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Transmission genetics of the somatic hybridization process in *Nicotiana*

1. Hybrids and cybrids among the regenerates from cloned protoplast fusion products

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Summary. Callus protoplasts of a *Nicotiana tabacum* chlorophyll-deficient mutant were fused with mesophyll protoplasts from one of following five sources: 4 cms-analogs of tobacco bearing the cytoplasms of *N. plumbaginifolia, N. suaveolens, N. repanda,* and *N. undulata,* respectively, as well as wild species *N. glauca.* In another series of experiments, callus protoplasts from the chlorophyll-deficient genome *Su/Su* mutant of tobacco were fused with mesophyll protoplasts of the wild species *N. glauca* and those of a plastome chlorophyll-deficient tobacco mutant. The screening of hybrids consisted of visual identification followed by mechanical isolation and cloning of heteroplasmic fusion products in microdroplets of nutrient medium. Studies of regenerated plants included the analyses of gross morphology of plants, leaf and flower morphology, analysis of chromosome size and morphology and chromosome numbers, studies of multiple molecular forms of esterase and amylase, analysis of chloroplast DNA restriction patterns and analyses of chlorophyll-deficiency controlled by *Su* and P^- genes. The study of progeny of 41 clones representing all species' combinations demonstrated that regenarants of most (63%) clones from intraspecific (for nuclear genes) combinations were cybrid forms, whereas in the case of the fusion *N. tabacum* + *N. glauca,* the true nuclear hybrids prevailed and the proportion of cybrids did not exceed 26%. Clones regenerating both hybrid and cybrid plants from the same fusion product were also found.

Key words: Somatic cell – Fusion – Inheritance – Nuclear genes – *Nicotiana*

Introduction

The method of hybrid plant production by induced fusion of isolated protoplasts followed by regeneration of heteroplasmic fusion products to entire plants is a newly elaborated alternative to sexual crossing in higher plants. This method is of special practical interest to geneticists and plant breeders, as it allows the creation of novel plant forms genetically different from analogical sexual hybrids. However, the transmission genetics of the process is still poorly understood, and the elucidation of regularities of parental genes transmission to the progeny upon parasexual hybridization is one of the most important and urgent tasks.

The major shortcomings of earlier studies were the application of hybrid screening methods which resulted in an automatic rejection of a proportion of the theoretically possible recombinant forms (thus, for example, the screening based upon genetic complementation of recessive genome mutations permits one to reveal only nuclear hybrids, cybrids forms are rejected by the screening procedure). Other screening methods involve in many cases different sorts of cell inactivation (irradiation, treatment with iodoacetate) of one or both parents, resulting in a modification of the gene transmission and, in some cases, impoverishment of the spectrum of produced forms. Moreover, the methods of somatic hybridization used permited only the investigation of genetic diversity of recombinant forms as calculated per one experiment rather than per one hybridization event.

Therefore, we performed a series of experiments aiming at a special study of the fate (behavior) of parental genes in the process of somatic hybridization. As a method for hybrid screening, visual identification of heteroplasmic fusion products followed by mechanical

Table 1. Genetic constitution of *Nicotiana* plants regenerated from individual protoplast fusion products

No.	Clone	Parental combination	Nuclear constitution*	Plastid constitution*	Mitochondrial constitution*
1	21a2	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	hybrid[a, b, c]	mixture[f]	*tabacum*[j]
2	21a4	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	cybrid[a, b, c] (Coks 187)	mixture[f]	
3	21b1	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	hybrid[a, b, c]	*suaveol.*[f]	
4	21c1	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	cybrid[a, b, c] (Coks 187)	mixture[f]	
5	21c3	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	hybrid[a, b, c]	mixture[f, h]	*suaveol.*[j]
6	21c5	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	hybrid[a, b, c]	mixture[f]	
7	21d2	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	cybrid[a, b] (Coks 187, Samsun)	mixture[f, g, h]	*suaveol.*[j]
8	21d5	*N. tabacum* (P^-) + *N. tabacum* (*suaveolens*)	cybrid[a, b, c] (Coks 187)	mixture[f]	*suaveol.*[j]
9	17a3	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	cybrid[a, b] (Hicks)	mixture[f, h]	mixture[j]
10	17a4	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	hybrid[a, b, c]		
11	17a6	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	cybrid[a, b] (Hicks)	mixture[f, h]	
12	17c2	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	cybrid[a, b, c] (Hicks)		
13	17c7	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	cybrid[a, b] (Hicks)		*tabacum*[j]
14	17d3	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	cybrid[a, b, c] (Hicks)	mixture[f, g, h]	*tabacum*[j]
15	17d4	*N. tabacum* (P^-) + *N. tabacum* (*undulata*)	hybrid + cybrid[a, b, c] (Hicks)	*undulata*[f]	*undulata*[j]
16	2c6	*N. tabacum* (P^-) + *N. tabacum* (*repanda*)	cybrid[a, b, c] (NC 95)	mixture[f, g, h, i]	*repanda*[j]
17	3a3	*N. tabacum* (P^-) + *N. tabacum* (*plumbaginifolia*)	hybrid[a, b, c]	mixture[f, g, h]	
18	22b2	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	mixture[f]	
19	22b4	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	mixture[f]	
20	22b6	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c]	*glauca*[f]	
21	22c3	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	mixture[f, g, h]	
22	22c5	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	*glauca*[f]	
23	22c7	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	mixture[f, g, h]	
24	22d1	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b]	*glauca*[f]	
25	22d2	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c]	mixture[f]	

isolation and cloning in microdroplets of nutrient medium has been utilized. The mentioned screening procedure is the most adequate, being not connected with evident selective pressure/screening of some recombinant forms out of the total number of hybridization products. Moreover, the selection of cells is not based on any inactivation/mutation influences. In addition, mechanical isolation of individual fusion products is at the same time a cloning procedure, theoretically permitting the investigation of a diversity of recombinant forms within a clone, as well as genetic distinctions in progeny of independently arised heterokaryocytes. As initial material for these experiments, the plant forms differing for a number of traits coded for by nuclear genes, as well as plasmagenes, have been utilized.

The main purpose of this communication is a general characterization of regenerates from 41 clones obtained from heteroplasmic products of protoplast fusion in seven parental combinations of *Nicotiana,* and, particularly, the study of cybrid/nuclear hybrid ratio in a total spectrum of hybrid forms.

Materials and methods

As sources of mesophyll protoplasts, aseptically grown plants of the following material were used: 1) *Nicotiana tabacum,* 'Coks 187' var., carrying cytoplasm of *N. suaveolens;* 2) *N. tabacum* 'Hicks' var., carrying cytoplasm of *N. undulata;* 3) *N. tabacum,* 'NC 95' var., carrying cytoplasm of *N. repanda;* 4) *N. tabacum,* 'Coks 187' var., carrying cytoplasm of *N. plumbaginifolia;* 5) *N. tabacum,* plastome chlorophyll-deficient mutant; and 6) wild species *N. glauca.* Cms-analogs and *N. glauca* plants were obtained from Dr. D. A. Evans (DNA Plant Technology Corp., Cinnaminson, New Jersey, USA) Seeds of the plastome mutant were originally provided by Prof. D. von Wettstein (Carsberg Research Laboratory, Copenhagen, Denmark). Aseptically grown plants of the mutant, carrying only defective plastids were obtained via tissue culture (Gleba et al. 1978). As an alternative source, a callus cell line of the same

Table 1 (continued)

No.	Clone	Parental combination	Nuclear constitution*	Plastid constitution*	Mitochondrial constitution*
26	22d3	*N. tabacum* (P^-) + *N. glauca*	hybrid[a, b, c, d]	mixture	
27	24a3	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e, c]		
28	24a4	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e]		
29	24a6	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e]		
30	24b4	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid + cybrid[e, c, d] (*glauca*)		
31	24b6	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e]		
32	24c1	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e]		
33	24c4	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid + cybrid[e, c, d] (*tabacum, glauca*)		
34	24d3	*N. tabacum* (*Su/Su*) + *N. glauca*	cybrid[e] (*tabacum, glauca*)		
35	9a1	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid + cybrid[e] (*glauca*)		
36	9b6	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid + cybrid[e, c] (*tabacum, glauca*)		
37	9d3	*N. tabacum* (*Su/Su*) + *N. glauca*	cybrid[e] (*tabacum, glauca*)		
38	9d5	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid + cybrid[e] (*glauca*)		
39	9d7	*N. tabacum* (*Su/Su*) + *N. glauca*	hybrid[e]		
40	26k	*N. tabacum* (*Su/Su*) + *N. tabacum* (*P*)	cybrid (Samsun)	mixture[f, g, i]	
41	27k	*N. tabacum* (*Su/Su*) + *N. tabacum* (*P*)	cybrid (Samsun)	mixture[f]	

* Conclusions on the basis of:
[a] Gross morphology; [b] Leaf morphology; [c] Chromosome number and morphology; [d] Analysis of multiple molecular forms of esterase and amylase; [e] Mode of *Su* gene expression; [f] Variegation (P^- gene expression); [g] Presence of heteroplastidic cells; [h] Presence of chloroplast DNAs of both parents; [i] Inheritance of variegation in analytical crosses; [j] Associated with male sterility flower morphology

plastome chlorophyll-deficient mutant was used. Calli were induced by culturing mesophyll protoplasts in NTmod (Gleba et al. 1978) medium; cells were grown thereafter on solid B_5 medium of Gamborg et al. (1968) containing 2 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l indole-3-acetic acid and 0.2 mg/l kinetin. As still another callus cell source, tobacco cell suspension culture, derived from yellow seedling of 'John Williams Broadleaf' var., homozygous for the *Su* gene, was used. Cells were provided by Dr. D. A. Evans; they were grown with short subculture intervals as described by Evans and Gamborg (1982).

Protoplast fusion was induced as described by Menczel et al. (1981). Isolation of protoplasts and mechanical isolation and culture of individual heterokaryocytes were performed as described by Gleba and Hoffmann (1978); 7p medium of Kao and Michayluk (1975) rather than the NTmod medium was used for culturing the hybrid cells. Colonies, developed from isolated fusion products within 2.5–3 months of culture, were then transferred onto regeneration medium (Gleba et al. 1978).

For chromosome analysis, excised roots were pretreated with colchicine (0.05%, 1 h), fixed in acetic acid : ethanol (1 : 3) for 12 h and stained with 1% acetoorcein for 2 h.

Electrophoretic analyses of multiple molecular forms of esterase and amylase were carried out according to Gleba et al. (1983).

Results

1 N. tabacum (plastome chlorophyll-deficient mutant) + N. tabacum (suaveolens cytoplasm)

Callus protoplast of plastome mutant were fused with mesophyll cells of cms-analog. A total of 13 cell clones were produced: 8 of them were analyzed at the stage of regenerated plants. In total, 149 developed plants were investigated. 'Coks 187' variety, used for obtaining the cms-analog, possessed a sessile type of leaf; the male sterility character of the analog was manifested by flower unisexuality (in most cases, absence of anthers or, more rarely, the availability of rudimentary and unfunctional anthers). As for their gross-morphology, some of the plants recovered from the fusion products were similar to the plants of either 'Coks 187' var. (sessile leaf type) or 'Samsun' var. (petiolated leaf type). Regenerates from four clones were morphologically intermediate and were classified as nuclear hybrids (Fig. 1, Table 1). Chromosome counts in 16 randomly

Table 2. Chromosome numbers in *Nicotiana* plants regenerated from individual protoplast fusion products

No.	Clone	Chromosome nos. in root cells	% of telocentrics
1	21a2	98, 96	
2	21a4	48, 48, 48, 48	
3	21b1	94, 92	
4	21c1	48, 48	
5	21c3	> 100	
6	21c5	> 86	
7	21d5	48, 48, 48, 48	
8	17a4	92	
9	17c2	48, 48, 48, 48, 48, 50, 52	
10	17d3	48, 48, 48, 48	
11	17d4	48, 48, ⟶ 88, 86	
12	2c6	48, 48, 48, 48, 48, 48, 48	
13	3a3	120, 118	
14	22b2	112, 112, 110	46
15	22b4	164, 162	61.4
16	22b6	84, 86	57.5
17	22c3	90, 90, 92, 88	60
18	22c5	138, 134	47
19	22c7	64, 64, 64, 64, 64, 64, 64	55
20	22d2	112, 112, 112, 114, 114	48
21	22d3	130, 132	48.5
22	24a3	92, 94	
23	24b4	79, 80	
24	24c4	126, 128, 128	
25	9b6	76	
26	26k	48, 48, 48, 48, 48	

selected plants revealed strictly diploid (2n = 48) chromosome numbers in three clones, as well as polyploid ones in four of them (Table 2). The conclusions concerning the nuclear status of different clones derived from analyses of plant morphology and chromosome counts were in a good agreement. Among the regenerants of 8 clones under investigation, in seven cases, green, albino and variegated plants were revealed. The variegation was connected with the availability of heteroplastidic cells in leaf tissues, and with the presence of chloroplast DNAs from both parental types. A more detailed analysis of plastome genes and chloroplast DNAs of the plants, both mentioned and those described below, is reported in the parallel work (Gleba et al. 1984). Among the regenerants, the fertile forms, as well as forms with different morphological types of male sterility (including the parental type) were revealed. A more detailed study of this character in all the plants, involving those listed below, is in progress.

2 N. tabacum (chlorophyll-deficient plastome mutant) + N. tabacum (undulata cytoplasm)

For fusion, callus protoplasts of plastome mutant and mesophyll protoplasts of cms-analog, 'Hicks' var., were used. A total of 15 clones were isolated, and 98 plants regenerated from 8 clones were analyzed. The cms-analog used as the parental form in these experiments possessed a sessile leaf type; male sterility was manifested as complete transformation and unfunctional state of anthers which were turned into petaloid structures. As for their morphology, most of the plants obtained from fusion products were similar to the plants of 'Hicks' var. and possessed the sessile leaf type. One nuclear hybrid clone was found and in still another clone, both hybrids and cybrids were revealed. Chromosome counting in randomly selected plants revealed diploid chromosome numbers in 5 clones, as well as polyploid numbers in plants that were classified as hybrids on the basis of their morphology (Tables 1 and 2). Among the regenerants from 3 studied clones, the variegated plants, as well as purely green and purely albino ones, were observed. Also the variegation was accompanied by the presence of heteroplastidic cells in leaf tissues. These plants comprised chloroplast DNAs from both parental species. Studies of the regenerates' flower morphology demonstrated, that among plants from different clones both fertile and morphologically different (parental and non-parental) sterile forms were available (Fig. 2, Table 1).

3 N. tabacum (plastome chlorophyll-deficient plastome mutant) + N. tabacum (repanda cytoplasm)

In this experiment, callus protoplasts from plastome mutants were hybridized with mesophyll protoplasts of a cms-analog, line NC 95. This line has a sessile leaf type; male sterility is expressed as either a total absence of anthers, or they are rudimentary and completely sterile in all cases. In our work, 8 cell clones were obtained, but on only one clone were detailed studies performed sorting out the large amounts of green, albino and variegated plants. All the plants recovered from this clone were morphologically similar to the parental NC 95 line: they possessed a sessile leaf type and were male sterile. However, a great variability was found for pistil length and flower coloration (from white to dark-rose). Seven studied plants from this clone all contained 48 chromosomes, i.e. they were strictly diploid. Similarly to the foregoing cases, the variegation was connected with heterozygocity for chloroplast DNA composition as well as with availability of heteroplastidic cells in leaves. After pollination of these plants with pollen of tobacco 'Dubeck 44' var., in the progeny all the phenotype classes (albino, green and variegated plants) were revealed.

4 N. tabacum (chlorophyll-deficient plastome mutant) + N. tabacum (plumbaginifolia cytoplasm)

For fusion, callus cells of plastome mutant and mesophyll cells of cms-analog, 'Coks 187' var., were utilized.

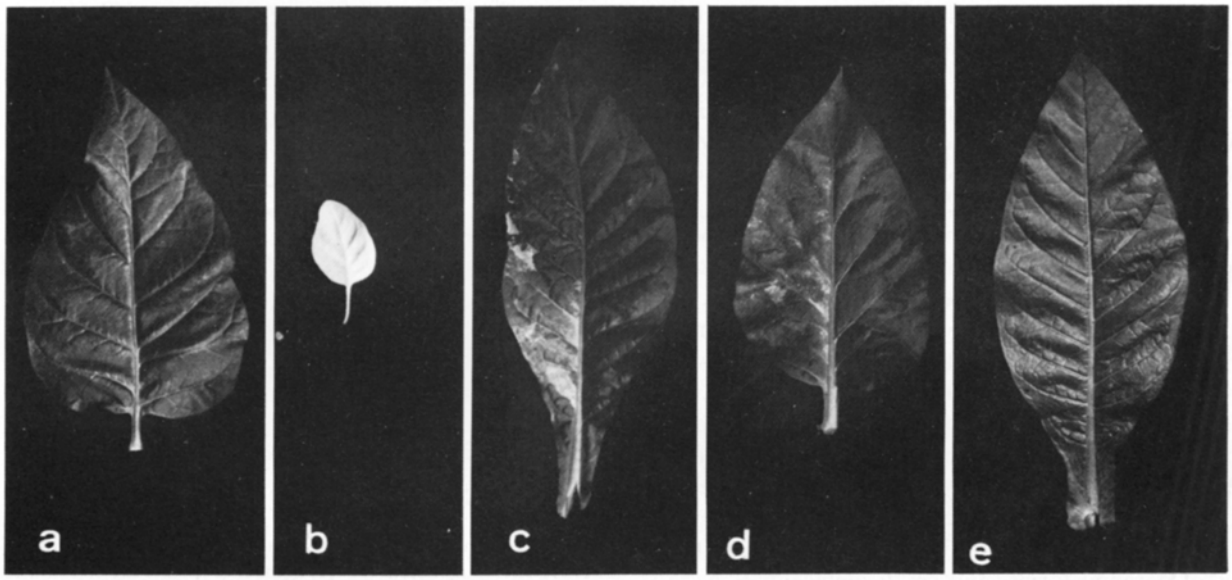

Fig. 1. Leaf shape in tobacco var. 'Samsun' (*a*), plastome mutant of tobacco var. 'Samsun' (*b*), cms-analog of tobacco var. 'Coks 187' (*e*), as well as in variegated cybrids regenerated from fusion clone 21d2 and showing either sessile 'Coks 187' (*c*) or petiolated 'Samsun' (*d*) leaf form

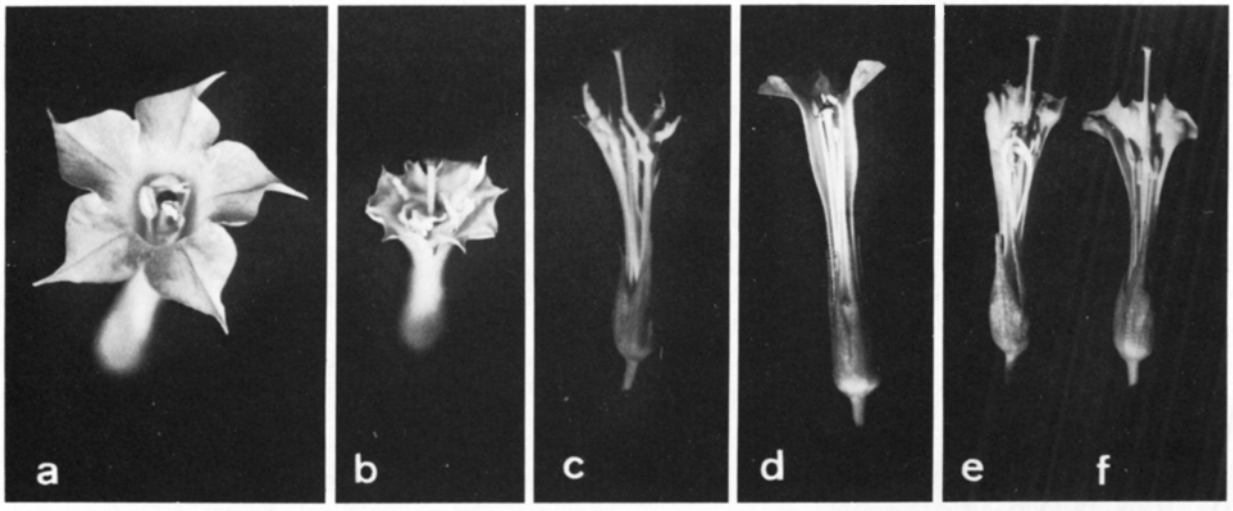

Fig. 2. Flower morphology in tabacco var. 'Samsun' (*a*), cms-analog of tobacco, carrying *undulata* cytoplasm (*b*), and sterile, clone 17d4 (*c*), fertile, clone 17c7 (*d*), as well as morphologically novel, clone 17a3 (*e, f*) cybrids

As has been mentioned above, this variety possesses the sessile leaf type. Sterility is expressed as a pollen degeneration in morphologically normal anthers which become brown at the time of pollen maturation. In this experiment, a total of 2 clones were obtained but we were successful in recovering plants from only one clone. All the regenerants turned out to be morphologically abnormal with traits charactristic for polyploids and aneuploids. Chromosome counting has confirmed this assumption (Table 2). Similarly to the foregoing experiments, the variegation of plants recovered from this fusion was accompanied by the availability of heteroplastidic cells and was related to the presence of chloroplast DNA from both parental types.

5 N. tabacum (chlorophyll deficient plastome mutant) + N. glauca

Callus protoplasts of plastome mutant and mesophyll protoplasts from wild species *N. glauca* were fused. A

total of 17 clones were isolated; the analysis was carried out for recovered plants (over 50 regenerates) from 9 clones. Morphologically, *N. glauca* differs from *N. tabacum:* it is a perennial arbuscular plant attaining 6 m in length, with small petiolate leaves covered with wax (that determines its grey coloration) and with very few trichomes; the flowers are yellow. All the plants regenerated from 9 heteroplasmic fusion products possessed an intermediate type of morphology, characteristic for nuclear hybrids between the two species (Fig. 3). *N. tabacum* and *N. glauca* differ for chromosome number and morphology. Tobacco possesses 2n = 48 chromosomes, 37.5% of them are telocentric. In *N. glauca,* 2n = 24 chromosomes, and telocentric chromosomes comprise 83.3%; small metacentric chromosomes are absent in *N. glauca.* The proportion of telocentric chromosomes in root cells of the studied plants from 8 clones was in all cases intermediate between the values of parental forms, and small metacentrics were invariably present (Fig. 5, Table 2). Biochemical studies of isoenzymes included electrophoresis of multiple molecular forms of esterase and amylase, and involved 24 plants from 8 clones. Plants recovered from fusion products comprised isoenzymes characteristic for both parental species (Fig. 4). Among the regenerants from 3 clones, numerous albino and variegated plants were revealed. In variegated plants, chloroplast DNAs from both parents were found; in leaf tissues, cells bearing both normal and mutant plastids were enregistrated (Gleba et al. 1984).

6 N. tabacum (chlorophyll-deficient Su/Su mutant) + N. glauca

Callus protoplasts from a diploid suspension cell line obtained from *Su/Su* homozygotes ('John Williams Broadleaf'), and mesophyll protoplasts from wild species *N. glauca* were fused. A total of 13 clones were isolated, and regenerates from 13 clones were obtained. Either small (clones of the experiment 24) or multinuclear (clones of the experiment 9) protoplast fusion products were observed. From cells of 6 clones, only yellow-green (*Su/Su*) forms were regenerated, giving evidence for their hybridity for nuclei. In 4 clones, besides the yellow-green forms, dark-green (*N. glauca*) or both dark-green (*N. glauca*) and yellow (*N. tabacum*) plants were also separating out. Regenerates from 3 clones were in each case mixtures of yellow and green plants. Limited cytogenetic (chromosome number and morphology) and biochemical (electrophoresis of multiple molecular forms of esterase and amylase) studies involving 8 yellow-green plants from clones 24b4, 24c4,

Fig. 3. Variegated somatic hybrid, obtained by cloning individual fusions of *N. tabacum* (plastome mutant) and *N. glauca* protoplasts

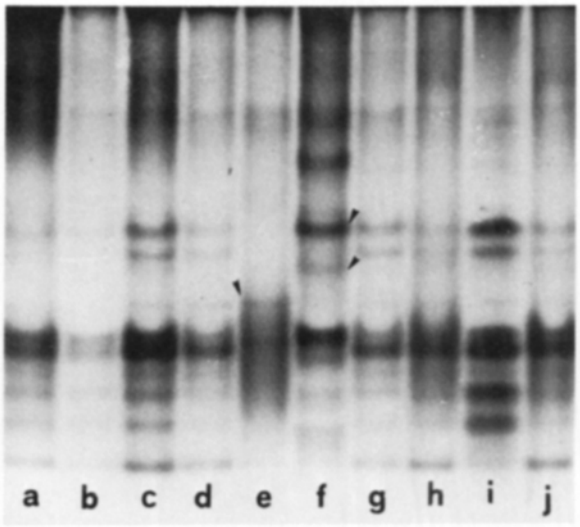

Fig. 4. Electrophoretic analysis of multiple molecular forms of esterase in *N. tabacum* (*f*), *N. glauca* (*e*), and eight somatic hybrids from clones 22c7 (*a, h*) 22c3 (*b, c, g*) and 22c5 (*i, j*). Species-specific bands are marked by *arrows*

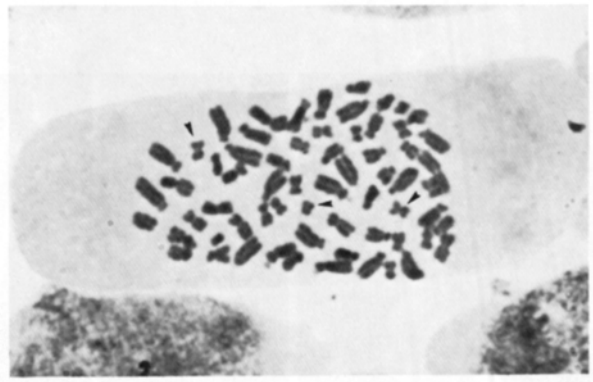

Fig. 5. Root cell metaphase in *N. tabacum* + *N. glauca* plant 22c7-02 with 64 chromosomes including 55% telocentrics. Tobacco small metacentrics are marked by *arrows*

24a3 and 9b6 confirmed the presence of chromosomes and isozymes from both parents in the material under investigation.

7 N. tabacum (chlorophyll-deficient Su/Su mutant) + N. tabacum (plastome chlorophyll-deficient mutant)

In this experiment callus protoplasts of the genome *Su/Su* mutant (genetically regular diploid suspension culture) were hybridized with mesophyll protoplasts of the plastome mutant. For fusion, plants which served as a source of mesophyll protoplasts were grown under dim light. Under these conditions their chloroplasts were partially green, making possible visual identification of heteroplasmic fusion products. Since, however, the distinctions mentioned disappeared after three days of culturing, the heterokaryocytes were picked up on the second day of culturing; a total of 10 heterokaryocytes were cultured together. As a result of such a cocultivation, two independent cell cultures were obtained. Upon regeneration, each of them turned out to be variegated and revealed, in addition to white, yellow-green and green partially variegated plants. We were not successful in rooting yellow-green plantlets; green plants (most of them turned later to be variegated) were brought to the stage of flowering (Table 2). Variegation of plants was connected with the presence of heteroplastidic cells in leaf tisues and was inherited by a part of sexual progeny (Gleba et al. 1984).

Discussion

The study of regenerated plants has demonstrated that most of them were recombinant forms bearing the genes from both parents involved in fusion. Since the mentioned recombinant forms were revealed to be in the absolute majority (39 out of 41) clones, the inference may be drawn about the high efficiency of the procedure of visual identification and mechanical isolation of heterokaryocytes as a screening method for somatic hybrids. This conclusion also is in a good agreement with the data from other works (Kao 1977; Gleba and Hoffmann 1978; Chien et al.1982; Gleba et al. 1982).

The present work is the first investigation of the fate of parental genes involving the cloning of heterokaryocytes with strict analysis of the characters encoded by nuclear genes and plasmagenes. Thus, a detailed study of nuclear behavior in heteroplasmic fusion products became possible. The main inference of this work is as follows: in the process of somatic hybridization, nuclear genetic determinants are inherited both bi- and uniparentally; uniparental inheritance of nuclear genes is evidently connected with genomic, rather than chromosome segregation in dividing cell fusion products. The latter conclusion is confirmed by the absence of any merozygotes (i.e. partial nuclear heterozygotes) and by sorting out of pure parental genomes for the traits studied. The several new modifications found in some cases (changes in flower coloration, lengths of pistils, leaf shape, etc.) cannot be explained by the action of nuclear genes only, and therefore are not taken into consideration.

From the data obtained, one can calculate the hybrid/cybrid ratio for different specific combinations. It is of interest that the rate of cybrids in intraspecific (for nuclear genes) combinations in high and attains 63%, whereas among fusion products between tobacco and *N. glauca*, nuclear hybrids prevail, the cybrid content being about 26% (we calculate complex segregants as both hybrids and cybrids).

The results obtained concerning such different ratios of cybrid versus hybrid forms in intra- and interspecific crosses are in good agreement with data from other investigators. Thus, an important or even prevailing proportion of cybrid, not nuclear hybrid forms, were found in all experiments involving fusion of different mutants and cms-analogs of tobacco, based on screening permitting the revelation of genomic segregates (Gleba et al. 1975, 1978; Belliard et al. 1977, 1978, 1979; Glimelius et al. 1981; Gleba and Sytnik 1982). On the contrary, analogical experiments on interspecific (for nuclear genes) hybridization, where no inactivation procedures such as irradiation of parental cells, were used, have in nearly all cases resulted in the formation of nuclear hybrids (to mention here only the experiments in which the cybrids were not automatically rejected by the screening procedure used: Kao 1977; Gleba and Hoffmann 1978; Sidorov et al. 1981; Gleba et al. 1982; Menczel et al. 1982; Gleba and Evans, unpublished). The most reasonable explanation of the difference discussed is selective multiplication and dominance of either cybrid or hybrid recombinants in the conditions used rather than the difference in actual rate of nuclear fusion in intra- versus interspecific heterokaryocytes.

Though mechanical isolation of fusion products is not a priori connected with any genetic event within a cloned product, the numerical ratio of produced (hybrid versus cybrid) recombinants determined can hardly be regarded as reflecting the situation in the population of fusion products after treatment with fusogen. It is evident that the method of mechanical isolation is nevertheless discriminative: as a rule, only actively dividing heterokaryocytes are selected (i.e. the heterotic variants have some advantages under the given conditions of culturing); there is also a tendency for picking up the major fusion products (whose "hybridity" is the most expressed) resulting in preferential screening of multicellular fusion products. Finally, only a proportion of the selected fusion products do survive in micro-

droplets, i.e. at this stage certain selection also takes place.

Another class of heterokaryocytes discovered in our work are those demonstrating complex segregation and sorting out of both simultaneously nuclear hybrid and cybrid combinations. Their presence due to mistakes in the mechanical isolation procedure is ruled out. They are especially abundant (3 out of 5 heterokaryocytes) in experiment 9, where multinuclear fusion products were preferentially isolated. Although detailed characterization of the progeny from these fusions has not yet been done, one can nevertheless state that multiple protoplast fusion products are viable and contribute to the parasexual progeny in experiments on somatic cell fusion.

The results obtained give good evidence that the spectrum of recombinant forms revealed by using the method of visual identification, mechanical isolation and cloning is much broader as compared to other existing procedures of hybrid screening. In particular, this method permits easy identification of the products of nuclear segregation, and we suggest using the method of mechanical isolation of cell hybrids as a simple and reliable procedure for transfer of cytoplasm. The previously proposed procedures were either of little efficiency and complicated (the use of cytoplasts – Maliga et al. 1982), or connected with the genetically confusing inactivation procedure (Zelcer et al. 1978; Sidorov et al. 1981; Menczel et al. 1982).

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