Theor Appl Genet (1983) 65:289–294

Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusions in *Nicotiana*

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Received October 14, 1982; Accepted February 2, 1983
Communicated by G. Wenzel and Y. Gleba

Summary. Streptomycin resistance was transferred by "donor-recipient" protoplast fusion from *Nicotiana tabacum* (SR-1) protoplasts into *Nicotiana tabacum* (cytoplasmic male sterile – Line 92) protoplasts in one case and into *Nicotiana sylvestris* protoplasts in another. It is demonstrated that streptomycin resistance (SR-1) is a chloroplast marker which segregates independently from a mitochondrial marker.

In the fusion experiment where *Nicotiana tabacum* (Line 92) was the recipient, microcalli were plated in the presence of streptomycin. In this case, chloroplast sorting out occurred at a stage preceeding plant regeneration, producing stable streptomycin resistant cybrids. In the fusion whre *Nicotiana sylvestris* was the recipient, no direct selection for streptomycin resistance was performed. In this case chloroplast sorting out was incomplete, thus producing cybrid plants with a mixed chloroplast population. In some plants, sorting out of streptomycin resistant and sensitive chloroplasts was still apparent in the second generation progeny.

Key words: Mitochondrial DNA – Somatic fusion – Organelle segregation – Chloroplast streptomycin resistance – Chloroplast DNA – Cybrids

Introduction

Protoplast fusion followed by plant regeneration, is a powerful tool for the study of organelle genetics (Galun and Aviv 1982). In tobacco, as in most angiosperms, chloroplasts and mitochondria are uniparentally (maternally) inherited (Kirk and Tilney-Basset 1978; Sears 1980). Protoplast fusion provides a means for introducing two populations of cytoplasmic organelles into one cell. The interaction between different organelles and novel nuclear-cytoplasm combinations can then be studied. Zelcer et al. (1978) have shown that by X-irradation of the donor protoplasts, a preferential transfer of organelles but not donor nucleus occurs. This procedure markedly increases cybrid plant production and is therefore particularly useful in the study of organelle transfer.

Recently, streptomycin resistance was transferred via protoplast fusion from *N. tabacum* (SR-1) to *N. knightiana* (Menczel et al. 1981), *N. sylvestris* (Medygesy et al. 1980) and *N. plumbaginifolia* (Maliga et al. 1982). From this and previous results (Yurina et al. 1978) it was suggested that streptomycin resistance resides in the chloroplast. As mitochondrial markers were not analysed the possibility that streptomycin resistance resides in mitochondria could not be excluded. Nagy et al. (1981) attempted to eliminate the possibility of streptomycin resistance linkage to mitochondrial DNA (mtDNA) but the evidence was not unequivocal due to exentsive rearrangement of the mtDNA in the somatic fusion progeny. It should be emphasized that the establishment of correct linkage groupings in cytoplasmic genetics is essential for full utilization of fusion techniques. Here we show that streptomycin resistance cosegregated with a chloroplast marker and independently segregated from a mitochondrial marker indicating that the streptomycin resistance of line SR-1 resides in the chloroplast.

The reported segregation of chloroplast types in hybrids following somatic fusion suggests that in most cases (involving either intraspecific or interspecific fusion) a rapid sorting out of organelles is achieved (Chen et al. 1977; Belliard et al. 1979; Melchers et al. 1978; Scowcroft and Larkin 1981). A model hypothesizing a transient state with very low organelle

number (Scowcroft and Larkin 1981) was put forth to explain the observed rapid sorting out and random fixation of one organelle type. We have transferred streptomycin resistance to acceptor protoplasts under different selective regimes to contrast the effect of marker selection on organelle segregation, thus we have obtained cybrid plants with mixed chloroplasts.

Materials and methods

N. tabacum, SR-1 (streptomycin resistant) and *N. tabacum* L-92 cytoplasmic male sterile (CMS) seeds were kindly provided by Drs. P. Maliga (Szeged) and A. Marani (Rehovot) respectively. Protoplasts were isolated from leaves of green-house grown plants of SR-1, L-92 and *N. sylvestris* according to a previously published method (Zelcer et al. 1978). Protoplast fusion by polyethylene glycol treatment was as described before (Aviv and Galun 1980). SR-1 donor protoplasts were X-irradiated 5 Kr in Experiment I (Rich Seifert & Co. Demoval apparatus) or γ-irradiated 100 kr in Experiment II (Cobalt 60, G.B. 150A Atomic Energy of Canada).

For the analysis of fusion-product plants, seeds of fusion product plants were tested for streptomycin sensitivity by germination on Nitsch agar plates (Nitsch 1969) containing 1 mg/ml streptomycin. Secondary leaves of resistant seedlings greened while sensitive ones became chlorotic. Tentoxin sensitivity was determined by germination of seeds in water containing 20 µg/ml tentoxin (Durbin and Uchytol 1977). Primary leaves of resistant seedlings greened while sensitive ones became chlorotic.

Chloroplast DNA (cpDNA) isolation and digestion with Bgl I endonuclease were as previously described (Aviv et al. 1980). MtDNA was extracted and digested with Xho I and Sal I endonucleases as described in Galun et al. (1982).

Results

Transfer of N. tabacum (SR-1) cytoplasm to N. tabacum (L-92) with selection for streptomycin resistance

SR-1 protoplasts were X-irradiated, fused with L-92 protoplasts and plated in Nagata and Takebe, media (Nagata and Takebe 1971), without streptomycin. Only L-92 nuclei were expected to divide as the irradiation inhibited SR-1 nuclear division. One month later, the plates were washed with Murashige Skoog medium (Murashige and Skoog 1962) and calli were checked for regeneration by individual transfer to the same medium containing 2 µg/ml kinetin, 0.8 µg/ml IAA and 0.5 mg/ml streptomycin.

In the presence of streptomycin, SR-1 calli regenerate green shoots while streptomycin sensitive calli produce albino shoots. Altogether, 4 calli producing green shoots were isolated from the original fusion plates. Two to six plants were grown from each of the calli and transferred to the green-house. Several characteristics of these plants are summarized in Table 1.

The general morphology and flower type of each plant was observed and noted. Leaves of SR-1 plants were tapered and slender, their influorescence profused and their anthers fertile. On the other hand, L-92 plants which are male sterile, had bigger and wider leaves, compact inflorescences and anthers which were petaloid – stigmatoid. All regenerated plants had the general morphology of recipient L-92 plants, as expected. Where chromosome number was determined (in 5 plants, derived from three different calli), their

Table 1. Characterization of *Nicotiana tabacum* cybrid plants derived from the fusion of X-irradiated protoplasts from a streptomycin-resistant (SR-1) plant and protoplasts of a cytoplasmic male-sterile L-92 plant. All regenerated plants had L-92 nuclear type morphology. Numbers in parentheses refer to number of plants tested

Callus designation	No. of plants regenerated	Chloroplast characters			Anther type	Chromosome No.[c]
		Streptomycin sensitivity[a]	Tentoxin sensitivity[a]	cpDNA restriction pattern[b]		
			Cybrids			
A	2	R (2)	R (2)	SR-1 (2)	sterile (1)	–
					fertile (1)	48 (1)
B	4	R (4)	R (4)	SR-1 (1)	sterile (4)	48 (2)
C	2	S (2)	S (2)	L-92 (1)	sterile (2)	–
D	6	R (6)	R (6)	SR-1 (2)	sterile (6)	48 (2)
			Parents			
SR-1		R	R	SR-1	fertile	48
L-92		S	S	L-92	sterile	48

[a] Seeds of all 14 plants were tested for streptomycin and tentoxin sensitivity (R, resistance; S, sensitivity)
[b] Chloroplast DNA was isolated from 6 plants and individually digested with Bgl I
[c] Chromosome number was determined for 5 plants

cells had consistently 48 chromosomes. The remaining nine plants similarly had normal morphology and were assumed to have the normal amphidiploid chromosome set of L-92 nuclei.

Twelve plants, derived from calli A, B and D produced seedlings which were resistant to both streptomycin and tentoxin, (SR-1 derived characters). CpDNA analysis of the streptomycin-resistant fusion plants revealed SR-1 cpDNA restriction endonuclease patterns as exemplified in Fig. 1. Thus we may designate the twelve fusion plants as cybrids because they contain the recipient L-92 nuclear characters, including in most cases, the recipient cytoplasmic male sterility but contained the donor SR-1 streptomycin-resistant chloroplasts.

Callus A was the only one that gave rise to two different types of plants. Both plants were streptomycin resistant and had the general morphology of L-92 plants. They were therefore assumed to be cybrids composed of L-92 nuclei and SR-1 chloroplasts. However, one plant, A-2 retained the recipient L-92 male sterility, while the other plant, A-1, had male fertility restored.

We have shown recently that in *Nicotiana* cpDNA- rather than mtDNA restriction patterns segregated independently from cytoplasmic male sterility, while most or all mtDNA restriction fragments cosegregated with this trait (Galun et al. 1982). MtDNA was isolated from D-4 and A-1 plants, digested with endonuclease and fractionated in 0.7% agarose gels (Fig. 2). The D-4 plants produced a mtDNA restriction pattern differing from both parental strains while mtDNA from A-1 type yielded a restriction pattern different from that of cytoplasmic male sterile L-92 and similar to that of

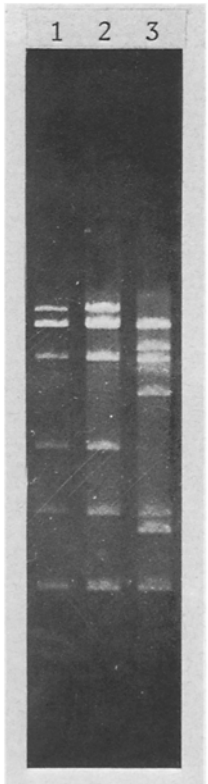

Fig. 1. Bgl I restriction endonuclease digest of cpDNA isolated from: *1 N. tabacum* (SR-1); *2* D-4; *3 N. tabacum* L-92. The species specific difference in *Nicotiana* of Bgl I digestion has been previously mapped (Fluhr and Edelman 1981)

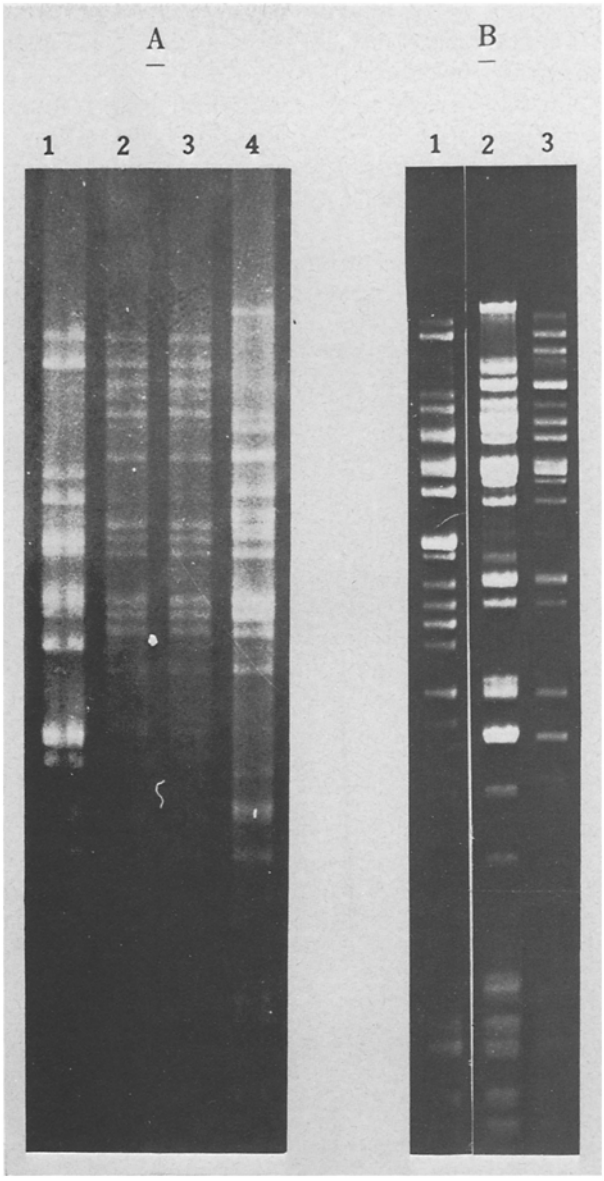

Fig. 2. **A** Sal I digest of mtDNA isolated from: *1 N. tabacum* (L-92); *2* and *3*, 0.5 and 1.5 μg DNA, of cybrid D-4 respectively; *4 N. sylvestris.* **B** Sal I digest of mtDNA isolated from: *1 N. tabacum* (L-92); *2 N. sylvestris; 3* cybrid A-1. We have previously shown that the Sal I digestion pattern of mtDNA from *N. tabacum* and *N. sylvestris* are identical (Galun et al. 1982)

male fertile SR-1. Male fertility restoration in cybrid A-1 appears consistant therefore with a change in mitochondrial population.

Callus C produced 2 plants which were identical with parental L-92 in every respect investigated (Table 1). Thus, this callus apparently resulted from a L-92 protoplast which either escaped streptomycin selection or resulted from a fusion product that later on re-segregated L-92 mitochondria and chloroplasts. This phenomenon was recently reported by Maliga et al. (1982) in selective transfer of SR-1 chloroplasts to *N. plumbaginifolia.*

Transfer of N. tabacum (SR-1) cytoplasm to N. sylvestris without selection for streptomycin resistance

In order to investigate the effect of organelle selection on the stability of the heteroplasmic state, we performed a somatic fusion in which selection for fused progeny would be independent of the streptomycin cytoplasmic marker. *N. tabacum* (SR-1) donor protoplasts were γ-irradiated and fused with *N. sylvestris* protoplasts. The fusion products were plated in Nagata and Takebe medium containing mannitol which was shown to inhibit initial cell division of *N. sylvestris* (Zelcer et al. 1978). Hence it was expected that only fusion products containing unirradiated *N. sylvestris* nuclei fused with SR-1 cytoplasms would develop.

Altogether 24 calli were isolated and plated on regeneration medium without streptomycin and a total of 52 plants were transferred to the greenhouse. All had typical *N. sylvestris* (nuclear) morphology. Seeds of selfed plants were collected and tested for streptomycin resistance. The results, divided into three groupings, are summarized in Table 2. In group I three independent calli yielded seedlings which exhibited resistance to streptomycin. It was concluded that this group of cybrids contained *N. sylvestris* nuclei and *N. tabacum* (SR-1) chloroplasts. In group II nine calli produced streptomycin sensitive plants only. These might have originated from early sorting out of streptomycin sensitive chloroplasts in cybrid calli, or from *N. sylvestris* protoplasts which had escaped mannitol selection. The third group, of twelve calli, produced mixed progeny, i.e. each callus produced at least one plant that among its seedlings either streptomycin sensitive, resistant or

Table 2. Streptomycin test of progeny of selfed cybrid plants derived from the fusion of γ-irradiated *N. tabacum* (SR-1) protoplasts and *N. sylvestris* protoplasts. All regenerated plants were of *N. sylvestris* morphology (i.e. with *N. sylvestris* nuclei)

Group	Callus designation	No. of plants regenerated	Plants yielding resistant seedlings only	Plants yielding sensitive seedlings only	Plants yielding resistant, sensitive and mixed seedlings
I	d-3	3	3	–	–
	d-14	2	2	–	–
	l-3	3	3	–	–
II	c-1	1	–	1	–
	c-8	2	–	2	–
	e-4	2	–	2	–
	f-4	1	–	1	–
	f-7	1	–	1	–
	f-9	1	–	1	–
	l-4	3	–	3	–
	a-1	3	–	3	–
	b-11	2	–	2	–
III	b-13	1	–	–	1
	d-4	3	–	2	1
	d-6	3	–	1	2
	d-8	4	–	–	4
	d-13	3	–	2	1
	d-15	1	–	–	1
	d-16	2	1	–	1
	e-8	3	–	2	1
	f-1	1	–	–	1
	f-3	2	–	1	1
	b-6	2	–	–	2
	b-8	3	–	–	2

mixed seedlings appeared. Thus under conditions of neutral selection one can readily obtain mixed chloroplast characters in progeny.

Discussion

Previous attempts to establish the cytoplasmic linkage of *N. tabacum* (SR-1) resistance showed that fusion plants which had SR-1 plastid types were resistant to streptomycin (Menczel et al. 1981). However, in this context it is of utmost importance to show that plastid and mitochondrial combinations in the resultant progeny are the result of independent segregation events; this was not previously done. We present here evidence using a mitochondrial marker for *N. tabacum,* cytoplasmic male sterility (Galun et al. 1982), which shows that, indeed, the segregation of these two cytoplasmic organelles in post fusion divisions is independent. In the fusion (Table 1) between male fertile *N. tabacum* (SR-1) and *N. tabacum* L-92 (CMS) 3 independent calli ware obtained producing eleven plants, all with SR-1 type chloroplasts but exhibiting CMS type mitochondria. In contrast in a different fusion experiment (Aviv and Galun 1980) we obtained a higher rate of male fertility restoration.

Mt-DNA analysis of one of these plants (D-4) showed novel mtDNA restriction patterns (Fig. 2) in line with previously reported results (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982). As the altered mtDNA patterns may represent recombination events between parental mitochondrial species, the possibility that streptomycin resistance in *Nicotiana* is a mitochondrial character which has independently recombined with the cytoplastic male sterile character in all fusion progeny cannot be ruled out, but we consider this remote.

In previously reported fusions where cybrids were produced (Chen et al. 1977; Melchers et al. 1978; Scowcroft and Lakin 1981; Iwai et al. 1980; Medgyesy et al. 1980, Douglas et al. 1981; Glimelius et al. 1981) a quick sorting out of plastid characters was obtained (however see Gleba 1979). Our experimental results suggest that given the proper experimental mode and sensitivity of assay for the presence of cybrids, it is not difficult to obtain mixed chloroplast cybrids from the original heterokaryons through sexual reproduction of the respective offsprings. For example when the heterokaryon is not subject to selection for streptomycin resistance as in the fusion between *N. tabacum* (Sr-1) and *N. sylvestris,* a large percentage of offsprings (above 50%) exhibited mixed plastid characteristics (Table 2). In contrast, in a fusion experiment reported by Medgyesy et al. (1980) using the same parental types but where the resultant heterokaryons were subsequently plated on streptomycin – containing media, only streptomycin resistant plants were regenerated.

We have obtained mixed cytoplasms in fusions of *N. tabacum* protoplasts containing white plastids with irradiated *N. tabacum* protoplasts containing green plastids (unpublished results, Galun and Aviv 1982). In these experiments many green calli gave rise to variegated plants in which the white portions consisted of a small fraction of the total leaf area. This suggests that in order to properly sample a regenerated plant for non-visual markers a large part of the plant should be used; or as we have done in the analysis of progeny in Table 2, many independent seed pods of the regenerated plants should be sampled. The importance of large sampling volume is illustrated by a report by Iwai et al. (1980) where in a fusion between *N. tabacum* and *N. rustica* one of the regenerated hybrids, examined by small scale sampling, showed only *N. tabacum* large subunit ribulose bisphosphate carboxylase patterns. However, out of nine androgenetic plants produced from the anthers of this hybrid two showed *N. rustica* large subunit carboxylase patterns (Iwai et al. 1981). This suggests to us that the sensitivity of the technique used was too low or the sampling volume too small for detection of low percent mixtures which were apparently present in the hybrids.

The high percentage of cytoplasms containing mixed chloroplasts obtained by our procedure is in contrast to the claim for rare or none existence of mixed chloroplasts, stated by Scowcroft and Larkin (1981) who did not detect mixed chloroplast populations in the six intraspecific hybrid plants obtained in their experiments. The reason for their failure is not clear.

Acknowledgements. We are grateful to Pazia Arzee-Gonen for the mtDNA analyses and to Shlomith Bleichman for technical assistance.

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