-resistant clones were obtained.

Parental protoplasts were also cultured and screened for streptomycin resistance. Among 2×10^4 SR1-A15 calli no green colonies were obtained. In LR400 cultures, among 3×10^4 calli 8 spontaneous streptomycin-resistant clones were found.

Classification of the Regenerated Plants. *N. tabacum*, *N. plumbaginifolia*, and their interspecific hybrid can be easily distinguished by their morphology (Fig. 1). Based on leaf shape and the color, size, and morphology of the flowers, plants in one clone, pt14, were classified as somatic hybrids. The somatic hybrids were similar to those described (22). In 7 clones *N. plumbaginifolia* regenerates were obtained. The remaining 15 clones were not studied.

Somatic hybrid plant pt14 was tested for streptomycin and lincomycin resistance by placing leaf sections onto selective media (Fig. 2). The plant was resistant to streptomycin and sensitive to lincomycin.

Characterization of the Chloroplast Genome of Line pt14. The cpDNA of the somatic hybrid was compared to that of *N. tabacum* and *N. plumbaginifolia*. *N. tabacum* cpDNA was prepared from the SR1 mutant since preparation of cpDNA in sufficient quantities from the albino mutant would have been difficult. *N. plumbaginifolia* cpDNA was prepared from line LR400 and wild-type *N. plumbaginifolia*. Possible restriction site differences between line SR1-A15 and its parental line, SR1, were excluded by Southern blot hybridization (next section). cpDNAs of line LR400 and wild-type *N. plumbaginifolia* were compared by restriction endonuclease fragmentation patterns (data not shown) and Southern blot hybridization (next section) and were identical with respect to all restriction sites studied.

Regions of the chloroplast genome will be referred to by their position in the *Sal* I map published for *N. tabacum* (23) (Fig. 3). Since the size and number of *Sal* I fragments are identical in SR1, wild-type *N. plumbaginifolia*, and pt14 lines (Fig. 4*C*) it was assumed that the *Sal* I maps of the three lines are identical. Parent-specific restriction sites were identified by comparing restriction endonuclease fragmentation patterns of total cpDNA (S5–S2 region) or those of cloned *Sal* I fragments (S4, S6–S8 region) from line pt14 and from SR1 and wild-type *N. plumbaginifolia*. Our mapping data agreed with those published for *N. tabacum* (23, 24) and *N. plumbaginifolia* (25, 26), except for one case.

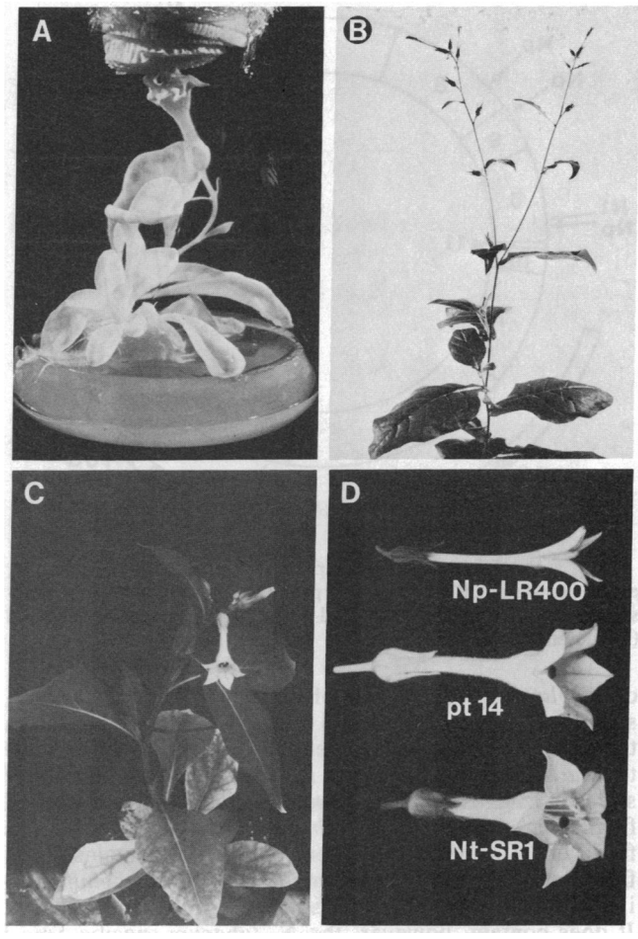

FIG. 1. *N. tabacum* SR1-A15 (*A*) and *N. plumbaginifolia* LR400 (*B*) parental plants and their somatic hybrid pt14 (*C*). Flower morphology of the lines is also shown (*D*).

In the S5–S2 region the *N. tabacum* and *N. plumbaginifolia* chloroplast genomes can be distinguished by the *Sma* I restriction endonuclease (ref. 26; Fig. 4*A*). The *N. tabacum* genome contains two more *Sma* I sites than does the genome

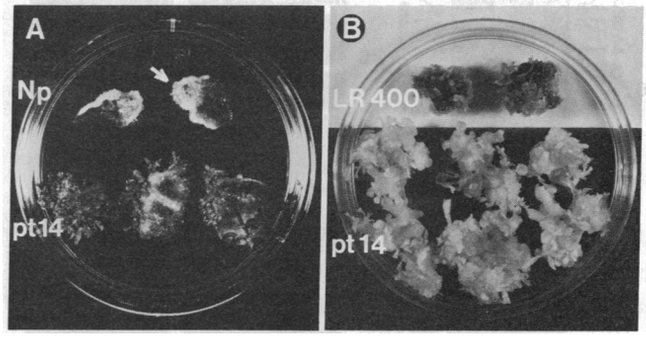

FIG. 2. Test of the pt14 plants for resistance to streptomycin (*A*) and lincomycin (*B*). As controls, *N. plumbaginifolia* (Np; streptomycin-sensitive) and LR400 (lincomycin-resistant) leaf sections were used. Note that callus developing at the edge of the Np leaf sections was white (arrow), whereas in the case of the resistant pt14 sections it was green and was forming shoots (*A*). The characteristic coloration in the lincomycin assay is more obvious since callusing of the leaf sections was more advanced (*B*).

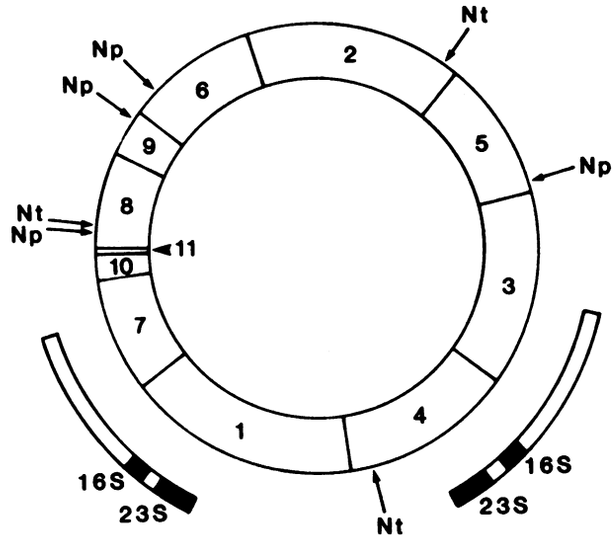

FIG. 3. *Sal* I restriction map of *N. tabacum* SR1 (Nt), *N. plumbaginifolia* LR400 (Np), and pt14 chloroplast genomes. Parent-specific restriction sites identified in the pt14 genome are indicated (for details see Fig. 5).

of *N. plumbaginifolia*. One of these is in the region corresponding to *Sma* I fragment 3 of *N. plumbaginifolia* (this is the *Sma* I site close to fragment S3, Fig. 5*A*) and gives rise to *Sma* I fragments 5 and 6 of *N. tabacum*. The other *Sma* I site is in fragment 6 of *N. plumbaginifolia* and yields *Sma* I fragments 8 and 11 of *N. tabacum* (data and designations from ref. 26). The *Sma* I pattern of line pt14 contains *Sma* I fragment 3 (Fig. 4*A*) characteristic of *N. plumbaginifolia*—i.e., the *Sma* I site characteristic for *N. tabacum* is absent. It does contain, however, the *N. tabacum* specific *Sma* I fragment 8 (Fig. 4*A*), indicating that in fragment S2 the additional *Sma* I site characteristic of *N. tabacum* is present. Restriction maps of region S5–S2 based on *Sma* I restriction endonuclease fragmentation patterns (Fig. 4*A*) and published maps of the parental species (26) are shown in Fig. 5*A*.

In fragment S4 the two parental species can be distinguished by an additional *Xho* I site in the *N. plumbaginifolia* genome (ref. 25; Fig. 5*B*). Based on comparing cloned S4 fragments, this site in pt14 is *N. tabacum*-type (Fig. 5*B*).

The S6–S8 region contains *Sal* I fragments S6, S9, and S8 (Fig. 3). This region was studied by comparing cloned *Sal* I fragments. In fragment S6 published data (25) indicated that the two parental species can be distinguished by a *Pst* I site absent in *N. tabacum*. Since this site was identified in line pt14 (Fig. 5*C*), pt14 was classified as *N. plumbaginifolia*-type.

In fragment S9 we identified an *Eco*RI site that is present only in *N. tabacum* (Fig. 5*C*). Since the diagnostic *Eco*RI site is absent in line pt14, it was classified *N. plumbaginifolia*-type with respect to this site (Fig. 5*C*).

By comparing cloned S8 fragments we found that the position of a *Pst* I site is species-specific (Fig. 5*C*), a fact not recognized earlier (25). In pt14 both parent-specific *Pst* I sites were present. Digestion of total cpDNA with *Pst* I therefore resulted in the excision of a unique 0.9-kilobase (kb) fragment that was absent from both parents (Figs. 4*B* and 5*C*). This nonparental fragment should contain a recombination site. Fragment S8 in SR1 contained a diagnostic *Kpn* I site. The map position of this site was identical with the diagnostic *Pst* I site in *N. plumbaginifolia* (Fig. 5*C*). In pt14 the *Kpn* I site was absent (and the *Pst* I site was present; see above); therefore, pt14 was classified as *N. plumbaginifolia*-type with respect to this site.

Localization of more than one recombination site has been hampered by the similarity of the parental DNA sequences. For example, fragment S8 contains restriction sites for 13 restriction endonucleases that were not suitable to differentiate the SR1, wild-type *N. plumbaginifolia*, and pt14 *Sal* I clones since the number of restriction sites and the sizes of the fragments were identical in all three lines. These enzymes were *Acc* I, *Bgl* I, *Bgl* II, *Eco*RI, *Eco*RV, *Hin*cII, *Nde* I, *Nru* I, *Pvu* I, *Pvu* II, *Sac* I, *Xba* I, and *Xho* I.

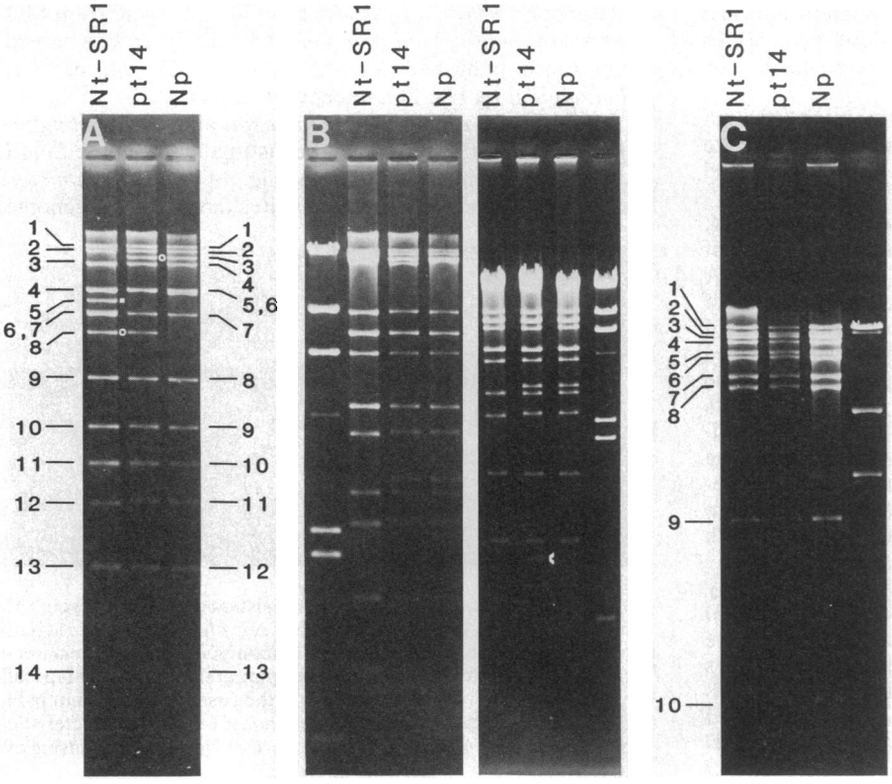

FIG. 4. Restriction pattern of the cpDNA isolated from *N. tabacum* SR1 (Nt), *N. plumbaginifolia* (Np), and pt14. Digestion was carried out with the restriction endonucleases *Sma* I (*A*), *Pst* I (*B*), and *Sal* I (*C*). Lanes contained ≈2 μg of DNA, except the *Pst* I digests (*B*), where 2 μg (left) and 4 μg (right) were loaded. Some of the parent-specific bands present (open circle) and absent (dot) in the *Sma* I digest of pt14 (*A*) and the nonparental *Pst* I fragment (*B*) are marked. λ DNA *Hin*dIII digests were run as DNA molecular weight standards (lanes not labeled). The diffuse band above λ fragment 1 in the chloroplast patterns is contaminating nuclear DNA. *Sma* I and *Sal* I fragments are numbered as in refs. 26 and 23, respectively. *Sal* I fragment 11 ran out of the gel shown in *C*.

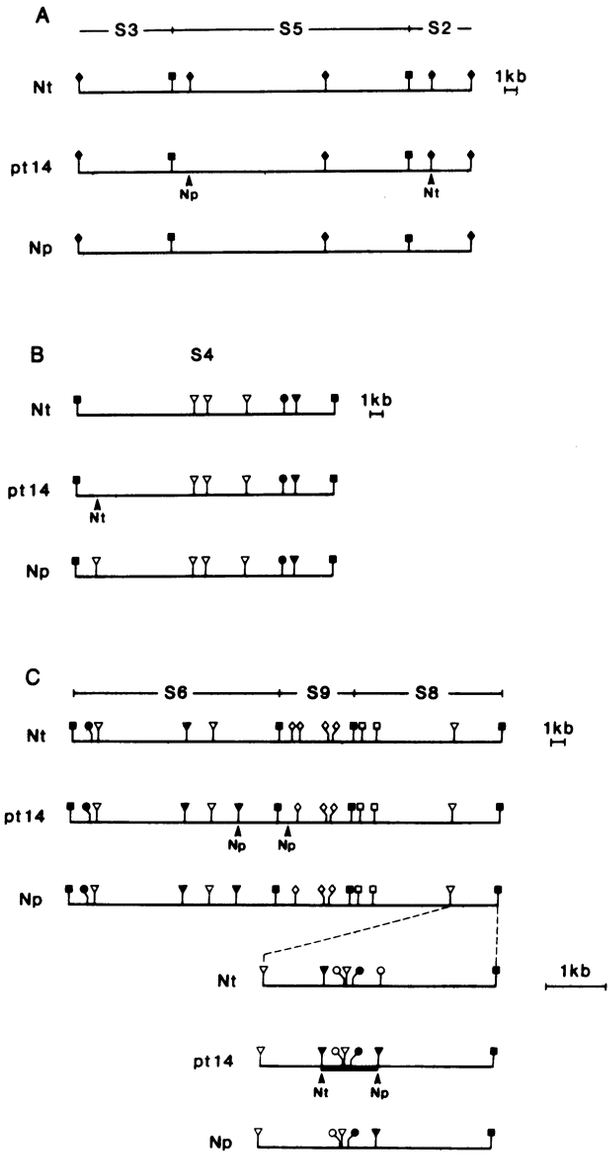

FIG. 5. Restriction maps of regions S5–S2 (*A*), S4 (*B*), and S6–S8 (*C*) of *N. tabacum* SR1 (Nt), *N. plumbaginifolia* (Np), and line pt14. For the location of the *Sal* I fragments, see Fig. 3 and ref. 23. ●, *Bgl* I; ◇, *Eco*RI; □, *Hin*dIII; ○, *Kpn* I; ▼, *Pst* I; ■, *Sal* I; ◆, *Sma* I; ▽, *Xho* I. Arrowheads below the line representing the pt14 genome indicate positions of parent-specific restriction sites. The nonparental *Pst* I fragment in *Sal* I fragment S8 of line pt14 is marked by a solid line (*C*).

Comparison of the Physical Map of the Mutants SR1-A15 and LR400 with Their Parental Lines. Species-specific restriction sites in the pt14 chloroplast genome were identified by comparison with the *N. tabacum* mutant SR1 and wild-type *N. plumbaginifolia* rather than with their mutant derivatives, the direct parents, SR1-A15 and LR400, respectively. It was important to show, therefore, that the species-specific restriction sites identified in the pt14 chloroplast genome (Figs. 3 and 5) had been present unaltered in the SR1-A15 and LR400 lines. To this end, total cellular DNA was prepared from SR1-A15 and LR400 calli, digested with *Sma* I, *Xho* I/*Sal* I, *Pst* I/*Sal* I, or *Eco*RI/*Sal* I restriction endonucleases, electrophoresed, and blotted onto nitrocellulose, and the blots were probed with cloned pt14 fragments S5, S4, S6 and S8, and S9, respectively. The number and size of hybridizing fragments were in agreement with the predictions of the maps shown in Fig. 5. In the following discussion, therefore, no distinction will be made between the mutants used in the fusion study and their parental lines with respect to their cpDNAs. Autoradiograms proving preservation of relevant restriction sites in fragments S5, S6, and S8 are shown in Fig. 6.

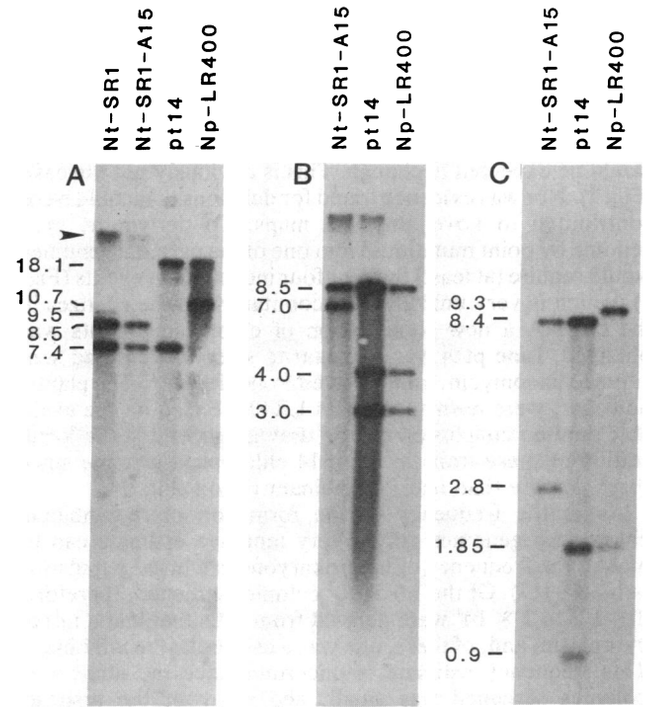

FIG. 6. Confirmation of parental-type maps in mutants *N. tabacum* (Nt) SR1-A15 and *N. plumbaginifolia* (Np) LR400. Total cellular DNA was digested with *Sma* I and probed with *Sal* I fragment S5 (*A*) or digested with *Pst* I/*Sal* I and probed with *Sal* I fragment S6 (*B*) or S8 (*C*). *Sal* I clones used as a probe were derived from line pt14. The molecular sizes are given in kb. Arrows indicate hybridization to nuclear DNA.

DISCUSSION

Appearance of streptomycin-resistant colonies after fusing the SR1-A15 and LR400 lines could have been the result of chloroplast recombination, forward mutation to streptomycin resistance in LR400, or reversion to wild type in SR1-A15. It was known from previous experience that protoplast fusion was followed by nuclear fusion in practically every clone in this species combination (16). Since protoplast fusion is the prerequisite of obtaining a mixed chloroplast population, recombinant chloroplasts were expected only in somatic hybrids.

The seven streptomycin-resistant *N. plumbaginifolia* lines identified in the fused protoplast population were most likely spontaneous mutants in the LR400 line. This interpretation is in line with finding several streptomycin-resistant lines in the control LR400 cultures. The presence of *N. plumbaginifolia*-type chloroplasts in the streptomycin-resistant *N. plumbaginifolia* plants was confirmed by *Pst* I, *Sma* I, and either *Xho* I or *Eco*RI restriction endonclease fragmentation patterns (data not shown). No SR1-A15 revertants were identified in this experiment.

The conclusion that the chloroplast genome of the pt14 somatic hybrid is a product of homologous recombination is based on the verification of three *N. tabacum* and four *N. plumbaginifolia* specific restriction sites in the pt14 chloroplast genome. The pt14 map can be derived from the two parental maps by assuming formation of six recombination sites. Easy identification of the parental regions within the pt14 chloroplast genome has been hampered by lack of restriction site polymorphism (see also ref. 25). So far only

one border fragment containing a recombination site has been identified in *Sal* I fragment S8, which is in the large unique region of the chloroplast genome (Figs. 3 and 5*C*).

The observed changes cannot be explained by intramolecular rearrangements in one of the parental chloroplast genomes. In case of inversion the order of restriction sites would be expected to change. This is obviously not the case (Fig. 5). Nor was evidence found for deletions that could have contributed to novel physical maps. To derive the pt14 genome by point mutations from one of the parental genomes would require (at least) three or four independent events (Fig. 3), which is very unlikely. Concomitant with the changes in the cpDNA a new combination of cytoplasmic traits was obtained. Line pt14 was resistant to streptomycin and sensitive to lincomycin, traits derived from SR1-A15. The plants, however, were normal green as LR400. Based on the available data no conclusion can be drawn concerning the localization of these traits in the pt14 chloroplast genome since there is more than one recombinant region (Fig. 3).

As to the frequency of the formation of recombinant chloroplast genomes only a very tentative estimate can be made. The frequency of heterokaryons in a fused population is 1–10% (17). Of the 1.9×10^5 colonies screened, therefore, 2×10^3 to 2×10^4 were derived from cells that had a mixed cytoplasm, and, of these, one was a chloroplast recombinant. This frequency estimate is uncertain since the number of colonies screened was small, and many of the resistant isolates (15 of 23) have not been studied.

In a mature *Nicotiana* leaf cell there are about 100 chloroplasts (6). To detect a recombinant visually as a green colony, the progeny of the chloroplast carrying the recombinant genome should become dominant within a cell, and the cell containing the resistant chloroplasts should have a competitive edge over the sensitive cells. The use of an efficient screening procedure, in our view, was the key in recovering the chloroplast recombinant. It also seems important that the sensitive cells are not killed by the drug so that there was sufficient time for the sorting-out process. In line pt14 the segregation of chloroplast genomes was complete, as evidenced by the fact that not all parent-specific fragments were present (Fig. 4 *A* and *B*).

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