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Nicotiana cybrids* with *Petunia* chloroplasts

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Summary. Protoplasts of a chloroplast-defective cultivar of *Nicotiana tabacum* were fused with gamma-irradiated protoplasts of *Petunia hybrida.* Over 100 photoautotrophic plants were regenerated; of these 94 were tested for *Petunia* chloroplast traits and all but one had *Petunia* chloroplasts based on their sensitivity to the fungal toxin, tentoxin. Chloroplast DNA was analysed for 3 of the sensitive plants and was shown to be identical to *Petunia* chloroplast DNA. Most of the plants (about 70%) appeared to be normal *N. tabacum* plants, based on morphology and chromosome number. They were fully fertile with normal pollen viability, seed set, and seed viability. The remaining 30% of the plants showed varying degrees of vegetative and reproductive abnormalities.

Key words: Chloroplast – ctDNA – Cybrid – *Nicotiana* – *Petunia* – Restriction endonucleases

Introduction

Evolutionary processes leading to reproductive isolation and speciation primarily result from changes in the nuclear genome, in which the number of genes for each trait is limited and therefore the expression of genetic change can be rapid. Chloroplasts are, in contrast to the nucleus, characterized by large gene copy numbers for each trait and, furthermore, by transmission from one generation to the next in large numbers during sexual reproduction. The recent development of restriction enzyme analysis of chloroplast DNA has enabled its use as a tool in investigations of evolutionary relationships among species of genera such as *Triticum* (Vedel et al. 1978), *Nicotiana* (Kung et al. 1982; Salts et al. 1984), *Lycopersicon* (Palmer and Zamir 1982), and *Brassica* (Palmer et al. 1983).

The fundamental differences in gene copy number and sexual transmission between nuclear and chloroplast genetic systems should be reflected in a lower rate of genetic change in chloroplast DNA. A slow rate of change, in turn, could also dictate a greater degree of genetic conservation of those nuclear genes necessary for chloroplast function and replication, since many polypeptides encoded in the nuclear genome function in multimeric complexes together with chloroplast gene products. Accordingly, the evolutionary range between the nucleus of one species and the chloroplasts of another species or genus, which are capable of compatible interactions, may be substantially broader than between two nuclear genetic systems (e.g., two related species or genera).

We have sought to test this concept in the Solanaceae with the genera, *Nicotiana* and *Petunia.* Although numerous researchers have attempted to produce sexual hybrids between these two genera, there is only one report of successful hybridization (Poliaga 1952) and this have not been repeated, even when using care to cross the same species (D. U. Gerstel, personal communication). In addition, neither somatic cell fusion (Binding 1976; Zenkteler and Melchers 1978) nor in vitro pollination and ovule culture (Zenkteler and Melchers 1978) of these two genera resulted in hybrid production. We describe here the production and partial characterization of cybrids between *Nicotiana*

* The techniques of somatic cell genetics have led to many possible nuclear-organellar combinations that may be considered as "cybrids". In this paper, we use the term to include the combination of nucleus from one species and chloroplast from another species

tabacum and *Petunia hybrida.* A preliminary account of these studies has already appeared (Glimelius et al., in press).

Materials and methods

Plant material

Seeds were collected from variegated plants of *Nicotiana tabacum* cv. 'Turkish Samsun' containing mixtures of normal and mutant chloroplasts, as described by Wong-Staal and Wildman (1973). These seeds were grown into plants in the greenhouse, selected and pruned to yield albino shoots supported by green shoots. *Petunia hybrida* cv. 'Comanche' plants were also grown from seed in the greenhouse.

Protoplast preparation and fusion

Protoplasts from leaves of the albino shoots of tobacco and from *Petunia* leaves were prepared as described by Glimelius and Bonnett (1981). *Petunia* protoplasts were gamma-irradiated by Cs^{137} with a total dosage of 8,000 R over a 10-min exposure. Albino protoplasts were mixed with the irradiated *Petunia* protoplasts in a mixture of approximately 4:1, respectively, and fused by the droplet method as described by Glimelius et al. (1978). After fusion and washing, gamma-irradiated albino protoplasts were added as feeder cells to increase the protoplast density.

Protoplast culture and plantlet regeneration

Protoplasts were grown to small colonies which were plated on agar medium and then transferred to organogenesis medium. Shoots which regenerated were rooted and plantlets were grown to the reproductive stage in the greeenhouse. The procedures used for these steps were as previously described (Glimelius and Bonnett 1981).

Analysis of plants

Seeds were collected from plants which were self-fertile. Those which did not set seed were pollinated with 'Turkish Samsun' pollen. Seeds were germinated in the presence of tentoxin in order to evaluate the resistance of chloroplast ATP synthetase (CF_1) to this toxin, as described by Glimelius et al. (1981). Chloroplasts of *N. tabacum* are resistant to the toxin whereas chloroplasts of *Petunia* are sensitive (Durbin and Uchytil 1977). Seedlings with resistant chloroplasts are green. Pollen viability measurements and chromosome counts of root tip cells were conducted as described by Glimelius and Bonnett (1981).

Chloroplast DNA analysis

Progeny of several plants were evaluated for chloroplast DNA by restriction enzyme analysis. Briefly, chloroplast DNA was isolated by (1) grinding leaf material in liquid nitrogen (Rhodes and Kung 1981); (2) suspending the powder in an isolation medium containing 0.35 M sucrose, 0.007 M Na_2EDTA, 0.05 M TRIS, 0.1% BSA, and 0.01 mM mercaptoethanol, pH 8; (3) differentially centrifuging for a chloroplast-enriched fraction; (4) purifying the chloroplasts on a discontinuous Percoll gradient (Walbot 1977) consisting of isotonic concentrations of Percoll of 7.5, 15, and 30% v/v in 0.3 M sucrose, 0.05 M TRIS, 0.007 M Na_2EDTA, 0.001 M $MgCl_2$, 0.1% BSA, and 0.01 mM mercaptoethanol; and (5) lysing the chloroplasts and purifying the DNA in CsCl (Rhodes and Kung 1981). The DNA was dialyzed to remove Cs, precipitated in ethanol, and restricted with EcoR1.

Results

Green calli were detected after about 15–20 days when protoplast-derived calli were cultured on agar in the light on hormonal mixtures favoring callus growth. Since gamma irradiation prevented growth of the *Petunia* protoplasts and albino protoplasts did not produce green calli, the green calli potentially represented the combination of the nucleus of tobacco and the chloroplasts of *Petunia.* When green calli had developed to a size of about 1–2 mm in diameter, they were subcultured on to organ-inducing medium. Since only a few green calli appeared on each 9-cm Petri dish, and since they appeared well apart from each other, each was assumed to have arisen from a separate fusion event. Most calli produced shoots which were grown into plants.

Four different experiments gave rise to green plants, with a total number of 109 plants (Table 1). Each plant listed in Table 1 was derived from a separate callus. On the basis of morphology and reproductive behavior, the plants have been separated into 3 groups. Plants in Group I had a morphology indistinguishable from 'Turkish Samsun' plants. They were fully fertile and produced seed pods of normal size. About 70% of the plants belong to this group. Group II contains plants which showed some degree of growth abnormality and/or failed to produce seeds without cross pollination. More than 70% of the plants in Group II have produced viable seeds, although only after cross pollination. This is a diverse group, representing fewer than 30% of the plants. It includes about 5 plants which appeared, on morphological grounds, to be normal tobacco plants with 48 chromosomes, but which still required cross pollination in order to set seed. Also included in this

Table 1. Response to tentoxin of seedlings of plants regenerated from the fusion of albino tobacco protoplasts with gamma-irradiated *Petunia* protoplasts

Experiment no.	Groupings of regenerated plants								
	I			II			III		
	S[a]	R	U	S	R	U	S	R	U
1	32	0	0	10	0	8	0	0	0
2	26	0	1	8	0	1	0	0	0
3	9	1	1	5	0	1	0	0	1
4	2	0	2	1	0	0	0	0	0

[a] S = tentoxin sensitive, R = resistant, U = untested. See text for description of groupings

group is a larger number of plants which displayed abnormal vegetative growth patterns, such as stunted growth and irregularly shaped leaves. These plants have flowered and, with cross pollination, have set viable seed. Finally, this group includes a few plants which were very abnormal in vegetative growth and which dropped all flower buds before opening. Group III is represented by only one plant which appeared to be a normal *Petunia* plant and which did not produce seeds.

Seeds from all plants were germinated in the presence of tentoxin. Combining the data from all groups, 93 plants produced seedlings which were sensitive to tentoxin. Only 1 plant, which was in Group I, produced seedlings which were resistant to tentoxin. No plants produced populations of seedlings with mixed response to tentoxin. Plants of Group I produced seeds which germinated to the same extent as is characteristic for 'Turkish Samsun' (over 90%). Plants from Group II produced seeds with reduced viability, usually ranging from 30–50%.

A few of the plants in Group I have been analyzed further. Chromosome numbers for 5 of the plants were determined to be 48, the typical number for *N. tabacum.* Chloroplast DNA of the progeny of 3 of the plants which produced seedlings sensitive to tentoxin has been isolated and restricted with EcoR1. Agarose gel electrophoresis of the restricted DNA enabled comparison to both tobacco and *Petunia* chloroplast DNA restricted with the same enzyme (Fig. 1). Scrutiny of these DNA banding patterns confirmed the tentoxin results, since chloroplast DNA of the tobacco cybrids precisely matched that of *Petunia* and differed in many locations along the gel profile from the profile of *N. tabacum* chloroplast DNA. Several of the more prominent differences are noted by small arrows. All three cybrids which were analyzed conformed precisely in the chloroplast DNA profiles of their progeny to *Petunia.*

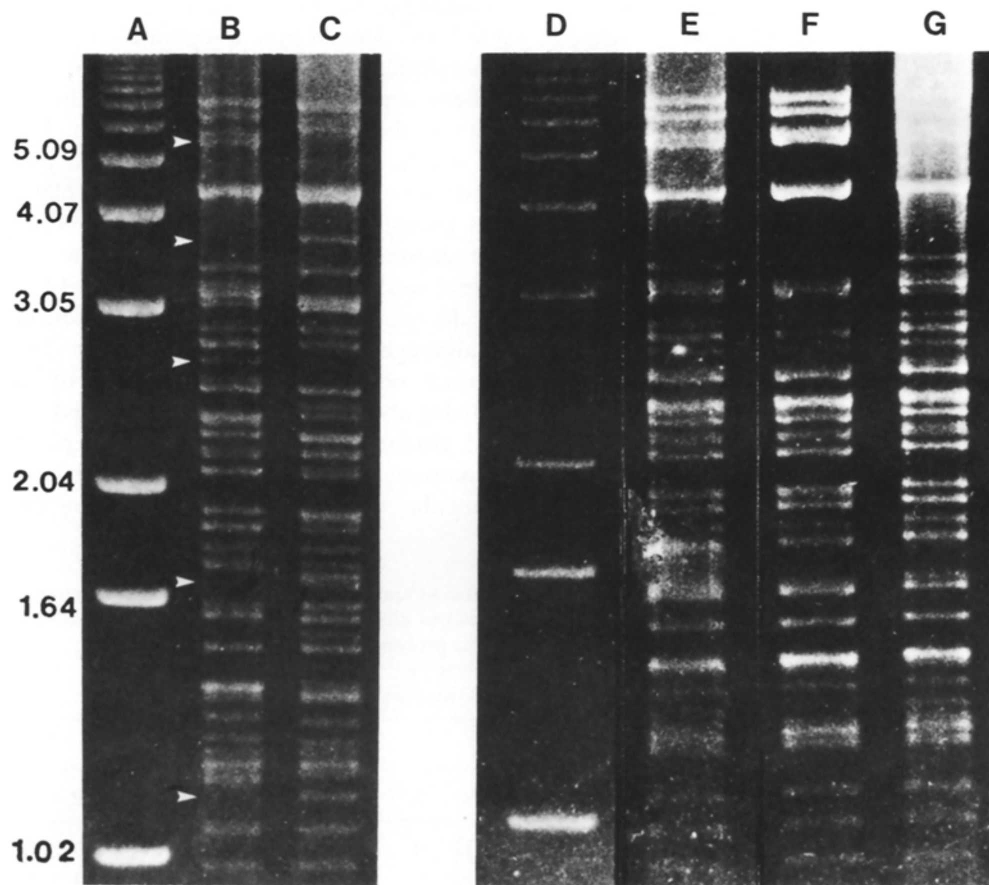

Fig. 1. EcoR1 restriction patterns of chloroplast DNA of parenal and cybrid plants. Fragment sizes are given in kilobase pairs in *lanes A* and *D.* In *lane B* chloroplast DNA from a cybrid plant is compared to tobacco chloroplast DNA in *lane C.* Some of the numerous differences between the two gel profiles are marked with white arrows. In *lanes E* and *G,* chloroplast DNA banding patterns from two other cybrids are compared to *Petunia* chloroplast DNA in *lane F.* The pattern of restriction bands for the cybrids is identical to that of *Petunia* throughout the gel

Discussion

The success of the *Petunia-Nicotiana* experiments demonstrates that protoplast fusion can be used to obtain vigorously growing intergeneric cybrids. However, these experiments depended on the development of techniques to achieve efficient interspecific organellar transfer in *Nicotiana.* Zelcher et al. (1978) used x-irradiation to inactivate the nucleus of the protoplast chosen as organelle donor species. Menczel et al. (1982) utilized gamma irradiation, which was also utilized to inactivate the *Petunia* nucleus in the experiments described here. Glimelius and Bonnett (1981) showed that the albino protoplasts could be restored to photoautotrophy by protoplast fusion, and Medgyesy et al. (1985) utilized such a chloroplast mutant together with gamma irradiation to select for interspecific chloroplast transfer by production of cybrids of *Nicotiana* resulting from somatic cell fusion.

Both nuclear and chloroplast genetic systems evolve and must do so harmoniously in order to maintain function. Analyses of chloroplast DNA of species representing all sub-genera of *Nicotiana* by several restriction enzymes have shown numerous species-specific differences (Salts et al. 1984). However, there was a high degree of conservation of arrangement and sequence of chloroplast DNA within the genus. Changes in restriction enzyme cleavage sites appeared to cluster in portions of the chloroplast DNA where change is more rapid. These portions may be of little functional significance. In a review of published reports of chloroplast DNA structure of more than 200 angiosperm species, Palmer (1985) concluded that the chloroplast genome is characterized by a slow rate of genetic change. The ability of the *Nicotiana* nucleus to support the development of fully functional chloroplasts of *Petunia* confirms that sufficient harmony exists at the intergeneric level to permit cybridization even when the nuclear genomes of the two genera have evolved to the point where they are incompatible. We must assume that all *Nicotiana tabacum* chloroplast polypeptides coded by the nucleus have those characteristics necessary for transiting the *Petunia hybrida* chloroplast membranes and interacting with *Petunia* chloroplast gene products in a fully functional manner.

Gamma irradiation was effective in eliminating the regeneration of *Petunia* protoplasts into plants, since only 1 of 109 plants was a *Petunia.* Without irradiation, *Petunia* protoplasts which did not fuse would have grown into plants under the experimental conditions used. However, transfer of some nuclear genetic material from the irradiated *Petunia* protoplasts cannot be excluded, particularly in Group II plants. The spectrum of altered vegetative and reproductive growth exhibited by these plants could be explained by aneuploidy due to changes in the tobacco chromosome complement during culture of the cybrids, by aneuploidy due to the introduction of alien chromosomes from the irradiated *Petunia* protoplasts, or by alteration of male or female fertility due to intergeneric nuclear-cytoplasmic interactions.

Even though protoplasts with albino chloroplasts were the recipient protoplasts, variegated plants containing both albino and *Petunia* chloroplasts were not observed. Furthermore, no albino leaf sectors have been observed in any of the hundreds of cybrid plants grown for organellar DNA characterization. In addition, all seedlings from a single plant were either sensitive or resistant to tentoxin. No variegation in response to tentoxin resistance has been noted either. Apparently, sorting of chloroplast type was complete in the cybrid plants.

Interspecific hybrids are fairly common within the genus *Nicotiana* and can be produced by pollinating many species with *N. tabacum* pollen. Alloplasmic cultivars of *N. tabacum* have been produced after a series of backcrosses of interspecific hybrids using *N. tabacum* as the recurrent male parent. Among the plants produced during the series of backcrosses, manifestations of male sterility frequently appear, depending on the species originally selected as the female parent (see Gerstel 1980).

One expectation of our program of intergeneric cybridization was that cytoplasmic male sterility would appear among plants regenerated from cybrid cells. In fact, plants in Group I showed no reduction in pollen viability, seed set, or seed viability. Many plants in Group II did show reductions in pollen production or viability, but these plants also showed reduced seed set after cross pollination. Moreover, seed germination rates in this Group were 50% or below, further suggesting a reduction in female fertility as well as male fertility. The sorts of abnormalities in stamen development which are characteristic of alloplasmic male-sterile cultivars of tobacco (e.g., petalode production, feminization of stamen structures, early abortion of stamens) have not appeared among any of the cybrids or their progeny.

We conclude that the production of intergeneric combinations of organellar and nuclear genomes, such as between *Petunia* and *Nicotiana,* respectively, supports the concept that organelle transfer can play a role, together with transformation (De Block et al. 1985), in the utilization of organellar genetic diversity in plant breeding.

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References

Binding H (1976) Somatic hybridization experiments in Solanaceous species. Mol Gen Genet 144:171–175

De Block M, Schell J, Van Montagu M (1985) Chloroplast transformation by *Agrobacterium tumefaciens.* EMBO J 4:1367–1372

Durbin RD, Uchytil TF (1977) A survey of plant insensitivity to tentoxin. Phytopathology 67:602–603

Gerstel DU (1980) Cytoplasmic male sterility in *Nicotiana* (a review). NC Agric Exp Stn Tech Bull 263:1–31

Glimelius K, Bonnett HT (1981) Somatic hybridization in *Nicotiana:* restoration of photoautotrophy to an albino mutant with defective plastids. Planta 153:497–503

Glimelius K, Wallin A, Eriksson T (1978) Concanavalin A improves the polyethylene glycol method for fusing plant protoplasts. Physiol Plant 44:92–96

Glimelius K, Chen K, Bonnett HT (1981) Somatic hybridization in *Nicotiana:* Segregation of organellar traits among hybrid and cybrid progeny. Planta 153:504–510

Glimelius K, Fahlesson J, Sjödin C, Sundberg E, Djupsjöbacka M, Fellner-Feldegg H, Bonnett HT (1986) Somatic hybridization and cybridization as potential methods for widening the gene-pools of crops within Brassicaceae and Solanaceae. Genetic manipulation in plant breeding. Int Symp Eucarpia (in press)

Kung SD, Zhu YS, Shen GF (1982) *Nicotiana* chloroplast genome. 3. Chloroplast DNA evolution. Theor Appl Genet 61:73–79

Medgyesy P, Golling R, Nagy F (1985) A light sensitive recipient for the effective transfer of chloroplast and mitochondrial traits by protoplast fusion in *Nicotiana.* Theor Appl Genet 70:590–594

Menczel L, Galiba G, Nagy F, Maliga P (1982) Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana.* Genetics 100:487–495

Palmer JD, Zamir D (1982) Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon.* Proc Natl Acad Sci USA 79:5006–5010

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Pogliaga HH (1952) Hybrido intergenerico (*Nicotiana* × *Petunia*). Rev Argent Agron 19:171–178

Rhodes PR, Kung SD (1981) Chloroplast deoxyribonucleic acid isolation: purity achieved without nuclease digestion. Can J Biochem 59:911–915

Salts Y, Herrmann RG, Peleg N, Lavi U, Izhar F, Frankel R, Beckmann JS (1984) Physical mapping of plastid DNA variation among eleven *Nicotiana* species. Theor Appl Genet 69:1–14

Vedel F, Quieter F, Dosba F, Doussinault G (1978) Study of wheat phylogeny by EcoR1 analysis of chloroplastic and mitochondrial DNAs. Plant Sci Lett 13:97–102

Walbot V (1977) Use of silica sol step gradients to prepare bundle sheath and mesophyll chloroplasts from *Panicum maximum.* Plant Physiol 60:102–108

Wong-Staal F, Wildman SG (1973) Identification of a mutation in chloroplast DNA correlated with formation of defective chloroplasts in a variegated mutant of *Nicotiana tabacum.* Planta 113:313–326

Zelcher A, Aviv D, Galun E (1978) Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and x-ray irradiated protoplasts of male-sterile *N. tabacum.* Z Pflanzenphysiol 90:397–407

Zenkteler M, Melchers G (1978) In vitro hybridization by sexual methods and by fusion of somatic protoplasts. Theor Appl Genet 52:81–90

Note added in proof

After acceptance of this article the results obtained by Pental et al. (1986) were published where they reported that shoots were obtained from one cell line grown from fusions between *N. tabacum* and *P. hybrida.* These shoots contained predominantly the nuclear genome of *Petunia* and chloroplasts of *Nicotiana.*

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida* recovery of plants with *P. hybrida* nuclear genome and *N. tabacum* chloroplast genome. Mol Gen Genet 202:342–347