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Morphological Characteristics and Chloroplast DNA Distribution in Different Cytoplasmic Parasexual Hybrids of *Nicotiana tabacum*

G. Belliard and G. Pelletier

Laboratoire d'Amélioration des Plantes, Bât. 360 – Université Paris Sud, F-91405 – Orsay, France

F. Vedel and F. Quetier

Laboratoire de Biologie Moléculaire Végétale, associé au C.N.R.S. (L.A. 40), Bât. 430, Université Paris Sud, F-91405 Orsay, France

Summary. Protoplast fusion makes possible the fusion of two different cytoplasms, allowing genetical analysis of cytoplasmic factors. Two varieties of *Nicotiana tabacum* differing by their cytoplasms have been used. Techne, the first variety, obtained by an interspecific cross between *N. debneyi* (female) and *N. tabacum* (male) is characterized by the nuclear *tabacum* genome inside the *debneyi* cytoplasm. Techne plants present abnormal flowers with cytoplasmic male sterility (cytoplasmic marker) and sessile leaves (nuclear marker). Techne leaf protoplasts were fused with leaf protoplasts of *N. tabacum* var. Samsun (or Xanthi). The last variety is characterized by petiolated leaves and normal flowers, because it possesses the nuclear *tabacum* genome associated with the *tabacum* cytoplasm. The nuclear marker (leaf shape) and the cytoplasmic one (flower shape inducing male sterility or fertility) have been used to distinguish among the whole regenerated plants the somatic nuclear hybrids and the cytoplasmic hybrids (cybrids) displaying the nuclear phenotype of one of the two parents associated with a modified flower type, intermediate between the parental ones.

The chloroplastic (cp) DNA contained in each parent has been specifically identified by using EcoRI restriction nuclease and gel electrophoresis. EcoRI fragment patterns of cp DNA isolated from the first progeny of the regenerated cytoplasmic hybrids revealed that only one of the two parental cp DNAs is present in all cases; neither mixture of both parental cp DNAs nor recombinant cp DNA molecules were observed. This indicates that a specific elimination of one or the other parental cp DNAs occurs after the initial mixing of the cytoplasms. The study of the association of the modified flower type with the cp DNA isolated from the corresponding plant showed that cp DNA seems independent from the mechanism of cytoplasmic male sterility in tobacco.

For offprints contact: G. Belliard

Introduction

Genetical studies of the somatic hybrids induced by protoplast fusion in higher plants have gained considerable interest since the demonstration of plant regeneration from protoplasts (Nitsch and Ohyama, 1971; Takebe et al., 1971). However, regeneration constitutes a critical step in parasexual hybridization and hybrid plants have been obtained only with some species of Solanaceae belonging to the *Nicotiana, Petunia* and *Datura* genera (Carlson et al., 1972; Melchers and Labib, 1974; Power et al., 1976; Schieder 1977a, b; 1978) and with carrots (Dudits et al. 1977). Most of the previous reports deal with the fate of the parental nuclei and chromosomes both during culture of hybrid cell lines and plant regeneration (Constabel et al., 1976; Dudits et al., 1976; Kao, 1977; Melchers and Sacristàn, 1977).

On the other hand, the cytoplasmic aspect of the protoplast fusion appears quite interesting in many respects; when the two cytoplasmic genetic informations are different, one can obtain cytoplasmic hybrids or cybrids (Gleba et al., 1975, 1977).

Recently, some of us succeeded in the regeneration of mature cybrids from fused protoplasts of two *Nicotiana tabacum* exhibiting different cytoplasms (Belliard et al., 1977). It has been shown that the protoplast fusion technique provides new kinds of plants, bearing modifications of the cytoplasmically inherited characters, i.e. floral morphologies associated to male sterility or fertility. Moreover, the male sterility or fertility can be transmitted from one variety of tobacco to another variety by protoplast fusion.

It has been demonstrated recently that the cp DNAs isolated from different species of *Nicotiana* can be specifically identified by using EcoRI restriction nuclease and gel electrophoresis (Vedel et al., 1976). The work described here was undertaken to characterize the chloroplastic (cp) DNA from the first progeny of various cytoplasmic hybrid plants by using

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this technique. This analysis should answer to the following questions; (1) do the cybrid plants contain the two parental cp DNAs or only one, as it is generally the case in sexual hybridization? (2) is cp DNA involved in the cytoplasmic male sterility in tobacco?

Materials and Methods

Plant Material

Protoplasts were isolated from three varieties of *Nicotiana tabacum* (2 n = 48). The varieties *Samsun (Sf)* and *Xanthi (Xf)* are characterized by petiolated leaves (a mendelian character) and flowers with normal shape (Fig. 1). *Techne (Ts)*, the third variety has been obtained by Tsikov et al. (1971) from the interspecific cross female *N. debeneyi* × male *N. tabacum*, followed by backcrosses with *N. tabacum* as the male parent; so these plants possess the *tabacum* genotype in *debneyi* cytoplasm. The *Techne* variety is characterized by sessil leaves and abnormal flowers (ribonned petals, lack of anthers) leading to male sterility. Therefore the shape of leaves is a nuclear marker and the shape of flowers is a cytoplasmic one.

Ts female × *Xf* male and *Ts* female × *Sf* male sexual hybrids present specific leaves, intermediary in shape when compared to the parental leaves, and are male sterile as the *Ts* parent (Fig. 1).

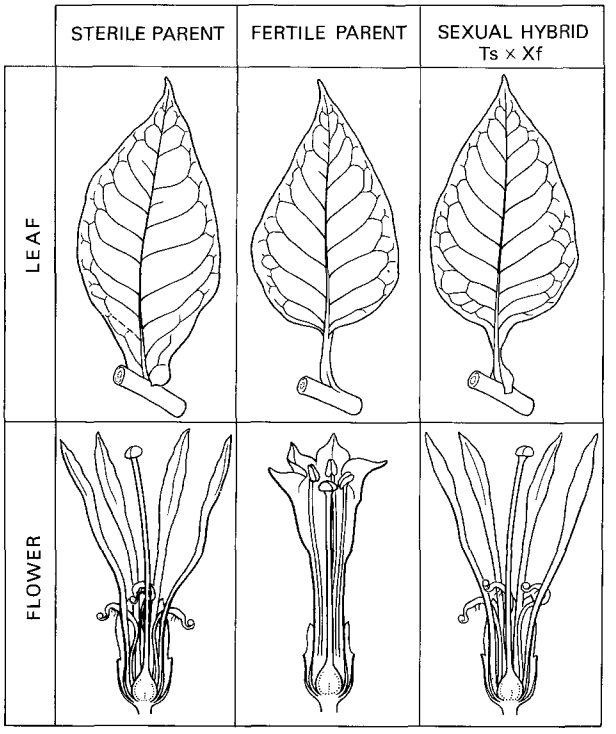

Fig. 1. Flower and leaf morphologies from respectively, the sterile parent: *N. tabacum* var. *Techne (Ts)*, the fertile parent *N. tabacum* var. *Xanthi* or *Samsun* (*Xf* or *SF*) and the sexual hybrid (*Ts* × *Xf* or *Ts* × *Sf*)

Isolation and Fusion of Protoplasts

Leaf protoplasts were isolated according to Chupeau et al. (1974) by using a mixed enzyme treatment. The peeled leaves were incubated in a plasmolyticum containing 0.2% cellulase R 10, 0.1% pectinase R 10, 11% mannitol, 6 mM $CaCl_2$, 0.7 mM Na_2HPO_4, pH 5.5, during 16 h at 20° C.

Fusions were achieved between protoplasts from one of the two fertile varieties (*SF* or *Xf*) and protoplasts from the cytoplasmic male sterile variety (*Ts*). In the first experiments, *Sf* protoplasts were used, and then were replaced by *Xf* protoplasts because plantlet regeneration in vitro from *Sf* protoplasts could not be obtained in our experimental conditions.

Protoplasts were induced to fuse according to Kao and Michayluk (1974). Each experiment was constituted by six kinds of assays in order to estimate the fusion ratio induced between *Ts* and *Xf* (or *Sf*) protoplasts by polyethylene glycol 1500 (Table 1) and to rule out artifacts induced by in vitro culture.

Protoplast Culture and Plant Regeneration

After the fusion treatment, protoplasts were cultured in the following medium: Murashige and Skoog macro-nutrients diluted at half; Heller micro-nutrients; Morel vitamines; 37.3 mg/l Na_2 EDTA plus 27.8 mg/l $FeSO_4$, 7 H_2O; 20 g/l sucrose; 100 g/l glucose; 750 mg/l bovine serum albumine fraction V; 1 mg/l naphtalene acetic acid (NAA) and 0.2 mg/l 6-benzyl-adenine, pH 5.8. The cell density of the culture was about 50,000 protoplasts by ml of liquid medium.

After three weeks, callus were plated on solid medium containing inorganic nutrients, vitamines, 20 g/l sucrose, 1 mg/l 6-benzyl-adenine, no glucose and no NAA. Buds were formed in about 2–3 weeks and were transferred for root initiation on solid medium without any growth substances. In these experiments we have only transferred the first bud formed from each callus. So we have kept only one regenerated plant from each protoplast.

Plantlets were transplanted in soil and raised until flowering. As mentioned above, leaf shape (a nuclear marker) and flower shape (a cytoplasmic marker) were used to distinguish the cybrids from the nuclear hybrids among the whole regenerated plants. Every time that a plant regenerated with modifications in flower morphology (intermediate shape between the parental ones) or/and in leaf morphology, a leaf was set apart for cutting and the chromosome number was determined in meristematic root cells.

Isolation of Chloroplastic (cp) DNA

The cp-DNAs were extracted as previously reported (Vedel et al., 1976). Chloroplasts prepared from deribbed leaves of tobacco were treated by DNase to remove nuclear DNA. The DNase-treated chloroplast pellet was lysed by sarcosyl-pronase treatment and the lysate centrifuged on a two-step CsCl-ethidium bromide gradient. The cp-DNA was recovered as the fluorescent bands and pelleted by ultracentrifugation. Ethidium bromide was removed by a short Dowex 50-Na^+ column chromatography.

EcoRI Cleavage and Electrophoresis of the cp DNA Restriction Fragments

EcoRI restriction enzyme was prepared according to Thomas and Davis (1975). The purified cp-DNA was exhaustively digested by EcoRI enzyme and the specific fragments separated by electrophoresis on 0.7% agarose, 40 cm long vertical slab gels as already described (Quétier and Vedel, 1977). Gels were stained with 2 μg/ml ethidium bromide and u.v. fluorescence (260 nm) photographs were taken using a MP4 Polaroid camera, 55 PN films and a red filter (Kodak Wraten n° 26).

Table 1. Fusion experiment (1) and control assays (2–6) of leaf protoplasts from *N. tabacum* var. *Techne (Ts)* and *N. tabacum* var. *Xanthi (Xf)*

Exp.	Protoplasts	Ratio	PEG treatment	Modified floral phenotypes in regenerated plants
1	*Ts*+*Xf*	1/1	+	+
2	*Ts*+*Xf*	1/1	−	−
3	*Ts* −	−	−	−
4	*Ts* −	−	+	−
5	*Xf* −	−	−	−
6	*Xf* −	−	+	−

936 plants have been regenerated from the fusion assay reported in experiment 1. These plants ranged into 3 classes: 654 identical to one of the parents in respect to both leaf and flower morphologies, 225 cybrids (213 *Ti* and 12 *Xi*) with leaf morphology identical to one of the parents and intermediate flower morphology, 57 characterized by both intermediate leaf and flower morphologies: *Hi* plants.
150 plants have been regenerated from each control assay (experiments 2–6).
Among the 225 cybrids, 10 appeared modified for other criteria than leaf and flower shapes (leaf fasciation, polyploidy) and were eliminated. Identical yield of such "abnormal" plants was obtained in each control experiment.
Fusion experiments were carried out on january 1976 and regenerated plants were transfered in greenhouse during march 1976. All *Ti* and *Xi* plants had flowered by december 1976

Results

Phenotypic Distribution of the Regenerated Plants

The regenerated plants were distinguished first according to the shape of leaves, allowing us to determine the variety (*Techne* or *Xanthi*) or the nuclear somatic hybrids, then, according to the shape of flowers and the male fertility or sterility.

The plants regenerated from either *Ts* or *Xf* protoplasts (cf. Table 1) appeared always identical to the initial plant in respect to the leaf and flower shapes. These controls indicate that the *in vitro* culture as well as the PEG treatment do not induce phenotypical modifications regarding only the markers retained. However, some phenotypical modifications involving criterias other than leaf and flower shapes and genotypical ones such as polyploidy could be observed (see the legend of Table 1 for the frequency of these modifications). These plants were eliminated.

The plants regenerated from the mixture of *Ts* and *Xf* protoplasts after PEG treatment ranged into 3 classes: (1) 654 were identical to one of the parents in respect to both leaf and flower morphologies. Consequently, these plants represented either sterile *Techne (Ts)* or fertile *Xanthi (Xf)* parent. (2) 225 were characterized by leaves identical to one of the parents and by flowers intermediate between the two parental ones. These plants exhibited the characteristic chromosome number of *N. tabacum* (2 n = 48) and were called modified *Techne, Ti*, and modified *Xanthi, Xi*. The most modified plants were respectively fertile *Techne* and sterile *Xanthi*. (3) 57 presented modified flowers like *Ti* and *Xi* but also intermediate leaves (petiolated with wings). They were called *Hi*. These regenerated plants possessed about 96 chromosomes and were both nuclear and cytoplasmic somatic hybrids. In a previous experiment, plants regenerated from the mixture of *Ts* and *Sf* protoplasts after PEG treatment ranged into the above 3 classes with the respective numbers: 451, 40 and 2.

In this work, the plants *Ti* and *Xi* (cybrids) characterized by the nuclear genome of one parent and hybrid cytoplasms, have been choosen to study the nature of their cp DNA.

Floral Phenotypic Distribution of Ti and Xi Plants and of Their Progenies

The shape of the flowers from *Ti* and *Xi* plants appeared modified in different ways involving corolla, stamen filament and anther. According to the good correlation found between the respective modifications of the flower morphology, the 253 *Ti* plants (213 from *Ts* × *Xf* plus 40 from *Ts* × *Sf* fusion experiments) obtained have been sorted out in 4 classes, as shown in Figure 2. *Ti* and *Xi* plants appeared randomly distributed between these 4 classes (see legend of Figure 2).

Plants belonging to class I were characterized by flowers bringing pollen; however, the fertility of these plants was always lower than that of the normal parent. Selfing required "manual intervention" because of the reduced length of the stamens. Plants from classes II, III and IV presented always male sterility. The majority of the *Ti* plants bears only one kind of flower; yet, about ten plants possessed two or three flower types, each located on different stems. For cp DNA analysis, we have taken into account plants bearing only one kind of flower.

Twelve *Xi* plants have been only obtained. An important variability of the floral phenotype occured as with *Ti* plants.

The offsprings of *Ti* and *Xi* plants have been studied through the following crosses:

female *Ti* × male *Tf*,
female *Ti* × male *Xf*,
female *Xi* × male *Tf*,
female *Xi* × male *Xf*.

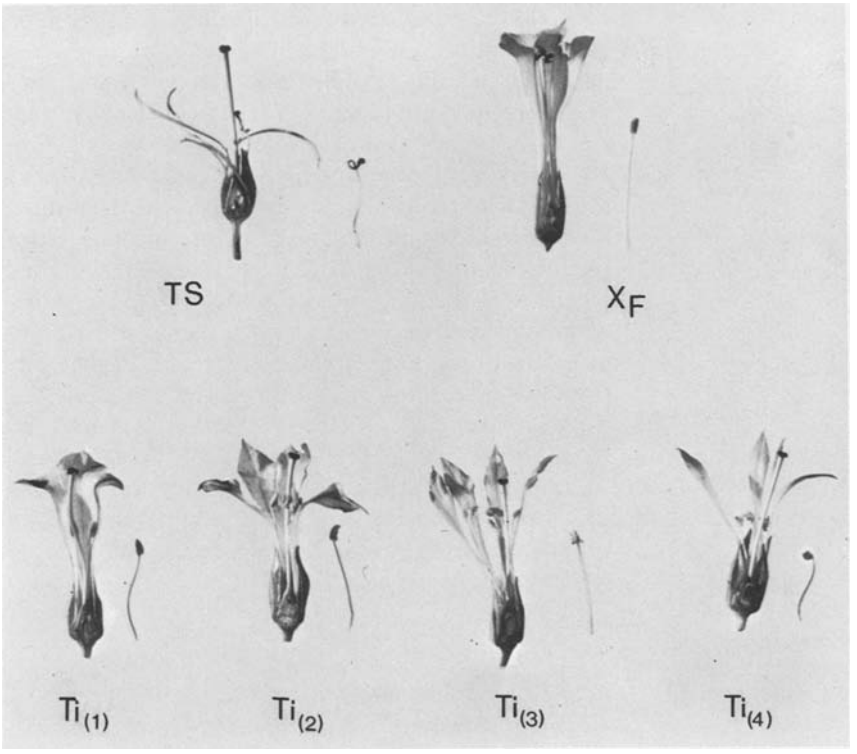

Fig. 2. The four classes of floral morphology in *Ti* plants. Distribution of the 253 (213+40) *Ti* plants: class 1:63, class 2:66, class 3:58, class 4:66. The floral morphology of the two parents, T_s and X_F are presented in the upper part

The selfing and the reciprocal crosses have been realized when the *Ti* plants were male fertile, as indicated below:

female *Tf* × male *Ti*,

female *Xf* × male *Ti*.

All the progenies appeared phenotypically homogeneous and identical to the mother plant. The same results were obtained with the second generation. These observations led to important statements: (1) the modified floral characters were cytoplasmically inherited, (2) the final stability of the new cytoplasms obtained from protoplast fusion was demonstrated.

Analysis of Chloroplastic DNA of Cybrids

cp DNA was analyzed only on cybrids characterized by flower morphology intermediate between the two parental ones, leaf morphology identical to one of the parents and by 2 n=48 chromosomes.

A prerequisite for such a study was to ascertain that each of the two parental cp DNA can be specifically identified after EcoRI cleavage and gel electrophoresis of the restriction fragments. Figure 3 shows that cp DNA from *N. tabacum* var. *Xanthi* (gel a) and cp DNA from *N. tabacum* var. *Techne* (gel b) led to two distinct diagrams. Consequently, cp DNA when cleaved by EcoRI enzyme appeared as a very useful genetic marker to characterize *debneyi* and *tabacum* cytoplasms. Cp DNA of *N. tabacum* var. *Samsun* was identical to cp DNA of *N. tabacum* var. *Xanthi*. On the other hand, the EcoRI electrophoretic pattern of the cp DNA from *N. tabacum* var. *Techne* (obtained by crossing female *N. debneyi* × male *N. tabacum* var. *Techne* and characterized by male sterility) was quite identical to the EcoRI electrophoretic pattern of the cp DNA from *N. debneyi*.

Figure 3 also shows the electrophoregrams of cp DNA isolated from the offsprings of the crosses: female *Ti* × male *Tf* (with the 4 classes mentioned above) and female *Xi* × male *Xf*. It is noteworthy that each class of offspring contained only one or the other of the two parental cp DNAs; no mixture nor recombination have been observed on the electrophoretic patterns. According to the technique used, a ten per cent mixture of the two cp DNAs would have been detected. The initial mixture of the two cytoplasms was thus followed by the elimination of one of the two parental cp DNA molecules.

Table 2 presents the comparison of the phenotypic marker of cytoplasm to the cp DNA for a given class of plants. It is clear that the flower morphology and the male sterility or male fertility could be changed whereas the cp DNA remained unmodified. This finding suggests that cp DNA is likely not involved or is not involved alone in the cytoplasmic male sterility of tobacco. However, it is noticeable that the greatest modifications, represented respectively by passages

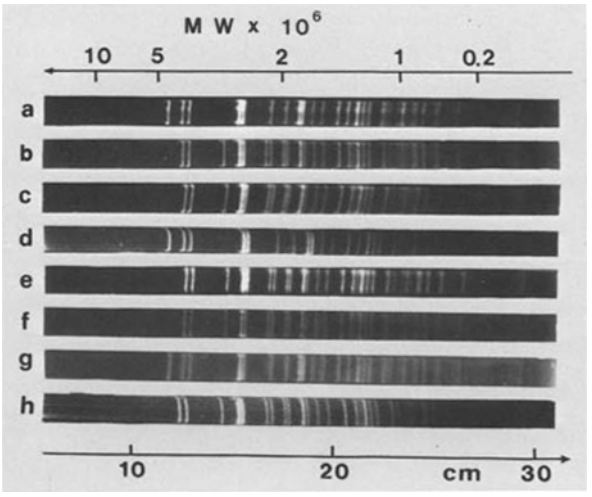

Fig. 3. Agarose slab gel electrophoresis of EcoRI digests of cp DNAs from: a, *N. tabacum* var. *Xanthi*; b, *N. tabacum* var. *Techne*; c, *N. debneyi*, and from some plants of *Ti* and *Xi* first-generation: d, T_{161}; e, T_{27}; f, T_{13}; g, X_6; h, X_7 (see table II for class correspondence)

Table 2. Relationship between floral phenotype and type of cp DNA (the corresponding electrophoregrams are shown on Figure 3)

Plants	Classes	Male organ	cp DNA
parents			
Ts	–	sterile	*debneyi D*
Xf or *Sf*	–	fertile	*tabacum T*
T_{161}	I	fertile	T[a]
T_{27}	I	fertile	D
T_{47}	I	fertile	D
T_1	II	sterile	D
T_{13}	III	sterile	D
T_{75}	IV	sterile	D
X_1	–	sterile	D[a]
X_7	–	sterile	D[a]
X_6	–	sterile	T

[a] These plants represent the change of sterile *Techne* into fertile *Techne* and of fertile *Xanthi* into sterile *Xanthi*

from sterile *Techne* to fertile *Techne* and from fertile *Xanthi* to sterile *Xanthi* (see asteriks on Table 2), are correlated with cp DNA changings. In these extreme cases, the fusion process would come to place the *tabacum* nuclear genome inside either the *tabacum* (T_{161} plant) or the *debneyi* (X_1 and X_7 plants) cytoplasms.

Discussion

In this work, protoplast fusion has been used to get cytoplasmic hybrids characterized by new and stable cytoplasms. Selection was performed among the regenerated plants only after flowering because no earlier screen was available. The cybrids were distinguished from other regenerated plants, namely nuclear hybrids, according to both leaf and flower morphologies. The cybrids we have retained were further characterized each by a chromosome number of 2n = 48, suggesting that these plants have retained only one nucleus belonging to one of the parents. We recognize which is it with the leaf shape specific of each parental variety. The plant material selected in this study offers the possibility to separate cytoplasmic events from the other ones, consecutively to the protoplast fusion.

This paper deals more precisely with the fate of the cp DNA in progenies of the cybrids. The nuclear hybrids, the caryological characteristics of which are in progress, are not taken into account here.

Analysis of the cp DNA using EcoRI specific cleavage has shown that the first generation progeny of *Ti* and *Xi* cybrid plants contained only one or the other of the two parental cp DNAs. A similar conclusion was drawn from the analysis of the polypeptide composition of fraction I protein isolated from parasexual hybrid plants derived from the fusion of protoplasts of *Nicotiana glauca* and *Nicotiana langsdorffii*. Kung et al. (1975) have reported that third-generation progeny of one parasexual hybrid produced from the last type of fusion, presented large subunit polypeptide (coded by cp DNA) of only one of the parental species. More recently, during the preparation of this manuscript, Chen et al. (1977) have presented results on first and second-generation progeny from *N. glauca* × *N. langsdorffii* parasexual hybrids. In the first-generation progeny, all but one of the sixteen different somatic hybrids analyzed for polypeptide composition of fraction 1 protein showed the large subunit pattern characteristic of one or the other parent. A single plant retained a mixture of the two parental large subunits, but this mixture was replaced by one or the other of the two parental subunits in the second generation progeny.

Results obtained from fraction 1 protein analysis and from EcoRI cleavage of cp DNA on parasexual hybrids show that the initial mixtures of two chloroplast types evolve into one type or the other. Several reasons for the rapid sorting out of the chloroplast types have been discussed by Chen et al. (1977). From quantitative considerations the authors suggest that occurence of plants containing either only *langsdorffii* or only *glauca* plastom could not be explained by "genetic drift" only. They suppose a selection pressure instead of exclusively sorting out of one or the other type of plastids only by chance. However cybrids described in the present work can be dis-

tinguished from parasexual hybrids obtained by Smith et al. (1976) and by Chen et al. (1977) regarding the chromosome number. Fraction 1 protein was analyzed from hybrids characterized by irregular chromosome numbers, ranging from 56 to 64. Since a simple addition of *N. glauca* (2 n = 24) and *N. langsdorffii* (2 n = 18) genomes gives 2 n = 42, these authors have suggested that the hybrids have resulted from triple fusions followed by loss of chromosomes during the subsequent divisions. In this case, the possibility remains, as previously discussed by Melchers (1977), that the non-homogeneous constitution of the nuclei could play a role in the selection process. In our case, cp DNA was analyzed on cybrids containing the normal chromosomal number (48) of *N. tabacum*. Results obtained with our cybrids strongly suggest that the sorting out of one or the other type of chloroplast occured without a participation of the nucleus. The simultaneous existence of plants like T_{161} and T_{27} or T_{47}, and like X_6 and X_1 or X_7 (see Table 2) shows that the two chloroplast types (*debneyi* and *tabacum*) may coexist each with the same nucleus. The analysis of cp DNAs of the Hi plants (which generally posses a variable chromosome number) should state precisely the relation beetwen the nature of cp DNA and the chromosomic constitution, and the influence of the non-homogenous constitution of the nucleus, on the chloroplast selection. As the two nuclear genomes of the *Techne* and *Xanthi* varieties are very similar we should see the polyploidisation effect on the selection of cytoplasmic organelles.

However, subsequent knowledge on the developmental events (like callus formation and plant regeneration) taking place after protoplast fusion, is required to elucidate the sorting out of the plastids.

Comparison of cytoplasmic male sterility or fertility to the type of cp DNA in cybrids indicates that cp DNA alone cannot be taken into account for the genetical support of male sterility in tobacco. Moreover, previous reports have shown that the cytoplasmic male sterility mechanism involved mitochondrial (mt) DNA in maize (Levings and Pring, 1976) and in wheat (Quétier and Vedel, 1977). Despite previous unsuccessful experiments related to polyphenol occurence and impossibility to get etiolated plants in tobacco, we hope to characterize mt DNA of tobacco cybrids by EcoRI specific cleavage.

If the two parental mt DNAs (*debneyi* and *tabacum*) can be specifically identified, such a study could allow

1) examination of the mt DNA in these cytoplasmic hybrids i.e. to know which of the three following situations occurs; only one parental mt DNA type, as in the case of the cp DNA; a mixture of the two parental mt DNAs; or a new type of mt DNA molecule arising from recombination.

2) the determination of the relation between the different classes of cytoplasmic hybrids, identified by their phenotype, and the mt DNA types.

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