

The use of streptomycin resistance and chlorophyll deficiency for selection of somatic hybrids between *Nicotiana tabacum* and *N. rustica*

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To prove the usefulness for somatic hybridisation of a *Nicotiana tabacum* L. double mutant (SA), which is both streptomycin resistant and chlorophyll deficient, protoplasts obtained from SA suspension cultures were fused via the dextran method with protoplasts isolated from *N. rustica* L. mesophyll cells. Prospective somatic hybrids were selected for streptomycin resistance and the ability to produce chlorophyll in regenerated plants. By using this selection system, green plants were recovered from 5 colonies. The hybrid nature of these plants was confirmed by morphological studies and isoelectric focusing of the Fraction 1 protein (RuBP carboxylase). The present results show evidence for the potential use of the double mutant in studies of somatic hybridisation.

INTRODUCTION

Recently, by using universal hybridisers, which have both positive and negative selection markers, some somatic hybrids have been obtained effectively (LoSchiavo *et al.*, 1983; Pental *et al.*, 1984; Ye and Widholm, 1986). In the preceding paper (Toki and Kameya, 1987), we reported production of a double mutant of *Nicotiana tabacum* (SA) carrying markers for streptomycin resistance and chlorophyll deficiency. Calli initiated from SA showed resistance to streptomycin, and the chlorophyll content in SA leaves was lower than that of the normal green plants. Therefore, it was expected that the double mutant SA could be useful in studies of somatic hybridisation. The present paper describes production of somatic hybrid plants between *N. tabacum* SA and *N. rustica* by using streptomycin resistance and chlorophyll deficiency for selection.

MATERIALS AND METHODS

Plant materials

A streptomycin resistant chlorophyll deficient mutant of *Nicotiana tabacum* L. (Toki and Kameya, 1987) and *N. rustica* L. were used for experimentation. A suspension culture of *N. tabacum* was prepared as described previously (Toki and Kameya, 1987).

Isolation of protoplasts

N. tabacum SA protoplasts were isolated from 16-month-old suspension cultured cells by incubating in a solution containing 9 per cent (W/V) mannitol, 0.1 per cent (W/V) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 per cent (W/V) macerozyme R10 and 3 per cent (W/V) cellulase Onozuka R10 for 12 hours at 25°C. *N. rustica* protoplasts were isolated from leaves by incubating in the same enzyme solution for 3 hours at 25°C.

In both cases, protoplasts were filtered through a mesh (56 µm), washed twice with a washing solution (containing 9 per cent (W/V) mannitol, 0.1 per cent $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), floated to the surface with 20% (W/V) sucrose, collected by centrifugation (80 g for 5 minutes) and resuspended in the washing solution.

Protoplast fusion

Protoplasts were fused according to the method of Kameya (1983) with minor modifications. One drop (about 0.2 ml) of fusion medium (containing 15 per cent (W/V) dextran and 6 per cent (W/V) NaCl) was placed in a Petri dish and one drop of the protoplast suspension containing *N. tabacum* SA and *N. rustica* cells was placed on the fusion medium. Then 3 drops of fusion medium were added to the protoplast suspension and mixed gently by shaking the Petri dish. Fifteen minutes

Table 1 Composition of culture media^a

Addenda	Medium A	Medium B	Medium C	Medium D	Medium E
Mineral salts ^b					
Vitamins ^c					
Sucrose(W/V)		3%	3%	3%	3%
Glucose(W/V)	9%	5%			
2,4-D ^d	1 mg/l	1 mg/l			
NAA ^e			0.05 mg/l		
IAA ^f				1 mg/l	
Kinetin	0.5 mg/l	0.5 mg/l		1 mg/l	
BAP ^g			1 mg/l		
Streptomycin sulfate		1000 mg/l			
Agar(W/V)			0.8%	0.8%	0.8%

^a pH was adjusted to 5.7. ^b Murashige and Skoog (1962) formulation. ^c Uchimiya and Murashige (1974) formulation. ^d 2,4-dichlorophenoxyacetic acid. ^e indole-3-acetic acid. ^f α -naphthaleneacetic acid. ^g benzylaminopurine.

later one drop of NaOH-NaCl solution (containing 0.1 N NaOH and 8 per cent (W/V) NaCl) was added. After 15 minutes, 1 ml of an elution medium (5 per cent (W/V) mannitol and 2 per cent (W/V) CaCl₂·2H₂O) was added six times at 5 minute intervals. The fused protoplasts were collected by centrifugation (80 g for 5 minutes).

Culture of protoplasts and plant regeneration

The fused protoplasts (1×10^5) were cultured in 4 ml of protoplast culture medium A (table 1) in plastic Petri dishes at 25°C. During the first 14 days, Petri dishes were incubated under continuous weak light (0.1 W/m²), and then transferred to strong light (4 W/m²). After 10 days of culture, the cell suspension was diluted with an equal volume of the selection medium (medium B, table 1), according to the selection scheme in fig. 1. After 20 days of culture, clusters consisting of 10–20 cells were mixed with equal volume of melted medium B containing 0.6 per cent agar, and placed in a Petri dish. After growth, calli about 1–2 mm in diameter, were transferred onto shooting medium (medium C or D, table 1). Regenerated shoots were transferred onto a rooting medium (medium E).

Analysis of fraction 1 protein

The polypeptide composition of Fraction 1 protein (RuBP carboxylase) of regenerated plants was determined by the procedure of Hirai (1982).

Streptomycin sensitivity of hybrid plant

The callus of putative somatic hybrid was induced from leaves on MS (Murashige and Skoog, 1962)

agar (0.8 per cent) medium with 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.1 mg Kinetin, then transferred onto the same medium containing 0–1000 mg/l streptomycin sulfate. The fresh weight was determined after 2 weeks.

RESULTS

Selection of somatic hybrids

After 1 month of culture, colonies (1–2 mm in diameter) appeared on the selection medium B (table 1) which contained 750 mg/l streptomycin sulfate as final concentration. The colours of the colonies were white, yellowish-green, green as well as intermediate colours.

Twenty colonies were transferred to shooting medium C. Sixteen of them produced small calli (2–3 mm in diameter). From these 2 calli (called H1 and H2) several green plants were regenerated, 1 callus regenerated an albino plant.

Forty colonies were transferred to shooting medium D. Eighty per cent of them were yielded small calli, but only 1 callus regenerated an albino plant. Others were then transferred to shooting medium C. Of these, 3 calli (called H3, H4 and H5) regenerated several green plants.

The green plants regenerated from selected calli were expected to be somatic hybrid plants from the selection scheme in fig. 1. So we transferred these green plants to the hormone free rooting medium E.

The plants regenerated from H2 calli (H2P) were rooted easily on medium E and grew vigorously. The plants regenerated from other calli were rooted only occasionally. But 1 year after fusion treatment, at least one plant from each callus had been rooted.

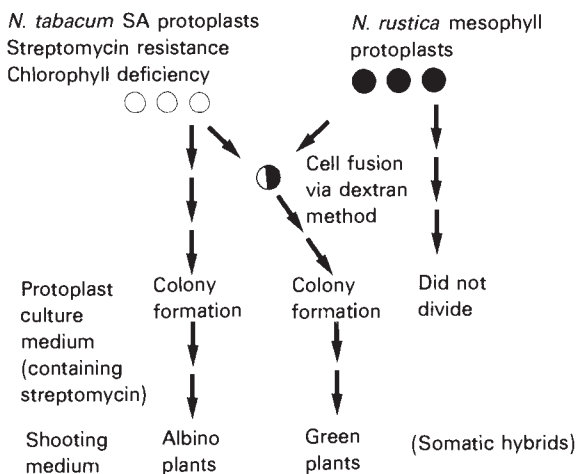


Figure 1 Selection scheme for hybrids between *N. tabacum* SA and *N. rustica*.

Characterisation of hybrids

Flower morphology of regenerated green plants were intermediate between *N. tabacum* and *N. rustica* for corolla length and width (fig. 2). While corollas of *N. tabacum* were pink and those of *N. rustica* were greenish-yellow, there were two types of plants among regenerated plants: one type had pinkish-yellow flowers, the other type, creamy-white. Pinkish-yellow flowers and creamy-white flowers were not found on an individual plant.

With respect to leaf morphology, *N. tabacum* lacks a petiole and possesses oval shaped leaves. *N. rustica* has a well formed petiole, egg shaped leaves and trichomes longer than that of *N. tabacum*. Regenerated green plants showed an intermediate morphology and had trichomes like a *N. rustica* but distribution of the trichomes was more random than that of *N. rustica* (fig. 3).

One plant of H2P was analysed for the polypeptide composition of Fraction 1 protein (fig. 4). The large subunit polypeptides of this plant corresponded to the *N. rustica* type, indicating the presence of chloroplasts from *N. rustica*. The small subunit polypeptides of this plant contained both parental types of polypeptides; one being characteristic of the protein from *N. rustica*, one of that from *N. tabacum* and the third being common to both species. This result indicate that the nuclear genomes from both species were present in this plant. Also esterase isozyme patterns provided evidence of hybridity for H2P (data not shown).

Streptomycin sensitivity of hybrid plant

The callus derived from one of H2P was sensitive to streptomycin like *N. rustica* callus (fig. 5). This indicates lack of *N. tabacum* chloroplasts which are resistant to streptomycin in this plant.

DISCUSSION

The colonies which appeared on the selection medium, containing 750 mg/l streptomycin sulfate, were expected to be *N. tabacum* colonies or somatic hybrid colonies. When we transferred some of these colonies to shooting medium, several albino plants and green plants were obtained. The latter were likely to be somatic hybrids. These plants expressed the morphology expected of a somatic hybrid and no evidence of unhybridised *N. rustica* was observed. Fraction 1 protein analysis of H2P also showed evidence for hybridity.

Fraction 1 protein analysis of H2P showed that the chloroplasts originated from *N. rustica*, and

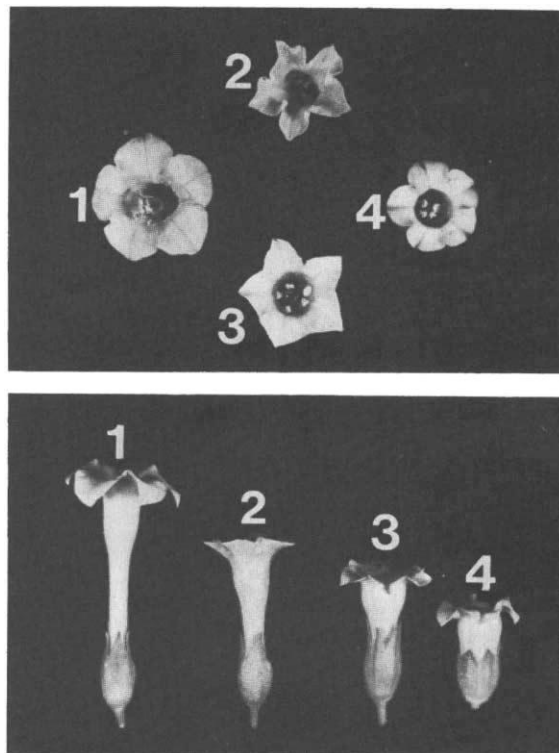


Figure 2 Flower morphology of somatic hybrids between *N. tabacum* SA and *N. rustica*, and parents. (1) *N. tabacum* SA, (2) a somatic hybrid (pinkish-yellow flower), (3) a somatic hybrid (creamy-white flower), (4) *N. rustica*.

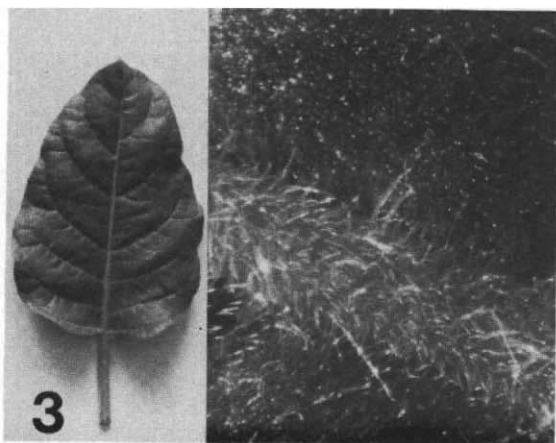
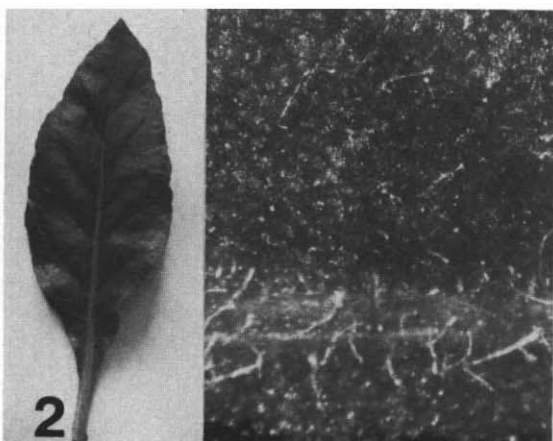
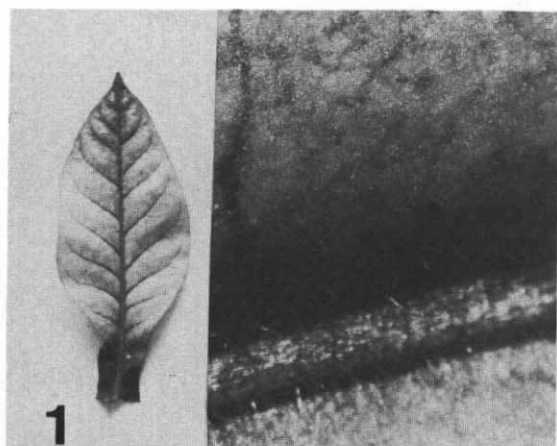


Figure 3 Leaf and trichomes on the leaf surface of a somatic hybrid between *N. tabacum* SA and *N. rustica*, and parents. (1) *N. tabacum* SA, (2) a somatic hybrid, (3) *N. rustica*.

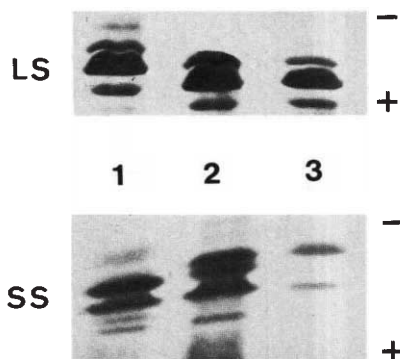


Figure 4 Large and small subunit polypeptides of Fraction 1 protein resolved by isoelectrofocusing polyacrylamide gel electrophoresis. (1) *N. tabacum* SA, (2) a somatic hybrid, (3) *N. rustica*. LS and SS denote large and small subunits of Fraction 1 protein, respectively.

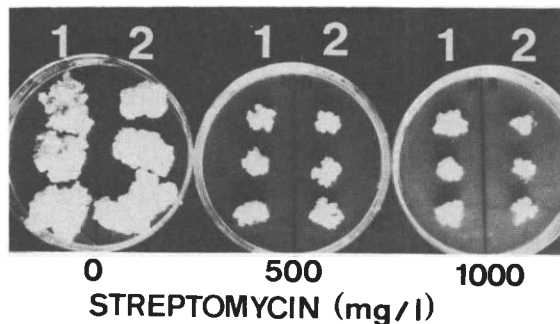


Figure 5 Streptomycin resistance test. (1) a somatic hybrid (H2P), (2) *N. rustica*.

callus obtained from H2P showed the presence of streptomycin sensitive chloroplasts of *N. rustica*. Both results showed that streptomycin sensitive chloroplasts had not been eliminated completely by growth on selection medium. Similar results have been reported recently by Bourgin *et al.* (1986). The chloroplasts constitution of other hybrid plants are now being investigated.

Somatic hybrids between *N. tabacum* and *N. rustica* have been produced using selection systems for hybrids, such as callus morphology by Nagao (1978) and genetic complementation for chlorophyll synthesis by Douglas *et al.* (1981). However, these selection systems seem to be limited to combinations of parental plants.

Pental *et al.* (1984), however, obtained hybrids by using a nitrate reductase deficient, streptomycin resistant double mutant (NR⁻ SR⁺) of *N. tabacum*. They selected hybrid colonies for nitrate reductase proficiency and streptomycin resistance, and then obtained hybrid plants on regeneration medium.

In our experiment using the double mutant *N. tabacum* SA, somatic hybrids could be selected initially by culture on a medium containing streptomycin and then by the green pigmentation of the regenerated plants. So we can conclude that *N. tabacum* plants can be used as a universal hybridiser, like NR^-SR^+ as established by Hamill *et al.* (1983), to hybridise with any streptomycin sensitive green plants.

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