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A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA

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Summary. Mitochondrial DNA (mtDNA) restriction patterns were studied in mutant, cybrid and somatic hybrid plants regenerated from *Nicotiana* protoplasts.

It has been shown that neither components of the culture media used for protoplast culture and plant regeneration, nor the antibiotics streptomycin and lincomycin used for the mutant selection induced alterations in the mtDNA. No rearrangements were detected in the mtDNA of plants derived from homoplasmic fusions where the mtDNA of the parents was identical as judged by mtDNA restriction patterns.

There were rearrangements, however, in the mtDNA of each of the cybrid plants derived from heteroplasmic fusions. Restriction patterns generated by BamHI and SalI restriction endonucleases were different from those of both parents, and were composed of parental and non-parental fragments.

Key words: *Nicotiana* – Mitochondrial DNA-cybrids – Somatic hybrids

Introduction

Cytoplasmic mixing and bi-parental transmission of the cytoplasmic organelles does not occur during sexual reproduction in the genus *Nicotiana,* or in many other flowering plant species (Sears 1980). Somatic cell fusion makes it feasable to produce cells with mixed "bi-parental" cytoplasm in these cases. Mitochondrial DNA (mtDNA) was studied in two types of plants derived from cell fusion. In somatic hybrids the chromosomes of both parents were present as the result of nuclear fusion in the heterokaryons. In the cybrids, chromosomes were retained only from one of the parents as the result of the segregation of nuclei in the primary protoplast fusion products. (Note that in the literature somatic hybrids may be referred to as cybrids in order to indicate the hybrid origin of the cytoplasm. This terminology does not distinguish the clones according to the fate of nuclei in the primary fusion product. Somatic hybrids are derived from cells with a mixed cytoplasm, therefore in those cases we do not use the term cybrid.)

MtDNA in *Nicotiana tabacum* (Belliard et al. 1979) and *Nicotiana sylvestris* (Galun et al. 1982) cybrids, and in *Nicotiana tabacum* + *Nicotiana knightiana* somatic hybrids (Nagy et al. 1981) was non-parental, as judged by mtDNA restriction patterns. In the case of the somatic hybrids, rearrangements in the mtDNA were confirmed by hybridizing *E. coli* ribosomal RNA genes to the restriction fragments. In *Petunia* interspecific somatic hybrids, the mtDNA restriction patterns were also non-parental (M. Hanson, personal communication; A. J. Kool, personal communication).

It has been shown in *Zea mays* that extensive rearrangements in the mtDNA may take place during cell culture, a phenomenon which does not normally occur in plants (Gengenbach et al. 1981; Kemble et al. 1982). In *Nicotiana* no studies have been made on the mtDNA of plants regenerated from unfused protoplasts. Therefore, the possibility that alterations in the mtDNA were induced by cell culture conditions has not been excluded. The effect of polyethylene glycol, the chemical agent used for fusion induction, has also not yet been studied. Furthermore, it has not been excluded whether streptomycin selection, employed in one of these studies (Nagy et al. 1981), is responsible for some of the changes in the mtDNA. Such a study as the present one is justified by reports showing that streptomycin induces mutations (Sager 1972) and re-

arrangements (Heizmann et al. 1982) in *Chlamydomonas* chloroplast DNA. With these studies on *Nicotiana* mutants, cybrids and somatic hybrids, we provide evidence that none of these factors induces changes in the mtDNA: rearrangements could be detected only in heteroplasmic fusion combinations.

Materials and methods

Plant material

The plants were maintained in sterile culture on the RM medium of Linsmaier and Skoog (1965) without vitamins and growth regulators. The cultures were illuminated 16 h daily and were kept at 28 °C.

The lines used in this study are listed in Table 1.

Isolation and restriction of mtDNA

MtDNA was isolated from the leaves of aseptically cultured plants according to the technique of Belliard et al. (1979). Mitochondria from 5–10 g leaves were isolated and purified on a sucrose step gradient. MtDNA was extracted and isolated by CsCl-ethidium bromide gradient centrifugation. The isolated mtDNA was digested by BamHI and SalI restriction endonucleases. Digestion with SalI was carried out in 150 mM NaCl, 6 mM β-mercaptoethanol, 10 mM Tris-HCl at pH 8.0, 5 mM $MgCl_2$ and 100 µg/ml gelatin. BamHI digestion was carried out in 10 mM $MgCl_2$, 20 mM Tris-HCl, at pH 8.0 and 150 mM NaCl. The endonuclease SalI was prepared and assayed according to Arrand et al. (1978). BamHI was a kind gift from P. Venetianer.

Agarose gel electrophoresis of mtDNA

After digestion with restriction endonucleases, fragments of mtDNA were separated on 0.7% agarose gels, stained with eithidium bromide and photographed as previously described (Nagy et al. 1981).

Results

1 MtDNA in mutant plants regenerated from tissue culture

MtDNA was studied in antibiotic resistant mutants isolated in *N. plumbaginifolia* protoplast cultures. No alterations were detected in the mtDNA of streptomycin resistant (five lines) and lincomycin resistant (four lines) plants after digestion with the BamHI (Fig. 1) and SalI (not shown) restriction endonucleases. For comparison, a pattern of mtDNA from a wild-type *N. plumbaginifolia* plant is also shown (Fig. 1). MtDNA of the streptomycin resistant *N. tabacum* mutant, SR1, isolated in callus culture, was also studied (from regen-

Table 1. Experimental plant material

Line	Code	Reference
Nicotiana tabacum cv 'Petit Havanna'	Nt	
Nicotiana tabacum cv 'Samsun' light sensitive mutant	Nt-1p	Wettstein 1965 Wong-Staal and Wildman 1973
Nicotiana tabacum cv 'Petit Havanna' streptomycin resistant mutant from callus culture	SR1	Maliga et al. 1973, 1975
Nicotiana sylvestris	Ns	
Nicotiana sylvestris + *Nicotiana tabacum* streptomycin resistant somatic hybrids with SR1 plastids	st1, st2, st3, st4, st5	Medgyesy et al. 1980
Nicotiana sylvestris cybrids with SR1 plastids	Ns(SR1)1; Ns(SR1)3; Ns(SR1)2; Ns(SR1)4; Ns(SR1)5; Ns(SR1)6; Ns(SR1)7	Medgyesy et al. 1980
Nicotiana plumbaginifolia	Np	
Nicotiana plumbaginifolia streptomycin resistant mutants from protoplast culture	SR402, SR414, SR426, SR435, S436	Maliga, unpublished
Nicotiana plumbaginifolia lincomycin resistant mutants from protoplast culture	LR403, LR411, LR443, LR450	Cséplö, unpublished
Nicotiana plumbaginifolia cybrids with Nt-1p chloroplasts	Np(Nt-1p)7; Np(Nt-1p)17; Np(Nt-1p)27; Np(Nt-1p)38; Np(Nt-1p)40; Np(Nt-1p)41	Sidorov et al. 1981
Nicotiana plumbaginifolia cybrids with SR1 plastids	Np(SR1)6; Np(SR1)16; Np(SR1)22; Np(SR1)30	Menczel et al. 1982
Nicotiana tabacum cybrids with *Nicotiana plumbaginifolia* cytoplasm	Nt105, Nt133	Maliga et al. 1982
Nicotiana plumbaginifolia intraspecific somatic hybrids	pp1; pp2	Marton et al. 1982, unpublished

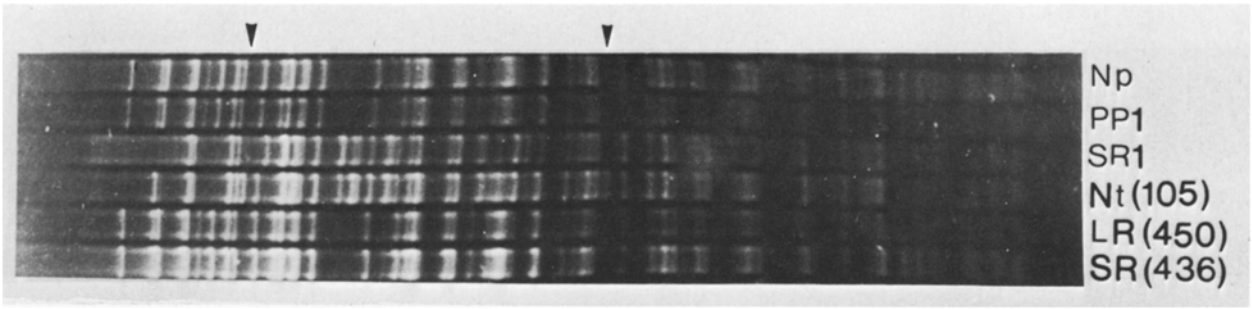

Fig. 1. BamHI restriction patterns of the mtDNAs from different *N. plumbaginifolia* mutants (*LR450, SR436*), or intraspecific *N. plumbaginifolia* somatic hybrid (*pp1*) and a *N. tabacum* cybrid (*Nt105*). Non-parental fragments in the mtDNA of the cybrid are marked (▼). Parental (*Np,* SR1) patterns are also shown. For details on the lines see Table 1

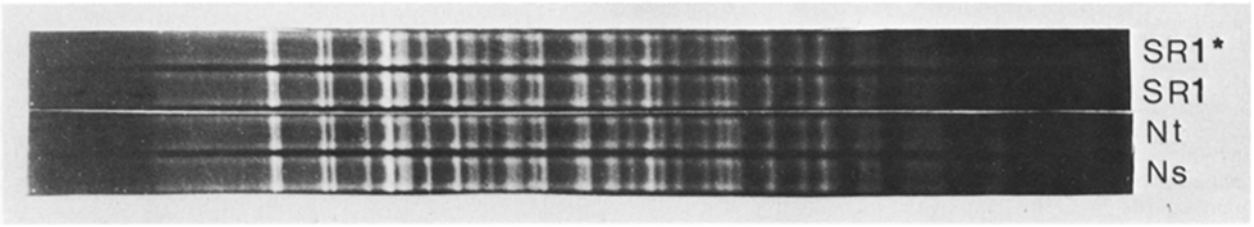

Fig. 2. BamHI restriction patterns of the mtDNAs from *N. sylvestris* (*Ns*), wild-type *N. tabacum* (Nt), *N. tabacum* SR1 plants (*SR1*) and suspension culture (*SR1**)

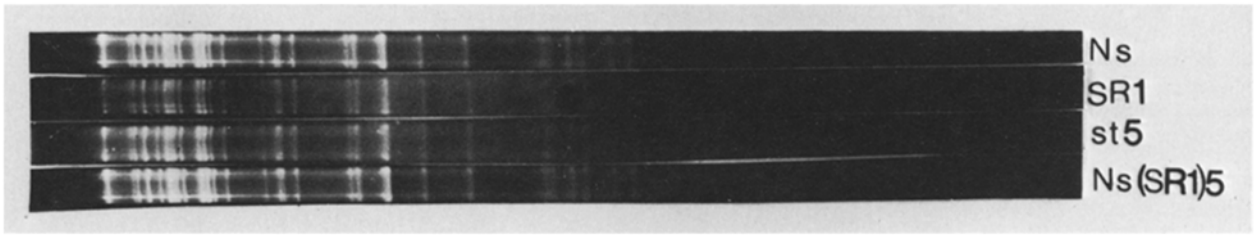

Fig. 3. SalI restriction patterns of the mtDNAs from a *N. sylvestris* cybrid Ns (*SR1*) 5, and from the somatic hybrid *st5,* both obtained by fusing *N. sylvestris* (*Ns*) and *N. tabacum* (SR1) protoplasts. Parental mtDNA patterns are also shown

erated plants and from suspension cultures) and compared to that of the wild type *N. tabacum* cv. 'Petit Havanna' plants. The three mtDNAs could not be distinguished with Bam HI (Fig. 2) or SalI (not shown).

2 MtDNA in plants derived from homoplasmic fusions

N. sylvestris is the cytoplasmic progenitor of the amphiploid species *N. tabacum* (Smith 1975). It is not surprising, therefore, that the mtDNA of the two species could not be distinguished after SalI or BamHI digestion (Figs. 2 and 3). Similar results were obtained by Galun et al. (1982) using XhoI and SalI restriction endonucleases.

The mtDNAs of five *N. tabacum* + *N. sylvestris* somatic hybrids and seven *N. sylvestris* cybrids (Table 1) were identical with each other and with the parental species. A SalI pattern of a cybrid and a somatic hybrid are shown in Fig. 3.

MtDNAs in two intraspecific somatic hybrids of *N. plumbaginifolia* were also investigated. Again, no changes were detected in the BamHI (Fig. 1) and SalI (not shown) patterns, as compared to those of the wild-type *N. plumbaginifolia.*

3 MtDNA in plants derived from the heteroplasmic Nicotiana plumbaginifolia and Nicotiana tabacum protoplast fusions

The mtDNA of *N. tabacum* and of *N. plumbaginifolia* are sufficiently different to allow easy identification by restriction endonucleases (Figs. 1 and 4).

MtDNA of *N. plumbaginifolia* cybrids, derived from fusing protoplasts of the two species, and selected for the acquisition of the *N. tabacum* chloroplasts (Nt-lp, six clones; SR1, four clones; Table 1), was studied in regenerated plants. The BamHI and SalI restriction patterns were non-parental in each of the ten cybrids,

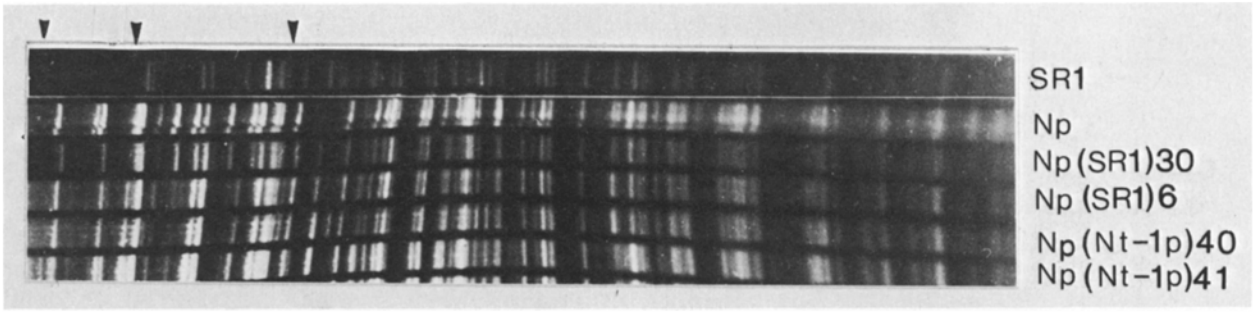

Fig. 4. BamHI mtDNA restriction patterns of *N. plumbaginifolia* cybrids. The cybrids contain streptomycin resistant (*Np(SR1)6, Np(SR1)30*) and light-sensitive (*Np(Nt-1p)40, Np(Nt-1p)41*) chloroplasts derived from *N. tabacum*. Parental patterns (*Np, SR1*) are also shown. *Arrows* indicate the position of non-parental fragments

Table 2. MtDNA in plants regenerated in tissue culture

Plant type	No. of lines	MtDNA [a]
Mutants		
Nicotiana plumbaginifolia streptomycin resistant mutants from protoplasts	5	P
Nicotiana plumbaginifolia lincomycin resistant mutants from protoplasts	4	P
Nicotiana tabacum SR1 streptomycin resistant from callus culture	1	P
Cybrids [b]		
Nicotiana sylvestris (SR1)	7	P
Nicotiana plumbaginifolia (SR1)	4	N
Nicotiana plumbaginifolia (Nt-1p)	6	N
Nicotiana tabacum (Np)	2	N
Somatic hybrids		
Nicotiana sylvestris + *Nicotiana tabacum*	5	P
Nicotiana plumbaginifolia + *Nicotiana plumbaginifolia*	2	P

[a] P = parental type; N = non-parental type
[b] In brackets the origin of cytoplasm is indicated. Lines marked as given in Table 1

and consisted of some (but not all) of the parental fragments, and a few novel fragments not present in any of the parents. Four of these, after digestion with BamHI, are shown on Fig. 4.

Two *N. tabacum* cybrids (Nt105, Nt133), derived from fusing *N. tabacum* and *N. plumbaginifolia* protoplasts, were also investigated. Cybrid plants Nt105 (Fig. 1) and Nt133 (data not shown) contained altered mitochondrial DNA.

Data concerning the mtDNA of various lines are summarised in Table 2.

Discussion

The study of nine antibiotic resistant mutants isolated in protoplast culture and one isolated in callus culture indicated that tissue culture conditions themselves are not sufficient for inducing changes in mtDNA – or at least such changes are so rare that they could not be detected in the present study (Table 2). *Nicotiana* plants in this respect, therefore, seem to be different from *Zea mays* in which rearrangements can be detected in regenerated plants. It should be noted, however, that the *Zea* plants in that study were selected by a mitochondrial resistance trait (Gengenbach et al. 1981; Kemble et al. 1982) so it cannot be excluded that random regeneration of plants in *Zea mays* would lead to results similar to ours.

Seven of the mutants (Tables 1 and 2) were exposed to streptomycin during selection for resistance in culture. Since the patterns from the mutants were identical with those of the original lines from which the cultures were initiated, it is concluded that exposure to streptomycin can not be responsible for alterations in the mitochondrial DNA in heteroplasmic fusions.

The species *N. tabacum* is alloploid and has the *N. sylvestris* cytoplasm (Smith 1975; "Results, section 2"), therefore fusion between these two species may be considered as a homoplasmic fusion. Intraspecific *N. plumbaginifolia* somatic hybrids (pp1, pp2) are also derived from homoplasmic fusions. No changes in the mtDNA were detected in any of these homoplasmic combinations.

MtDNA was rearranged, however, in each of the lines derived from the heteroplasmic *N. tabacum* – *N. plumbaginifolia* fusions (Table 2, Fig. 4). MtDNA in plants derived from heteroplasmic fusions was non-parental in each of the clones studied so far (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982). Formation of a mixed mitochondrial population subsequent to cell fusion, therefore, as a rule, seems to lead to rearrangements in the mtDNA. This observation makes it tempting to speculate on the existance of a mitochondrium-specific system which induces alterations in the mtDNA in heteroplasmic combinations.

Rearrangement in the mtDNA has been interpreted as an indication of recombination between parental mtDNA molecules (Belliard et al. 1979). Reshuffling of a heterogenous parental mitochondrial DNA population (and recombinations) has also been considered (Nagy et al. 1981). Indeed, circular mtDNA molecules of different sizes have been isolated from *N. tabacum* suspension culture cells (Sparks and Dale 1980; Dale 1981), although the presence of different size circles in differentiated plants has not been confirmed. In homoplasmic fusions, in the absence of genetic markers, both homologous genetic recombination and new combinations of parental mtDNA molecules would remain undetected. Further experiments on the fate of mtDNA in these homoplasmic cases are necessary, therefore, to show whether or not fusion-induced rearrangements in the mt-DNA are limited to heteroplasmic combinations.

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