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Intersectional cytoplasmic hybrids in *Nicotiana*

Identification of plastomes and chondriomes in *N. sylvestris* + *N. rustica* cybrids having *N. sylvestris* nuclear genomes

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Summary. Cybrid plants having the nuclear genomes of one species and either or both plastomes and chondriomes of another species were obtained by fusing protoplasts of *Nicotiana sylvestris,* as "recipients", with X-irradiated protoplasts of *N. rustica* as "donors" of chloroplasts and mitochondria. Forty-nine flowering plants, derived from 28 calli, were analysed. As expected, they all had *N. sylvestris* (i.e. "recipients") morphology. Chloroplast DNA restriction patterns indicated that 8 and 41 plants had *N. rustica* and *N. sylvestris* plastomes, respectively. Some of the plants with either type of plastomes produced sterile pollen but none showed anther malformation typical to alloplasmic male sterility. Chondriome identification by mitochondrial DNA restriction analysis of cybrid plants revealed only restriction patterns which were either similar or identical to those of *N. sylvestris* while no cybrids with *N. rustica* restriction patterns were detected.

Key words: Protoplast fusion – Cybrids – Restriction pattern – Chloroplast – Mitochondrion

Introduction

Chondriome and plastome coded polypeptides which constitute subunits of organelle enzymes are complemented by nuclear coded subunits introduced into the organelles and assembled there to composite proteins (see reviews of Galun 1982; Galun and Aviv 1983). Thus, for the production of fully functional organelle-enzymes a concerted subunit synthesis, programmed by organelle and nuclear genomes, is required. Subunit misfits were probably eliminated in the process of evolution and ramification of species. One way to investigate the interactions between organelle and nuclear gene products is to expose either or both mitochondria and chloroplasts of a given species to alien nuclear "environments". This can now be achieved in angiosperms by somatic hybridization (Zelcer et al. 1978; Aviv et al. 1980; Sidorov et al. 1981; Galun et al. 1982a; Menczel et al. 1982), as detailed recently in Galun and Aviv (1983). Although cytoplasmic exchange can be achieved by breeding, the protoplast fusion method is not only much less time consuming than repeated backcrossing, it also furnishes the only means to unidirectional transfer in either, rather than both, organelle types.

Generally, interspecific protoplast fusion (Schieder and Vasil 1980; Schieder 1982) resulted commonly in plants with hybrid nuclear genomes and mixed or sorted-out organelles. Hence, although novel organelle compositions may be achieved this heteroplasmonic state will be accompanied by hybrid nuclei. On the other hand, the "donor-recipient" protoplast fusion technique (Zelcer et al. 1978), when applied interspecifically, will produce cybrid plants having nuclei of the "recipient" species only. These plants may have various organelle/nuclear combinations (Galun et al. 1982), including cybrids with "recipient" nuclei but either or both mitochondria and chloroplasts of the "donor" species. The latter type of cybrids are thus efficient potential tools with which to investigate nuclear – organelle interactions.

In the present study we produced cybrids by using two species of different *Nicotiana* sections: *N. sylvestris* (Subgenus Petunioides, Section Alatae) as "recipient" and *N. rustica* (Subgenus Rustica, section Rusticae) as organelle "donor". We intended to study whether plants having *N. sylvestris* nuclei in combination with either or both *N. rustica* chloroplasts and mitochondria could be achieved. Furthermore, provided that such viable cybrids are obtained, will alloplasmic malformations – such as changes in anther differentiation – be correlated to certain nuclear/mitochondrial or nuclear/

chloroplast combinations. A preliminary account of the results has been reported earlier (Galun et al. 1982 b).

Materials and methods

Plant material

Nicotiana rustica was obtained from Dr. G. A. White (USDA/SEA, Beltsville, USA) and *N. sylvestris* was originally received from Dr. P. Maliga (Szeged, Hungary). Both species are sexually propagated in our laboratory.

Protoplast isolation and fusion

Protoplasts were isolated from greenhouse grown *N. sylvestris* and *N. rustica* plants by overnight incubation of leaves with a mixture of macerozyme, driselase and cellulase as described previously (Aviv and Galun 1980). *N. rustica* protoplasts were X-irradiated (5Kr) as described in Aviv and Galun (1980) prior to fusion. Protoplasts of *N. sylvestris* and *N. rustica* were mixed in 1:1 ratio and fused by PEG in the presence of DMSO as described by Medgyesy et al. (1980). After the fusion the protoplasts were plated in either NT agar (Nagata and Takebe 1971) or NT liquid medium which does not permit the development of unfused *N. sylvestris* protoplasts.

Isolation of calli and plant regeneration

Fusion plates were gradually diluted with MS medium (Murashige and Skoog 1962) starting 18 days after fusion. Colonies of 2 to 3 mm diameter were then transferred to fresh MS agar medium and after reaching about 1 cm in diameter were transferred to regeneration medium (MS medium containing 2 μg/ml kinetin, 0.8 μg/ml IAA). Four to five shoots from each callus were transferred to Nitsch agar medium (Nitsch 1969) for rooting. Plantlets were transferred to the greenhouse for further growth.

Determination of chloroplast DNA type

Chloroplast DNA (cpDNA) was prepared according to Fluhr and Edelman (1981). Total DNA extraction and the characterization of cpDNA in this extract were essentially as detailed by Fluhr et al. (1983 b). Briefly, total DNA was extracted from 200 mg of leaf tissue, digested with Pvu II, run on agarose gel, transferred to nitrocellulose and hybridized with nick-translated cpDNA probe (pBa 1–9) which was kindly provided by R. Fluhr.

Determination of mitochondrial DNA type

Maternal progenies of plants resulting from protoplast fusion were raised in the greenhouse. Mitochondrial DNA (mtDNA) was isolated from about 250 g leaf tissue of 2–3 months old plants, as previously described (Galun et al. 1982). Parental (i.e. *N. sylvestris* and *N. rustica*) mtDNA was extracted from the respective cell suspensions according to Sparks and Dale (1980), digested with either SalI or XhoI, run on agarose gel and further processed as previously described (Galun et al. 1982).

Results

In order to obtain cybrids which will contain *N. sylvestris* nuclei but various combinations of *N. rustica* and *N. sylvestris* organelles, we used the "donor-recipient" system in combination with selection against plant regeneration from unfused (and homo-fused) *N. sylvestris* protoplasts. In our experimental design we fused *N. sylvestris* protoplasts, henceforth defined as syl/syl/syl (for nuclear genome/plastome/chondriome composition), with X-irradiated *N. rustica* protoplasts (rus/rus/rus) in a 1:1 ratio. Controls consisted of unfused, X-irradiated *N. rustica* protoplasts and unfused *N. sylvestris* protoplasts plated in NT medium as well as mixed *N. rustica* + *N. sylvestris* protoplasts without the addition of PEG. No callus development occurred in any of these controls (in other experiments not reported here, *N. sylvestris* protoplasts occasionally escaped the mannitol inhibition resulting in a few colonies).

We regenerated 119 plantlets of which 54 were grown to maturity. Of these, 49 plants, resulting from 28 calli, attained flowering and were further analyzed (Table 1). All plants were morphologically identical to *N. sylvestris* and none had floral malformations. Since the morphologies of *N. sylvestris* and *N. rustica* differ considerably, we concluded that the regenerated plants had essentially syl nuclei, but we cannot exclude the possibility that some

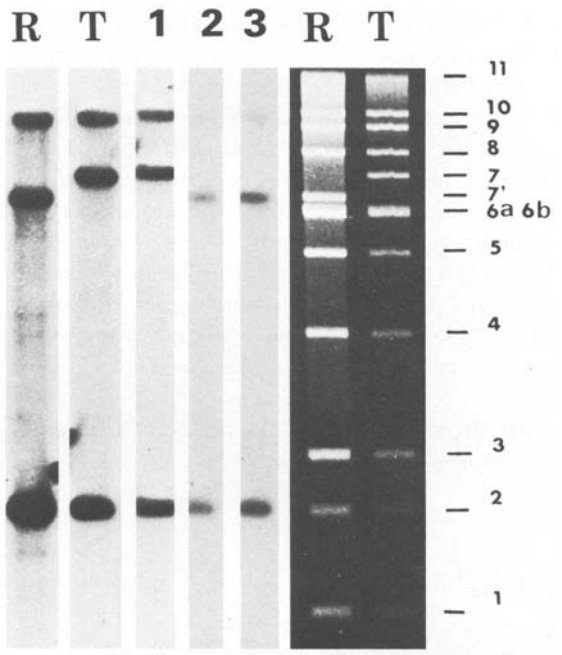

Fig. 1. Chloroplast DNA identification. cpDNA of *N. rustica* (*R*), *N. tabacum* (*T*, identical to cpDNA of *N. sylvestris*) and whole cell DNA from three cybrids (*1–3*) were digested with Pvu II run on an agarose gel and either stained with ethidium bromide (two right slots) or transferred to nitrocellulose paper and hybridized with a radioactive cpDNA probe (clone pBa *1–9*, consisting of *E. coli* plasmid pBR322 containing *N. tabacum* chloroplast insert complementary to Pvu II fragments P2, P7 and P10). Parental cpDNAs differ in location of fragment P7 (for fragment designation see Fluhr and Edelman 1981). Analyzed cybrids: *1* c-1-1; *2* d-1-6; *3* d-2-2

rus nuclear DNA having no morphological expression was transferred to the regenerated plants. Based on the inability of control syl/syl/syl (i.e. normal *N. sylvestris*) to form calli in NT medium we assumed that most or all these plants developed from heterofusion products and will thus be termed cybrids.

Table 1. *N. rustica* + *N. sylvestris* cybrids, having *N. sylvestris* nuclear features, which attained flowering

1. Plants with *N. sylvestris* chloroplasts
   a. Fully self-fertile cybrids
      b-5-2 b- 7-4 b-12-2 b-14-5 b-26-1
      b-5-3 b-10-4 b-13-2 b-17-2 b-32-2
      b-7-1 b-12-1 b-14-1 b-25-3 b-33-1
   b. Slightly self-fertile cybrids
      b- 5-5 b-24-3 b-29-1 b-34-1
      b-18-4 b-25-1 b-31-1
      b-24-1 b-28-2 b-31-2
   c. Self-sterile cybrids
      b- 6-4 b-14-4 b-22-1 b-26-2 b-30-4 c-1-2
      b- 9-3 b-19-1 b-23-3 b-26-3 b-33-2
      b-11-3 b-19-2 b-23-4 b-28-4 b-37-2
2. Plants with *N. rustica* chloroplasts (all self-sterile cybrids)
      b-6-1 d-1-3 d-2-1
      b-6-3 d-1-4 d-1-2
      d-1-1 d-1-6

Table 2. Characterization of cybrids mt DNA based on the analysis of Sal I fragments. Fragment numbers are based on Fig. 2; additional fragments are designated according to location relative to Sal I *N. sylvestris* mtDNA fragments, i.e. additional fragment No. 1/2 is located between fragment No. 1 and fragment No. 2 of *N. sylvestris* mtDNA

Cybrids	Chloroplast type	Fragments differing from *N. sylvestris* mtDNA	
		Missing fragments	Additional fragments
b- 5-2	*syl*	none	none
b- 6-4	*syl*	3; 5; 7; 9	1/2; 1/2; 22/23
b- 7-1	*syl*	none	1/2[a]; 13/14
b-11-3	*syl*	none	12/13; 13/14; 21/22
b-14-5	*syl*	none	none
b-17-2	*syl*	none	none
b-26-1	*syl*	none	none
b-26-3	*syl*	none	none
b- 6-1	*rus*	3; 4; 5; 7; 9; 12; 13	1/2[a]; 1/2[a]; 1/2; 3/4; 12/13; 17/18[a]
b- 6-3	*rus*	2; 7; 8; 18	1/2[a]; 12/13
d- 1-1	*rus*	8	1/2[a]; 1/2[a]; 22/33
d- 1-3	*rus*	2; 8	1/2
d- 1-4	*rus*	2; 7; 9	1/2; 28/29[a]
d- 1-6	*rus*	none	none
d- 2-2	*rus*	2; 8	13/14[a]

[a] Fragment located at same position as a Sal I *N. rustica* mtDNA fragment

Analysis of cpDNA, using whole cell DNA, was performed by hybridization to a plastome probe. Parental cpDNA's differ in one fragment (P7) when digested with Pvu II (Fig. 1). Thus, cybrids containing either *N. rustica* cpDNA (Fig. 1, slots 2, 3) or *N. sylvestris* cpDNA (Fig. 1, slot 1) could easily be identified. By this analysis we found that 8 cybrids (from 3 calli) had rus plastomes while 41 cybrids (from 25 calli) retained syl plastomes (Table 1).

The mitochondrial compositions of the cybrids were identified by their respective mtDNA restriction patterns. The Sal I digested mtDNAs of *N. sylvestris* and *N. rustica* provide very different restriction patterns (Figs. 2 and 3): only 8 relatively light syl fragments comigrate with rus fragments. We found that the cybrids with syl plastomes had mtDNA restriction patterns which were either identical or similar to those of *N. sylvestris*. One such cybrid (b-6-4, Fig. 3, slot 4) lacked some syl fragments and had 3 additional fragments; two other cybrids (b-7-1, b-11-3) had also additional fragments (not shown) while the restriction patterns of other cybrids of this group e.g. b-5-2, b-14-5 or b-26-1 (not shown) were identical to the *N. sylvestris* mtDNA pattern either when digested with Sal I (Table 2) or when digested with Xho I (data not shown).

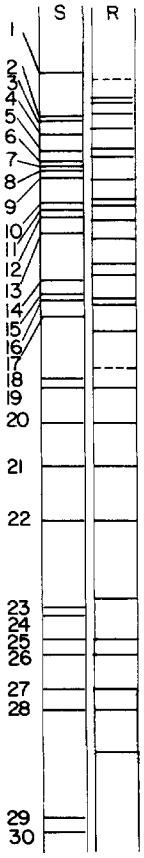

Fig. 2. Schemes of mtDNA restriction patterns of *N. sylvestris* (*S*) and *N. rustica* (*R*) after digestion with Sal I. Sal I fragments of *N. sylvestris* were numbered consecutively starting from the slowly migrating fragments. Some very light, fast migrating and very faint fragments are not shown.

When the mtDNA of the cybrids with rus plastomes were similarly analyzed we found (Figs. 3 and 4; Table 2) that none had rus mtDNA restriction patterns. One of these cybrids (d-1-6) had a restriction pattern which was identical to syl mtDNA when digested with Sal I (fig. 4, slot 3) but differed from syl when digested with Xho I (Fig. 4, slot 8). Other cybrids had patterns which were similar to syl but they lacked some fragments and had one or more additional fragments, e.g. d-1-4, Fig. 3 slot 3; d-1-1, Fig. 4 slot 2 (additional fragments are marked by white squares and missing fragments are marked by white circles).

Most of the regenerated plants had 2n = 48 chromosomes, and thus were tetraploids. This polyploidization

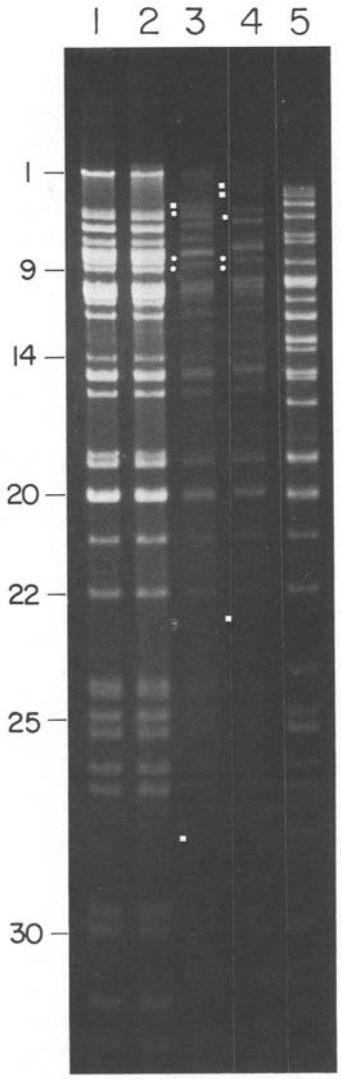

Fig. 3. MtDNA restriction patterns, after Sal I digestion of the parental species *N. sylvestris* (*slot 1*) and *N. rustica* (*slot 5*) and of three cybrids: b-17-2 (*slot 2*), d-1-4 (*slot 3*) and b-6-4 (*slot 4*). Missing bands are marked by *white circles* and additional bands by *white squares*. Band numbers are as in Fig. 2

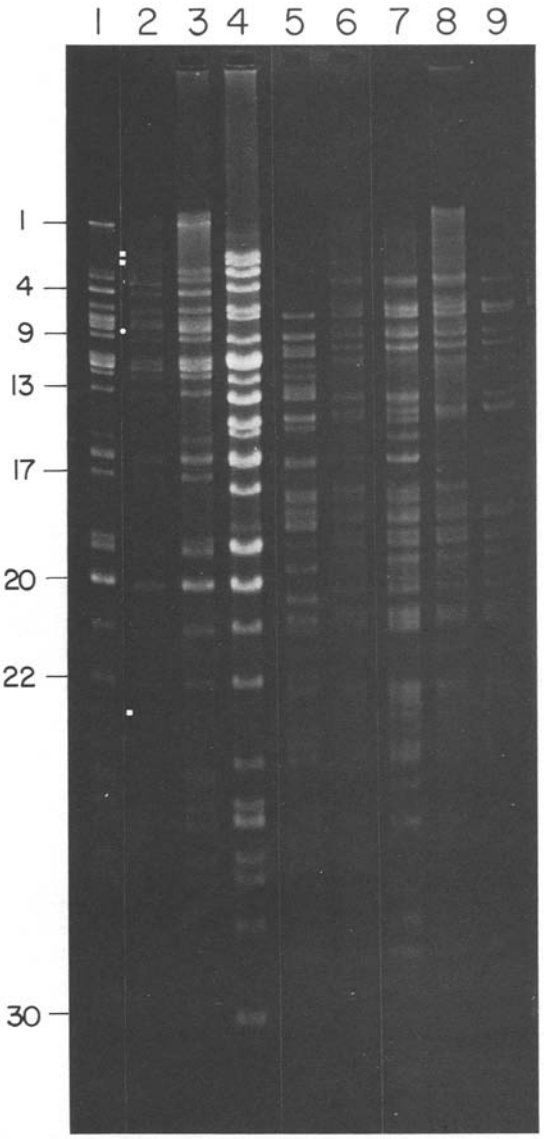

Fig. 4. MtDNA restriction patterns after Sal I (slots 1–4) and Xho I (*slots 5–9*) digestions of the parental species *N. sylvestris* (*slots 1 and 9*) and *N. rustica* (*slots 4 and 5*) and of three cybrids with *N. rustica* plastomes: d-1-1 (*slots 2 and 6*), d-1-6 (*slots 3 and 8*) and d-1-3 (*slot 7*). Missing band is marked by *white circle* and additional bands by *white squares*. Band numbers as in Fig. 2

of protoplast derived plants was expected in *N. sylvestris* (Zelcer et al. 1978). Although all the cybrids had morphologically normal anthers, pollen viability, as evaluated by pollen germination tests, was variable. Some plants produced only non-viable pollen. There was no correlation between pollen viability and plastome composition: there were syl/syl/syl plants with either viable (e.g. b-5-2) or non-viable pollen (e.g. b-6-4). Furthermore, several functional male sterile cybrids

(e.g. b-6-4, d-1-1, d-1-6, d-2-2) were pollinated with *N. sylvestris* and their progeny were tested again for pollen viability. All were found to produce only non-viable pollen suggesting that the plants are cytoplasmic male sterile.

Discussion

Heteroplasmonic fusion products which, through culture and regeneration, lead to plants having novel chloroplast/mitochondrion combinations can be obtained in angiosperms by somatic hybridization (Galun 1982). Galun and Aviv (1983) recently reviewed studies in which the chloroplast compositions of plants resulting from somatic hybridization were analysed. The hybrids (including cybrids) commonly contained pure populations of chloroplasts from either one of the two fusion partners but persistent heteroplastomic hybrids have also been reported (Gleba 1979; Fluhr et al. 1983 a, b).

Nagao (1978) and Iwai et al. (1980, 1981) as well as Douglas et al. (1981) fused *N. tabacum* and *N. rustica* protoplasts and obtained plants with hybrid nuclear genomes. Eighteen plants were analyzed in both of these studies and the authors used isoelectric focussing of the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxigenase for chloroplast identification. Their results indicated that each hybrid contained either the LS of *N. rustica* (13 plants) or the LS of *N. tabacum* (5 plants). Flick and Evans (1982) recently reported on somatic hybridization between *N. tabacum* and each of three wild species – *N. nesophila, N. glauca* and *N. otophora* – resulting in 5, 1 and 2 hybrid plants, respectively. They used tentoxin – towards which the wild species have maternal inherited sensitivity while *N. tabacum* is resistant – to identify the chloroplasts of the hybrids. The *N. glauca* + *N. tabacum* hybrid was tentoxin resistant (i.e. *tabacum* chloroplasts) while the hybrids from *N. otophora* + *N. tabacum* and *N. nesophila* + *N. tabacum* were tentoxin sensitive (i.e. *otophora* and *nesophila* chloroplasts, respectively). The authors considered the results of the latter two hybridizations to indicate "non-random segregation of cytoplasmic DNA" and to be related to a nuclear/chloroplast incompatibility which precludes existence of *N. tabacum* chloroplasts with hybrid nuclei containing nuclear material of *N. otophora* or *N. nesophila.* It should be noted that the mitochondrial composition of the hybrids was not analyzed. Thus, whether the claimed cytoplasmic/nuclear incompatibility is actually based on chloroplasts or on mitochondria is an open question.

A serious drawback of somatic hybrids with respect to investigating possible nuclear/cytoplasmic incompatibility is that the nuclei contain the genomes of both fusion partners. As pointed out by us in the past (Galun and Aviv 1978) an efficient way to avoid this complication is to produce cybrids with nuclei containing the genome of only one fusion partner (the "recipient") and organelles of the other fusion partner (the "donor"). Such cybrids have been produced by us (Zelcer et al. 1978; Aviv and Galun 1980; Galun et al. 1982 a, b, Fluhr et al. 1983 a) as well as by others (Belliard et al. 1978; Sidorov et al. 1981; Menczel et al. 1982; Maliga et al. 1982) and the same strategy was utilized in the present study.

In this study we showed that fully functional and morphologically normal cybrids with *N. sylvestris* nuclei and *N. rustica* chloroplasts can be obtained. The base sequence of the plastomes of these two species, as expressed in the respective cpDNA restriction patterns, are different. In spite of this difference no nuclear/chloroplast incompatibility was detected in the syl/rus (genome/plastome) combination. We do not have an unequivocal explanation for this compatibility. The two most plausible possibilities, not mutually exclusive, are that (a) the differences in base sequence are at sites which are not involved in nuclear/plastome coordination; (b) the plastomes of syl and rus do code for polypeptides with different amino-acids sequences but these differences do not cause mis-fits between sub-units coded by the syl genome and the rus plastome, respectively.

This study also showed that all 8 cybrids which had syl/rus (genome/plastome) composition had syl rather than rus chondriomes and can thus be abbreviated as syl/rus/syl. No syl/rus/rus cybrids were detected. Are nuclear genome/chondriome compatibility requirements more stringent than genome/plastome requirements? Our data is not sufficiently extensive to furnish a decisive answer to this question but we should recall that in previous *Nicotiana* systems studied by somatic hybridization (Belliard et al. 1979; Galun et al. 1982) chondriom – rather than plastome/nuclear exchange-caused anther malformations. Thus an affirmative answer to the above posed question should be seriously considered and a major role of mitochondrial mutation in the evolution of new species is plausible.

As for the cause of cytoplasmic male sterility, the above results support ours' and others' previous results where it was shown that plastome/nuclear interactions is not involved. On the other hand, we still do not have enough data to unravel the role of chondriome/nucleus interaction in causing male sterility. None of the cybrids reported in Table 2 contain *N. rustica* mitochondria. Their mitochondria are either identical or very similar to *N. sylvestris.* The mitochondria of all fertile cybrids but one (b-7-1) are identical with *N. sylvestris.* The mitochondria of all sterile cybrids but (b-26-3, d-1-6) differ from *N. sylvestris* in respect to addition or omission of several fragments. There is no apparent correlation between the type of rearrangement and pollen sterility.

Several cybrids (e.g. b-5-2, b-14-5, b-17-2 and b-26-1) have Sal I and Xho I mt DNA patterns which are identical with the respective *N. sylvestris* patterns (Table 2). As all these plants have *N. sylvestris* plastomes (and all are fertile) therefore we cannot exclude the possibility that they are *N. sylvestris* escapees. However, they also could have resulted from segregating heterofusion products. In favor of the second alternative are the following points: 1. Cybrid b-7-1 is also a syl/syl/syl type and is fully fertile and yet it has a slightly changed Sal I mt DNA pattern. 2. Cybrid d-1-6, which clearly resulted from heterofusion as it is a syl/rus/syl type has identical Sal I pattern as *N. sylvestris* although it has a

slightly changed Xho I pattern. Recently Maliga and coworkers found (personal communication) that every heterofusion leads to mtDNA rearrangements. Our previous results (Galun et al. 1982 a) as well as the present ones suggest that although very often a heterofusion leads to mtDNA rearrangements, the degree of rearrangements is variable from very pronounced to minor ones and possibly to those which are difficult to detect.

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