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Generation of heteroplastidic *Nicotiana* cybrids by protoplast fusion: analysis for plastid recombinant types

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Summary. Protoplasts of a mutant line of *Nicotiana tabacum* having a maternally-transmitted chlorophyll deficiency were fused with protoplasts of two alloplasmic-male-sterile *Nicotiana* lines by the "donor-recipient" technique. In both fusion experiments variegated plantlets were regenerated which were shown to contain cytoplasms of mixed chloroplast nature. This confirms that with the "donor-recipient" method one can obtain mixed cytoplasms of genetically different chloroplasts. We present a convenient system to assay for genetic recombination between chloroplasts by combining use of several cytoplasmic markers: vis. chlorophyll pigmentation, chloroplast DNA restriction patterns, tentoxin resistance and male sterility. Within the limits of the experiment no recombinant types were recovered.

Key words: Tobacco – Chloroplast DNA – Cybrids – Male sterility – Tentoxin resistance – Plastid recombinants – *Nicotiana*

Introduction

In the majority of reports where chloroplast markers have been assayed the resultant fused plants showed homogeneous cytoplasms representing only one of the parental types. This was the case whether chloroplasts of the same or different genotypes were used (Medgyesy et al. 1980; Scowcroft and Larkin 1981; Menczel et al. 1982; Schiller et al. 1982). However, in two reports (Glimelius et al. 1981; Fluhr et al. 1983) cybrids of mixed cytoplasm were observed in regenerated plants. In the first case the cybrids were of an undetermined nuclear background. In addition, in the first report no clear correlation was shown between the chloroplast markers used. In the second report, cybrids were described with well-defined nuclear backgrounds which contained mixtures of streptomycin resistant and sensitive chloroplasts. These cybrids were produced using the "donor-recipient" method. In this procedure irradiated protoplats of one species are fused with non-irradiated protoplasts of another species. Thus, cell division in the irradiated parent (donor) is inhibited, while for the other parent (recipient) a cytoplasmic feature of selection exists, e.g. streptomycin sensitivity or plastid pigmentation. Plants regenerated from such a fusion will often exhibit a uninuclear background of the non-irradiated "recipient" nucleus and mixed or sorted-out cytoplasms of either parental species (Zelcer et al. 1978; Aviv et al. 1980).

In the present work we have further refined the cybrid selection process by using plastid-dependent chlorophyll deficiency as a convenient and sensitive marker for direct selection. Along with this system, we employed chloroplast DNA (cpDNA) restriction-digest-patterns and plastid-dependent tentoxin sensitivity to enable direct assaying for plastid recombinant types.

Materials and methods

Plant material

Seeds from a variegated *N. tabacum* mutant, DPI (Burk et al. 1964), containing defective plastids in the pale sectors of its leaves, were kindly provided by Dr. L. G. Burk, U.S. Dept. of Agriculture, Tobacco Research Laboratory, Oxford, North Carolina, and raised to maturity. Seed pods were individually analysed and those with pure seed populations, resulting in either only albino or only normal green plants, were utilized to establish, respectively, albino or green shoot tips. The shoot tips were maintained in MS medium (Murashige and Skoog

Table 1. Nuclear and cytoplasmic characteristics of mutant lines

Mutant plant designation	Source	Nuclear characters	Cytoplasmic characters	
			Chloroplast	Anther type
VCMSG	Chen (unpublished)	*N. tabacum:* long internodes, sessile leaves, 2n = 48	wild-type (green), tentoxin sensitive, cpDNA-*suaveolens* type	no anthers; sterile
VBW	Burk et al. 1964	*N. tabacum:* long internodes, petiolated leaves, 2n = 48	mutant (albino), tentoxin resistant, cpDNA-*tabacum* type	potentially fertile but plants do not attain flowering stage
VBG	Burk et at. 1964	*N. tabacum:* long internodes, pink flowers, 2n = 48	wild type (green), tentoxin resistant, cpDNA-*tabacum* type	fertile
f29s	Aviv et al. 1980	*N. sylvestris:* short internodes, rosette growth, white flowers, 2n = 24	wild type (green), tentoxin sensitive, cpDNA-*undulata* type[a]	stigmatoid anthers; sterile

[a] In our previous publications this cytoplasm was termed as L-92. According to the source (Prof. A. Marani, Hebrew University, Rehovot) it was thought to be *suaveolens* type. However, after detailed comparative analysis of cpDNA restriction pattern and anther type we have reclassified it *undulata* type

1962) containing 2 mg/l and 0.8 mg/l of kinetin and indoleacetic acid, respectively. No reversion from albino to green was observed after two years of subculture. The albino and green shoot tips were designated VBW and VBG, respectively. Seeds of a variegated, alloplasmic male-sterile *N. tabacum* line having *N. suaveolens* cytoplasm were kindly provided by Dr. K. Chen, Dept. of Biological Sciences, University of Maryland, Baltimore County, Maryland. This line was maintained by pollination with *N. tabacum* cv. 'Xanthi' and similarly cloned into green and white tip culture shoots designated VCMSG and VCMSW, respectively. Another alloplasmic male-sterile line, f29s, having *N. sylvestris* nuclei and an alien cytoplasm (probably, *N. undulata*) was established by a "donor-recipient" fusion experiment (Aviv et al. 1980) and was maintained by pollinations with *N. sylvestris*. The characteristics of the plant materials used in this report are summarized in Table 1.

Protoplast manipulations

Protoplast isolation, X-irradiation, fusion and regeneration were as described previously (Aviv and Galun 1980).

Isolation of chloroplast DNA

CpDNA was isolated from white and green areas of variegated plants as previously described (Fluhr and Edelman 1981 a). Small scale samples of total DNA were extracted from leaf tissue in the following manner. One hundred mg of leaf tissue were ground, along with 100 mg of polyvinyl-polypyrrolidone, in 5 ml of buffer containing: 0.3 M sorbitol, 50 mM 2-N-morphinoethanesulfonic acid (MES), 2 mM EDTA, 2 mM isoascorbate, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.8 mM K_2HPO_4, 10 mM KCl, 5 mM B-mercaptoethanol, pH 6.1. Four hundred μg/ml ethidium bromide was added as well (Kislev et al. 1980). The solution was brought to 0.5% sodium dodecyl sulfate and proteinase K was added to a concentration of 50 μg/ml. Incubation was for 1 h at 37 °C. The solution was extracted once with freshly-neutralized distilled phenol, once with chloroform:isoamyl alcohol (24:1) and once with distilled ether. The extract was transferred to a polyallomer SW40 tube (Beckman) and centrifuged at 35,000 rpm for 5 h at 4 °C. The nucleic acid pellet was then resuspended overnight in 200 μl of 10 mM Tris pH 8.0, 1 mM EDTA. Restriction endonuclease digestion, agarose gel fractionation, transfer to nitrocellulose and hybridization were as previously described (Fluhr and Edelman 1981 a).

Tentoxin test

Resistance or sensitivity to tentoxin was determined as described (Galun 1982). Briefly, seeds were germinated in the light (1,000 lux) on discs containing 20 μg/ml of tentoxin (Durbin and Uchytil 1977). For leaf tests, a few hundred microliters of the tentoxin solution were injected directly into the intact leaf of greenhouse cultured plants.

Results

Fusion of VCMSG and VBW protoplasts resulting in cybrids with mixed cytoplasms

VCMSG ("donor") protoplasts were X-irradiated (5 krad), fused with albino VBW ("recipient") protoplasts and plated on NM media (Nagy and Maliga 1976). The green protoplasts were not expected to divide as they were irradiated; the white protoplasts were expected to divide freely but form white colonies. Thus, any green colonies should result from a partial or complete transfer of VCMSG cytoplasm to VBW protoplasts. Three such green calli were isolated independently. Plants were regenerated from them and analyzed for nuclear and cytoplasmic characteristics (Table 2).

Table 2. Characteristics of plants derived from fusion between X-irradiated VCMSG protoplasts and VBW protoplasts

Callus	No. plants regenerated	Nuclear character	Cytoplasmic characters	
			Chloroplast	Anther type
1	1	sessile leaf	green	sterile
2	12	petiolated leaf, 2n = 48 (3)[a]	all green, tentoxin sensitive	all sterile
3	14	petiolated leaf, 2n = 48 (3)[a]	12 green + 2 variegated, all tentoxin sensitive	13 sterile, 1 fertile

[a] No. in parenthesis refers to number of plants analyzed

The single plant regenerated from callus 1 showed only the parental characteristics of the "donor" fusion partner and was therefore considered to be the result of an unfused VCMSG protoplast escaping X ray-induced division arrest. All 26 plants regenerated from calli 2 and 3, on the other hand, had petiolated leaves and were morphologically similar to VBG plants. Six of these plants were analysed and had the expected 2n = 48 chromosome number; thus, it was surmised that all plants from calli 2 and 3 contained only the nucleus of the recipient VBW protoplast.

All 12 plants derived from callus 2 were male-sterile and green. We concluded that these cytoplasmic characteristics were transferred from "donor" VCMSG into "recipient" VBW protoplasts to form cybrid plants. None of the "recipient" cytoplasmic markers were recovered in these plants.

Eleven plants derived from callus 3 were also concluded to be cybrids containing "recipient" VBW nuclei with cytoplasmic markers of the VCMSG "donor". The one fertile plant of callus 3 was considered to be a cybrid in which the "donor" contributed only the chloroplast markers while the mitochondria were apparently of the fertile "recipient" type. The two variegated cybrid plants resulting from callus 3, E-7-5 and E-7-12 (Fig. 1 A), were considered

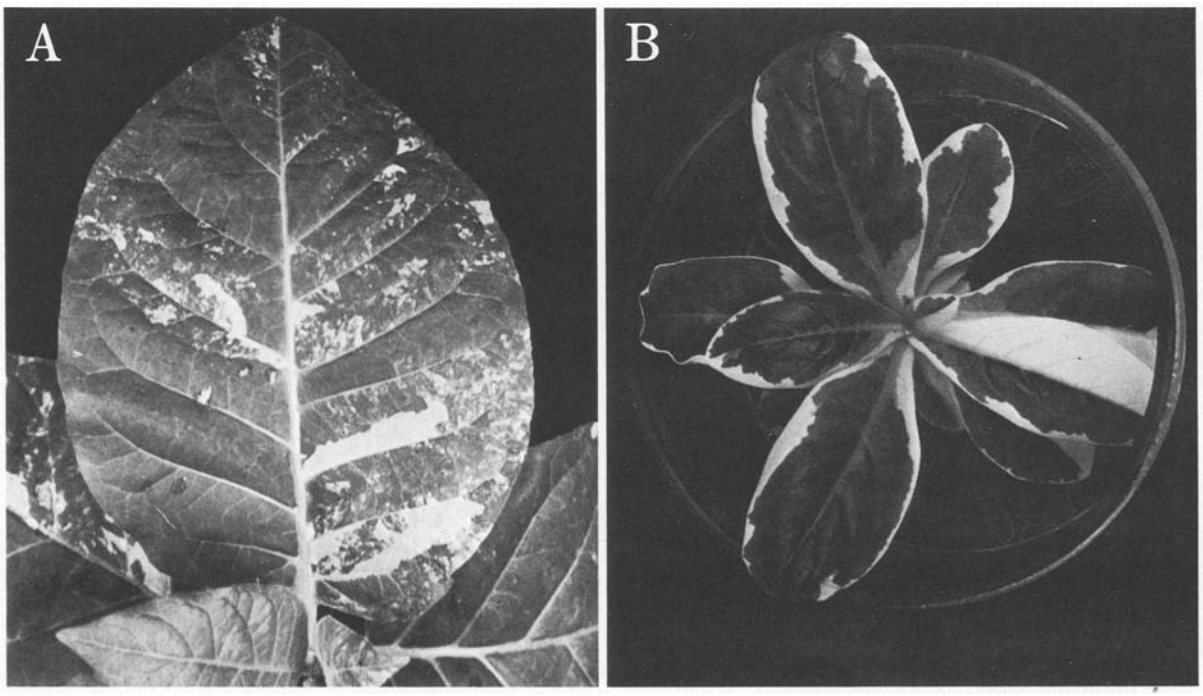

Fig. 1 A, B. Leaf variegation patterns in somatic fusion cybrids. **A** variegated leaf from somatic fusion cybrid E-7-5. Note as well petiole leaf morphology; **B** leaf of a rosette type plant derived from callus group D (Table 3) exhibiting margin and sectored variegation

to contain both "recipient" and "donor" chloroplasts but only "donor" mitochondria, as they both were male sterile.

The variegated plants, E-7-5 and E-7-12, were pollinated with *N. tabacum* cv. 'Xanthi' to test whether they contained cells with mixed plastid types. When the resulting seed were germinated on Nitsch agar plates (Nitsch 1969), hundreds of green, white and variegated seedlings were obtained. Their relative ratios varied from pod to pod from almost all green plantlets to all variegated to all white. These results proved that indeed E-7-5 and E-7-12 plants contained cells of mixed cytoplasms in their L_{II} layer. Moreover, when cpDNA was separately isolated from white and green leaf tissues of the variegated plants and the samples digested with endonuclease, a clear difference in cpDNA type was detected between these two parts corresponding to the original parental patterns (Fig. 2). It is also evident from Fig. 2 that the mutant VBW cpDNA-restriction-pattern does not differ at this level of detection from that of the wild-type VBG. Thus, no major deletion in cpDNA sequence is responsible for the mutant plastid phenotype.

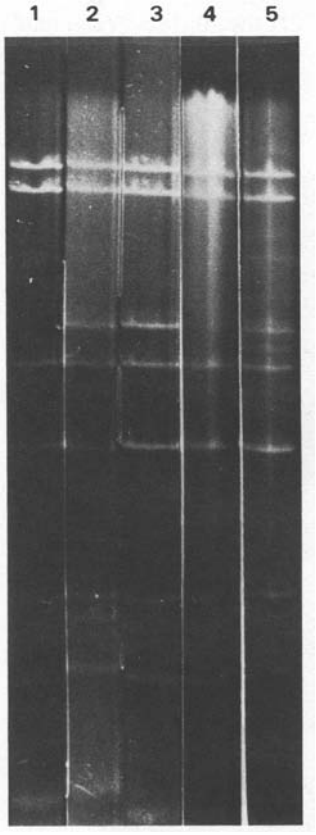

Fig. 2. BamHI restriction pattern of cpDNA (lug/lane) isolated from VCMSG (*lane 1*); VBW (*lane 2*); VBG (*lane 3*); green part of a variegated leaf from somatic fusion hybrid E-7-5 (*lane 4*); white part of variegated leaf from E-7-5 (*lane 5*)

Table 3. Characteristics of plants derived from fusion between X-irradiated VBW protoplasts and f 29s protoplasts

Callus group	No. of calli	Growth habit	Chloroplast type	Fertility of regenerated plants
A	3	rosette	albino (2)[a]	no flowering[b]
B	2	rosette	albino (2) green (14)[c]	no flowering male-fertile (1), male-sterile (13)
C	4	rosette	green (11)	male-fertile (3), male-sterile (8)
D	1	rosette	albino (10), green (10), variegated (7)	no flowering, male-fertile, male-fertile

[a] No. of plants regenerated from original calli are given in parenthesis
[b] Plants which did not flower were maintained by tip-culture
[c] All green types expressed tentoxin sensitivity upon injection with tentoxin or upon seed germination in tentoxin

Fusion of f29s and VBW protoplasts resulting in chimeric apical

In the previous fusion (VCMSG + VBW) the number of green calli recovered was lower than expected. One possibility was that VBW colonies which were not selected against, reduced the survival of the green cybrid colonies. To test this we mixed non-irradiated green mesophyll protoplasts with VBW protoplasts in a 1 : 500 ratio. Indeed, we found that the ratio of green to white colonies was much lower than the original plating ratio (data not shown). To circumvent this, we designed a different set of fusion conditions in which the "donor" were the albino VBW protoplasts which were X-irradiated and thus could not divide. The green "recipient" protoplasts were f29s which contained *N. sylvestris* nuclear characters (Table 1) and were selected against by plating in mannitol (Zelcer et al. 1978). Under these conditions f29s protoplasts would divide only if fused with VBW protoplasts, the cytoplasmic complement of which are not inhibited by mannitol.

A total of ten calli were isolated and about ten plants regenerated from each callus. The calli were arranged into four groups according to the characteristics of the plants derived from them (Table 3). In group A three calli produced only albino plantlets whose growth were of the rosette leaf type. We concluded that these were true cybrids containing f29s nuclei and the defective plastids of VBW. In group B two calli produced green and albino plantlets. The albino ones were of the rosette type described above. The green ones produced mostly plants identical with f29s, including the character of male sterility, however

several of them were male fertile. We therefore propose that these two calli contained only cells with f29s nuclei but were heterogenous in respect to chloroplast types (green and albino) and mitochondria (male sterility/fertility). In group C four calli produced exclusively green plants; most of them were identical to f29s but several were male fertile. Thus the recipient f29s contributed the nuclei, the normal green chloroplasts and in some plants also the mitochondria, while VBW contributed only its mitochondria to certain cybrids. Apparently, as the mitochondria segregated out in these four calli male fertility was restored in the resultant plants. In group D a single callus produced variegated rosette type plantlets, together with plantlets which were either all green or all albino. This callus was subcultured over a two year period. During that time it regenerated variegated plants of a similar nature (Fig. 1 B). When small amounts of total DNA of variegated tissue were analysed both parental chloroplast DNA patterns were recovered (Fig. 3).

Over 20 variegated plants were transferred to the greenhouse but only two (C-4-15 and C-4-16) flowered and set seeds. From these, seeds from many pods were germinated on Nitsch agar plates. Either green or white seedlings were produced but no variegated ones were observed. It seems that, in contrast to plants E-7-5 and E-7-12 which were discussed previously, the variegated plants in this experiment did not contain cells with mixed cytoplasms but rather apical meristems with two cell types. Such meristems apparently arose as a result of early and complete cytoplasmic sorting out at the cellular level.

Testing for chloroplast recombinant types

We attempted to detect plastid recombinant types in variegated plant progeny resulting from cells which maintained mixed cytoplasms, using the following strategy. VCMSG chloroplasts are sensitive to tentoxin and produce white chlorotic seedlings, while VBG chloroplasts are tentoxin resistant and produce normal green seedlings (Table 1). VBW chloroplasts, although white in leaf tissue, are of *N. tabacum* origin and, thus, presumably carry the tentoxin resistance locus.

In order to test for possible plastid recombination between the markers of tentoxin sensitivity and chlorosis following protoplast fusion, seeds derived from pods which produced over 50% variegated E-7-12 plantlets were plated on Nitsch agar containing tentoxin. The seedlings were kept until the first leaves appeared. Under these conditions control VBG seedlings developed green cotyledons and green leaves, control VCMSG seedlings developed white cotyledons and white leaves, and control VBW seedlings developed light-green cotyledons but white leaves (upon full expression of the albino mutation). Altogether two hundred and fifty E-7-12 seedlings were tested on tentoxin plates. In the cotyledon stage many had light green spots but all produced white leaves; thus, no recombinants were obtained. When these plantlets were transferred to Nitsch agar without tentoxin their variegated status was re-established. This indicated the continuing presence of mixed cytoplasm. The light

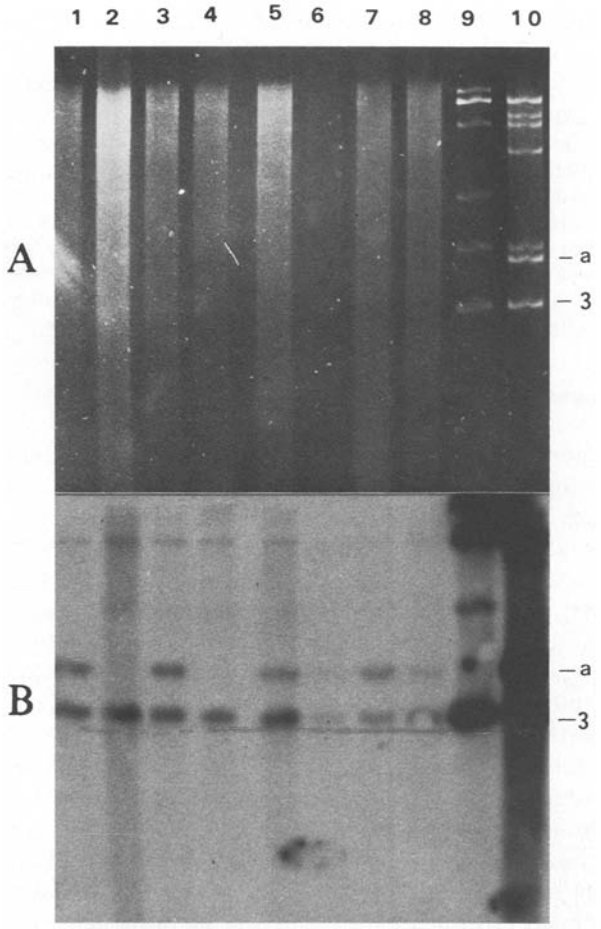

Fig. 3 A, B. Hybridization of whole cell DNA from somatic cybrids with cloned cpDNA probes which differentiate between "donor" and "recipient" lines. **A** *lanes 1–8* show small scale preparations of total plant DNA digested with BglI; *lanes 9,10* show similarly digested cpDNA samples (lug/lane). The sources of DNA for the individual lanes are: f29s (*lane 1*), VBW (*lane 2*), green plantlets regenerated from callus group D (*lane 3*), white plantlet regenerated from callus group D (*lane 4*), variegated portions of leaf tissue regenerated from callus group D (*lanes 5–8*), *N. tabacum* cpDNA (*lane 9*), *N. tabacum* line 92 cpDNA (*lane 10*); **B** autoradiography of a nitrocellulose blot of the gel in 3A hybridized to clone pBa 2–14 consisting of *E. coli* plasmid pBR322 containing a *N. tabacum* chloroplast rRNA gene insert as well as an additional, nonselective cpDNA insert (Fluhr, unpublished). The rRNA gene probe hybridizes to *N. tabacum* cpDNA BglI fragment 3 and to *N. tabacum* line 92 BglI fragments 3 and a. For fragment designations and map positions, see Fluhr and Edelman 1981 b

green areas on the cotyledons of E-7-12 were therefore due to sectors composed of cells with VBW (i.e. tentoxin resistant) plastids, while the rest of the cells contained VCMSG plastids.

In another approach, plastid recombination types were sought in variegated portions of leaves from somatic-fusion hybrid E-7-5 which were excised and subcloned through protoplast formation. Variegated leaves contain cells with mixed chloroplast populations (Burk et al. 1964). After two weeks, approximately one thousand colonies were spread on NM agar media with tentoxin, however no green regenerating calli were recovered.

Discussion

Fusion between plant protoplasts having different plastomes has been commonly observed to result in a quick sorting out of plastid characters in the respective hybrid progeny (Chen et al. 1977; Melchers et al. 1978; Iwai et al. 1980; Medgyesy et al. 1980; Douglas et al. 1981; Scowcroft and Larkin 1981; Flick and Evans 1982; Menczel et al. 1982). Our experimental results suggest that the "donor-recipient" fusion technique enables one to readily obtain cybrids with mixed chloroplasts. By utilizing appropriate markers such as greenness/whiteness, (Gleba 1979) or streptomycin resistance/sensitivity (Fluhr et al. 1983), both of which can be assayed visually, one can select directly for variegated progeny. Variegation can arise from mixed cells which have been maintained in mixed cytoplasm for many cell generations, or as a result of the presence of different types of homoplastic cells forming a chimera at the growing tip (Kirk and Tilney-Bassett 1978). In the first case, for which plants E-7-5 and E-7-12 are representatives, leaves are variegated in a spotty fashion due to plastid sorting out. In addition, the seeds of these plants may be similarly variegated. In the second case, for which plants C-4-15 and C-4-16 are representative, variegation is cleary sectored and seed progeny are of either white or green plastid type. In this instance early and complete sorting out occurs and no cells with mixed cytoplasm are present.

In contrast to chloroplast markers, cytoplasmic male sterility, which is a mitochondrial marker in *Nicotiana* (Belliard et al. 1979; Galun et al. 1982), has not been detected by us, or others, to undergo sorting out in the somatic plant state. Thus, following *Nicotiana* interspecific fusions and regeneration, no individual plants were observed to have both fertile and sterile flowers (Tables 2 and 3). However, in a recent report in which cytoplasmic male sterility was transferred from *N. tabacum* to *N. plumbaginifolia* via somatic fusion, individual plants with flowers of varying fertility types were recovered (Menczel et al. 1983).

In the examples where plastid variegation arose from cells which had been maintained with mixed cytoplasm for many cell generations, we assayed for the possibility of marker recombination at two different developmental stages. In one instance somatic cells of the original fused plants were subcloned and regenerated in the presence of tentoxin, while in the other, seeds from pods which yielded variegated seedlings were germinated in the presence of tentoxin. In no case was recombination observed between the tentoxin locus and a plastomic genetic lesion causing albinism. It should be noted that no major deletion in cpDNA sequence was responsible for the white plastid phenotype (Fig. 2). Although we have assayed less than three hundred seed and approximately one thousand regeneration calli, we assume the chloroplast population screened to be much larger due to the multiplicity of chloroplasts per cell and the number of somatic cell cycles. However, if recombination events are limited to a critical temporal span of plant development (e.g. meiosis), then, at best, each germinating plantlet will represent the sum of plastid exchanges at that time of development, and many more seeds will need to be analysed.

In a fusion between *N. sauveolens* having tentoxin sensitive chloroplasts and a *N. tabacum* nitrate-reductase nuclear mutant reported by Glimelius et al. (1981), no marker recombination between the large subunit of ribulosebisphosphate carboxylase and tentoxin resistance was detected. The chlorophyll pigment marker used in our study enables a much more sensitive assay for recombination, as even small clusters of individual tentoxin-resistant green cells can be visually detected against the background of white tentoxin-sensitive leaf material. Direct attempts to detect recombination between plastids of higher plants were performed in the genus *Oenothera* where biparental inheritance of plastid characters exists (Kutzelnigg and Stubbe 1974). In this case plastid mutants of unknown lesions were crossed, however no recombinant types were recovered. On the other hand, recombination of chloroplast characters is a well established phenomenon in the uniplastidal alga, *Chlamydomonas rhinehardii* (Gillham 1978). The difficulty in detecting plastid recombination in higher plants may partially lie in a relative rarity of chloroplast fusion events in cells having numerous plastids (although it has been suggested that such fusion has been observed in higher plants (Vaughn 1981)). No doubt future progress in this type of cytoplasmic genetics will be dependent upon an increased use of plastid mutants for which sensitive assays can be devised.

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