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Organelle transfer by microfusion of defined protoplast-cytoplast pairs

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Summary. Defined cybridization was performed by one-to-one electrofusion (microfusion) of preselected protoplast-cytoplast pairs of male-fertile, streptomycin-resistant *Nicotiana tabacum* and cytoplasmic male-sterile, streptomycin-sensitive *N. tabacum* cms (*N. bigelovii*), followed by microculture of the fusion products until plant regeneration. Dominant selectable markers, namely, kanamycin resistance (*nptII*) and hygromycin B resistance (*hpt*) genes had been previously integrated in the nuclear genomes of the otherwise almost fully isogenic parental strains using direct gene transfer to protoplasts. In addition to chromosome counts indicating the expected allotetraploid tobacco count of 48, the absence of the nucleus from the cytoplast donor line was confirmed by Southern blot hybridization using *nptII* and *hpt* probes, as well as by an in vitro selection test with leaf explants and the corresponding enzyme assays for 30 cybrids. The cytoplasmic composition of the cybrids obtained was analyzed for chloroplast type using the streptomycin resistance/sensitivity locus. The fate of mitochondria in cybrids was checked by species-specific patterns in Southern analysis of restriction endonuclease digests of total DNA with *N. sylvestris* mitochondrial DNA probes.

Key words: One-to-one electrofusion – Cybridization – Cytoplast (enucleated protoplast) – Cytoplasmic male sterility – *Nicotiana*

Introduction

It has been shown that the use of protoplast fusion for transfer of alien cytoplasms is an important way to increase the genetic diversity of extranuclear genomes in plants, mainly because of its potential to allow for biparental inheritance of cytoplasmic genomes. As maternal inheritance of organelles predominates among angiosperms during sexual reproduction, with only a few species exhibiting biparental inheritance (Sears 1980), studies on organelle genetics via somatic hybridization are novel, since protoplast fusion combines cytoplasmic organelles from both parents (Gleba and Sytnik 1984). The development of the one-to-one electrofusion technique provided a new possibility for the predictable transfer of partial genomes by using subprotoplasts (cytoplasts and karyoplasts), as it is so far the only method available which can a priori guarantee that the desired subcellular compartments are involved in the fusion event.

This is not the case for example, in experiments dealing with organelle transfer by mass fusion of protoplast-cytoplast populations because: (a) none of the protocols so far available for subprotoplast isolation yield pure subprotoplast preparations, and (b) protoplasts commonly contaminating cytoplast preparations are known to be more stable and efficient in the fusion process than the cytoplasts, and therefore preferentially participate in the fusion events. Keeping this in mind, attempts in the past to transfer organelles by protoplast-cytoplast fusions performed at the population level might be interpreted mainly as normal protoplast+protoplast fusions, followed by nonfusion of nuclei and nuclear segregation of one fusion partner rather than true transfer of partial genomes (Maliga et al. 1982; Gleba and Sytnik 1984).

The individual production and microculture of fusants arising from a single cytoplast and a protoplast, a karyoplast and a protoplast, or a karyoplast and a cytoplast (electrofusion-mediated cell reconstitution), by using the one-to-one microfusion approach (Koop and Schweiger 1985a; Koop and Spangenberg 1989) based on

a micromanipulation setup (Schweiger et al. 1987), were first reported for *Brassica napus* (Spangenberg and Schweiger 1986). However, no experimental proof for the predictable transfer of partial genomes, e.g., chondriome and plastome, and neither genetic nor molecular characterization of the fate of organellar and nuclear genomes upon protoplast-cytoplast microfusion and cybrid plant regeneration have yet been provided. Here we report on the controlled transfer of alien cytoplasms by defined protoplast-cytoplast microfusion in an interspecific *Nicotiana* combination, and provide confirmative evidence for the defined cybridization by genetic and molecular characterization of independent, regenerated cybrid plants.

Materials and methods

Plant material

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

Plasmid constructs

Plasmid pGL2 was obtained by cloning the *hpt* gene as a BamHI fragment derived from pGL88 (Blochinger and Diggelmann 1984) into the BamHI site of plasmid pDH51 (Pietrzak et al. 1986), as described by Datta et al. (1990). Plasmid pHP23 was obtained by cloning the *nptII* gene as an EcoRV fragment derived from plasmid pABDI (Paszkowski et al. 1984) into the SmaI site of plasmid pDH51, as described by Paszkowski et al. (1988).

Production of kanamycin-resistant and hygromycin B-resistant tobacco strains

Kanamycin-resistant strains of *N. tabacum* cv Petit Havana SR1 and hygromycin B-resistant strains of *N. tabacum* cms (*N. bigelovii*) were obtained by direct gene transfer with plasmids pHP23 and pGL2, respectively, to protoplasts using the polyethylene glycol chemical method (Negrutiu et al. 1987) and selection with 50 mg/l kanamycin and 25 mg/l hygromycin B, respectively, in a bead-type culture technique (Shillito et al. 1983).

A representative kanamycin-resistant (Km^r) transgenic plant of *N. tabacum* SR1 ($SR1^{Km}$) and a hygromycin B-resistant plant of *N. tabacum* cms (*N. bigelovii*) (cms big^{Hm}) were characterized for the integration of the full-length gene into the genome and for its expression at the molecular level, as well as for the stable transmission of the foreign gene as a single-locus in a Mendelian manner, as described by Potrykus et al. (1985).

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

Mesophyll protoplasts from tobacco strains $SR1^{Km}$ and cms big^{Hm} were isolated as described earlier (Shillito et al. 1983; Potrykus and Shillito 1986). An enriched cytoplast (enucleated protoplast) fraction was recovered after step gradient centrifugation of protoplasts, as previously reported (Spangenberg et al. 1986). Individually selected protoplast-cytoplast pairs were transferred into 0.5-µl microdroplets of 0.4 *M* mannitol in microfusion chambers, using the experimental setup described by Schweiger et al. (1987). Microelectrofusion was induced by single or multiple dc-pulses (50 µs; 0.8–1.8 kV cm^{-1}) after mutual dielectrophoresis (1 MHz; 65–80 V cm^{-1}) for a few seconds. The defined fusion products obtained were transferred to microculture chambers (Koop and Schweiger 1985 b) under the experimental conditions reported elsewhere (Spangenberg et al. 1990). Microcallus formation and plant regeneration from microfusion products were achieved as previously described (Spangenberg et al. 1990).

Fluorochrome nuclear staining of selected protoplasts/cytoplasts

The presence or absence of nuclei in freshly isolated protoplasts and cytoplasts was assessed by staining with the Hoechst 33342 dye. Freshly isolated protoplasts were incubated for 30 min in culture medium PNT (Spangenberg et al. 1990) containing 0.01% (w/v) bis-benzimide Hoechst 33342 (Sigma). After selection of individual protoplasts and cytoplasts according to morphological criteria using a micromanipulation setup, the presence or absence of nucleus was checked under Nomarski optics with a Zeiss IM35 microscope. In addition, selected cells were analyzed under UV light in a Zeiss standard microscope with the appropriate filter combination for bis-benzimide fluorescence (filter no. 30/G365 LP430).

DNA analysis

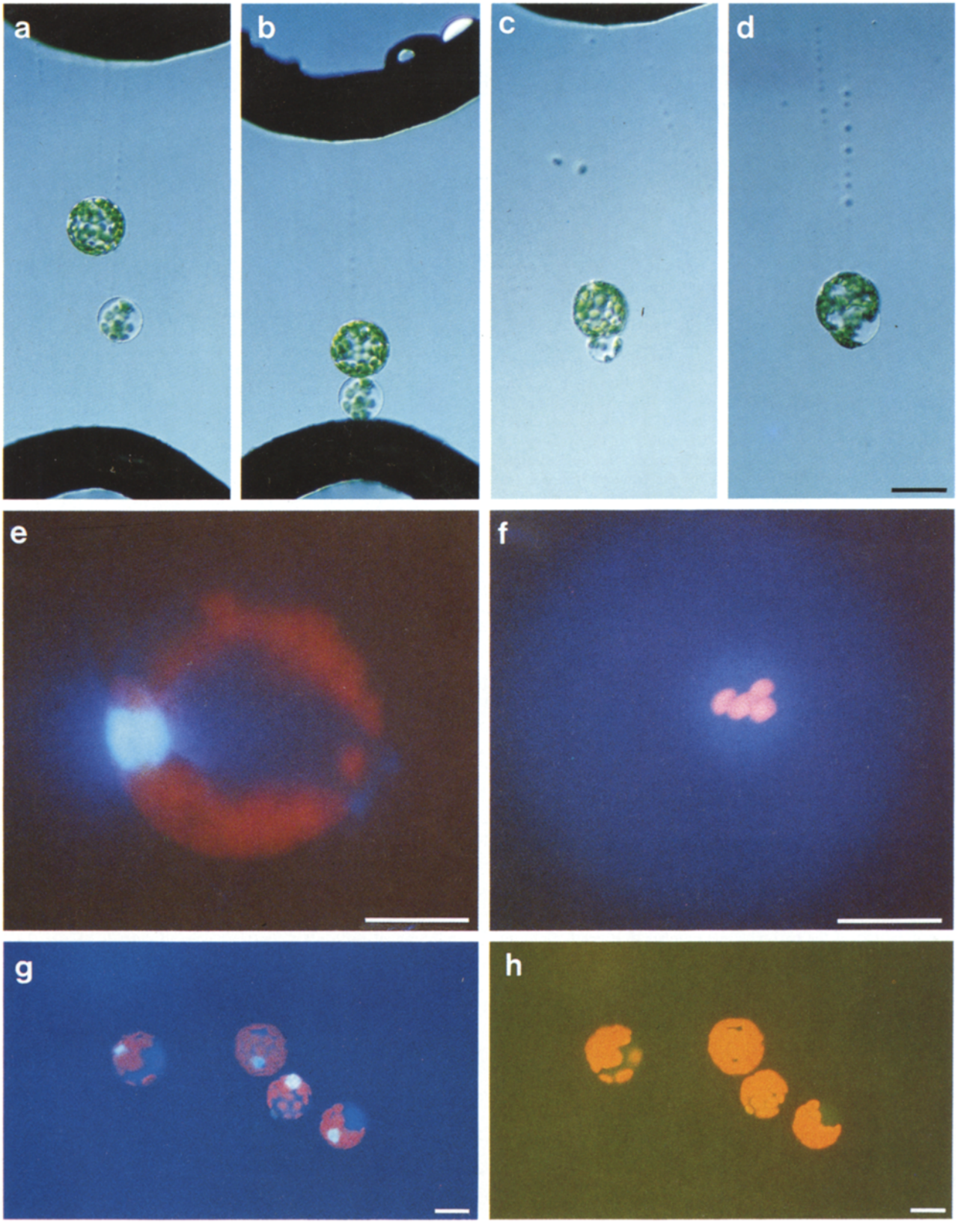
Total DNA was isolated from leaf material of shoot cultures according to Lichtenstein and Draper (1985). DNA was digested with various restriction endonucleases following the conditions recommended by the manufacturers and electrophoresed in 0.8% agarose gels.

Multiprime labelling of three mitochondrial DNA probes – pmtSylSa-1, pmtSylSa-2, and pmtSylSa-8 – as discrete SalI fragments from clones of *N. sylvestris* mtDNA (Aviv et al. 1984) and of the *nptII* gene probe as a HindIII fragment from plasmid pHP23 (Paszkowski et al. 1988), and of the *hpt* gene probe as a BamHI fragment from plasmid pGL2 (J. Paszkowski, personal communication), was performed as described by Feinberg and Vogelstein (1983). Southern blotting to Hybond N nylon filters (Amersham), hybridization, and washing conditions were carried out as described by Maniatis et al. (1982) and according to the manufacturer's manual.

Neomycin phosphotransferase II activity dot assay

The dot assay for neomycin phosphotransferase II activity detection in plant extracts using 0.1–0.2 g (fresh weight) leaf ma-

Fig. 1 a–h. Microfusion of selected protoplast-cytoplast pair. **a–d** Protoplast-cytoplast microfusion of a selected mesophyll protoplast of *Nicotiana tabacum* cv Petit Havana SR1 ($SR1^{Km}$) and a cytoplast from *N. tabacum* cms (*N. bigelovii*) (cms big^{Hm}); bars: 50 µm. **e–f** Fluorescence micrograph from a selected protoplast stained with Hoechst 33342 dye showing red autofluorescence from chloroplasts and bright-blue fluorescence from stained nucleus (**e**), and corresponding image for a selected cytoplast containing four chloroplasts (**f**); bars: 25 µm. **g** Four selected protoplasts visualized under fluorescence microscopy using filter combination as **e** and **f** bar: 50 µm. **h** Same image as **g** using filter combination, only allowing for visualization of red chloroplast autofluorescence; bar: 50 µm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terial from sterile shoot cultures was performed according to McDonnel et al. (1987). Plant extracts were adjusted to the same protein content after protein quantification following Bradford (1976). Representative kanamycin-resistant lines were tested in addition for neomycin phosphotransferase II activity following the enzymatic in situ assay, essentially according to Reiss et al. (1984) and Schreier et al. (1985).

Assay for detection of hygromycin phosphotransferase activity

Hygromycin phosphotransferase activity was detected in extracts from 0.1 to 0.2 g leaf material of sterile shoot cultures following Cabanes-Bastos et al. (1989) modified, according to personal communication, by R. D. Shillito (CIBA Geigy, Research Triangle Park/NC, USA). Leaves from sterile shoot cultures were frozen in liquid nitrogen and ground with mortar and pestle in extraction buffer (0.05 *M* TRIS-HCl, pH 7.0; 10% glycerol, 0.1 m*M* phenylmethyl sulphonyl fluoride) (100–200 mg tissue/100 µl), in the presence of acid-washed sea sand at 4 °C. The samples were then centrifuged at 14,000 rpm for 5 min at 4 °C and the supernatant was used for the reaction. The enzyme reactions were carried out in 10-µl vol. containing 50 m*M* TRIS-maleate, pH 7.0, 50 m*M* $CaCl_2$, 0.05 m*M* ATP, 0.4 µl γ^{32}P ATP (10 mCi/ml; 3,000 Ci/mmol), 62 µg hygromycin B, and 5.6 µl crude extract. Reactions were carried out with and without hygromycin B. Incubation of the reaction mix was for 30–45 min at 37 °C. Aliquots (0.8 µl) from the reaction mixtures were applied to a PEI cellulose F TLC plate (Merck), which was developed in 50 m*M* sodium formate/formic acid, pH 5.4. The plates were air-dried prior to autoradiography.

Kanamycin, hygromycin B, and streptomycin resistance/sensitivity test for leaf explants and seedlings

Leaf explants from sterile shoot cultures were incubated for 7–8 weeks, 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 50 mg/l kanamycin, 25 mg/l hygromycin B, 1 mg/ml streptomycin, or no antibiotics. Seeds were selected on MS macronutrients, MS half-strength micronutrients, 2 g/l sucrose, 6 g/l agar containing 200 mg/l kanamycin, 100 mg/l hygromycin B, or 0.5 mg/ml streptomycin; they were then evaluated 3–4 weeks after germination.

Chromosome counts

Chromosomes were counted in root tips from aseptically grown plants. Root tips were treated for 3 h with 0.2% α-chloronaphthalene and stained with 1% orcein in 45% acetic acid. For determination of the chromosome number of a given plant, at least three well-spread mitotic figures were examined.

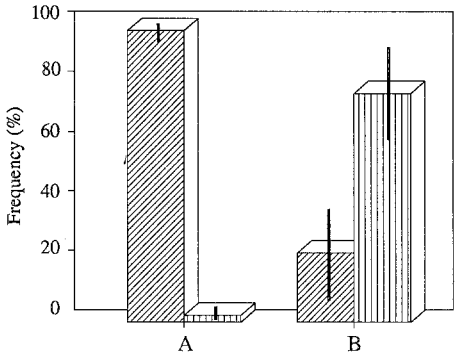

Fig. 2. Reliability of manual selection for protoplasts and cytoplasts. Frequency distribution of protoplasts (*A*) and cytoplasts (*B*) selected with micromanipulation setup using Normarski optics, checked for presence or absence of nuclei after staining with fluorescent dye Hoechst 33342. Protoplast showing light-blue fluorescence (nucleus) and red autofluorescence (chloroplasts) ▨; cytoplasts without light-blue fluorescence (enucleated) and showing red autofluorescence of chloroplasts ▥. Columns with bars representing mean value ±SD from three independent experiments, including 100 selected cells each

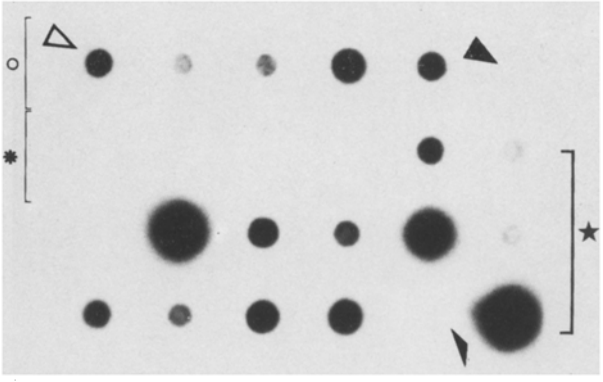

Fig. 3. Dot assay for neomycin phosphotransferase II activity from parental form $SR1^{Km}$ (△), four independent regenerated plants derived from microcultured protoplasts of $SR1^{Km}$: SK 1, SK 2, SK 3, and SK 4 (○); parental from cms big^{Hm} (▲), four independent regenerated plants derived from microcultured protoplasts of cms big^{Hm}: BH 1, BH 2, BH 3, and BH 4 (✱); independent protoplast-cytoplast derived cybrids SKBH (★); control double transformant (Hm^r, Km^r) (◗). Hm^r: hygromycin resistant; Km^r: kanamycin resistant

Results and discussion

Protoplast-cytoplast microfusion and microculture of fusants

Defined pairs of protoplasts from a kanamycin-resistant strain of *N. tabacum* SR1 ($SR1^{Km}$) and cytoplasts from a hygromycin-resistant strain of *N. tabacum* cms (*N. bigelovii*) (cms big^{Hm}) were individually selected with a micromanipulation setup into microfusion chambers and microelectrofused (Fig. 1 a–d).

In order to assess the reliability of the manual selection for protoplasts and cytoplasts while using Nomarski optics, individually selected protoplasts and cytoplasts were stained with the fluorescent dye Hoechst 33342 and checked for the presence or absence of nucleus (blue fluorescence of stained nuclei and red autofluorescence of cytoplasts, Fig. 1 e–f) by fluorescence microscopy. Over 96% of the selected protoplasts were indeed protoplasts, while on the average 73% and up to 84% of the selected cytoplasts did not show nuclear blue fluorescence, but still showed chloroplast red autofluorescence (Fig. 1 e–f, Fig. 2). A total of 978 protoplast-cytoplast pairs from 25 independent experiments was selected and

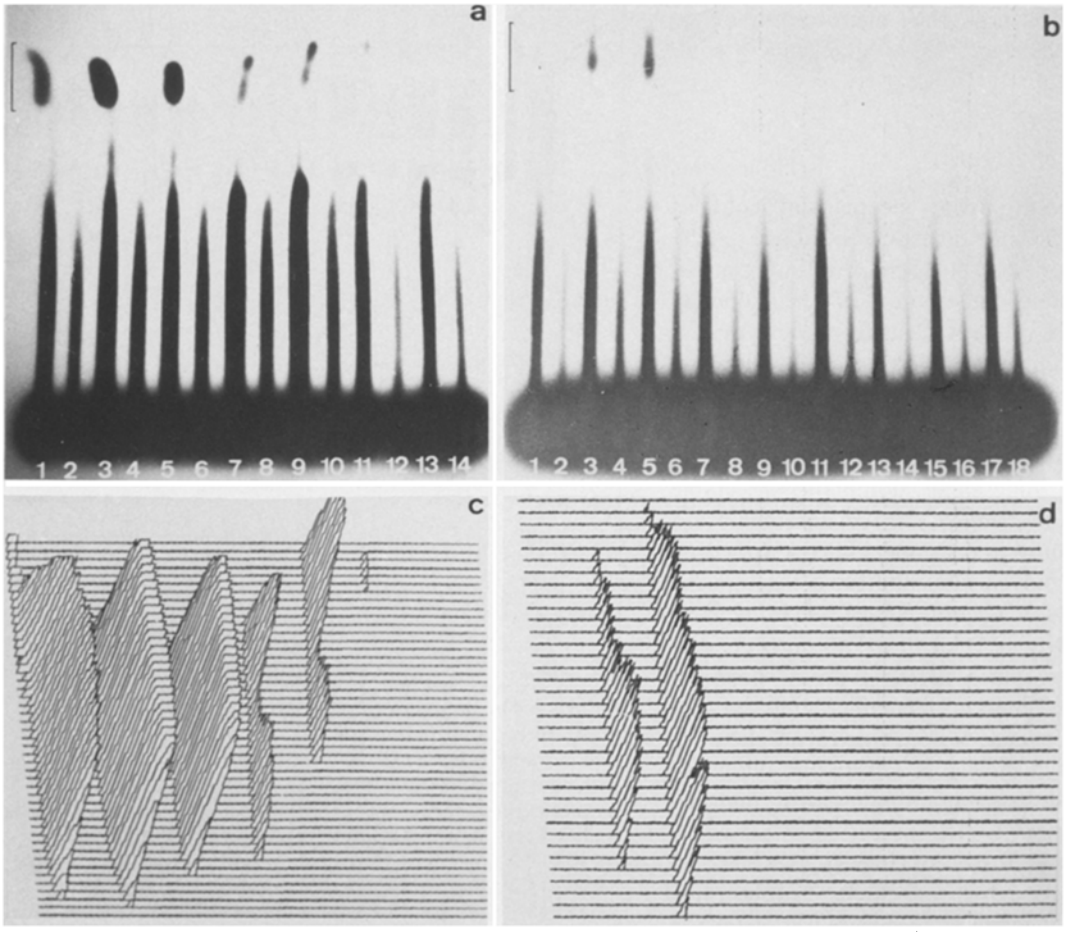

Fig. 4a–d. Enzyme assay for hygromycin B phosphotransferase activity. **a** Assay for Hm^r parental form *N. tabacum* cms (*N. bigelovii*) (cms big^{Hm}) (*lanes 1* and *2*), and from four regenerants derived from microcultured cms big^{Hm} parental protoplasts BH 1 (*lanes 3* and *4*), BH 2 (*lanes 5* and *6*), BH 3 (*lanes 7* and *8*), and BH 4 (*lanes 9* and *10*); Km^r Hm^r control double transformed *N. tabacum* cv Petit Havana SR1 ($SR1^{KmHm}$) (*lanes 11* and *12*) and Km^r parental form *N. tabacum* cv Petit Havana $SR1^{Km}$ (hygromycin sensitive) (*lanes 13* and *14*). *Lanes 1, 3, 5, 7, 9, 11,* and *13:* assay performed with hygromycin B; *lanes 2, 4, 6, 8, 10, 12,* and *14:* assay performed without hygromycin B. **b** Hygromycin B phosphotransferase activity assay for protoplast-cytoplast, microfusant-derived cybrids: control Km^rHm^S parental form $SR1^{Km}$ (*lanes 1* and *2*); control Km^sHm^r parental form cms big^{Hm} (*lanes 3* and *4*); microfusant-derived cybrid Km^sHm^r SKBH 4.2 (*lanes 5* and *6*); protoplast-cytoplast, microfusant-derived Km^rHm^s cybrid SKBH 7.1 (*lanes 7* and *8*); cybrid SKBH 10.2 (*lanes 9* and *10*); cybrid SKBH 11.3 (*lanes 11* and *12*); cybrid SKBH 13.3 (*lanes 13* and *14*); cybrid SKBH 14.2 (*lanes 15* and *16*); and cybrid SKBH 19.2 (*lanes 17* and *18*). *Lanes 1, 3, 5, 7, 11, 13, 15,* and *17:* assay performed with hygromycin B; *lanes 2, 4, 6, 8, 10, 12, 14, 16,* and *18:* assay performed without hygromycin B. **c** Scanning quantification of autoradiograph marked with [on **a**, showing peaks of phosphorylated hygromycin B corresponding to *hpt* enzyme activities detected in sample from *lanes, 1, 3, 5, 7,* and *9* from Hm^r cms big^{Hm} lines; corresponding activity from Km^rHm^r double transformant $SR1^{KmHm}$ (**a**, *lane 11*) over background from negative control $SR1^{Km}$ (**a**, *lane 13*). **d** Scanning quantification of autoradiograph marked with [on **b**. Detectable levels of activity corresponding to *lanes 3* and *5*; no detectable activity over background for *lanes 1* and *7–18*. Hm^r: hygromycin resistant; Hm^s: hygromycin sensitive; Km^r: kanamycin resistant; Km^s: kanamycin sensitive

microfused. From these, 214 microfusants were obtained and transferred to microculture, representing an average microfusion frequency of 22% and reaching values of up to 80% in individual experiments. Thus, these results are in the range of previous studies for *Brassica napus* protoplast-subprotoplast microfusion, where fusion yields of up to 60% and routinely in the range of 15–45% were reported (Spangenberg and Schweiger 1986). For tobacco mesophyll protoplasts, microfusion frequencies between 20 and 50% have been indicated (Koop et al. 1983), reaching values of up to 90% in individual experiments (Koop and Schweiger 1985b). In the present study, after 3–4 weeks of microculture, microcalli derived from, on average, 14% (and up to 60% in individual experiments) of the microcultured protoplast-cytoplast microfusants could be transferred onto solidified culture medium for further growth and, finally, plant regeneration. Similar behavior in microculture has been

observed for protoplast-protoplast microfusants of tobacco (Koop and Schweiger 1985a; Spangenberg et al. 1990).

Nuclear composition of cybrids

Independent regenerants from independent colonies derived from microfused protoplast-cytoplast pairs in the genotypic combination SR1^{Km}(+) cms big^{Hm} were investigated for the genetic constitution of their nuclear genomes. The absence of the nucleus from the cytoplast donor line [*N. tabacum* cms (*N. bigelovii*), hygromycin resistant] and the presence of the nucleus from the protoplast donor line (*N. tabacum* SR1, kanamycin resistant) were confirmed by assessing the absence and presence, respectively, of the functional, dominant, selectable marker gene *nptII* (conferring kanamycin resistance) and *hpt* (conferring hygromycin resistance) in corresponding enzyme assays. A neomycin phosphotransferase dot assay from leaf extracts of 12 representative cybrids, confirming enzyme activity in 11 of them (no *nptII* enzyme activity observed for cybrid SKBH 4.2), is shown in Fig. 3. Independent regenerants from microcultured protoplasts of the kanamycin-resistant line (protoplast donor) *N. tabacum* SR1^{Km} showed neomycin phosphotransferase activity, while corresponding control material from the cytoplast donor *N. tabacum* cms (*N. bigelovii*)^{Hm} was negative in the enzyme assay (Fig. 3).

An enzyme assay for hygromycin B phosphotransferase activity, complementing the information on the type of nuclear genome present in the protoplast-cytoplast, microfusion-derived cybrids, is shown in Fig. 4. Out of 30 cybrids analyzed thus far from the protoplast-cytoplast combination, all but 1 (namely, cybrid SKBH 4.2) showed no *hpt* enzyme activity (Fig. 4a), while exhibiting neomycin phosphotransferase activity (Fig. 3).

Control hygromycin-resistant parental (cytoplast donor) line cms big^{Hm} and independent regenerants from microcultured protoplasts derived therefrom showed significant hygromycin B phosphotransferase activity (Fig. 4a), while no hygromycin B phosphorylating activity above background (from quantitatively scanned images of the autoradiograms) was present in the kanamycin-resistant, parental (protoplast donor) SR1^{Km} line or independent regenerants derived from it by microculture of protoplasts (data not shown).

In order to exclude possible misinterpretations due to transgene inactivation or artifacts in the in vitro enzyme assays, Southern blot hybridization of total DNA isolated from the cybrids was probed with *hpt*- and *nptII*-specific probes, and in vivo selection assays using leaf explants on kanamycin- and hygromycin B-containing medium were performed.

Southern blot hybridization of total DNA isolated from 25 independent cybrids, digested with HindIII and

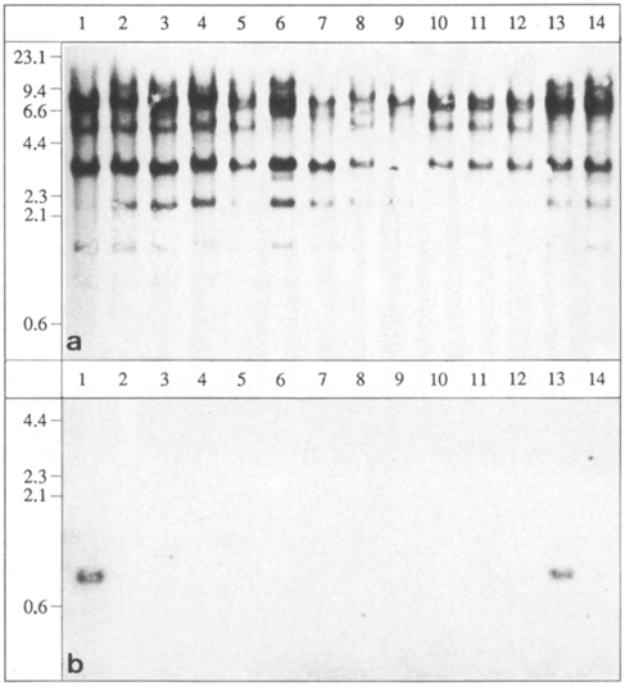

Fig. 5a and b. Southern blot analysis from protoplast-cytoplast, microfusant-derived cybrids. **a** Southern blot hybridization of BamHI-digested total DNA from defined cybrids probed with mitochondrial probe pmtSylSa-8 (compare Table 1, mtDNA pattern type). *Lane 1:* cybrid SKBH 4.2; *lanes 2–10:* SR1^{Km} protoplast + cms big^{Hm} cytoplast-derived cybrids SKBH 7.1 (*lane 2*), SKBH 10.2 (*lane 3*), SKBH 11.3 (*lane 4*), SKBH 13.3 (*lane 5*), SKBH 14.2 (*lane 6*), SKBH 16.2 (*lane 7*), SKBH 19.2 (*lane 8*), SKBH 23.1 (*lane 9*), SKBH 26.1 (*lane 10*); Km^r parental form *N. tabacum* cv Petit Havana SR1 (SR1^{Km}, *lane 11*); Km^s *N. tabacum* cv Petit Havana SR1 (*lane 12*); Hm^r *N. tabacum* cms (*N. bigelovii*) (cms big^{Hm}, *lane 13*), and Hm^s *N. tabacum* cms (*N. bigelovii*) (cms big, *lane 14*). **b** Southern blot hybridization of BamHI-digested total DNA from protoplast-cytoplast, microfusant-derived cybrids and parental forms as **a**, hybridized with *hpt* probe (BamHI fragment of pGL2) (compare Table 1, nuclear marker)

with BamHI, hybridized with *nptII* gene specific probe (HindIII fragment of pHP23) and with *hpt* gene specific probe (BamHI fragment of pGL2), was performed to confirm the physical presence and absence, respectively, of the corresponding selectable marker genes, thus providing additional information on the type of nucleus present in the cybrids. All analyzed DNA samples from 25 independent cybrids digested with HindIII and hybridized with *nptII*-probe, with the exception of cybrid SKBH 4.2, showed the diagnostic 0.80-kb band corresponding to the internal fragment of the *nptII* gene from pHP23 (data not shown), but did not show the diagnostic 1.03-kb band corresponding to the internal fragment from the *hpt* gene from pGL2 after digestion with BamHI (Fig. 5b). Only cybrid SKBH 4.2 (Fig. 5b, lane 1) showed the 1.03-kb band indicating the presence of the hygromycin resistance gene from plasmid pGL2 trans-

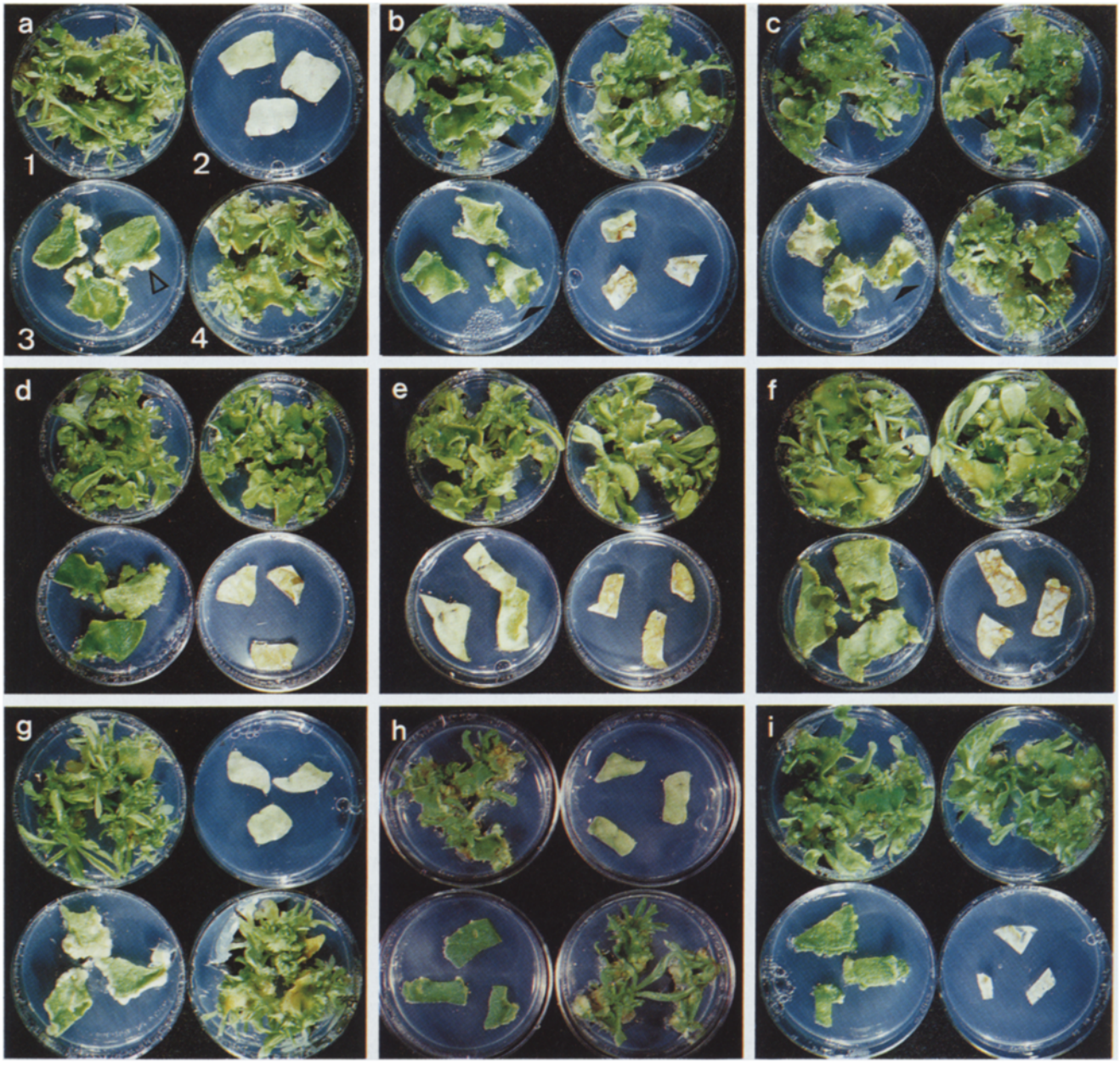

Fig. 6a–i. In vivo assay for kanamycin, hygromycin B, and streptomycin resistance/sensitivity with leaf explants from protoplast-cytoplast, microfusant-derived cybrids and parental forms. **a** Parental form *N. tabacum* cms (*N. bigelovii*) transformed with pGL2 (cms bigH^{m}): *dish 1* (control without selection), *dish 2* (with 50 mg/l kanamycin), *dish 3* (with 1.0 mg/ml streptomycin), *dish 4* (with 25 mg/l hygromycin); **b** parental form *N. tabacum* cv Petit Havana SR1 transformed with pHP23 (SR1^{Km}); **c** control double transformant Km^{r}Hm^{r} *N. tabacum* cv Petit Havana SR1 (SR1^{KmHm}); **d** regenerant from microcultured protoplast from parental form SR1^{Km} (SK 5.5); **e** protoplast-cytoplast, microfusant-derived cybrid SKBH 7.1 (Km^{r}, Str^{s}, Hm^{s}); **f** cybrid SKBH 20.1 (Km^{r}, Str^{r}, Hm^{s}); **g** regenerant from microcultured protoplast from parental form cms big^{Hm} (BH 1.2); **h** cybrid SKBH 4.2 (Km^{s}, Str^{s}, Hm^{r}); and **i** cybrid SKBH 3.3 (Km^{r}, Str^{r}, Hm^{s}). *Arrows* indicate green (↙) or white (▷) formed callus on leaf explants while assessing streptomycin resistance and sensitivity, respectively

ferred to the cytoplast donor line cms big^{Hm}. However, cybrid SKBH 4.2 showed neither the *nptII* gene specific 0.8-kb hybridizing band nor neomycin phosphotransferase activity (Fig. 3), and therefore it is not a kanamycin^{r} hygromycin^{r} somatic hybrid.

The corresponding 0.8-kb and 1.03-kb hybridizing bands in Southern blots from HindIII- and BamHI-digested DNA samples from control regenerants from microcultured, parental SR1^{Km} and cms big^{Hm} tobacco strains were present in all cases analyzed, after hybridiza-

Table 1. Characteristics of somatic cybrid plants regenerated from microfusion products of *N. tabacum* SR1^{Km} (+) *N. tabacum* cms (*N. bigelovii*)^{Hm}

Somatic cybrid	Nuclear marker		Chloroplast $Str^{R/S}$	mtDNA pattern type		
	$Km^{R/S}$	$Hm^{R/S}$		pmtSylSa-1	pmtSylSa-2	pmtSylSa-8
SKBH 1.3	R	S	S	sb	sb	s
SKBH 3.1	R	S	R	sb	b	sb
SKBH 3.2	R	S	S	new	sb	sb
SKBH 3.3	R	S	R	sb	sb	sb
SKBH 4.2	S	R	S	new	sb	s
SKBH 7.1	R	S	S	sb	sb	sb
SKBH 7.2	R	S	S	new	sb	new
SKBH 10.2	R	S	S	sb	sb	sb
SKBH 11.3	R	S	S	new	b	sb
SKBH 13.2	R	S	S	sb	sb	sb
SKBH 13.3	R	S	S	new	new	sb
SKBH 14.2	R	S	S	sb	sb	new
SKBH 14.3	R	S	S	sb	new	sb
SKBH 16.2	R	S	S	new	sb	b
SKBH 19.2	R	S	S	new	sb	new
SKBH 20.2	R	S	R	b	b	b
SKBH 20.3	R	S	R	new	sb	sb
SKBH 23.1	R	S	S	sb	b	b
SKBH 26.1	R	S	S	new	sb	s

R resistant
S sensitive
s SR1 type pattern
b cms big type pattern
sb additive pattern (SR1 + cms big)
new novel pattern

tion with *nptII* and *hpt* specific probes, respectively (data not shown). The presence of the kanamycin resistance phenotype (nuclear marker from the protoplast donor line SR1^{Km}) and the absence of the hygromycin resistance phenotype (nuclear marker from the cytoplast donor line cms big^{Hm}) could be observed in an in vitro selection assay with leaf explants for 29 out of 30 tested cybrids (Fig. 6), with the exception of cybrid SKBH 4.2; this showed the reverse phenotypes (Fig. 6h) and thus confirmed the molecular data presented. In addition, chromosome counts for all protoplast-cytoplast derived regenerants tested indicated the expected allotetraploid tobacco count of 48.

Thus, it is evident that most regenerants (24 out of 25 analyzed in detail) could be molecularly identified as being cybrids of the desired fusion combination protoplast of SR1^{Km} (+) cytoplast of cms big^{Hm}.

Keeping in mind that the selection reliability for cytoplasts using the micromanipulation setup with Nomarski optics described here is not absolute (namely, 73–84% of the selected cytoplasts are indeed enucleated, the rest being contaminating protoplasts) (Fig. 2), the exceptional case of cybrid SKBH 4.2 (Km^sHm^r) could be interpreted as a microfusant of a protoplast-protoplast pair in the genotypic combination SR1^{Km} (+) cms big^{Hm}, where a genomic segregation (loss of nucleus from SR1^{Km}) has taken place. This type of nuclear segregant (cybrid) of tobacco has been reported upon protoplast-protoplast microfusion in an analogous genotypic combination with frequencies of up to 40% (Spangenberg et al. 1990) and up to 62% for protoplast mass fusion, followed by microisolation of fusants in interspecific *Nicotiana* combinations (Gleba et al. 1985; Gleba and Sytnik 1984).

Cytoplasmic composition of cybrids

Independent regenerants from 28 individual colonies derived from microfused protoplast-cytoplast pairs were investigated for the genetic constitution of their cytoplasmic factors: chloroplasts and mitochondria.

Chloroplast type. For the analysis of chloroplast type in recovered plants from protoplast-cytoplast microfusion, the resistance or sensitivity to streptomycin was determined using leaf explants from primary regenerants (R_0) and seeds (R_1) derived therefrom. Seedlings of *N. tabacum* SR1^{Km} showed resistance to the antibiotic, and green regenerants could be induced from leaf explants on 1 mg/ml streptomycin, whereas those of *N. tabacum* cms (*N. bigelovii*)^{Hm} showed sensitivity.

In most cases, either streptomycin-resistant or streptomycin-sensitive primary regenerants and R_1 offspring

were found (Table 1, Fig. 6). However, in a few cases, segregational events among the R_1 progeny from the cybrids and chimeric structures (green and white patches on cotyledons or seedlings with green cotyledons and streptomycin-sensitive primary leaves) were observed (data not shown).

The fact that a heteroplastomic state following protoplast microfusion in *Nicotiana* is quite prevalent is in agreement with previous reports after somatic hybridization in tobacco via protoplast mass fusion. Gleba and coworkers (1984, 1985) reported on variegation in *Nicotiana* somatic hybrids derived from fusions in which one partner had a plastome deficiency. Similar results concerning heteroplasticity in somatic hybrids have also been described for other *Nicotiana* combinations (Fluhr et al. 1983; Böttcher et al. 1989).

Mitochondria type. In order to assess the mitochondrial constitution of the cybrids, species-specific patterns obtained by using total DNA digested with BamHI or HindIII and hybridized with three different mtDNA probes from *N. sylvestris* were used (Table 1).

For individual protoplast-cytoplast, microfusion-derived cybrids, different patterns, i.e., either additive, parental *N. tabacum* $SR1^{Km}$, parental *N. tabacum* cms $(N. bigelovii)^{Hm}$, or novel were detected (Fig. 5a, Table 1).

Novel patterns were due to the appearance of new nonparental bands or to the disappearance of parental-specific bands, involving qualitative and/or quantitative changes in restriction fragments. These novel patterns were not detected when individually regenerated plants derived from microcultured parental protoplasts were analyzed, and thus cannot be explained as a consequence of simple, protoclonal variation (data not shown). Similar results for analogous *Nicotiana* combinations and using the same mtDNA hybridization probes have been reported for the analysis of chondriomes of cybrids obtained after donor-recipient protoplast mass fusion (Galun et al. 1982; Aviv et al. 1984; Aviv and Galun 1987).

Finally, the present study demonstrates that microfusion of preselected protoplast-cytoplast pairs can be used for defined cybridization in higher plants. Microfusion allows for a good control of the type of cell, e.g., protoplasts, cytoplasts, and karyoplasts, participating in the fusion event, as well as for a quantifiable transfer of organelles, e.g., definable number of chloroplasts transferred with individual cytoplasts (Fig. 1f).

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