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Streptomycin Resistant and Sensitive Somatic Hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: Correlation of Resistance to *N. tabacum* Plastids

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Summary Protoplasts of *Nicotiana tabacum* SR1 (streptomycin resistant) and of *Nicotiana knightiana* (streptomycin sensitive) were fused using polyethylene glycol treatment. From three heterokaryons 500 clones were obtained. From the 43 which were further investigated, 6 resistant, 3 sensitive, and 34 chimeric (consisting of resistant and sensitive sectors) calli were found. From eight clones, a total of 39 plants were regenerated and identified as somatic hybrids. Chloroplast type (*N. tabacum* = NT or *N. knightiana* = NK) in the plants was determined on the basis of the species specific EcoRI restriction pattern of the chloroplast DNA. Regenerates contained NT (13 plants) or NK (15 plants) plastids but only the plants with the NT chloroplasts were resistant to streptomycin. This finding and our earlier data on uniparental inheritance points to the chloroplasts as the carriers of the streptomycin resistance factor.

Key words: *Nicotiana* – Somatic hybrids – Streptomycin resistance – Plastid segregation – Plastid DNA

Introduction

Streptomycin is an inhibitor of protein synthesis in bacteria. In bacteria the ribosomes are similar in structure to that of chloroplasts and mitochondria in plants (Pestka 1971; Kirk and Tilney-Bassett 1978). In the green alga *Chlamydomonas*, streptomycin resistance is controlled by nuclear and cytoplasmic (maternally inherited) factors (Adams et al. 1976). Chloroplast DNA is generally accepted as the location of the cytoplasmic mutations. Circumstantial evidence, however, indicates that, in at least some cases, mutations in mitochondrial DNA make both chloroplasts and mitochondria resistant (Boynton et al. 1973; Conde et al. 1975; reviewed in Harris et al. 1976).

Uniparentally inherited streptomycin resistance mutations have also been described in a flowering plant, *Nicotiana tabacum* (Maliga et al. 1973, 1975; Umiel 1979). One of them, the SR1 mutant has been characterized and shown to be similar to cytoplasmic mutants in algae. In this line the chloroplasts seem to be affected by the mutation since at least one chloroplast ribosomal protein was shown to be altered (Yurina et al. 1978; Capel et al. 1979). In this paper we provide evidence that SR1 chloroplasts are the carriers of the resistance gene since the resistant phenotype is in each case correlated with chloroplasts from the resistant parent in somatic hybrid plants.

The experimental design is based on the observation that chloroplasts in fused protoplasts quickly segregate, resulting in cells with only one type of parental chloroplasts (e.g. Chen et al. 1977; Belliard et al. 1978; Melchers et al. 1978). Published data suggest that mixed mitochondrial populations are also not maintained. Mitochondrial DNA restriction patterns, however, were different from the parental types (Belliard et al. 1979).

Protoplasts of *Nicotiana knightiana* (streptomycin sensitive) were fused with those of streptomycin resistant *Nicotiana tabacum* (SR1). The heterokaryons were isolated mechanically and cultured in the absence of selection pressure so that the organelles could freely segregate. At a later stage resistance or sensitivity of the clones was established and chloroplasts (and mitochondria) were identified in order to clarify their role in determining the resistant phenotype. Identification was based on the species specific organelle DNA fragmentation patterns generated by restriction endonucleases.

Materials and Methods

1 Culture Media and Conditions

Calli for protoplast isolation were initiated from leaves and subcultured once in the dark on Linsmaier and Skoog's (1965) RM medium supplemented with 0.04 mg kinetin, 0.1 mg 2,4D and 3.0 mg

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indole-3-acetic acid per liter (RMNO). Streptomycin resistance tests and shoot induction were carried out in the light (2000 lux, 16 h daily) on RM medium containing 2.0 mg indole-3-acetic acid and 0.5 mg benzyladenine per liter (RMO). The RM medium supplemented with only 1.0 mg benzyladenine per liter (RMB) was also used for shoot induction. Shoots were rooted and maintained on RM salt solution containing 3% sucrose. All cultures were kept at 28°C. Protoplasts were cultured in the K_3 medium of Kao as modified by Nagy and Maliga (1976) except that 0.4 M glucose was used as osmotic stabilizer and $CaHPO_4$ omitted from the medium.

2 *Isolation of Protoplasts*

N. knightiana protoplasts were isolated from leaves cut into narrow strips (1 mm) using 0.5% driselase dissolved in K_3 medium containing 0.4 M sucrose (pH 5.6). *N. tabacum* SR1 protoplasts were prepared from leaf calli grown on RMNO medium using 1.5% cellulase 'Onozuka' R10 + 0.5% macerozyme R10 in 0.4 M sucrose containing K_3 medium (pH = 5.6). In both cases digestion was carried out overnight (16-18 h) at 25°C in the dark. Protoplasts were filtered through a mesh (63 μ) and subsequently floated to the surface by centrifugation (300 g, 2 minutes). Before fusion, the protoplasts were washed once with the W5 solution (154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl; 5 mM glucose; pH = 5.6).

3 *Protoplast Fusion*

Two drops of a mixture of NK (green) and NT (white) protoplasts (1:2) were placed in a plastic dish (35 mm in diameter) and the cells allowed to settle (20 min). One drop of PEG40 solution (40% polyethylene glycol, Mw = 6000; 0.3 M glucose; 66 mM $CaCl_2$; pH = 6) was then gently added. Ten to fifteen minutes later the fluid over the cells was removed and two drops of W10 solution were added. W10 was freshly prepared by mixing 9 parts of stock A (0.4 M glucose; 66 mM $CaCl_2$; 10% dimethylsulfoxide) with one part of stock B (0.3 M glycine-NaOH buffer, pH = 10.5), both filter sterilized. After 20 minutes the fluid was diluted with 1 ml of K_3 culture medium with 0.4 M glucose. It was replaced 10 minutes later by fresh K_3 medium. The cultures were incubated in dim light (100 lux) at 25°C. This procedure gave consistently high fusion frequencies in the range of 10 to 20 percent.

4 *Isolation and Culture of Fusion Products*

Fusion of green mesophyll and white callus protoplasts yielded fusion products which could easily be recognized by the presence of differentiated plastids and numerous cytoplasmic strands from the mesophyll and callus protoplasts, respectively. Fusion products were isolated 72 hours after PEG treatment using a micropipette under microscopic control, and cultured using a modified version of the microdroplet technique of Gleba (1978). Thirty five isolated fusion products were put into a 9 μl droplet of K_3 medium with 0.4 M glucose in a plastic dish (35 mm in diameter) which contained balance drops to prevent drying. The culture was incubated in dim light (100 lux) at 25°C.

5 *Cytology*

Visualization of heterochromatic blocks in interphase nuclei by Giemsa staining, and counting chromosomes in root tips were carried out as described previously (Maliga et al. 1978).

6 *Analysis of Chloroplast DNAs*

Chloroplasts were prepared according to Bottomley et al. (1974), purified in sucrose gradients (Tewari and Wildman 1969) and washed with the buffer used for the endonuclease treatment (0.2 M NaCl; 20 mM $MgCl_2$; 10 mM Tris; adjusted with HCl to pH 7.8). EcoR1 was prepared from *Escherichia coli* strain RY13 according to Joshimori (1971), and had an activity of about 2 units/μl. Endonuclease treatment of chloroplasts was carried out as described by Atchinson et al. (1976) but chloroplast suspensious equivalent to 800 μg chlorophyll were used for each sample. The samples were run on 2% agarose slab gels (40 cm × 16 cm × 0.3 cm). In some cases chloroplast DNA, purified by caesium chloride density gradient ultracentrifugation, was also tested.

Results

1 *Establishment of the Clones*

Thirty five fusion products were isolated and cultured in a microdroplet (cf. Materials and Methods). Three of them divided and formed small colonies. When the culture medium was depleted, cell division ceased and the cells increased in size until they separated. These cells formed new calli upon dilution with fresh medium. This procedure was repeated. Finally the colonies were plated onto K_3 medium (plus 0.4 M glucose) solidified with agar. In this way, three months after fusion, 500 small (2-3 mm in diameter) calli were obtained. From these, 43 were randomly removed for further studies, and termed clones. They will be referred to as tk1 to tk43.

2 *Streptomycin Resistance in the Clones*

The selected calli were tested for streptomycin resistance by growing them on RMO medium containing streptomycin sulphate (1 mg ml^{-1}). Sensitive calli are white while resistant calli are green on this selective medium (Fig. 1a, c). Using this test, from the 43 clones, 6 resistant and 3 sensitive calli were found. The rest of the clones (34 calli) were chimeric, forming green and white sectors (Fig. 1b). These sectors were separated, and will be termed subclones. Codes for the subclones are derived by adding the letters R (for resistants) or S (for sensitives) to the codes of the original clones.

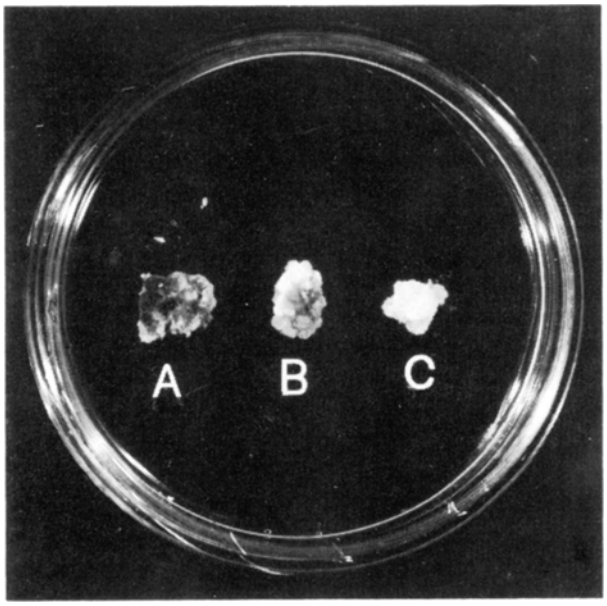

Fig. 1. Streptomycin resistance test. Calli were grown on RMO medium with 1 mg ml^{-1} streptomycin sulphate. *A* resistant; *B* chimeric; *C* sensitive

3 *Plant Regeneration and their Identification as Somatic Hybrids*

Plant regeneration was carried out in ten clones and subclones, from which altogether 39 plants were obtained (Table 1). The morphology of the regenerates is intermediate between the parental species. The flowers on the plants are red like those of *N. tabacum*, a trait dominant over the yellow-green colour of *N. knightiana*. Leaf esterase patterns are identical with those of *N. tabacum*, as is the case with the sexual hybrids (data not shown). Large heterochromatic blocks in the interphase nuclei, however, could also be detected, which is a feature characteristic of *N. knightiana*. Many of the regenerates have 72 chromosomes (Table 1), which could be obtained by a simple addition of the parental chromosome sets: 2n = 4x = 48 chromosomes from *N. tabacum* and 2n = 2x = 24 chromosomes from *N. knightiana*. The identification procedure is described here only briefly because it has been discussed in detail in a previous publication (Maliga et al. 1978) reporting on the isolation of different somatic hybrid lines of the same two species.

Table 1. Streptomycin resistance, chloroplast type and chromosome numbers in the hybrid plants

Streptomycin	resistance[b,c]	Chloroplast type[b,d]	Chromosome number
tk5	R (3)	NT (1)	72
tk7	R (5)	NT (5)	72
tk9[a]	S (7)	NK (7)	72
tk15R	R (1)	NT (1)	72
tk15S	S (4)	NK (3)	66
tk17R	R (6)	NT (6)	72
tk20[a]	S (4)	NK (1)	68
tk26R	R (2)	–	120
tk26S	S (2)	NK (1)	104–115
	R (1)	–	–
tk33S	S (4)	NK (1)	72

[a] Regenerated from calli not previously tested for streptomycin resistance;
[b] In brackets the number of tested plants are given;
[c] R, streptomycin resistant; S, streptomycin sensitive;
[d] NT, *N. tabacum* type; NK, *N. knightiana* type

4 *Streptomycin Resistance in the Plants*

Streptomycin resistance of the regenerates was tested by culturing leaf pieces on selective RMO medium. Callus formed on the cut edges of the resistant leaves was green, whereas it was white in the case of the sensitive leaves (Fig. 2). No sector formation was observed in the developing calli. In all cases but one the phenotype of the plants was identical with that of the callus from which the plants had been regenerated. The only exception was regeneration of a resistant plant from a subclone (tk26S) classified as sensitive. This may have been the result of the incomplete separation of the resistant and sensitive sectors or formation of a new sector from cells containing a few resistant plastids on the selective medium. Results are summarized in Table 1.

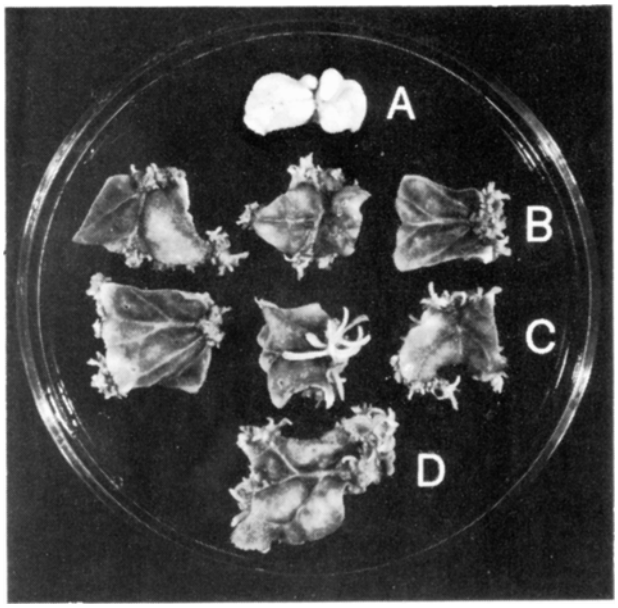

Fig. 2. Streptomycin resistance test. Leaf pieces from regenerated plants were cultured on RMO medium with 1 mg ml^{-1} streptomycin sulphate. *A* sensitive parent; *B* tk7; *C* tk15R; *D* resistant parent

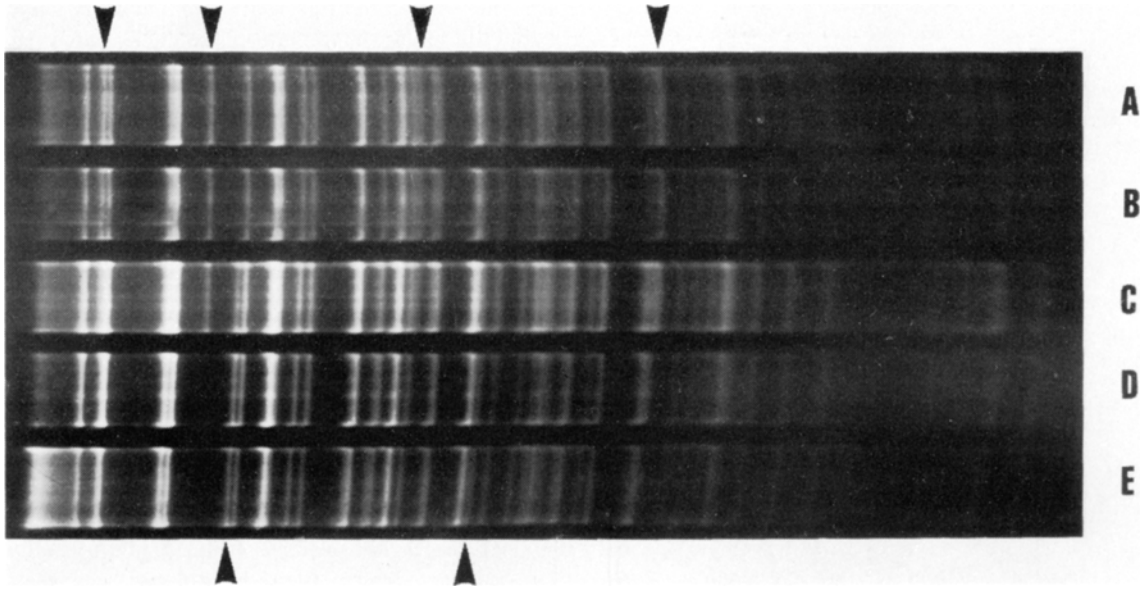

Fig. 3. EcoRI restriction pattern of plastid DNA. Arrows indicate differences in the parental patterns. *A* tk15R; *B N. tabacum*; *C N. tabacum* + *N. knightiana*; *D N. knightiana*; *E* tk9

5 Chloroplast Type in the Plants

EcoRI fragmentation patterns of *N. tabacum* and *N. knightiana* chloroplast DNAs are characteristically different on agarose gels (Fig. 3). There are at least three bands which are suitable for the identification of the chloroplast types. Patterns observed in the regenerates were, in each case, identical with one of the parents (Table 1). There was no indication for the presence of a mixed chloroplast population.

Discussion

The somatic hybrid plants of *N. tabacum* and *N. knightiana*, in general, are similar to those previously described from this laboratory (Maliga et al. 1978). This time, however, most of the plants have 72 chromosomes, a simple addition number. This number is stable, unlike in the previous case in which it was variable (44-131 chromosomes) even within the same plant. It is likely, therefore, that the cultured cells involved in fusion in the first experiment were aneuploids and this resulted in an unusually high degree of chromosome instability in the regenerates. Another difference is that isoenzyme patterns of the somatic hybrids are identical with that of the sexual hybrid (or *N. tabacum*) while they were different (additive) in the previous case. Data on the new stable hybrids confirm that differences seen in the previous case were most probably due to gene-dose effects.

Somatic hybrid plants have been obtained with both NT or NK plastids. In the previous case all hybrid regenerates (28 plants in three independent clones) contained NK chloroplasts (Maliga et al. 1980). A possible explanation may be that in the first case only cells with the NK plastids regenerated into plants.

Plants with NT chloroplasts are resistant, while those with NK plastids are sensitive, to streptomycin. The conclusion, however, that resistance is correlated to the NT plastids can be drawn only if organelle combinations in the clones are the result of independent segregation events. We believe this to be the case although plants have been regenerated from only three heterokaryons. In the progeny of these, mixed organelle populations must have been maintained at least until a total of 500 cells had been obtained, as indicated by the formation of sensitive and resistant sectors in most of the calli derived from this cell population. Furthermore, patterns of mitochondrial DNA (data to be published elsewhere) indicate that plants with the same type of chloroplasts contain different mitochondria: the five lines studied so far have individual patterns composed mainly from the parental Sal I fragments. These data, and maternal inheritance of streptomycin resistance in the SR1 line collectively reinforce our view that chloroplast DNA is the carrier of the genetic information determining streptomycin resistance in this *N. tabacum* mutant. The involvement of mitochondria, however, can be excluded beyond doubt only if segregants with unaltered parental mitochondria will be found.

Streptomycin resistance and male sterility were found to segregate independently after fusing the same fertile

streptomycin resistant mutant (SRI) with sensitive, cytoplasmic male sterile cells (D. Aviv, R. Fluhr, E. Galun, personal communication). Since cytoplasmic male sterility is not associated with chloroplasts (Belliard et al. 1978; Aviv and Galun 1980) independent segregation of the two traits is in agreement with streptomycin resistance being coded by the chloroplasts.

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