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Somatic hybridization by microfusion of defined protoplast pairs in *Nicotiana*: morphological, genetic, and molecular characterization

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Summary. Somatic hybrid/cybrid plants were obtained by microfusion of defined protoplast pairs from male-fertile, streptomycin-resistant *Nicotiana tabacum* and cytoplasmic male-sterile (cms), streptomycin-sensitive *N. tabacum* cms (*N. bigelovii*) after microculture of recovered fusants. Genetic and molecular characterization of the organelle composition of 30 somatic hybrid/cybrid plants was performed. The fate of chloroplasts was assessed by an in vivo assay for streptomycin resistance/sensitivity using leaf explants (R_0 generation and R_1 seedlings). For the analysis of the mitochondrial (mt) DNA, species-specific patterns were generated by Southern hybridization of restriction endonuclease digests of total DNA and mtDNA, with three DNA probes of *N. sylvestris* mitochondrial origin. In addition, detailed histological and scanning electron microscopy studies on flower ontogeny were performed for representative somatic hybrids/cybrids showing interesting flower morphology. The present study demonstrates that electrofusion of individually selected pairs of protoplasts (*microfusion*) can be used for the controlled somatic hybridization of higher plants.

Key words: Electric field-induced microfusion – Microculture – *Nicotiana* – Somatic hybridization – Cytoplasmic male sterility

Introduction

Protoplast fusion represents a powerful approach to organelle genetics in higher plants. Due to the uniparental maternal inheritance of organelles in most higher plants including the majority of cultivated crop species, with a few exceptions such as *Phaseolus* and *Secale*, the genetic and physiological effects of a heterozygotic organelle condition on the phenotype of plants have only been poorly studied (Kumar and Cocking 1987). Most of our limited knowledge is based on the few species that show biparental inheritance of cytoplasmic organelles, among others, *Epilobium*, *Oenothera*, and *Pelargonium* (Hagemann 1979).

With somatic hybridization via protoplast fusion, it has become possible to extend organelle genetic studies to several species that show strictly uniparental (maternal) inheritance of organelles during sexual hybridization. *Microfusion* of defined, preselected protoplast pairs has been recently developed and seems a priori to be an extraordinarily valuable tool for somatic hybridization (Koop and Spangenberg 1989). The strength of this technique lies in the fact that it does not restrict the spectrum of recovered somatic hybrids/cybrids, as is inherently the case in all other available methods for somatic hybridization that use *mass fusion* of protoplasts, followed by enrichment or selection of hybrid somatic cells (Gleba and Sytnik 1984).

The development of a microculture system (Koop et al. 1983a) for the culture of individually selected protoplasts leading to calli formation and, finally, plant regeneration made possible a series of manipulations at the single cell level: *micromanipulations* (Koop et al. 1983b; Spangenberg et al. 1986a; Schweiger et al. 1987; Spangenberg and Neuhaus 1988). Defined pairs of protoplasts, protoplast and cytoplast, or cytoplast and karyoplast (cell reconstitution) could be microfused, and the fusion products could be individually grown in a further improved version of the microculture system (Koop and Schweiger 1985a, b; Spangenberg and

Abbreviations: ac – alternate current; BAP – benzyl aminopurine; cms – cytoplasmic male sterile; dc – direct current; NAA – naphthalenacetic acid; SEM – scanning electron microscopy

Schweiger 1986). With the development of this microfusion technique, the production of parasexual hybrids under controlled conditions, namely, control of the number and type of cells participating in the fusion event, became feasible (Koop and Spangenberg 1989).

However, up until now a thorough characterization of regenerants from microfusants has not been available. The reports dealing with *Nicotiana*, *Brassica*, and *Funaria* protoplast microfusion and microculture (Koop and Schweiger 1985b; Spangenberg et al. 1985, 1986b; Mejia et al. 1988) have been mainly directed towards the development and optimization of the micromanipulation techniques involved, but they totally lack information concerning the genetic constitution of the recovered plants that could verify their somatic hybrid/cybrid nature.

In the case of *N. tabacum*, homofusion of two indistinguishable mesophyll protoplasts has been performed (Koop and Schweiger 1985b), thus making any further analysis for somatic hybrid verification practically impossible. Here we report the morphological, genetic, and molecular characterization of somatic hybrid/cybrid plants obtained by microfusion of defined protoplast pairs of male-fertile and cytoplasmic male-sterile *N. tabacum*. In addition, a detailed histological and scanning electron microscopy (SEM) study concerning flower organ ontogeny in recovered somatic hybrid/cybrid plants is presented and discussed, in terms of their use for further analysis of floral development and cytoplasmic male sterility in *Nicotiana*.

Materials and methods

Plant material

Shoot cultures from *N. tabacum* L. cv Petit Havana SR1 (male fertile, full corolla, streptomycin-resistant) and *N. tabacum* cms (*N. bigelovii*) (cms: petaloidy, split corolla, streptomycin-sensitive) were used for leaf protoplast isolation (Maliga et al. 1973; Gerstel 1980).

Protoplast isolation, microfusion, and microculture of fusants until plant regeneration

Mesophyll protoplasts were isolated as described earlier (Shillito et al. 1983; Potrykus and Shillito 1986). Individually selected pairs of mesophyll protoplasts were transferred into microfusion chambers with the setup previously described (Schweiger et al. 1987). Micro-electrofusion was induced by single or multiple, negative dc-pulses (50 µs; 0.8–1.8 kV/cm) after mutual dielectrophoresis (1 MHz; 65–80 V/cm) for a few seconds.

The fusion products obtained were individually transferred into 60 nl microdroplets of modified Kao medium (macro-, micronutrients, vitamins, sugars, and organic acids from Kao and Michayluk 1975; 0.4 *M* glucose, 0.5 mg/l NAA, 1.0 mg/l BAP) in microculture chambers (Koop and Schweiger 1985a). Microcalli obtained were transferred for morphogenesis onto modified MS medium (Murashige and Skoog 1962) consisting of MS macro- and micronutrients, 2 m*M* $CaCl_2 \cdot 2H_2O$, 100 mg/l inositol, 0.1 mg/l thiamine, 0.1 mg/l pyridoxine, 1 mg/l Ca-panthotenate, 0.1 mg/l nicotinic acid, 30 g/l sucrose, 1 mg/l BAP, and 0.1 mg/l NAA solidified with 6 g/l low-melting-point agarose. Shoots derived from regenerating calli were rooted on MS macro-, micronutrients, vitamins, 10 g/l sucrose solidified with 9 g/l agar.

Streptomycin resistance/sensitivity test for leaf explants and seedlings

Leaf explants from sterile shoot cultures were incubated for 7–9 weeks at 21 °C 16/8 h photoperiod on modified RM medium (MS macro- and micronutrients, 100 mg/l inositol, 0.4 mg/l thiamine, 1 mg/l BAP, 30 g/l sucrose) solidified with 8 g/l agar and containing 1 mg/ml streptomycin (Maliga et al. 1975). Seeds were selected on MS macro-, MS half-strength micronutrients, 2 g/l sucrose, 6 g/l agar containing 0.5 mg/ml streptomycin, and were evaluated 2–3 weeks after germination.

DNA analysis

Total DNA was isolated from leaf material of shoot cultures according to Lichtenstein and Draper (1985). Mitochondrial DNA was isolated from leaves from pot-grown plants according to Hakansson et al. (1988). DNA was digested with various restriction endonucleases following the conditions recommended by the manufacturers and electrophoresed in 0.8% or 1% agarose gels.

Multiprime labelling of three mitochondrial DNA probes – pmtSylSa-1, pmtSylSa-2 and pmt SylSa-8 – as discrete SalI fragments from clones of *N. sylvestris* mtDNA (Aviv et al. 1984) was performed as described by Feinberg and Vogelstein (1983). Southern blot hybridizations and washing conditions of hybridized Hybond N-nylon filters (Amersham) were carried out as described by Maniatis et al. (1982) and according to the manufacturer's manual.

Chromosome counts and histological preparations

Chromosomes were counted in root tips from aseptically grown plants. Root tips were treated for 3 h with 0.2% alpha-chloronaphthalene and stained with 1% orcein in 45% acetic acid. For determination of the chromosome number of a given plant, at least ten, well-spread mitotic figures were examined. For histological preparations from floral organs, samples were treated for 24 h in FAA fixative, washed in 70% ethanol, dehydrated by a tert-butanol series, stained with eosin, and paraffin-embedded; 5-µm thick sections were prepared and stained with 1% safranin for 8–12 h, washed in an ethanol series, stained with 0.1% fast green, rinsed in 95% isopropanol, and embedded in Canada balsam.

Scanning electron microscopy

Floral buds and mature floral organs were fixed in 3.5% glutaraldehyde in 0.01 *M* cacodylate buffer, pH 7.2, overnight at 4 °C. After washing in cacodylate buffer and distilled water, the samples were either frozen in liquid propane and freeze-dried, or dehydrated in acetone series, transferred to pure acetone, and critical-point-dried. The dried samples were mounted on SEM stubs, microdissected, and sputter-coated with gold. Observations were made with a Jeol SM T220 Scanning Electron Microscope at 10–30 kV, and images were recorded on Kodak P-120 film.

Results

Microfusion and microculture

Defined pairs of mesophyll protoplasts from *N. tabacum* SR1 and *N. tabacum* cms (*N. bigelovii*) were individually

Table 1. Characteristics of somatic hybrid/cybrid plants regenerated from microfusion products of *N. tabacum* SR1 (+) *N. tabacum* cms (*N. bigelovii*)

Somatic hybrid/cybrid	Corolla type	Fertility/ sterility	Chromosome number	Chloroplast type		mtDNA pattern		
				(a)	(b)	pmtSylSa-1	pmtSylSa-2	pmtSylSa-8
SB 1-1	SR1	(+)	47±1.1	S	S, R, V	new	sb	new
SB 1-2	cms big	(−0)	48±1.0	R	R	new	sb	new
SB 1-3	SR1	(+)	48±1.4	R	R	new	sb	new
SB 1-4	SR1	(+)	48±1.0	R	R	new	sb	s
SB 1-5	SR1	(+)	48±1.6	S	S	new	new	s
SB 1-6	SR1	(+)	48±1.5	S	S	new	sb	s
SB 1-7	SR1	(+)	48±0.8	R	R	sb	sb	new
SB 1-9	SR1	(+)	47±1.7	S, R	R	new	sb	s
SB 1-10	SR1	(+)	48±1.5	R	R	new	sb	s
SB 2-1	SR1	(−)	96±5.2	S	S	new	sb	sb
SB 2-3	SR1	(−)	96±3.8	S	S	new	sb	new
SB 2-4	SR1	(−)	96±2.3	S	S	sb	sb	sb
SB 2-7	SR1	(−)	94±1.9	S	S	new	sb	new
SB 2-8	cms big	(−)	96±2.2	S	S	sb	sb	sb
SB 2-10	cms big	(−)	96±2.1	S	S	sb	sb	sb
SB 2-12	SR1	(−)	95±2.3	S, R	S	sb	sb	sb
SB 3-1	SR1	(−)	96±2.5	S	S	new	sb	new
SB 3-3	new	(−)	96±2.1	S	S	new	sb	sb
SB 3-4	SR1	(−)	96±1.4	S	S, R, V	new	b	s
SB 3-8	SR1	(−)	96±1.4	S	S	new	sb	new
SB 3-9	SR1	(−)	96±2.2	S	S	new	b	s
SB 4-2	SR1	(−)	96±2.2	S	S, R, V	new	b	sb
SB 4-4	cms big	0	96±4.0	R	R	new	b	new
SB 4-7	SR1	(+)	96±2.3	R	R	new	b	new
SB 4-9	SR1	(+)	95±1.2	R	R	new	b	sb
SB 4-10	SR1	(−)	96±2.7	R	R	new	b	new
SB 5-2	cms big	0	47±2.8	S	S	new	new	new
SB 5-3	SR1	(−)	48±2.4	S	S	new	sb	new
SB 5-4	cms big	0	47±1.7	S	S	new	sb	s
SB 5-6	cms big	0	48±2.0	S	S	new	new	new

Corolla type:	SR1	– full corolla from *N. tabacum* cv Petit Havana SR1
	cms big	– split corolla from *N. tabacum* cms (*N. bigelovii*)
Fertility/sterility:	0	– cms, no microspores, cms big-type, petaloid stamens
	(−0)	– cms, few nonfunctional microspores, partially restored, cms big-type, petaloid stamens
	(−)	– not self-fertile, no functional pollen, SR1-type anthers
	(+)	– self-fertile, functional pollen, SR1-type anthers
Chloroplast type:	(a)	– leaf explant test: S: sensitive; R: resistant
	(b)	– seedling test: S: sensitive; R: resistant; V: variegated
mtDNA pattern:	s	– SR1-type pattern
	b	– cms big-type pattern
	sb	– additive pattern (SR1+cms big)
	new	– novel pattern

selected with a micromanipulation setup into microfusion chambers and micro-electrofused. A total of 1,582 protoplast pairs from 18 independent protoplast isolation experiments were selected and microfused; 242 microfusants were obtained and transferred to microculture, representing an average microfusion frequency of 15.3% and reaching values of up to 42% in individual experiments.

Individually selected parental protoplasts from either *N. tabacum* SR1 or *N. tabacum* cms (*N. bigelovii*), as well as the recovered microfusants *N. tabacum* SR1 (+) *N. tabacum* cms (*N. bigelovii*), were cultured in microculture chambers. In all cases, first cell divisions were observed 4 days after transfer to microculture. Multiple divisions of the individually selected protoplasts or microfusants were obtained starting 2–4 days later at frequencies in the range of 17 to 20%.

After 3–4 weeks of microculture, microcalli of the microcultured fusants could be transferred onto solidified culture medium for plant regeneration. Microcalli were obtained at an average of 4.5% and up to 32% of the microcultured fusants in some experiments.

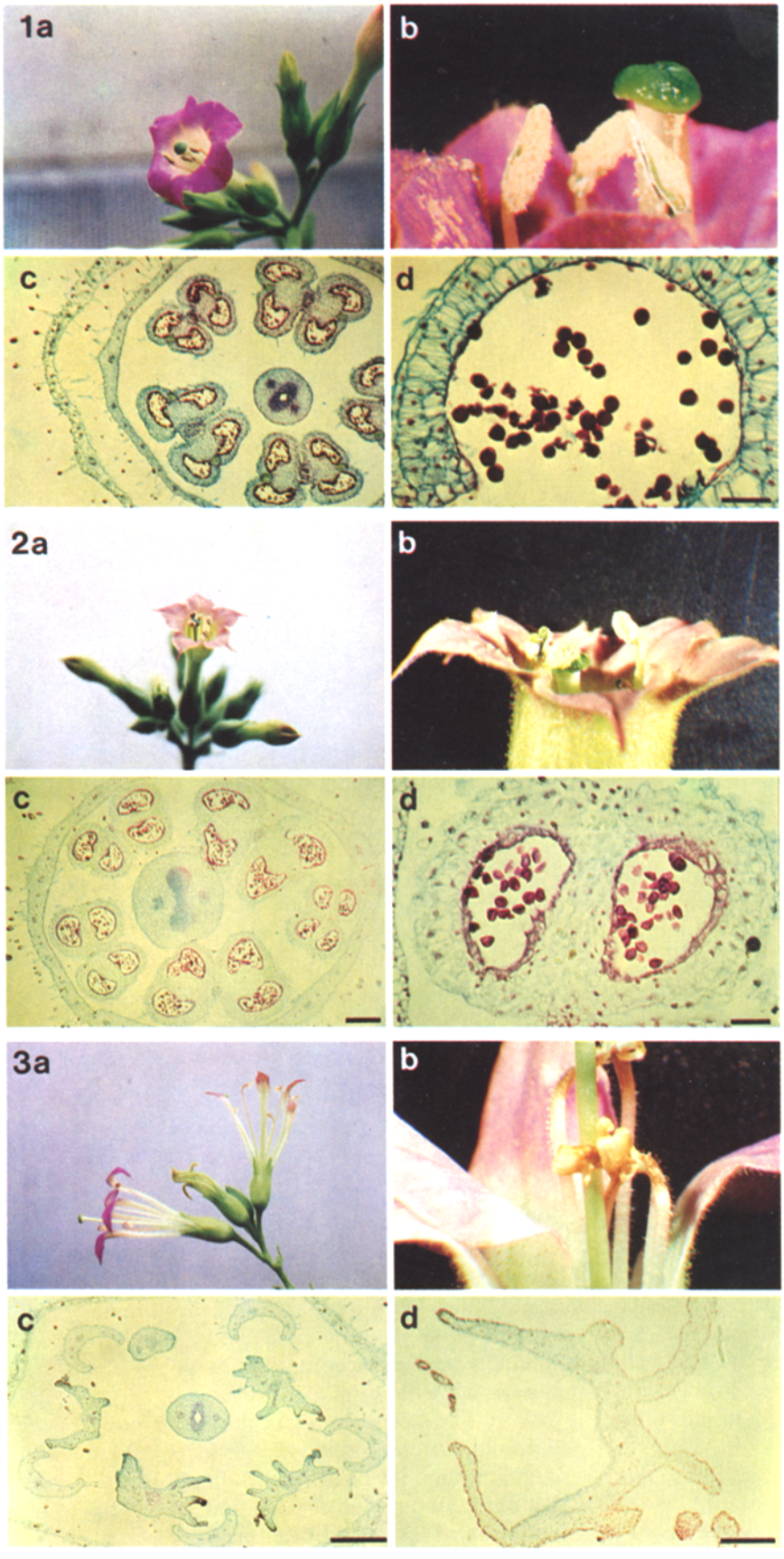

Fig. 1.1–1.3. Flower morphology and floral developmental histology of representative somatic hybrids/cybrids *N. tabacum* SR1 (+) *N. tabacum* cms (*N. bigelovii*). **1.1** Flower type class A – male-fertile SR1 type, e.g., somatic cybrid SB 1.10: *a* full, not split corolla; *b* detailed view from *a*, showing dehisced anthers and stigma; *c* histological section through floral bud, bar = 500 μm; *d* partial view of anther loculament containing normal microspores, bar = 100 μm. **1.2** Flower type class B – normal anther type with no functional pollen, e.g., somatic hybrid SB 4.9: *a* inflorescence showing flowers with normal SR1-type corolla; *b* detailed view from *a*, showing deshisced anthers and stigma; *c* histological section through floral bud, bar = 500 μm; *d* partial view of anther loculaments showing abnormal nonfunctional collapsed microspores, bar = 100 μm. **1.3** Flower type class C – cms big-type, e.g., somatic cybrid SB 5.4: *a* inflorescence showing cms big-type flowers with full split corolla; *b* detailed view from *a*, showing sterile, leaf-like structures at the tip of abnormal stamens; *c* histological section through floral bud, bar = 500 μm; *d* partial view of cms big-type antheroid in histological section, bar = 100 μm

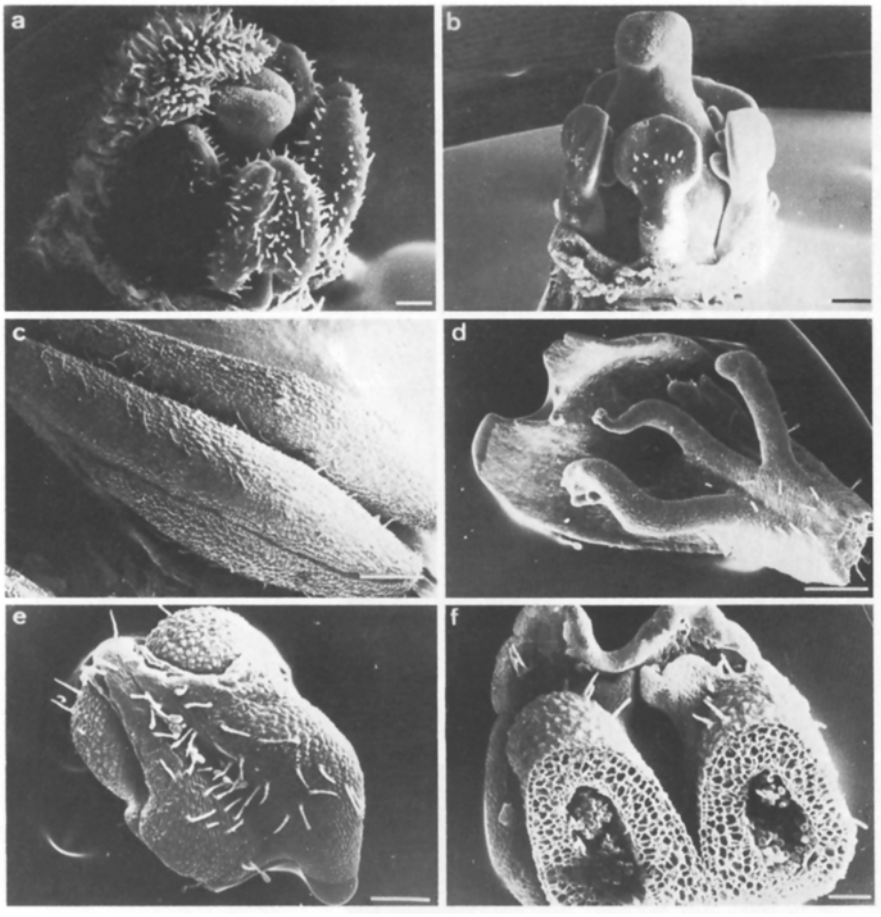

Fig. 2a–f. Scanning electron microscopy study of flower ontogeny of representative somatic hybrids/cybrids. **a** Oblique view of SR1-type flower bud from somatic cybrid SB2.1 at stage of style elongation, bar = 250 µm. **b** Side view of cms big-type flower bud from cybrid SB1.2 at stage of style elongation, bar = 250 µm. **c** Developed SR1-type anther from somatic hybrid SB2.12, bar = 500 µm. **d** cms big-type anther from cybrid SB5.4, bar = 250 µm. **e** Partially restored intermediate-type anther(oid) from cybrid SB1.2, bar = 250 µm. **f** Section through intermediate antheroid from **e**, showing loculaments containing developing microspores, bar = 250 µm

Morphological characteristics of flowers from regenerants

A total of 30 regenerated somatic hybrid or cybrid plants showing tetraploid (4n = 96) or diploid (2n = 48) chromosome numbers, respectively, and covering variation within and among independent microfusant colonies was grown to maturity for detailed morphological, genetic, and molecular characterization (Table 1). As control material, to check for the effects of tissue-culture-induced somaclonal variants of different ploidy level, independent regenerants from microcultured parental protoplasts or homofusants with diploid and tetraploid chromosome numbers of *N. tabacum* SR1 and *N. tabacum* cms (*N. bigelovii*) were included in the flower morphological comparison. All regenerants were scored for flower morphology, fertility/sterility, and anther(oid)-type. Selected regenerants were analyzed in detail for flower development by means of histological sections and SEM at different bud stages up to mature floral organs.

Four classes of anther(oid)-type and fertility/sterility phenotypes were detected among the somatic hybrid/cybrid plants analyzed: (A) male-fertile SR1-type (Fig. 1.1); (B) normal anther type with no functional pollen (Fig. 1.2); (C) cms big-type (Fig. 1.3); and (D) novel, partially restored type (Fig. 2e, f) (Table 1).

For the most part, the somatic hybrid/cybrid plants presented floral phenotypes corresponding to the parental forms (classes A and C), as could be confirmed by SEM studies of floral ontogeny (Fig. 2a–d). Anther types of class B appeared in somatic hybrids (with the tetraploid chromosome number 4n = 96). The same lack of pollen function was observed in tetraploid homofusants of SR1. This suggests that the increased ploidy level was responsible for this phenotype. Antheroid-type class D was shown only by somatic cybrid SB 1.2. This plant had a cms big-type, split corolla and partially restored antheroids, with rudimentary loculaments containing a few collapsed, nonfunctional microspores. Otherwise, floral bud development in cybrid SB 1.2 was completely normal (cms big-type), and stamen development ended with the formation of intermediate antheroids, thicker than the parental cms big-type, as confirmed by SEM studies (Fig. 2e, f).

All phenotypes described above had good penetrance. All flowers of the analyzed R_0 plants showed the

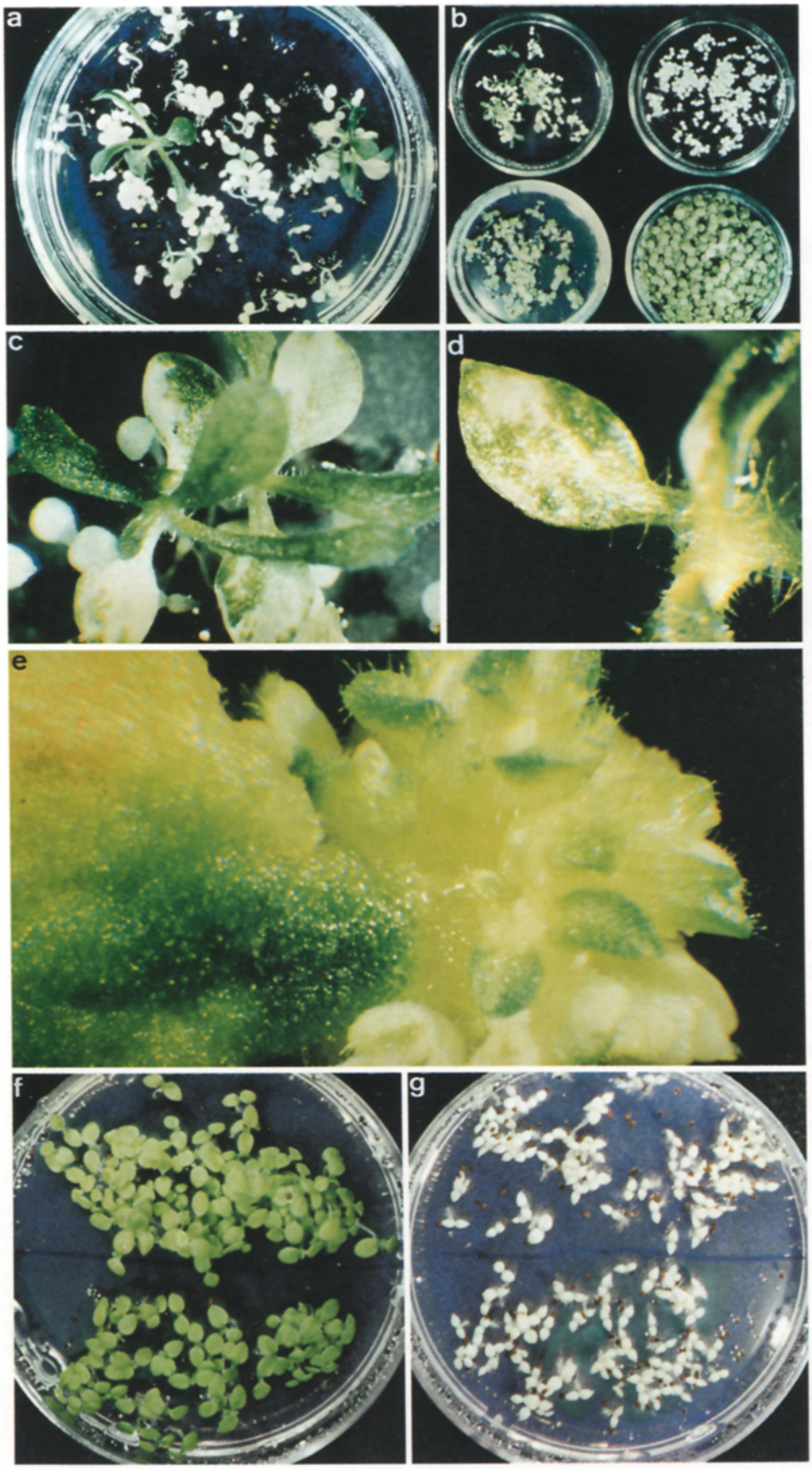

Fig. 3a–g. Streptomycin assay for assessing chloroplast type in somatic hybrid/cybrid primary regenerants and their offspring. **a** Segregation of streptomycin-resistant and -sensitive R_1 seedlings, offspring of cybrid SB1.1. **b** Segregation of streptomycin-sensitive and -insensitive R_1 seedlings after selfing SB1.1 (*upper row, left*); streptomycin-sensitive R_1 seedlings from the sexual cross SB1.1 × SR1 (*upper row, right*); controls without streptomycin, respectively (*lower row*). **c** Sectored R_1 seedlings from cybrid SB1.1 germinated in the presence of 0.5 mg/ml streptomycin, one rescued with green primary leaves. **d** Detailed view from sectored cotyledons from **c**. **e** Sectored regenerating leaf explant from somatic hybrid SB2.12 grown in the presence of 1 mg/ml streptomycin. **f** Streptomycin-resistant R_1 seedlings from somatic hybrid SB4.9. **g** Streptomycin-sensitive R_1 seedlings from somatic hybrid SB2.12

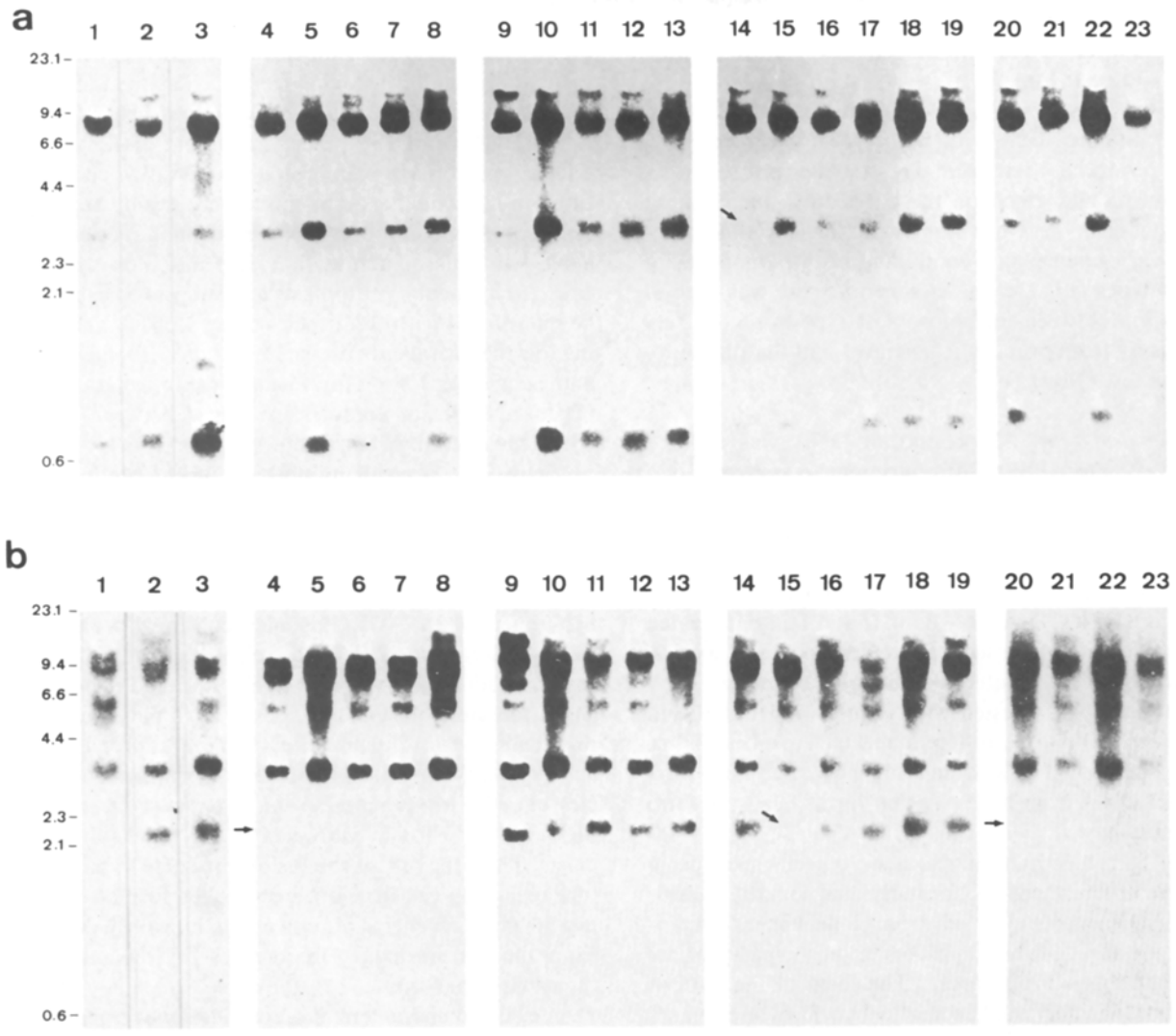

Fig. 4a and b. Mitochondrial genome analysis of the parental forms and independent regenerants from microfusion products. **a** Hybridization patterns of BamHI-digested total DNA from individual regenerants from microcultured fusion products with mtDNA probe pmtSylSa-1. *Lanes: 1* SR1; *2* cms big; *3* 1:1 mix SR1: cms big; *4* fusion product SB1.3; *5* SB1.4; *6* SB1.5; *7* SB1.6; *8* SB 1.7; *9* SB2.7; *10* SB2.8; *11* SB2.10; *12* SB2.11; *13* SB2.12; *14* SB4.3; *15* SB4.4; *16* SB4.5; *17* SB4.7; *18* SB4.8; *19* SB4.9; *20* SB5.2; *21* SB5.3; *22* SB5.4; and *23* SB5.6. **b** Hybridization patterns of BamHI-digested total DNA from individual regenerants from microcultured fusion products with mtDNA probe pmtSylSa-8. Same lane distribution as in **a**. *Arrows* indicate absence of parental-specific band. Four micrograms of total DNA has been digested and loaded on each lane

same morphology and these phenotypes were stably transferred to the R_1 offspring analyzed.

Cytoplasmic constitution of somatic hybrids/cybrids

Independent regenerants from individual colonies, derived from microfused protoplasts showing different flower morphologies, were investigated for the genetic constitution of cytoplasmic factors.

Chloroplast type. For the analysis of chloroplast type, the sensitivity to streptomycin was determined using leaf explants from primary (R_0) regenerants and R_1 seeds. Seedlings of *N. tabacum* SR1 showed resistance to the antibiotic, and green regenerants could be induced from leaf explants on 1 mg/ml streptomycin, whereas seedlings of *N. tabacum* cms (*N. bigelovii*) showed sensitivity and no green regenerants could be obtained from explants.

In most cases, either entirely streptomycin-resistant or entirely streptomycin-sensitive, primary, somatic hybrid/cybrid regenerants and R_1 offspring were found (Table 1, Fig. 3). In a few cases, segregational events among the progeny of the regenerated somatic hybrids/cybrids and chimeric structures within the progeny and in

somatic tissue of the primary regenerants were observed (Fig. 3f, g). Three such clones are SB 1.1, SB 3.4, and SB 4.2 (Table 1, Fig. 3a, b).

This R_1 segregation implies that the hybrid/cybrid clones contained cells with both types of chloroplasts. SB 1.1 produced a few seedlings showing sectored areas of chlorosis and green on the cotyledons, indicating a mosaic of cell types, thus supporting the view of streptomycin-variegated plants containing both parental chloroplast types (Fig. 3c, d). In general, there was a good correlation between the chloroplast type in the leaf explant assay from primary regenerants and that in the R_1 seedling test (Table 1).

Mitochondria type. Mitochondrial DNA was isolated from leaf material of the primary regenerants and used for mtDNA restriction pattern analysis. In addition, Southern hybridization of mtDNA and total DNA with DNA probes of *N. sylvestris* of mitochondrial origin (pmtSylSa-1, pmtSylSa-2, pmtSylSa-8) was carried out. Digestions of mtDNA with BamHI and HindIII yielded different restriction patterns for the two parental species.

Species-specific patterns could also be obtained by using total DNA digested with BamHI or HindIII and hybridized with three different mtDNA probes. These species-specific RFLPs could still be detected after mixing total DNA from both parental forms in a 1:50 ratio (data not shown).

Southern blot hybridizations were performed, using the three different probes, for individual somatic hybrid/cybrid regenerants derived from independent microfusion products (Table 1). Either additive, parental, or novel patterns were observed. The form of the pattern obtained for individual, somatic hybrid/cybrid plants depended on the probe used (Fig. 4). The novel patterns took the form of either the appearance of new nonparental bands or the disappearance of parental-specific bands (Table 1, Fig. 4). These novel patterns were not detected when individually regenerated plants derived from microcultured, parental protoplasts were analyzed (data not shown).

The presence of additive, parental-like, or novel patterns among the somatic hybrids/cybrids could also be seen in restriction digest patterns of purified mtDNA. Novel patterns involved qualitative and quantitative changes in restriction fragments.

Discussion

The present study demonstrates that electrofusion of individually selected pairs of protoplasts (*microfusion*) can be used for controlled somatic hybridization of higher plants. Microfusion allows for a perfect control of the type and number of cells participating in the fusion process; it therefore does not require any selective method for fusion product enrichment, provided it is combined with a tissue culture cloning step, such as the individual cell culture technique (*microculture*) used here.

An average of 15% and a maximum of 42% of the selected protoplast pairs could successfully be microfused in individual experiments. These results are in the range of previous reports for other plant species using analogous micromanipulation systems. For example, *Brassica* hypocotyl protoplasts and subprotoplasts could be microfused with frequencies of up to 60% and varying, on the average, between 15 and 45% (Spangenberg and Schweiger 1986); for *Funaria* protonemata protoplasts, microfusion frequencies ranged between 11 and 25% (Mejia et al. 1988).

Since only a small number of fusion products per experiment can be achieved by microfusion, an efficient culture system for small numbers of cells is required. One particularly attractive method is to combine the microfusion with an individual cell culture technique (Koop and Schweiger 1985a). With this microculture approach, the individual culture of defined, selected single cells (e.g., fusion products) in volumes in the nl-range is possible. Thus, the same optimal cell density as in mass culture is provided and a similar callus formation frequency is achievable. We obtained microcallus formation frequencies of 17% for parental *N. tabacum* cv Petit Havana SR1 and 3.5% for *N. tabacum* cms (*N. bigelovii*) protoplasts. The behavior of the fusion products in microculture regarding cell division, microcallus formation and, finally, plant regeneration was not significantly different from the corresponding frequencies for the microcultured parental forms.

A detailed characterization of individual regenerants from independent microfusion products regarding chromosome number, chloroplast type, mtDNA restriction and hybridization patterns, morphological, histological, and scanning electron microscopy studies of flower development was performed. Chromosome counts from a random selection of regenerants from microfusion showed mainly either the normal allotetraploid *N. tabacum* chromosome count of 48 or the expected additive of 96 for a somatic hybrid. Hence, some of the plants analyzed in this paper could be initially identified as cybrids and others as somatic hybrids. In all cases a genomic segregation, probably due to nonfusion of nuclei upon microfusion, rather than a chromosomal segregation was observed, as no proven intermediate chromosome counts were detected.

Thus, two-fifths of the regenerated lines were found to be nuclear segregants (cybrids) of tobacco and the rest could be identified as nuclear hybrids. Similar observations have been reported after protoplast mass fusion followed by microisolation of fusion products in interspecific *Nicotiana* combinations, where three out of eight

evaluated clones obtained were nuclear hybrids, the rest being nuclear segregants (cybrids) (Gleba and Sytnik 1984).

These results give independent evidence for the occurrence of nonfusion and segregation of nuclei in fusion products after the use of nonselective methods for hybrid screening (Gleba et al. 1985), e.g., microisolation or microfusion and microculture, and differ from the exclusive recovery of nuclear fusion products consistent with the use of selective conditions (e.g., use of universal hybridizers, hormone autotrophy).

The identification of the chloroplast type in different regenerants from fusion products was based on the streptomycin resistance locus – a mutation in chloroplast genome that has been reported to code for a chloroplast ribosomal protein – of *N. tabacum* cv Petit Havana SR1 (Maliga et al. 1973, 1975; Yurina et al. 1978). This streptomycin test – on leaf explants and R_1 seedlings – provided clear information on chloroplast composition of the recovered somatic hybrid/cybrid plants.

The fact that a mixed chloroplast type population can exist in somatic hybrid/cybrid plants regenerated from microfusion products is shown by the presence of chimaeric (streptomycin-sensitive and -insensitive) callus in the leaf explant assay for regenerants SB 1.9 and SB 2.13. In addition, segregation of chloroplasts may not be completed in the R_0 generation, as could be observed in the streptomycin seedling test for regenerant SB 1.1.

It is thus apparent that segregation to the organelle type of either fusion parent can occur in clones regenerated from a single microfused cell or that both fusion parent chloroplast types can persist in a regenerated plant, even through meiosis.

The segregation of chloroplasts in R_1 seedlings can most probably be explained by a maternal meiotic product containing both plastid types in the same cell, as plastids are known to be mostly strictly maternally inherited in *Nicotiana* (Flick and Evans 1982). A rare transfer of paternal chloroplasts in *Nicotiana* during sexual cybridization as shown by Medgyesy et al. (1986) seems to be improbable in the case of the cybrid SB 1.1 because of the following evidence: (1) all tested R_1 seedlings from the sexual cross with *N. tabacum* SR1 were streptomycin sensitive; (2) only in seedlings derived from one capsule after selfing SB 1.1 were segregational events observed; and (3) very high segregation (18 seedlings out of 187 tested, equivalent to 9.6%) showed this phenotype, compared to corresponding values between 0.07 and 2.5% for intra- and interspecific crosses from the data of Medgyesy et al. (1986), and these low frequencies were obtained exclusively after application of high selection pressure in tissue culture phase while inducing callus from the seedlings on streptomycin-containing medium (Medgyesy et al. 1986). As the mixed sensitivity to streptomycin was primarily observed in the cotyledons in the seedling assay, it it not surprising that some of the R_1 plants rescued from the test did not contain both parental chloroplast types in the mature plant, as was observed for a few cybrid SB 1.1 R_1 offspring. This may be due to the independent origin of the cotyledons and shoot apex in tobacco embryogenesis, and in these cases chloroplast segregation might have been completed in those cells, giving rise to the shoot apex.

All the results obtained after microfusion of defined protoplasts fully agree with published observations concerning chloroplast segregation and plastome heterozygosity after somatic hybridization in *Nicotiana* via protoplast mass fusion followed by different hybrid screening methods or microisolation of fusion products (Gleba et al. 1984, 1985; Flick et al. 1985).

Restriction analysis and Southern blot hybridizations indicated that the mitochondrial genomes of either male-sterile or male-fertile somatic hybrid/cybrid plants regenerated from microfusion products in intraspecific *Nicotiana* combination were modified from the parental ones, as has been described in many reports after *Nicotiana* somatic hybridization mediated by mass fusion (Aviv et al. 1984; Aviv and Galun 1987; Belliard et al. 1979; Kumashiro et al. 1988, 1989; Nagy et al. 1983).

This modified mitochondrial genome organization in somatic hybrids/cybrids involved qualitative as well as quantitative differences in mtDNA restriction patterns among independent regenerants derived from the same or different microfusion products. In addition, in most cases the mtDNA pattern from male-fertile microfusion regenerants was similar to the male-fertile parental form *N. tabacum* SR1, and that from the male-sterile somatic hybrids/cybrids was similar the the cms parental form *N. tabacum* cms (*N. bigelovii*). These observations for microfusion products are in agreement with similar results reported after donor-recipient protoplast mass fusion for analogous *Nicotiana* cybrid combinations (Galun et al. 1982).

Southern blot hybridizations of mtDNA from microfusion-derived somatic hybrids/cybrids with different hybridization probes showed, with one probe, a pattern similar to one parental form while, with another hybridization probe, a pattern similar to the other parental line became evident. Similar analysis with mtDNA from shoot cultures of the parental forms used in the present study or from plants regenerated from microcultured protoplasts of the parental forms did not reveal novel RFLP patterns, while new bands appeared or parental ones disappeared in the mtDNA restriction analysis of microfusion products.

Regarding putative correlations between floral phenotypes observed in somatic hybrids/cybrids obtained by protoplast microfusion and the characterization of their mitochondrial and chloroplast composition, it can be stated that (a) male fertility could be restored in alloplas-

mic cms tobacco plants by (micro)fusion of their protoplasts with protoplasts from a male-fertile line, and (b) a segregation of chloroplast type independent from the male fertility/sterility phenotype took place. Both results have been previously observed while analyzing somatic hybrids/cybrids obtained by protoplast mass fusion in tobacco (Aviv and Galun 1980).

Herewith, no simple direct correlation of floral phenotypes (cms or male fertile) with a particular mtDNA arrangement could be found. Similar results have been reported for the comparison of floral phenotypes and mtDNA restriction patterns for different alloplasmic cms tobacco lines with cytoplasms of *N. suaveolens*, *N. repanda*, and *N. debneyi* (Hakansson et al. 1988). In addition, as mitochondrial genome recombination (Nagy et al. 1983) may alter the segregation of cms, cms segregation is therefore not indicative of mitochondrial segregation and, hence, there is no basis for predicting segregation of the cms trait (Flick et al. 1985).

Thus, for an understanding of the molecular basis of the developmental abnormalities of cms homeotic flower mutants in *N. tabacum*, additional experimental approaches are necessary, e.g., the analysis of mitochondrial transcripts and in organello translation products of male-fertile, cms, and restored forms, for somatic hybrids/cybrids with the corresponding floral phenotypes, or for fertile, restored cybrids after complementation via protoplast fusion of two cms types (Aviv and Galun 1986; Kofer et al. 1990). For these purposes, the production of defined strains via microfusion of protoplast + protoplast or protoplast + cytoplast pairwise combinations is an attractive alternative.

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