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Somatic Hybridization Between Male Sterile *Nicotiana tabacum* and *N. glutinosa* Through Protoplast Fusion

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Summary. Protoplasts derived from suspension cultured cells of cytoplasmic male sterile *Nicotiana tabacum (N. debneyi* cytoplasm) and of fertile *N. glutinosa* were fused with the aid of polyethylene glycol (PEG). Out of 1,089 colonies developed from PEG-treated protoplasts, 29 restored whole plants.

A somatic hybrid plant was selected on the basis of isoelectrofocusing analysis of Fraction I protein in leaves of regenerated plants. A newly created hybrid contained small subunits of both parents but only a *N. glutinosa* type large subunit.

Male sterile character was conserved in a hybrid plant while leaf morphology was intermediate between the parents. By tobacco mosaic virus infection tests, the hybrid's leaves showed resistant symptoms, hypersensitive local lesions, which were due to *N. glutinosa* nuclear genome expression.

Key words: Protoplast fusion – Cytoplasmic male sterility – Tobacco – Fraction I protein – TMV resistance

Abbreviations

PEG Polyethylene glycol
TMV Tobacco mosaic virus

Introduction

Recent progress in somatic cell engineering technology using plant protoplast fusion has made it possible to create hybrids containing different types of cytoplasms which otherwise would never occur through sexual crosses. Using cytoplasmic male sterile *Nicotiana tabacum,* Belliard et al. (1978) and Gleba et al. (1978) were able to obtain new cytoplasmic hybrids (=cybrids) which contained nuclear information from the same species. In addition Aviv et al. (1980) reported the production of cybrids by fusing protoplasts of *N. sylvestris* and X-irradiated cytoplasmic male sterile *N. tabacum.* Similar hybridization was also reported in *Petunia* species (Izhar and Power 1979).

In this paper, the creation of a new hybrid containing two different cytoplasms and a mixture of nuclear genomes of two tobacco species, *N. tabacum* and *N. glutinosa,* is presented.

Materials and Methods

Plant Materials: A cytoplasmic male sterile line, *N. tabacum* containing *N. debneyi* cytoplasm (2 n=48), and *N. glutinosa* (2 n=24) were used for experimentation.

Callus and Cell Culture: Callus was induced from in vitro germinating seedlings placed on an agar medium (Table 1). Viable callus mass was transferred into a liquid medium (Table 1), then subcultured for at least three passages (two week-intervals of each) before being used for experimentation.

Isolation of Protoplasts and Fusion Treatments: Protoplasts were prepared from suspension cultured cells according to a method reported previously(Uchimiya and Murashige 1974). Protoplasts of two tobacco species were mixed together, then induced to fuse with the aid of PEG(Kao and Michayluk 1974; Uchimiya and Wildman, 1979). To achieve maximum protoplast fusion, 40% PEG(M.W.6,000), 100 mM $CaCl_2$, 50 mM Hepes buffer at pH 6.5 was mixed with an equal volume of protoplast suspension (10^5–10^6 protoplasts/ml). After 15 min, PEG solution was diluted with 0.5 M mannitol containing 50 mM $CaCl_2$. PEG was then removed by a centrifugation at 350 xg for 2 min.

Culture of PEG Treated Protoplasts and Plant Regeneration: Washed protoplasts were cultured in a plastic petri dish containing protoplast culture medium (Table 1). After 2 weeks culturing, clusters consisted of 10–20 cells were mixed with a melted agar medium (protoplast medium containing 0.6% agar), and plated in a petri dish. Calli, about 2–5 mm in diameter, were transferred onto a shooting medium (Table 1). Regenerated shoots were then placed on a rooting medium (Table 1). Rooted plants were transferred into soil contained in a pot and allowed to grow until flowering.

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Table 1. Composition of culture media[a]

Addenda	Callus and cell culture	Protoplast culture	Shooting medium	Rooting medium
Mineral salts[b]				
$NaH_2PO_4 \cdot H_2O$			170	
IAA			2	2
2,4-D	1	1		
Kinetin	0.1	0.5	0.2	
Thiamine · HCl	10	10	10	0.5
Nicotinic acid	5	5	5	
Pyridoxine · HCl	10	10	10	
Myo-inositol	100	100	100	100
Glycine	2	2	2	
Sucrose	30 000		30 000	30 000
Glucose		90 000	36 000	
Adenine sulfate			80	
L-Tyrosine			50	
Difco Bacto agar[c]	8 000		8 000	8 000

[a] pH was adjusted to 5.7 with 0.1 N NaOH.
[b] Murashige and Skoog (1962) formulation.
[c] For cell culture, agar was omitted

Analysis of Fraction I Protein: Polypeptide compositions of Fraction I protein of regenerated plants were determined using a rapid method reported elsewhere (Uchimiya et al. 1979).

TMV Infectivity Test: TMV resistance was assessed by swabbing leaves with TMV (200 μg/l) suspension. After 2–3 days, appearance of necrotic local lesions in TMV infected leaves was observed.

Counting Chromosome Numbers: Chromosome numbers were counted using root-tips of a hybrid plant. Fixation and staining of tissues were carried out by following the method of Imamura and Harada (1980).

Results and Discussions

The purpose of this experiment was to create new hybrid plants which possess different nuclear as well as cytoplasm backgrounds for the investigation of nuclear-cytoplasm interactions. In the present study cytoplasmic male sterile *N. tabacum* and male fertile *N. glutinosa* were selected. Since both tobacco species contain different types of large and small subunits of Fraction I protein, it was possible to identify the nuclear and cytoplasm type of somatic hybrid plants.

N. debneyi, an Australian tobacco species, was crossed with a male partner, *N. tabacum.* The hybrids were then subsequently backcrossed repeatedly with *N. tabacum.* Resulting backcrossed hybrids were characteristic in male sterility and split-blossom appearance (Sand and Christoff 1973). The analysis of polypeptide compositions of Fraction I protein suggests that this type of cytoplasmic male sterile tobacco possesses *N. debneyi* cytoplasm and *N. tabacum* nuclear genome (Chen et al. 1976). Since the sexual cross of this male sterile plant with fertile *N. glutinosa* as the pollen donor had not previously been possible, the use of the protoplast fusion technique is of special importance.

Ultimately, with the use of about 10^5–10^6 protoplasts/ml for the fusion treatment, 1,089 colonies were obtained from protoplast population treated with PEG. All the colonies were transferred to a new medium which stimulated shoot regeneration from callus. *N. glutinosa* callus hardly differentiated shoots on the same medium, whilst male sterile *N. tabacum* callus developed shoots. Eventually, 28 colonies resulted in shoot formation; these were then transferred to the rooting medium.

The key to determining the true make-up of hybrid plants comes from the analysis of the polypeptide compositions of Fraction I protein, whose small subunit is coded by the nuclear genome whereas the large subunit is under chloroplast DNA control (Chan and Wildman 1972). Since we have developed a rapid procedure to analyze polypeptides of Fraction I protein (Uchimiya et al. 1979), it was possible to determine the Fraction I protein type of 28 regenerated plants within a few days. Consequently, one plant contained both parent types of small subunits, indicating the presence of nuclear information from both parents (Fig. 1). In fact, the chromosome number of this hybrid plant appeared to be the sum of the parents. However, only the *N. glutinosa* type large subunit was seen in this same hybrid plant. The other 27 colonies all possessed the same polypeptide compositions of Fraction I protein as the male sterile tobacco. In view of the expression of chloroplast DNA in somatic hybrids, only one or the other of the two parent types has been expressed (Belliard et al. 1978; Chen et al. 1977; Evans et al. 1980; Kung et al. 1975; Melchers et al. 1978). In contrast, Gleba et al. (1978) observed a mixture of two different types of large subunits of Fraction I protein in somatic hybrids of *Nicotiana* species.

Observation was also made on the morphological characteristics of a whole plant. A male sterile *N. tabacum* lacks a petiole and possesses oval shaped leaves; *N. glutinosa* has a distinctive petiole and egg-shaped leaves. The hybrid created in this investigation showed an intermediate leaf morphology (Fig. 2). With respect to flower morphology, the narrow split-blossom of a male sterile *N. tabacum* was no longer seen in the hybrid flowers (Fig. 3). The hybrid while possessing deeply lobed blossoms and a stigmatic antheloid, lacked a complete anther (Fig. 4).

By pollinating a hybrid's stigma with either *N. glutinosa* pollen or *N. tabacum* var. 'Samsun' pollen, viable seeds could be produced. We have carried out such back-crossing for two consecutive generations, but so far have never observed the restoration of fertile anthers

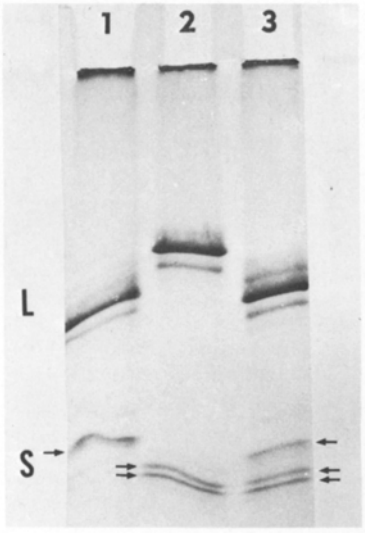

Fig. 1. Large and small subunit polypeptides of Fraction I protein resolved by isoelectrofocusing gel electrophoresis (*1*) *N. glutinosa,* (*2*) *N. tabacum* (*N. debneyi* cytoplasm) male sterile line, and (*3*) a somatic hybrid. L and S denote large and small subunit of Fraction I protein, respectively. Arrows indicate positions of each small subunit polypeptides

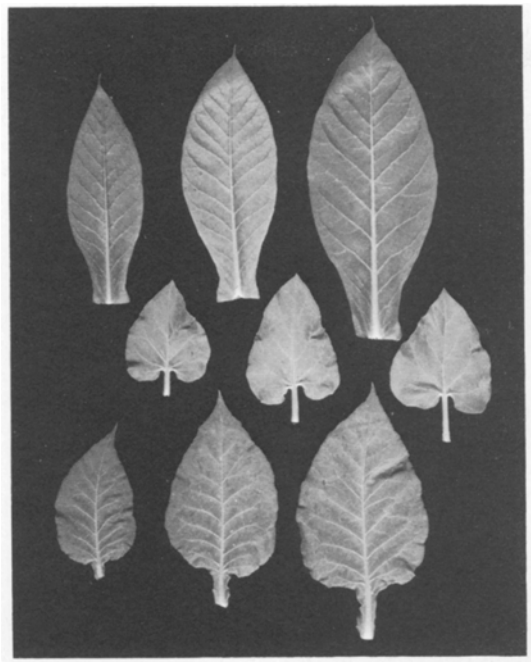

Fig. 2. Leaf morphology of a somatic hybrid and parents. Top: *N. tabacum* (*N. debneyi* cytoplasm) male sterile line. Middle: *N. glutinosa.* Bottom: a somatic hybrid

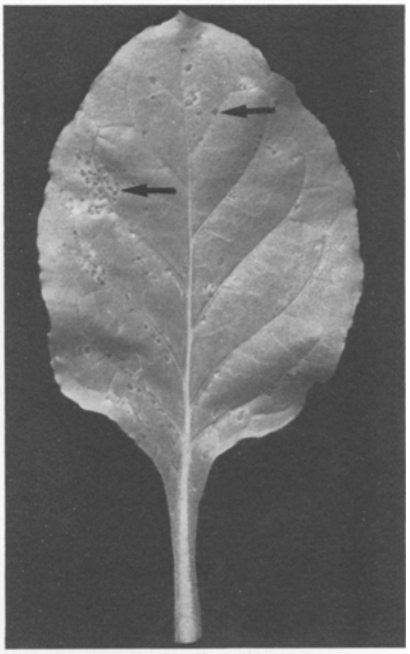

Fig. 5. TMV resistant symptom in a s matic hybrid's leaf. Upper leaf surfa of a hybrid plant was swabbed wi TMV suspension, and observation w made after 2–3 days. Arrows indica typical necrotic local lesions in tl TMV-infected leaf

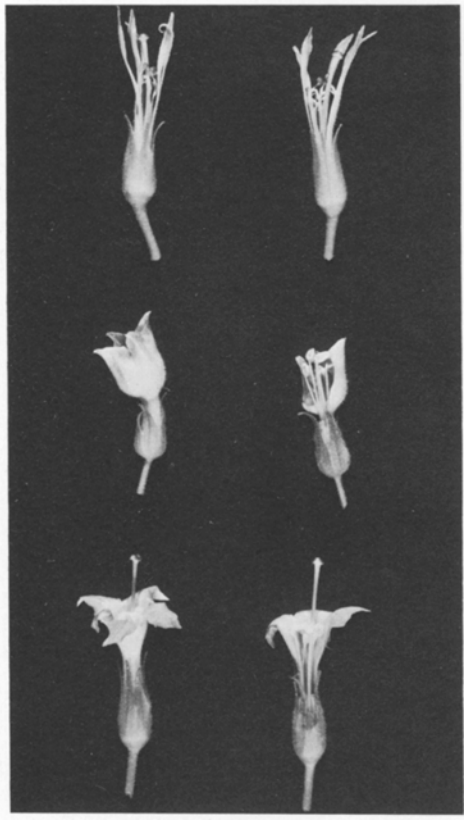

Fig. 3. Flower morphology of a somatic hybrid and parents. Note the hybrid possessing a deeply lobed blossom. Top: *N. tabacum (N. debneyi* cytoplasm) male sterile line. Middle: *N. glutinosa.* Bottom: a somatic hybrid

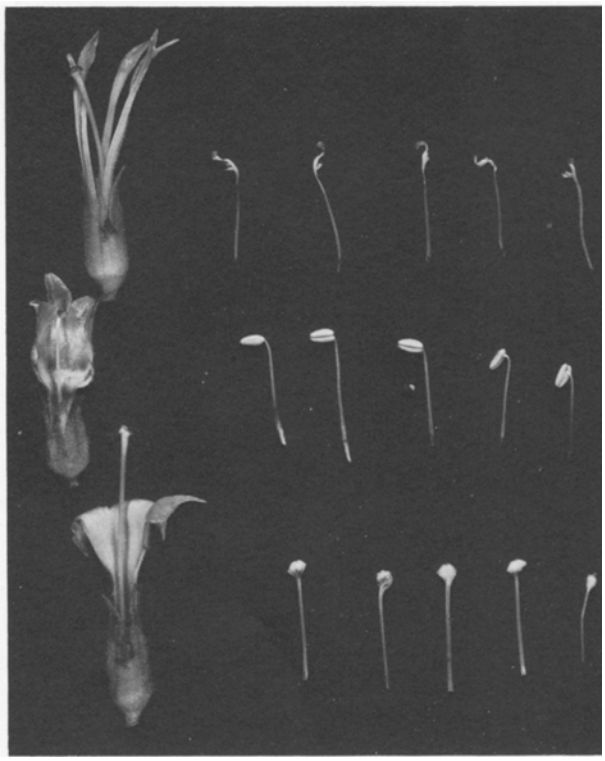

Fig. 4. Morphology of floral organs of a somatic hybrid and parents. Note the hybrid possessing stigmatic-antheloid lacking complete anthers

in the progenies. Therefore, cytoplasmic male sterile character seems to be conserved in the newly created hybrid.

It is a well known fact that *N. glutinosa* possesses a single dominant gene which shows localized necrotic lesions in TMV infected leaves (Clausen and Cameron 1957). Thus, it was of particular interest to determine whether such a disease resistant trait was still conserved in the hybrid plant. The hybrid's leaves showed distinctive TMV resistance (Fig. 5).

Using male sterile somatic hybrid plants, Belliard et al. (1979) made the significant finding that mitochondrial DNA could play a role in cytoplasmic male sterility in *Nicotiana.* The hybrid reported in this paper possesses two cytoplasmic markers, i.e. stigmatic-antheloid lacking anthers and *N. glutinosa* chloroplast genes in addition to a combination of two nuclear genomes of *N. tabacum* and *N. glutinosa.* Therefore, it should be worthwhile to analyze the mitochondrial DNA in this hybrid plant.

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