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Chondriome analysis in sexual progenies of *Nicotiana* cybrids

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Summary. We studied the chondriomes (the mitochondrial genomes) of sexual-progeny plants derived from eleven *Nicotiana* cybrids which resulted from donor-recipient protoplast fusions. The recipients were either *N. tabacum* or *N. sylvestris* and the donor (of the cytoplasm) was *N. bigelovii*. The chondriomes were characterized by the mitochondrial DNA (mtDNA) restriction-patterns. The differences in mtDNA restriction patterns were revealed after *Sal* I digestions and probing the respective Southern-blots with three mtDNA fragments. The hybridization patterns of mtDNAs from 35 second-generation plants (i.e. the sexual progeny derived from the cybrid plants) indicated only minor variations between plants derived from the same cybrid but pronounced variations among sibs derived from different cybrids. The mtDNA of 32 second-generation plants varied from both original fusion partners but the mtDNA of one (male-sterile) plant was apparently identical with the mtDNA of one of the original donor (*N. bigelovii*) and the mtDNA of two other (male-fertile) plants was apparently identical to the mtDNA of an original recipient (*N. sylvestris*). Generally, the mtDNAs of male-fertile, second-generation plants were similar to the mtDNAs of the original recipients while the mtDNAs of the male-sterile second-generation plants were similar to the mtDNA of the donor (*N. begelovii*). The analyses of mtDNAs from the third-generation plants indicated stabilization of the chondriomes; no variations were detected between the mtDNAs of plants derived from a given second-generation plant.

Key words: Mitochondrial DNA – Southern-blot hybridization – *Nicotiana* cybrids

Introduction

Belliard et al. (1979) reported on novel mitochondrial DNA (mtDNA) restriction patterns in cytoplasmic hybrids of *Nicotiana* which indicated that chondriome recombination resulted from fusion between protoplasts having different mitochondrial compositions.

Additional information substantiated this report in different *Nicotiana* cybrids (Galun et al. 1982; Nagy et al. 1983; Aviv et al. 1984a, b; Medgyesy et al. 1985) and also in *Petunia* (Boeshore et al. 1983, 1985) in *Brassica* (Chetrit et al. 1985) and in *Daucus* (Matthews and Widholm 1985).

In previously reported cases the mtDNA restriction pattern was visualized by ethidium bromide staining of mtDNA isolated from leaves of several cybrid progenies which were pooled (Belliard et al. 1979; Galun et al. 1982; Nagy et al. 1983; Aviv et al. 1984a). This procedure could have masked variability among individual sibling plants. Only in two studies (Boeshore et al. 1983; Aviv et al. 1984b) was the mtDNA extracted from cell suspensions, which were originally derived from individual cybrid plants. However, only a few attempts were made to compare mtDNA patterns among sibling plants and we are not aware of previous studies in which the mtDNAs analysis of sexual progenies derived from somatic-hybrids or cybrids was based on individual plants.

To study the stability of recombinant mtDNA in the sexual progenies of cybrids, we undertook the analysis of individual sibling plants of such progenies. As the amount of mtDNA obtained from leaves of a single plant is small, visualization of the respective restriction pattern was obtained by Southern-blot hybridization to radioactive mtDNA probes.

In several cases where variability in hybridization patterns was found among siblings, third generation siblings were analyzed to determine the stability of the novel patterns.

Materials and methods

Plant material

Sexual progenies. Cybrid plants were obtained by the donor-recipient method (Galun and Aviv 1983). Donor protoplasts were derived from *N. bigelovii* and recipient protoplasts were derived from either an albino *N. tabacum* line termed VBW (Table 1, Group A) or *N. sulvestris* (Table 1, Group B) as described by Aviv et al. (1984 b). Seeds from 11 different cybrid plants, resulting from self pollination of male-fertile cybrids, or manual cross-pollination of male-sterile cybrids with either *N. tabacum* (Group A) or *N. sylvestris* (Group B) were planted and grown to maturity in the greenhouse.

Protoplast derived plants. Protoplasts prepared from leaves of the cybrid 2–4 (Table 1) were plated, cultured and regenerated into plants as previously described (Aviv et al. 1984 b). Plants a and c (Table 1) were derived from the same callus (i.e. originated from the same protoplast) while plants b and d were derived from different calli.

Preparation of mtDNA and Southern blot-hybridization

Mitochondrial isolation, DNA extraction, digestion with *Sal* I, gel-electrophoresis and Southern hybridization were performed as previously described (Aviv et al. 1984 b) and recently detailed (Galun and Aviv 1986). Mitochondria were isolated separately from 40 g of deveined leaves derived from each of the progeny plants described in Table 1.

Table 1. Cybrid plants and their second generation sexual progenies and protoplast-derived plants utilized for mitochondrial DNA analysis

Designation of cybrid plants	Chloroplast type	Stamen character-ization	Designation of second generation siblings
	Group A- *N. tabacum* nuclei		
2-4	*N. bigelovii*	sterile	a^1, b^1, c^1, d^1
2-8	*N. bigelovii*	sterile	b, c, d
3-14	*N. bigelovii*	fertile[2]	a^2, b^2, c^2, d^2
B-32	*N. bigelovii*	fertile[4]	a^4, b^4, c^4, d^4
	Group B- *N. sylvestris* nuclei		
1-10-1	*N. sylvestris*	sterile	a, b, c
1-11-2	*N. sylvestris*	sterile	b, c, e
5-7-1	*N. sylvestris*	sterile	b, d, f
5-7-7	*N. sylvestris*	sterile	d, e
4-12-1	*N. bigelovii*	fertile[3]	b^2, c^2
4-12-2	*N. bigelovii*	fertile[4]	a^2, c^3, d^2, e^2
4-12-3	*N. sylvestris*	fertile[2]	a^2, c^2, d^2
4-12-4	*N. sylvestris*	fertile[4]	a^2, c^2

First generation cybrid plants were derived from fusion of irradiated *N. bigelovii* donor protoplasts with either *N. tabacum*, VBW protoplasts (group A) or *N. sylvestris* (group B) recipient protoplasts

[1] Protoplast derived progenies (see "Materials and methods"); all other second-generation plants were sexually-derived; [2] normal anthers with abundant pollen grains but not self-fertile; [3] normal anthers with abundant pollen grains but very low self fertility (few seeds per pod); [4] fully self-fertile

Mitochondrial DNA probes

The mitochondrial DNA probes were previously described (Aviv et al. 1984 b). They are discrete *Sal* I fragments of *N. sylvestris* mtDNA, cloned in pBR322. For brevity the clones *pmtSylSa*-1., *pmtSylSa*-2 and *pmtSylSa*-8, shall be referred below as probes 1, 2 and 8, respectively.

Results

The first generation cybrids used in this study were obtained by the donor-recipient technique (Aviv et al. 1984 b) and contained either *N. tabacum* (Table 1, Group A) or *N. sylvestris* nuclei (Table 1, Group B). All Group A cybrids contained donor *N. bigelovii* chloroplasts. Among Group B, two cybrids contained *N. bigelovii* chloroplasts and six cybrids contained recipient *N. sylvestris* chloroplasts. Two cybrids in Group A and four in Group B were cytoplasmic male-sterile (CMS) having defective anthers which did not produce pollen. All other cybrids had normal anthers and produced abundant pollen grains, but they differed with respect to self-fertility: ranging from complete fertility (e.g. cybrid B-32, Group A) through low seed production (e.g. cybrid 4-12-1, Group B) to the absence of self-fertility (e.g. cybrid 4-12-3, Group B).

Mitochondrial DNA was extracted from single progeny plants, derived either by protoplast culture or sexual propagation, as described below. The mtDNA was digested with *Sal* I, separated by gel-electrophoresis transferred to nitrocellulose paper and hybridized to three different *N. sylvestris* mtDNA probes. Several hybridization patterns, obtained with probes 1, 2 or 8 are demonstrated in Fig. 1. When evaluated by only one probe, some progeny-plants had hybridization patterns identical to the donor-parent *N. bigelovii* (e.g. panel B, lanes 9, 10 and 11), certain other progeny plants had hybridization patterns which seem to be identical with the pattern of the recipient-parents *N. tabacum* or *N. sylvestris* (e.g. panel A, lanes 5 and 6). Yet another probe revealed that some progeny plants had novel hybridization patterns (e.g. panel C, lanes 14, 16 and 19).

The hybridization patterns of all the analyzed progeny-plants are schematically presented in Figs. 2 and 3 for Group A and Group B plants, respectively. Note that plants a, b, c, and d of cybrid 2–4 (Table 1, Fig. 2) originated from protoplast-culture rather than from sexual propagation of this cybrid plant. A cell-suspension was also prepared from cybrid 2–4. Ethidium bromide staining of a *Sal* I digest of mtDNA extracted from this suspension revealed complete identity with the *Sal* I digest of mtDNA from intact *N. bigelovii* plants (Fig. 4). Furthermore, the hybridization patterns of *N. bigelovii* mtDNA and the mtDNA extracted from 2–4 cell suspension provided identical hybridization patterns

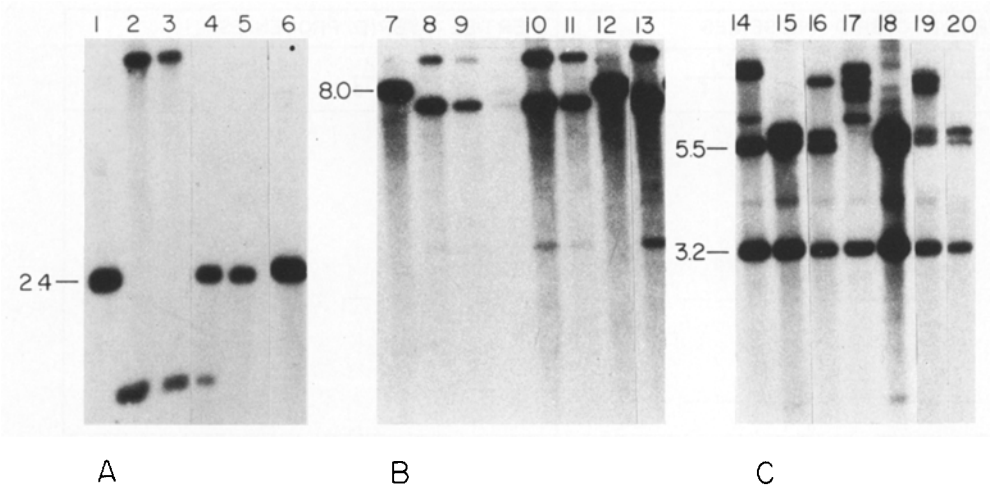

Fig. 1. Southern blot-hybridization of mtDNA isolated from sexual and protoplast-derived progenies of somatic cybrids. MtDNA was isolated from individual plants and digested with *Sal*I. Following gel-electrophoresis the blots were hybridized to mtDNA probe 1 (panel *A*) probe 2 (panel *B*) or probe 8 (panel *C*). Parental species: *N. sylvestris* (=*N. tabacum*) (lanes *1, 7* and *18*), *N. bigelovii* (lanes *2, 13* and *17*). Cybrid progeny: 2-4 (lane *3*), 5-7-1 (d) (lane *4*), 4-12-1 (a) (lane *5*) 1-10-1 (b) (lane *6*), 2-4 (d) (lane *8*), 2-4 (c) (lane *9*), 5-7-7 (d) (lane *10*), 5-7-1 (f) (lane *11*), 4-12-3 (a) (lane *12*), 5-7-7 (d) (lane *14*), 4-12-3 (a) (lane *15*), 4-12-2 (c) (lane *16*), 1-11-2 (b) (lane *19*), 4-12- 4 (c) (lane *20*). Numbers to the left of each panel refer to apparent molecular weight ($\times 10^{-6}$) of DNA standards. See Table 1 for description of the analyzed plants

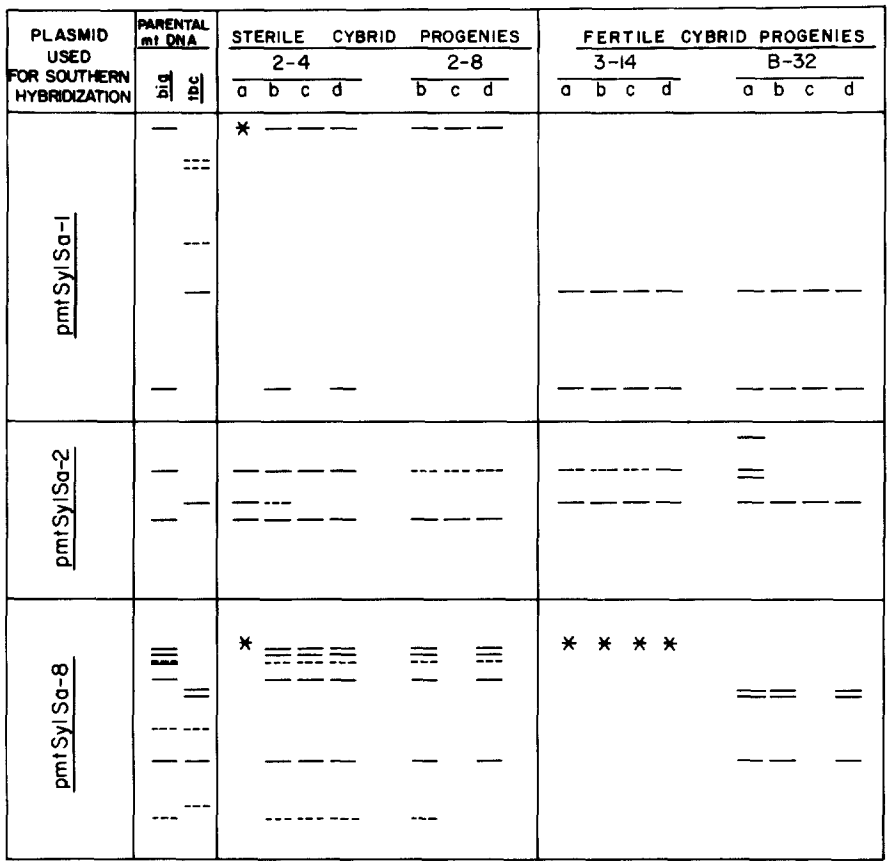

Fig. 2. Schematic presentation of Southern hybridization patterns of mtDNA isolated from sexual and protoplast-derived progenies of cybrid plants. The cybrids originated from fusion of irradiated *N. bigelovii* protoplasts with *N. tabacum*, VBW protoplasts. Broken lines refer to faint hybridization. Numbers refer to cybrid designations; letters (a–d) refer to second generation progeny-plants; designations *big* and *tbc* are abbreviations of *N. bigelovii* and *N. tabacum* respectively

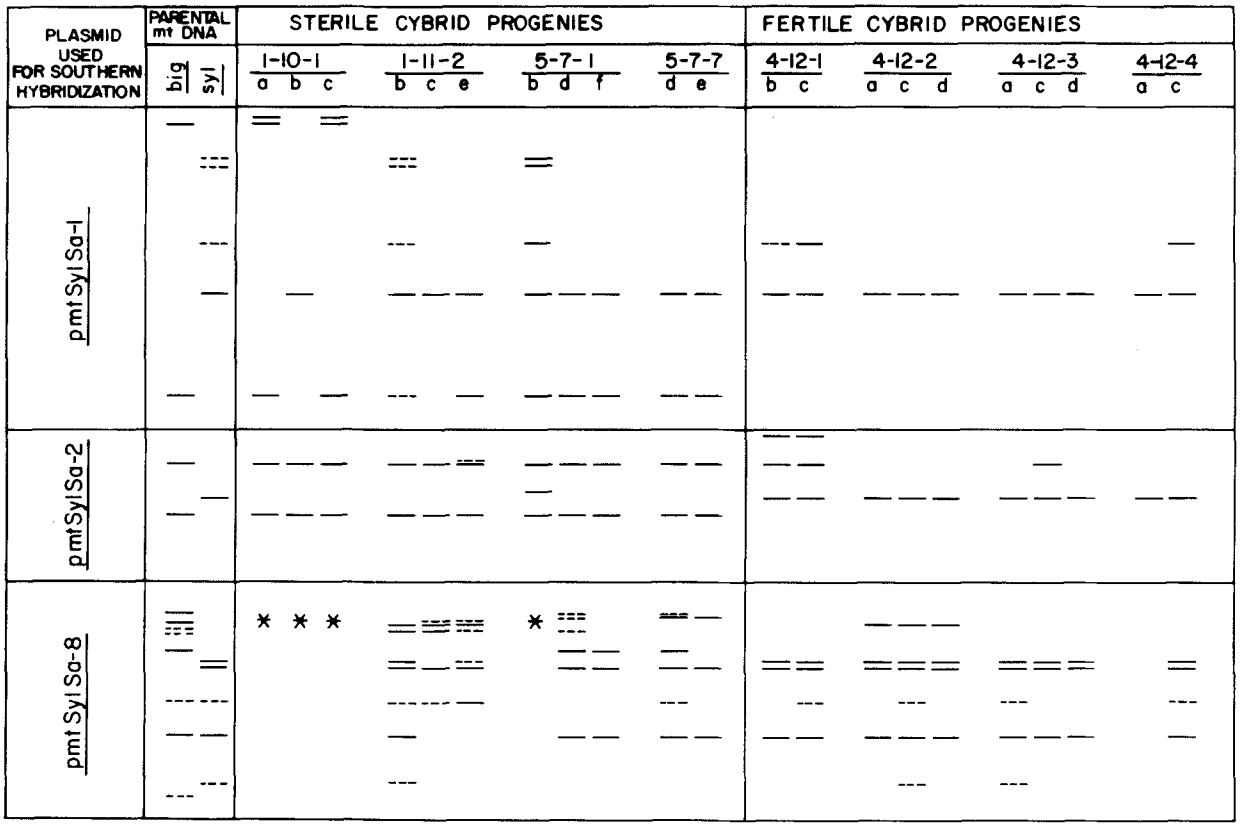

Fig. 3. Schematic presentation of Southern hybridization patterns of mtDNA isolated from sexual progenies of cybrid plants. The cybrids originated from fusion of irradiated *N. bigelovii* protoplasts with *N. sylvestris* protoplasts. Broken lines refer to faint hybridization. Numbers above the line refer to cybrid designation; letters (a–f) refer to second generation progeny-plants; designations *big* and *syl* are abbreviations of *N. bigelovii* and *N. sylvestris* respectively

with any of 3 different probes (not shown). These data furnish a clear indication that neither the culture of protoplasts nor the in vitro culture of cell-suspensions caused changes in the mtDNA restriction profiles.

Among protoplast derived plants of cybrid 2–4 (Fig. 2) only one plant (d) showed mtDNA hybridization-patterns which were identical with *N. bigelovii* when hybridized to any of the three probes. Plant c showed identity with *N. bigelovii* when hybridized with probes 2 and 8 but minor variation with probe 1. Plant b seemed to be identical to *N. bigelovii* when hybridized with probes 1 and 8 but the hybridization pattern was slightly different from that of *N. bigelovii* when analysed with probe 2. Plant a, which was tested only with probe 2 showed an additional hybridization band.

Comparison of mtDNA hybridization patterns among the sexual progeny of another cybrid with *N. tabacum* nuclei (Fig. 2) revealed that the mtDNA of all three siblings of cybrid 2–8 is almost identical to that of *N. bigelovii.* Thus, mtDNA derived from the two cytoplasmic-male sterile progenies (either sexual or via protoplasts) is identical or very similar to mtDNA of *N. bigelovii* (the original organelle-donor). Sexual-progeny plants derived from the fertile cybrid 3–14 had mtDNA which was identical among the siblings but varied from the mtDNA of *N. tabacum* and *N. bigelovii.* Among the progeny of the fertile cybrid B-32 one plant (a) differed from its siblings only when analysed with probe 2. Plants B-32(b) and B-32(d) appeared to have a *N. tabacum* (= *N. sylvestris*) mtDNA pattern when hybridized with either probe 2 or probe 8. When hybridized with probe 1 all four siblings demonstrated a novel pattern.

Similar analysis of mtDNA hybridization patterns of Group B plants (Fig. 3) demonstrated that variations among siblings do occur (e.g. siblings of cybrid 5-7-1 with probe 1 or siblings of cybrid 4-12-3 with probe 2), that most of the variations are detected with only one of the probes and that they exist among sterile progeny-plants as well as among fertile progeny plants. Only two of the fertile progeny plants, 4-12-3(a) and 4-12-3(d), had hybridization patterns which were identical with *N. sylvestris* for all 3 probes. The rest of the fertile progeny plants were similar to *N. sylvestris* but showed different degrees of variations. Among the cytoplasmic male sterile cybrids, in contrast to Group A, none was identical with *N. bigelovii* but all exhibited apparent recombination

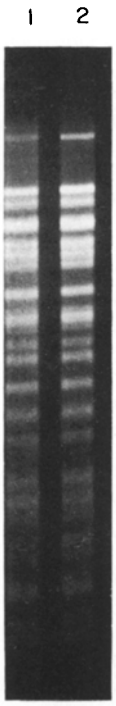

Fig. 4. Ethidium bromide staining of *Sal*I digests of mtDNA isolated from *N. bigelovii* (lane *1*) and cybrid 2–4 (lane *2*) cell suspensions

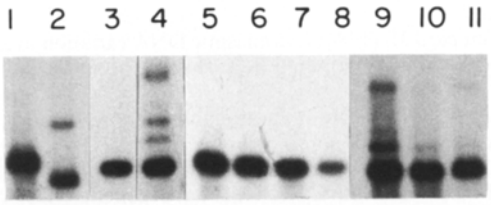

Fig. 5. Blot hybridization of *Sal*I digests of mtDNA from parental mtDNA as well as from second and third generation plants derived from a *N. tabacum* (recipient) + *N. bigelovii* (donor) cybrid to probe 2. Lane *1- N. tabacum,* lane *2- N. bigelovii,* lane *3-* second-generation cybrid B-32 (c); lane *4-* second generation cybrid B-32 (a); lanes *5 to 8-* four siblings of the third generation resulting from sexual propagation of B-32 (c); lanes *9 to 11-* three siblings of the third generation resulting from the sexual propagation of B-32 (a)

patterns between *N. sylvestris* and *N. bigelovii* chondriomes.

Plants B-32(a) and B-32(c) were chosen for the mtDNA analysis of siblings from the third generation (i.e. after two generations of sexual-propagation, following cybridization). While the mtDNA of these two second-generation plants showed identical patterns with probes 1 and 8 they differed in respect to probe 2 (Fig. 2). In B-32 (c), like in *N. tabacum,* probe 2 hybridized to one band only (Fig. 5, lane 3) whereas in B-32(a) this probe hybridized to three additional bands (Fig. 5, lane 4). All four siblings which resulted from sexual propagation of B-32(c) had mtDNA blot hybridizations patterns which were identical with those of their parent plant (Fig. 5, lanes 5–8). Among the three siblings which resulted from B-32(a) one exhibited a similar pattern to its parent (Fig. 5, land 9) while in the other two, there were additional very faint hybridization bands.

Discussion

MtDNA of higher plants displays complex restriction patterns (see reviews by Hanson and Conde 1985; Pring and Lonsdale 1985; Sederoff 1984). Hence, the elucidation of the chondriome structure in these plants presented difficulties. Recently the structure of the *Brassica campestris* mitochondrial genome was described by Palmer and Schields (1984). These authors suggested that the mtDNA is organized in three physically distinct circular molecules. The largest of these circles constitute the entire genome and its size is 218 kb. The sizes of the other two circles are 135 kb and 83 kb and each of them comprises only part of the genome. All three circles seem to contain identical repeat elements which enables them to interconvert via reciprocal recombination.

The structure of the mitochondrial genome of other angiosperm plants may be similar or even more complex than that of *B. campestris,* as was suggested by Lonsdale (1984) for maize. Assuming that the mitochondrial genome of *Nicotiana* is also composed of a "master-genophore" which interconverts into smaller genophores via reciprocal recombinations this may explain the observed mitochondrial recombination patterns in heterofusion plants. Probably the degree of homology between the repeating elements of mitochondrial genomes of different species determines the rate of mitochondrial recombination in the different interspecific cytoplasmic hybrids.

In maize it was reported that mere in vitro culture may induce variations in mtDNA restriction patterns (Gegenbach et al. 1981; McNay et al. 1984). Similarly, Kemble and Shepard (1984) reported mtDNA variations in protoplast derived potato plants. During the last few years we repeatedly extracted mtDNA from several cell suspension lines and obtained a stable mtDNA pattern typical of each of the lines. Nagy et al. (1983) reported no rearrangements of mtDNA in *Nicotiana* plants derived from either protoplasts or homofusion events. Our (unpublished) results from analysis of several protoplast derived plants indicate that minor variations may occur occasionally. While Nagy et al. (1983) noted that heterofusion invariably lead to extensive mitochondrial recombination, our data suggest that such a recombination does occur in most but probably not in all cybrid plants; a few of the cybrids did retain mtDNA hybridization patterns which were apparently identical to those of one of the parents. One example was described in this paper in which cybrid 2–4 and its progeny plant (d) are apparently identical with the *N. bigelovii* parent and cybrids 4-12-3(a) and 4-12-3(d) are probably identical with *N. sylvestris* parent. It should be noted, though, that the impact of protoplast culture on the mtDNA stability in alloplasmic substitution lines requires further

investigation. In another case, one of the cybrids derived from the fusion of *N. rustica* and *N. sylvestris* contained a mtDNA pattern which was identical to that of the *N. sylvestris* parent (Aviv et al. 1984a). More recently we reported on serveral cybrid plants, exhibiting nuclear morphology of the recipient parent *N. tabacum*, and containing mtDNAs which was identical with the donor parent, *N. rustica*. (Aviv et al. 1986) albeit in this particular case it might be as a result of the rhodamine 6-G pretreatment.

It should be kept in mind that the three different probes used for the Southern blot hybridization analysis represent only a small fraction of the mtDNA genome and therefore negative results, i.e., no change in hybridization pattern, does not necessarily exclude a change in other regions of the chondriome.

Comparison of mtDNA of second and third generation siblings in the present investigation demonstrate that in the majority of the studied cases there was no detectable variability in mtDNA among sibling plants. Similar results were recently obtained when we compared the mtDNA hybridization patterns between second and third generation siblings of another fusion combination (Aviv and Galun 1986).

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