PD-AAW-687

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 9/2/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 3. F-29
July 1 - Dec 31, 1986

Attachment

1 cy

The Weizmann Institute of Science
Department of Plant Genetics
Rehovot, Israel

Dr. Lloyd R. Fredrick
U.S. Agency for International Development
S&T/Ag: Room 420A SA-IP
Washington, D.C. 20523, U.S.A.

19 January, 1987
Ref: 1791/776

Grant No. DFE 5542-G-55-4030-00

Dear Lloyd,

Attached please find our Progress Report for the period of July 1, 1986 to devember 31, 1986 on the above noted grant, entitled:. Utilization of Plant Protoplast Biotechnologies for the Transfer of Organelles.

We are rather pleased with the 'yield' of this half year in which we made substantial progress in the understanding of transfer of chloroplasts and mitochondria by the donor-recipeint protoplast-fusion methodology as well as in the more applied trend of this research-- the transfer of cytoplasmic-male-sterility into potato cultivars. The latter part of our work is, as you probably recall, done in coordination with the International Potato Center (CIP). Moreover, you will read in the Progress Report that we reached the phase of real collaboration: novel potato cybrids which resulted from the fusion of "recipient" protoplasts of cultivars which were received from CIP, with "donor" protoplast of a CMS line - - were produced (see previous Progress Report). Shoot-cultures of these cybrids were given to Dr. Dodds of CIP when he visited us in April 1986. The cybrids' shoot-cultures were first analyzed at CIP to assure absence of viral infection. After being cleared they were further propagated and plated in the field under conditions which induced optimal flowering. We are now waiting for the results from CIP. If some of these cybrids will be CMS while retaining the expected nuclear-coded traits of the recipients cultivars - a major goal of our research will be achieved. this will cause a vast saving of efforts at CIP because the above mentioned potato cultivars are the "seed-parents" of hybrid, true-seed potato (F_1) varieties and presently they have to be emasculated manually before pollination by the "pollen-parent" of these hybrids.

Two articles on the fundamental aspects or organelle-transfer by the donor-recipient protoplast-fusion methodology were recently accepted for publication (see Appendix to the Progress Report); we acknowledged the AID support in these publications (reprint and preprint of these publications are attached).

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Finally there is a good chance that all the goals set at the beginning of this research will be met till the termination of this grant (May 1987). We hope to receive further support from the AID to launch the next phase of research. The respective grant-proposals are now at a final stage of consideration in Washington.

With kind regards from Dr. Dvora Aviv and myself.

Very truly yours,

Esra

Esra Galun
Professor of Biology

PROGRESS REPORT

3. F 29

Grant No. DFE 5542-G-55-4030-00

Utilization of Plant Protoplast Biotechnologies
for the Transfer of Organelles

by

DVORA AVIV AND ESRA GALUN

Period: July 1 to December 31, 1986

During the last six months we devoted most of our efforts to the more directly-applicable research line of our project, namely the work which is coordinated with the International Potato Center (CIP) where the main present goal is to exploit the possible application of our organelle-transfer biotechnology to potato. More specifically we focussed on the transfer of cytoplasmic male sterility (CMS) into potato cultivars which are used at CIP as seed-parents for the production of hybrid, true potato-seed, varieties.

We performed additional protoplast-fusion experiments with potato lines. The resulting hybrid-plants will be converted into shoot cultures and, as previously done, these will be shifted to CIP for final evaluation. We did find that our technique requires further modifications, based on more basic knowledge, improved experimental material (i.e. organelle mutants such as albino recipients and drug-resistant donors) and refined organelle analysis procedures. We therefore devoted efforts to produce albino mutants and to characterize chloroplast DNA and mtDNA of cultivated potatoes and of other species of *Solanum* which may be used as protoplast-donors.

The *Nicotiana* system offers advantages for gaining basic understanding on the fate of mitochondria and chloroplasts after the donor-recipient protoplast fusion; especially when either or both the recipient and the donor protoplasts are treated before fusion. We shall briefly report on one experiment in which the effect of prefusion treatment with Rhodamine 6-G on the transfer of male-sterility and mtDNA composition, was investigated in the resulting cybrids.

We are rather satisfied with the progress of this project as the main goals of furnishing CIP with cybrid potatoes and vastly improving our understanding of organelle transfer by protoplast manipulation – will obviously be achieved within the next few months. Moreover our working relation and coordinated research with CIP attained a level which assures productive future collaboration.

In the following we shall report on specific research lines.

Donor-recipient protoplast-fusion as a means to transfer organelles and organelle-controlled traits: Rhodamine 6-G (R6G) pretreatment

Recently we studied in *Nicotiana* the possibility of controlling the type of mitochondria in cybrid plants by R6G treatment of either donor or recipient protoplasts, prior to fusion. As mentioned in the last progress report and also published by us recently (Aviv et al., *Plant Cell Rep.* 3: 227–230, 1986), preliminary results suggested that indeed R6G pretreatment could affect the mitochondrial composition of cybrid plants. To better understand this phenomenon we launched upon a large scale study whereby donor protoplasts from the mesophyll of the green and CMS plants 92 *lin*R-17 exposed to 10, 15, 20 or 25μg/ml R6G prior to their X-irradiation and later fused with recipient protoplasts of the chlorophyll-deficient line SRI *pig*$^-$35.

The number of calli and flowering plants derived from each R6G treatment as well as floral characteristics of these cybrid plants are summarized in Table 1. The analysis of mtDNAs by Southern blot hybridization of selected cybrids is summarized in Table 2.

Table 1: Distribution of fertile plants and cytoplasmic male sterile (CMS) plants among *Nicotiana* cybrids derived from the fusion of Rhodamine 6-G treated donor protoplasts of 92*lin*^{R}-17 (CMS) with iodoacetate treated recipient protoplasts of SR1 *pig*$^{-}$35.

Rhodamine 6-G level (μg/ml)	Total number of calli which produced flowing cybrid plants	Number of calli which produced only CMS plants	Number of calli which produced only fertile plants	Number of calli which produced CMS and fertile plants	Total number of cybrid plants which attained flowering	Number of cybrid plants in which mtDNA analysis was performed
10	13	1	6	6	42	5
15	6	0	0	6	22	9
20	15	4	4	7	56	8
25	10	4	2	4	45	6

The major conclusions derived from this experiment are the following:

1. Elevated R6G levels did not cause a substantial increase of the ratio of calli producing fertile plants among the cybrid progeny. There was apparently a small increase in the number of calli producing only sterile cybrids and a similar decrease in the number of calli producing fertile cybrids, as R6G dose is increased, but these changes are obviously not significant.

2. The three DNA probes used by us for mtDNA analysis (Table 2) were not specific enough to differentiate between sterile plants, fertile plants and mixed cybrid plants. Thus on the one hand some sterile, fertile and mixed cybrid plants showed identical hybridization patterns. On the other hand different patterns were observed among a group of fertile cybrids or a group of sterile ones. Consequently we could not find a clear correlation between floral appearance and a particular mtDNA pattern. Nevertheless it was observed that frag-

Table 2: MtDNA analyses by Southern blot hybridization of cybrid plants derived from the fusion of Rhodamine 6-G (R6G) treated donar protoplasts of 92*lin*^{R}-17 (CMS) with recipient protoplasts of SR1 *pig*$^{-}$35.

Plant designation	R6G conc. (μg/ml)	Anther type	Hybridization pattern with radioactive probes								
			pm Syl Sa-1			*pm Syl Sa*-2			*pm Syl Sa*-8		
			T	U	Novel	T	U	Novel	T	U	Novel
a22	10	fertile	+++	+	–	+++	–	–	+++	–	–
a23	10	sterile	+++	+	–	NT	NT	NT	+++	–	–
a31	10	mixed	+++	+	–	+++	–	–	+++	–	–
a52	10	mixed	+++	+	–	+++	–	–	+++	–	–
a53	10	sterile	+++	+	–	+++	–	–	–	+++	++
b13	15	sterile	+++	+	–	NT	NT	NT	NT	NT	NT
b16	15	sterile	+++	+	–	NT	NT	NT	NT	NT	NT
b21	15	fertile	+++	+	–	NT	NT	NT	NT	NT	NT
b25	15	mixed	+++	+	–	NT	NT	NT	NT	NT	NT
b42	15	fertile	+++	+	–	+++	–	–	+++	–	–
b44	15	fertile	+++	+	–	+++	–	–	+++	–	++
b45	15	sterile	+++	+	–	NT	NT	NT	NT	NT	NT
b11(G)	15	sterile	+++	–	–	+++	–	–	+++	–	–
b12(G)	15	mixed	+++	–	–	+++	–	–	+++	–	–
c13	20	sterile	+++	+	–	–	+++	–	+++	–	–
c22	20	fertile	+++	+	–	NT	NT	NT	+++	–	–
c25	20	sterile	+++	+	–	+++	+++	novel	+++	–	–
c26	20	mixed	+++	+	–	NT	NT	NT	+++	–	–
c34	20	fertile	+++	+	–	+++	–	–	+++	–	–
c65	20	Mixed	+++	+	–	+++	–	–	+++	–	–
c75	20	fertile	+++	+	–	+++	+++	–	+++	+	–
c92	20	fertile	–	+	++	+++	–	–	+++	–	–
d12	25	sterile	+++	+	–	NT	NT	NT	+++	–	–
d26	25	sterile	+++	+	–	+++	–	–	+++	–	–
d12(G)	25	sterile	+++	+	–	+++	–	–	+++	–	–
d13(G)	25	sterile	+++	+	–	+++	–	–	+++	–	–
d34(G)	25	fertile	+++	–	–	+++	–	–	+++	–	–
d51(G)	25	fertile	+++	–	–	+++	–	–	+++	–	–

T = *N. tabacum*-pattern; U = *N. undulata* (=line 92) pattern; (–) = no bands; (+) = faint bands; (+++) = strong bands; (NT) = not tested.

ments typical of the recipients mtDNA are much more prevalent than fragments typical of the R6G treated donor (irrespective of R6G dose used). This latter observation indicated that in future experiments of this kind it is essential to include a control of donor protoplasts without R6G pretreatment.

3. The lowest concentration of R6G used (10μg/ml) was already sufficient to eliminate the donor's mtDNA fragments but due to high frequency of recombination many of the cybrid plants were not fully fertile, as expected, but rather mixed or even fully sterile.

Whether R6G treatment by itself (without fusion) may affect the mtDNA restriction pattern is yet unknown. Further study is needed before a firm conclusion could be reached concerning the possible use of R6G pretreatment for specific elimination of either donor or recipient mitochondrial genomes.

Organelle Transfer in Potato

Follow up on previous fusion experiments. As reported previously (Report on January 1 to June 30, 1986) we performed several donor-recipient protoplast fusions between cytoplasmic donors and recipient cultivars. These fusion experiments had two purposes. Either to cause cytoplasmic male sterility (CMS) in potential seed-parents of true-potato seed (TPS) hybrids or to confer better pollen production in potential pollen-parents of TPS hybrids. After discussions at CIP in Lima (March 1986, see special report of E.G. on this visit) we decided to focus our attention on the first aim. Thus shoot-cultures from the donor-recipient fusion of protoplasts from the CMS line Y245.7 (as donor) with the potato cultivar Atzimba were given to Dr. John

Dodds of CIP during his visit in Rehovot (April 1986). These shoots were first tested (at CIP) to assure lack of pathogens and then multiplied, transferred to turf-pots and planted in one of the CIP stations in Peru where optimal conditions to induce flowering are usually obtained (Huancayo). We expect to receive the results from CIP in the near future. If some of the plants will have the combination of morphological (nuclear-coded) features of Atzimba with CMS, the goal of these fusion experiments will be achieved: such plants will be true cybrids having Atzimba nuclei but with at least some cytoplasmic components (i.e. mitochondrial DNA) from the CMS donor — Y245.7. Figures 1 and 2 are recent photographs taken at CIP. The fusion-derived plantlets are at the stage of shoot multiplication.

In parallel, fusion-derived potato plants were grown in Rehovot (in the Phytotron of the Hebrew University's Faculty of Agriculture) under long-day (16 h light) conditions (22°/17°— day/night). Such conditions are considered optimal for floral induction. Flowering was obtained and the pollen of these plants was examined to detect male-sterility. It should be recalled that male sterility in the fusion-donor, Y245.7, is expressed as arrest of pollen development at the tetrad stage. Such tetrads do not germinate. Pollen samples were observed microscopically and germinated in "hanging-drops" in the usual manner. The mineral composition of the drops' solution was (in ppm): 100 H_3BO_3, 300 $Ca(NO_3)_2$, 200 $MgSO_4 \cdot 7H_2O$, 100 KNO_3. The solution contained 20% sucrose and was adjusted to pH 5.5.

Table 3 summarizes the pollen analyses. Plants from callus H were probably "escapeese" of donor plants and not fusion-derived, because they had the

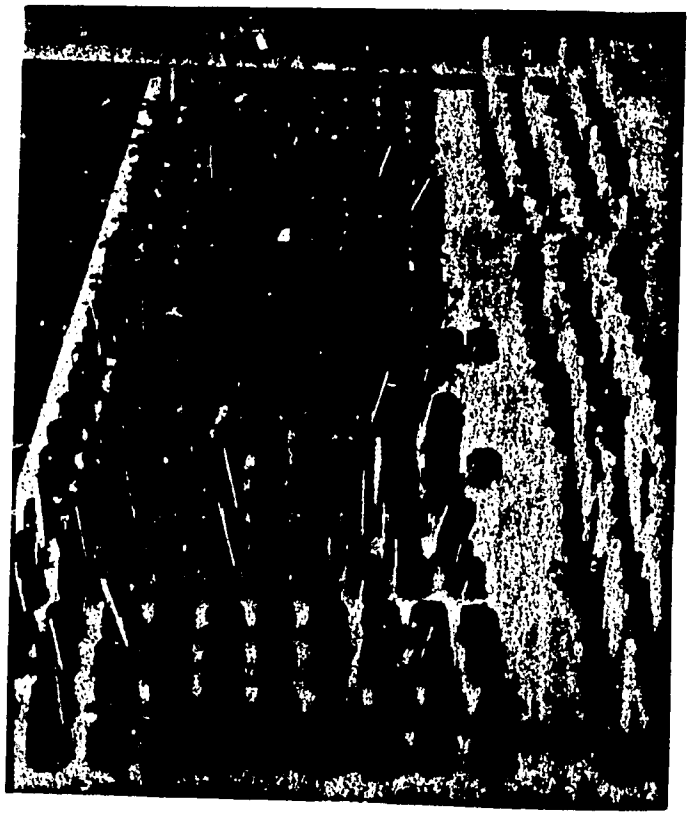

Figure 1. Young potato plants ready for field evaluation at the International Potato Center (CIP). The plants are cybrids which resulted from the fusion of (recipient) protoplasts fo the cultivar "Atzimba" with (donor) protoplasts of the cultivar malesterile (CMS) line Y.245.7. The plants will be analysed, at flowering, for CMS. Those being CMS and retaining all other traits of Atzimba will serve in the future for the production of TPS hybrids.

Figure 2. A close look at three of the cybrid plants shown in Figure 1.

Table 3. Characterization of pollen from fusion-derived plants resulting from the donor-recipient fusion between Y245.7 (donor) protoplasts and *S. tuberosum* cv *Atzimba* (recipient) protoplasts. Plants were regenerated from 9 fusion-derived calli; each callus is a result of a different fusion event.

Fusion-partner or fusion-derived callus	Designation of callus	Regenerated plants		
		Flower pigmentation	Pollen shape	Pollen germination
Donor: Y245.7	–	Violet-white	Tetrades	–
Recipient: Atzimba	–	White	Round	–
Fusion-derived callus	B	White	Round	–
Fusion-derived callus	C	White	Round	+
Fusion-derived callus	E	White	Round	+
Fusion-derived callus	F	White	Round	+
Fusion-derived callus	G	Violet-white	Tetrades + Round	–/+
Fusion-derived callus	H	Violet-white	Tetrades	–
Fusion-derived callus	P	White	Round	–
Fusion-derived callus	S	White	Round	+
Fusion-derived callus	X	White	Round	–

flower-pigmentation (a nuclear coded trait) of the donor. Plants from the calli C, E, F and S had flower pigmentation of the recipient and were male-fertile. They could either be as "escapeese" of non-fused recipient protoplast or they are fusion-derived but do not include the mitochondrial component which induces male-sterility. Plants from calli B, P and X are most probably true cybrids. Their pollen did not germinate but it lacked the typical tetrad-shape of pollen from Y245.7. They shall be tested further. Plants from callus G are unique. They have the CMS-donor's flower pigmentation but produced two types of pollen: tetrads which did not germinate and nor-

mal pollen grains which did germinate. The former were the vast majority. These plants are being further analysed.

New fusion combinations. In order to transfer CMS into other varieties, and to investigate nuclear/cytoplasmic compatibility between potato and alien *Solanum* species we conducted the fusion experiments described in Table 4.

Table 4. Current donor recipient protoplast fusion experiments. In all cases the donor protoplasts were X-irradiated and the recipient protoplasts were treated with Rhodamine-6-G.

Fusion Partners		Aim of fusion	Stage of fusion experiment
Donor	Recipient		
Y 245.7	*S. tuberosum* cv Atlantic	Transfer of CMS	Calli in regeneration medium
Y 245.7	*S. tuberosum* cv Kufri-Jyoti	Transfer of CMS	Calli in regeneration medium
Y 245.7	*S. tuberosum* cv Atzimba	Transfer of CMS	Plants being acclimatized
S. commersonii	*S. tuberosum* cv Ticahuosi	Nucl/cytopl compat.	Calli in regeneration medium
S. brevidens	*S. tuberosum* cv Desirée	Nucl/cytopl compat.	Calli in regeneration medium
S. demissum	*S. tuberosum* cv Desirée	Nucl/cytopl compat.	Calli in regeneration medium

Potato-organelle characterization

We continued to refine our methods to characterize the chloroplast genomes of potato and other *Solanum* species by ctDNA restriction patterns. Furthermore, we are developing similar procedures to characterize the mitochondrial genomes by mtDNA restriction patterns. The latter procedures are especially useful because they will provide means to identify positively also cybrids from fusions between closely related *Solanum* species.

Appendix to

PROGRESS REPORT

Grant No. DFE 5542-G-5-4030-00
Period: July 1 to December 31, 1986

The following publications were accepted by scientific journals:

1. Aviv, D., Chen R., Galun E. (1986): Does pretreatment by rhodamine 6-G affect the mitochondrial composition of fusion-derived Nicotiana cybrids? - Plant Cell Reports, 3:227-230

2. Aviv, D., Galun, E. (1986): Restoration of male fertile Nicotiana by fusion of protoplasts derived from two different cytoplasmic male-sterile cybrids.
   - Plant Molecular Biology, 7:411-417

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7: 411-417

Plant Molecular Biology ~~00: 000–000~~, *1986*
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Restoration of male fertile *Nicotiana* by fusion of protoplasts derived from two different cytoplasmic male-sterile cybrids*

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Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Keywords:

Summary

Using the 'donor-recipient' protoplast-fusion technique, we have recently constructed several alloplasmic-like lines of *Nicotiana* in which the original cytoplasms (or part of them) of either *N. tabacum* or *N. sylvestris* were replaced respectively, either by *N. undulata* or by *N. bigelovii* cytoplasms. These cybridizations resulted in two kinds of cytoplasmic male-sterile (CMS) cybrid plants: *N. tabacum* with *N. undulata*-like cytoplasm and *N. sylvestris* with *N. bigelovii*-like cytoplasm. Fusion of protoplasts, derived from the above two CMS types, by the 'donor-recipient' technique, lead to the recovery of 21 cybrid calli. One of these regenerated a cybrid with fertile pollen but having shortened filaments and slighly tapered anthers. Self pollination of the latter cybrid resulted in a second generation progeny having almost normal filaments and anthers. Further selfings produced a third generation in which numerous plants had normal stamens and fertile pollen. Mitochondrial DNA (mtDNA) analysis of second and third generation progenies revealed a novel pattern which differed from each of the parental CMS cybrids and also from the mtDNA of normal, male-fertile *Nicotiana* species. The results suggest that mtDNA recombination between different types of CMS can lead to restoration of male-fertility.

Introduction

Alloplasmic plants, i.e. plants in which the original cytoplasm was replaced by the cytoplasm of another species, were traditionally produced by an interspecific cross followed by recurrent backcrosses to the pollen parent (see review 14). The production of alloplasmic plants often lead to cytoplasmic male sterility (CMS) resulting in seed-parent lines, which are useful in the production of commercial F_1 hybrid seeds. Recently, alloplasmic-like lines were recovered from various protoplast fusion experiments. Most of the reported cases involved *Nicotiana* (e.g. 1, 5, 19, 25). The donor-recipient methodology (12, 13) was found to be particularly efficient in producing alloplasmic-like lines. The major difference between sexual-crosses' derived alloplasmic lines and alloplasmic-like lines obtained via protoplast-fusion is that in the former all the cytoplasmic components (i.e. both the chloroplasts and the mitochondria) are contributed solely by the alien (original female-parent) species while in the latter, the first event is a co-existance of the two cytoplasms in the same fusion-product cell followed by partial or total sorting out of cytoplasmic organelles. Therefore, in alloplasmic-like lines the complete replacement by the alien (donor) cytoplasm is expected to be rare; in most cases, novel chloroplasts/mitochondria combinations will

* Paper presented at the 1st Plant Molecular Biology Congress, Savannah, Georgia, Oct./Nov. 1985.

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be produced e.g. recipients chloroplasts with donor's mitochondria, recipients mitochondria with alien chloroplasts. Moreover, the initial cytoplasmic co-existance may lead to recombination of organellar DNAs. While there is a very limited evidence for chloroplast DNA (cpDNA) recombinations (18), novel mitochondria DNA (mtDNA) restriction patterns, which suggested mtDNA recombinations, were frequently observed (e.g. 6, 7, 8, 11, 17).

As no correlation was found between chloroplast type and CMS it is currently assumed that nuclear-mitochondrial interaction is controlling male sterility/fertility. This assumption is based on results with *Nicotiana* (e.g. 2, 5, 15) *Petunia* (9) and *Brassica* (23).

Male-fertility is commonly restored by certain nuclear (restorer) genes. Alternatively, we have shown that restoration to fertility can be achieved by transfer of the cytoplasm from a fertile donor into an alloplasmic CMS recipient (3). Here we report the first case of male-fertility restoration by an apparent recombination of mtDNAs from two different CMS *Nicotiana* cybrid lines.

Material and methods

Plant material

B-C-1-2 is an alloplasmic-like CMS cybrid line of the type *tbc/und/und-tbc* i.e. it is composed of *N. tabacum* nuclei, the chloroplasts of *N. undulata* and mtDNA which is similar but not identical to the mtDNA of *N. undulata*. B-C-1-2 was derived from the fusion of irradiated *N. undulata* protoplasts with *N. tabacum* VBW (albino) protoplasts (1). J-5-5-2 is an alloplasmic-like CMS cybrid-line of the type *syl/big/big-syl*, i.e. it is composed of *N. sylvestris* nuclei, the chloroplasts of *N. bigelovii* and its mtDNA is similar, but not identical to the mtDNA of *N. bigelovii*. This cybrid-line was derived from the fusion of X-irradiated *N. bigelovii* protoplasts with *N. sylvestris* protoplasts (1). The lines B-C-1-2 and J-5-5-2 were maintained as aseptic shoot-cultures.

Protoplast fusion and plant regeneration

Protoplasts were isolated from shoot-cultures of B-C-1-2 and J-5-5-2. B-C-1-2 protoplasts were X-irradiated (10 krad) and fused with iodoacetate treated J-5-5-2 protoplasts in the presence of polyethylene glycol by the donor-recipient technique as previously described (4).

Developing calli were transferred onto MS medium (20). After reaching a diameter of about 5 mm the calli were transferred to MS medium containing 2 g/ml kinetin and 0.8 μg/ml IAA, for shoot regeneration. One to 4 regenerated shoots per callus, were rooted and transferred to the greenhouse. Plant and flower morphology were recorded.

Chromosome analysis

Root tips were incubated in a 0.1% colchicin solution for 3 h, transferred to 2% acetocarmine in 45% acetic acid and the metaphases of at least 10 cells were scored.

Chloroplast DNA analysis

The procedure of chloroplast DNA (cpDNA) analysis was previously described (13). In brief, total DNA was extracted from 200 mg leaf samples, digested with *Sal*I, run on a slab-gel, blotted to a nitrocellulose paper and hybridized to a plasmid containing a cpDNA fragment which revealed different hybridization patterns when either *N. bigelovii* or *N. undulata* DNA was blotted on the paper.

Mitochondrial DNA analysis

The procedure of mtDNA analysis was described previously (13). In brief, mitochondria were isolated from 40–80 g of leaves derived from single plants. MtDNA was extracted, digested with *Sal*I or *Pst*I and run on a slab-gel. The restriction pattern was visualized either by ethidium bromide staining or by Southern-blot hybridization with either *pmtSylSa*-2 or *pmtSylSa*-8 (plasmids containing *Sal*I fragments from *N. sylvestris* mtDNA).

Results

The B-C-1-2 cybrid (*tbc/und/und-tbc*) has stigmatoid-petaloid stamens and does not produce any pollen (Fig. 1,A). The J-5-5-2 cybrid

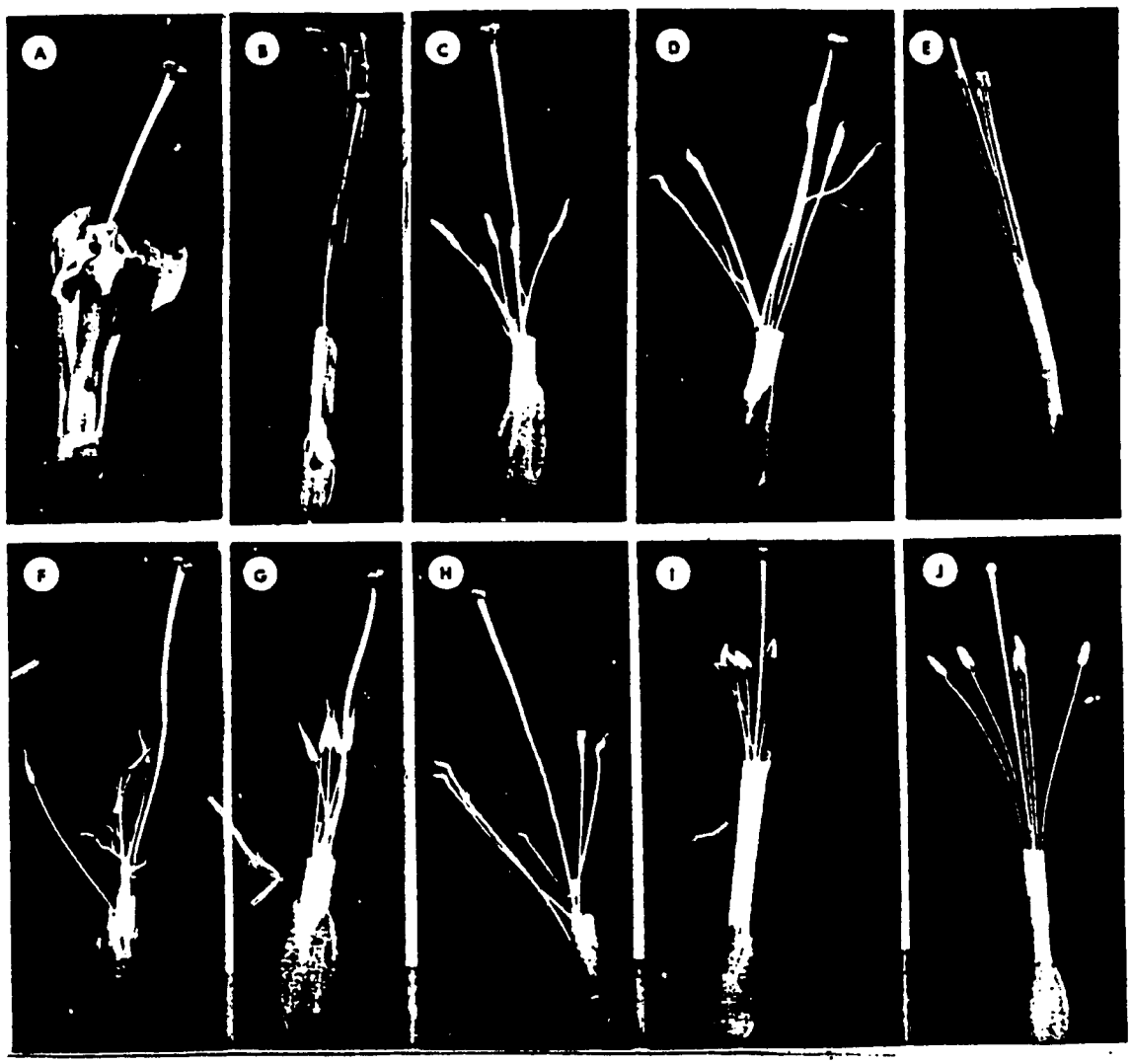

Fig. 1. Floral morphologies of parental fusion-partners and their cybrids; A. CMS fusion-partner (donor) B-C-1-2; B. CMS fusion-partner (recipient) J-5-5-2; C. first-generation cybrid G-1-6-2; D. first-generation cybrid G-3-1-1; E. third-generation cybrid G-2-1-1 (c-3) at anthesis; F. first-generation cybrid G-3-1-4; G. first-generation cybrid G-3-1-2; H. first-generation cybrid G-5-1-1; I. first-generation cybrid G-2-1-1; J. third-generation cybrid G-2-1-1 (c-3) before anthesis.

(*syl/big/big-syl*) has feathery anthers and also does not produce any pollen (Fig. 1,B). Protoplasts derived from B-C-1-2 (donor) were X-irradiated and fused with iodoacetate-treated protoplasts of J-5-5-2 (recipient). Twenty-one calli were recovered and transferred onto regeneration medium. Sixty-four of the regenerated plants reached flowering in the greenhouse. All the 64 cybrid plants had recipient's plant morphology i.e. rosette growth and typical vegetative traits of *N. sylvestris*. The fusion-derived plants were divided into four groups according to stamen morphology (Fig. 2). The largest group (I) was composed of 34 plants which were male-sterile cybrids with typical recipient's feathery anthers. Another group (III, 11 plants) was composed of male-sterile cybrids with tapered anthers (Fig. 1,G). Tapered anthers are typical of alloplasmic-like CMS *N. sylvestris* containing *N. undulata* cytoplasm (1, 25). A third group (II, 18 plants) was composed of male-sterile cybrids with

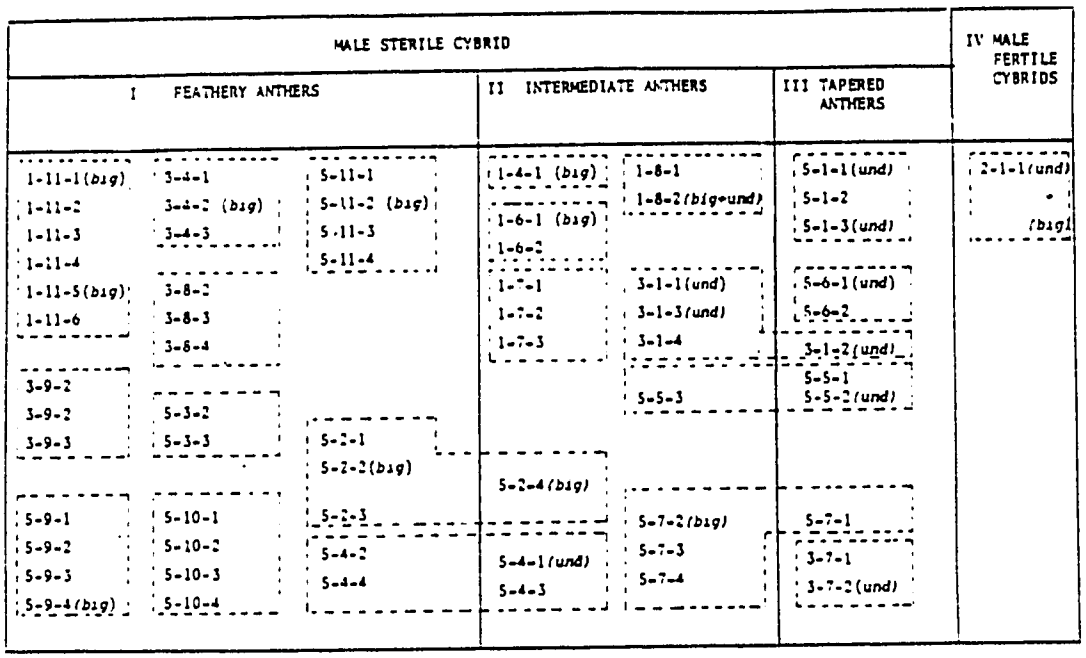

Fig. 2. Male-sterile and male-fertile cybrid plants which resulted from a donor-recipient protoplast-fusion between two male-sterile plants: X-irradiated B-C-1-2 (*tbc/und/und-tbc*) and iodoacetate-treated J-5-5-2 (*syl/big/big-syl*). The cpDNA constitution of the analysed cybrids are presented in parentheses, e.g. (*big*) or (*und + big*). Cybrids which resulted from the same callus are grouped within dashed frames. The prefix 'G' was omitted from all the cybrids, for brevity.

intermediate stamen-morphology (Fig. 1,C, D, F & H). The fourth group (IV) included only one cybrid plant (G-2-1-1) which grew slowly. It had almost normal, albeit somewhat tapered, anthers (Fig. 1,I). G-2-1-1 did produce viable pollen but had short stamen-filaments; it was self-pollinated manually to obtain seeds.

The chloroplast-compositions were determined by cpDNA analysis in 21 of the cybrids. The results are indicated in Fig. 2. Nineteen of the analysed cybrids, had either *big* cpDNA (10 plants) or *und* cpDNA (9 plants). While two cybrids had a mixture of *Big + und* cnloroplasts (G-1-8-2 and G-2-1-1).

The self-pollination progeny of G-2-1-1 was grown to maturity. Four plants of this progeny (e.g. G-2-1-1 (c), G-2-1-1 (k), G-2-1-1 (m) and G-2-1-1 (u)) had normal (or almost normal) *N. sylvestris* plant morphology. They also had normal anthers and produced abundant pollen but had shorter than normal stamen-filaments and were self-pollinated manually. Chromosome counts of several second generation progeny revealed a wide range of chromosome numbers. Some of the plants were euploids ($2n$-24) but the majority were aneuploids (ranging from $2n = 26$ to $2n = 58$).

The mtDNA restriction patterns of *N. bigelovii* and *N. undulata* are rather different (Fig. 3) and both patterns differ from that of *N. sylvestris* (1). Furthermore the mtDNA restriction patterns of the two parental lines used in this study, B-C-1-2 (Fig. 3) and J-5-5-2 (not shown) differ from *N. undulata* and *N. sylvestris* (not shown). Finally the second-generation cybrid G-2-1-1 (u) in which male-fertility was restored, had a novel mtDNA restriction pattern.

Southern-blot-hybridization substantiated the above mentioned results. By using the mtDNA probe *pmtSylSa*-8 (Fig. 4,A) we found that the hybridization patterns of mtDNA from B-C-1-2 and J-5-5-2 differed from those of *N. undulata* and *N. bigelovii* and that the hybridization pattern of mtDNA from the fertile cybrid G-2-1-1 (u) was novel but identical to its sib G-2-1-1 (k).

Analysis of third-generation progeny of G-2-1-1 obtained by self pollinations of the four fertile plants (G-2-1-1 (c), G-2-1-1 (k), G-2-1-1 (m) &

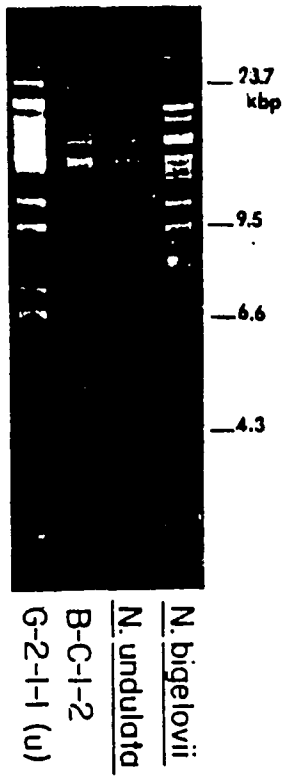

Fig. 3. Restriction-patterns of mtDNAs from *N. bigelovii*, *N. undulata*, one of the parent-lines (B-C-1-2) and a second-generation fertile cybrid (G-2-1-1 (u)). MtDNA was digested with *Sal*I and stained by ethidium bromide.

G-2-1-1 (n)) revealed that: (1) all plants of this generation had normal diploid chromosome constitutions ($2n = 24$); (2) all these plants had normal anthers and abundant pollen. In about half of the plants of this generation the stamen filaments were of normal length, thus the anther level reached the level of stigma. These plants were therefore naturally self-pollinators. In the other plants the filaments were shorter than normal requiring manual pollination for self-fertilization. Southern-blot hybridization revealed that the mtDNA of third-generation cybrid plants was identical with second-generation plants (Fig. 4B shows part of this data, other data-not shown).

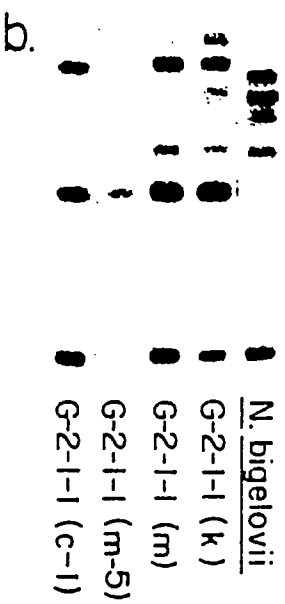

Fig. 4. Southern-blot-hybridization of mtDNAs. a. *N. undulata* and *N. bigelovii*, the two CMS parental lines J-5-5-2 and B-C-1-2 as well as two second-generation fertile cybrids G-2-1-1 (u) and G-2-1-1 (k); mtDNA was digested with *pst*I and hybridized with *pmtSylSa*-2. b. *N. bigelovii*, two second-generation fertile cybrids G-2-1-1 (k), G-2-1-1 (m) and two third-generation fertile cybrids, G-2-1-1 (m-5), G-2-1-1 (c-1); mtDNA was digested with *Sal*I and hybridized with *pmtSylSa*-8.

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Discussion

This study showed that donor-recipient protoplast-fusion between two alloplasmic-like CMS plants can result in a cybrid plant having restored male-fertility. It should be noted that the two partners of this fusion (J-5-5-2 and B-C-1-2) were cybrids in which the CMS resulted from the combination of mitochondria (or mitochondrial components) from normal, fertile donor *Nicotiana* species (*N. bigelovii* and *N. undulata* respectively) with nuclei of alien fertile *Nicotiana* species (*N. sylvestris* and *N. tabacum* respectively). The restoration of male-fertility in the first-generation cybrid was expressed in full pollen-fertility but their stamen-filaments were shortened and the anthers were slightly tapered. Two rounds of self-pollination resulted in completely normal male-fertile plants in the third generation. Chromosome analyses indicated various degrees of aneuploidy in the second generation while the third-generation plants which were normal in respect to male-fertility were also euploid. The ploidy of the original G-2-1-1 cybrid was not checked. Whether or not there is a causal relation between normal stamen-structure and euploidy cannot be determined because a concurrent stabilization of mtDNA was also observed among the third-generation plants in which stamen morphology was completely restored (e.g. Fig. 4B). However, it seems likely that in cybrid G-2-1-1 two opposite phenomena took place simulataneously, i.e. mitochondrial recombination restored fertility and at the same time aneuploidy had an adverse effect on the stamen structure. Only in subsequent generations, when euploidy was restored, the novel mitochondrial mtDNA genophore could be fully expressed leading to complete male fertility.

Since male-fertility/sterility in *Nicotiana* and several other genera (see: 13, 24, for reviews) is a consequence of nuclear-genome to mitochondrial-genome interactions – the restoration of male-fertility which resulted from the donor-recipient fusion between two CMS partners can be explained in two ways. A change in the nuclear-genome of the recipient partner may have caused the establishment of a nuclear restorer-gene or the novel mtDNA sequence may have rendered it 'compatible' with the recipient's nuclear genome. It is unlikely that the male-fertile plants derived in this study contain a nuclear restorer-gene since when one of these plants (G-2-1-1 (k-8)) was used as recipient in a donor-recipient protoplast fusion with a CMS donor (Line-92) the cybrid progeny was mostly sterile and had the recipients nuclear genome.

The mtDNAs of cybrid G-2-1-1 as revealed by restriction-pattern and probing with specific mtDNA fragments, differed from either of the fusion partners. Furthermore, all the individual plants derived from this cybrid by self-pollination revealed identical mtDNA patterns.

Novel mtDNAs, as revealed by restriction-patterns and Southern-blot hybridization with mtDNA probes were reported as a common result of somatic-hybridization and cybridization in higher plants (1, 6, 7, 8, 11, 17, 21, 22). The fate of mtDNA in subsequent (sexual) generations of somatic hybrids and cybrids was not reported in these studies. The present study (as well as our unpublished results) indicate that the mtDNA of cybrids is 'stabilized' in subsequent generations.

Interspecific cell-fusions in mammalian cell lines such as between mouse and human cells, where chromosomes of one of the fusion-partners are gradually eliminated from the derived hybrid cell-line, result in a concurrent elimination of one of the mitochondrial genomes without mtDNA recombination (e.g. 10). Generally mtDNA recombination is rare in somatic-hybrids of mammals and was reported only in a few cases (e.g. 16). There is no unequivocal explanation for this difference: massive recombination (or rearrangement) of mtDNAs in somatic-hybrid plants as opposed to the non-existing or rare mtDNA recombination in mammalian hybrid-cells. Structural rearrangements of mtDNAs were reported to be a common and natural phenomenon in plants and their rearrangements are made possible by direct-repeat sequences on the plants' mtDNA genophore (see: 24 for review). Such direct repeats were not reported in mammalian mtDNAs. Whether or not fusion-derived mtDNA recombination in plants is dependent on their direct-repeat sites may be revealed in future studies. Furthermore, it should be noted that mtDNA recombination, in fusion-derived plants, was not unequivocally demonstrated.

Although substantial progress was recently made in elucidating the molecular structure of the mitochondrial 'chromosome' the mechanism of male-sterility in plants is yet to be deciphered. Our

results suggest that in *Nicotiana* at least two different 'alleles' on the mitochondrial 'chromosome' are involved with male-sterility, because a recombination between the mtDNAs derived from two different CMS types lead to restoration of male fertility.

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References

1. Aviv D, Arzee-Gonen P, Bleichman S, Galun E: Novel alloplasmic *Nicotiana* plants by 'Donor-Recipient' protoplast fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genomes and either or both plastomes and chondriomes from alien species. Mol Gen Genet 196:244 – 253, 1984.
2. Aviv D, Fluhr R, Edelman M, Galun E: Progeny analysis of the interspecific somatic hydrids: *Nicotiana tabacum* (CMS) + *Nicotiana sylvestris* with respect to nuclear and chloroplast markers. Theor Appl Genet 56:145 – 150, 1980.
3. Aviv D, Galun E: Restoration of fertility in cytoplasmic male-sterile (CMS) *Nicotiana sylvestris* by fusion with X-irradiated *N. tabacum* protoplast. Theor Appl Genet 58:121 – 127, 1980.
4. Aviv D, Galun E: An *in vitro* procedure to assign pigment mutations in *Nicotiana* to either the chloroplast or the nucleus. J Hered 76:135 – 136, 1985.
5. Belliard C, Pelletier G, Vedel F, Quetier F: Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of *Nicotiana tabacum*. Mol Gen Genet 165:231 – 237, 1978.
6. Belliard G, Vedel F, Pelletier G: Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature 281:401 – 403, 1979.
7. Boeshore ML, Lifshitz I, Hanson MR, Izhar S: Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190:459 – 467, 1983.
8. Chetrit P, Mathieu C, Vedel F, Pelletier G, Primard C: Mitochondrial DNA polymorphism induced by protoplast fusion in *Cruciferae*. Theor Appl Genet 69:361 – 366, 1985.
9. Clark EM, Izhar S, Hanson MR: Independent segregation of the plastid genome and cytoplasmic male sterility in *Petunia* somatic hybrids. Mol Gen Genet 199:440 – 445, 1985.
10. Clayton DA, Teplitz RL, Nabholz M, Dovey H, Bodmer W: Mitochondrial DNA of human-mouse cell hybrids. Nature 234:560 – 562, 1971.
11. Galun E, Arzee-Gonen P, Fluhr R, Edelman M, Aviv D: Cytoplasmic hybridization in *Nicotiana*: Mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions. Mol Gen Genet 186:50 – 56, 1982.
12. Galun E, Aviv D: Cytoplasmic hybridization: genetic and breeding applications. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of Plant Cell Culture. Macmillan Publishing Corp., New York, 1983, pp 358 – 392.
13. Galun E, Aviv D: Organelle transfer. Methods in Enzymology 118:595 – 611, 1986.
14. Gerstel DU: Cytoplasmic Male Sterility in *Nicotiana* (A Review). Tech Bull No. 263, 31 pp, North Carolina Agr. Res. Service, 1980.
15. Glimelius K, Chen K, Bonnett HT: Somatic hybridization in *Nicotiana*: segregation of organellar traits among hybrid and cybrid plants. Planta 153:504 – 510, 1981.
16. Horak I, Coon HG, Dawid IB: Interspecific recombination of mitochondrial DNA molecules in hybrid somatic cells. Proc Natl Acad Sci USA 71:1828 – 1832, 1974.
17. Matthews BF, Widholm JM: Organelle DNA compositions and isoenzyme expression in an interspecific somatic hybrid of *Daucus*. Mol Gen Genet 198:371 – 376, 1985.
18. Medgyesy P, Fejes E, Maliga P: Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960 – 6964, 1985.
19. Menczel L, Nagy F, Lazar G, Maliga P: Transfer of cytoplasmic male sterility by selection for streptomycin resistance after protoplast fusion in *Nicotiana*. Mol Gen Genet 189:365 – 369, 1983.
20. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473 – 497, 1962.
21. Nagy F, Lazar G, Menczel L, Maliga P: A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. Theor Appl Genet 66:203 – 207, 1983.
22. Nagy F, Torok I, Maliga P: Extensive rearrangements in the mitochondrial DNA in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*. Mol Gen Genet 183:437 – 439, 1981.
23. Pelletier G, Primand C, Vedel F, Chetrit P, Remy R, Rouselle P, Renard M: Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244 – 250, 1983.
24. Pring DR, Lonsdale DM: Molecular biology of higher plant mitochondrial DNA. International Rev Cytol 97:1 – 46, 1985.
25. Zelcer A, Aviv D, Galun E: Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male-sterile *N. tabacum*. Z Pflanzenphysiol 90:397 – 407, 1978.

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Does pretreatment by rhodamine 6-G affect the mitochondrial composition of fusion-derived *Nicotiana* cybrids?

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ABSTRACT

Rhodamine-6G(R6G), a lipophilic dye which degrades mammalian mitochondria, was shown to arrest the division of Nicotiana protoplasts. When albino recipient-protoplasts were treated with R6G and fused with X-irradiated (green) donor- protoplasts, only green cybrid plants were obtained. The mtDNA of the cybrids was analyzed by Southern-blot hybridization. We found that cybrids which resulted from N. rustica (donor) protoplasts, fused with R6G-treated albino protoplasts, had only parental-type mtDNA. When another donor, with N. undulata mtDNA, was used, most of the resulting cybrids contained non-parental mtDNA. Only one cybrid (out of 12) had N. undulata -type (donor) mtDNA.

ABBREVIATIONS: big, N. bigelovii ; IAA, indoleacetic acid; mtDNA, mitochondrial DNA; R6G, rhodamine 6G; tbc, N.tabacum und, N. undulata,

INTRODUCTION

R6G is a lipophilic dye which was shown to be a potent inhibitor of mitochodnrial oxidative phosphorylation (Gear, 1974) through the blocking of adenine nucleotide translocate. The specific binding of R6G to the mitochondrial membranes was utilized by Ziegler and Davidson (1981) to control the mitochondrial input in fused mammalian cells. They showed that exposure of hamster cells to R6G and fusion of these cells to mouse cells resulted in hybrid cell-lines containing mouse mitochondria. Smith et al.(1983) utilized R6G prefusion treatment to demonstrate a close correlation between the maternally transmitted antigen (Mta, a murine cell surface molecule) and the mitochondrial compositions in the resulting somatic-hybrid cell-lines.

The chloroplast-composition of cybrid plants which resulted from the donor-recipient protoplast-fusion procedure, could be controlled by proper choice of fusion partners (review: Galun and Aviv, 1986). For example, cybrids with donor's chloroplasts were obtained by using recipients having pigmentation-deficient plastomes (Aviv et al., 1984) or donors with streptomycin-resistant plastomes (Fluhr et al., 1983), resulted in cybrids with donor's chloroplasts.

The lack of mitochondrial mutants in higher plants, prompted us to test R6G utilization to control the input of mitochondria in fused plant protoplasts.

MATERIALS AND METHODS

Green plants: 92 lin^R-17 is a plastome-coded lincomycin-resistant mutant induced by N-nitroso N-methylurea (Fluhr et al., 1985) in N. tabacum, line 92 (L-92). The latter line is cytoplasmic male-sterile with N. tabacum nuclei and the plasmone (plastome and chondriome) of N. undulata. It's designation is therefore tbc/und/und to denote for nuclear genome of N. tabacum /plastome of N. undulata /chondriome of N. undulata. I-2-4 is a cybrid line which originated from a donor-recipient protoplast fusion and is composed of N. tabacum nuclei and chloroplasts and mitochondria from N. bigelovii (tbc/big/big, Aviv and Galun, in preparation).

N. rustica, I-2-4, and 92lin^R-17 were kept as aseptic shoot cultures on Nit agar medium (Nitsch, 1969), at 25°C with 16h/d illumination.

Albino plants: SR1 V35 and VBW are both chloroplast encoded albino N. tabacum mutants. The former was cloned from a variegated plant derived from a streptomycin resistant line (SR1) (Fluhr et al., 1985) and the latter was cloned (Flunr et al., 1984) from a variegated DP1 mutant (Burk et al., 1964). The shoot cultures were kept on MS agar medium (Murashige and Skoog, 1962) supplemented with 2 µg/ml IAA and 0.2 µg/ml kinetin.

Protoplast manipulations: Protoplast isolation from green (donor) and albino (recipient) leaves, X-irradiation of donor protoplasts (50 Gy), fusion with polyethylene glycol and culture of fused protoplasts in VKM growth medium were previously described (Aviv and Galun, 1985). Cybrids containing the recipient's nuclear-genome were obtained by the donor-recipient protoplast fusion (Galun and Aviv, 1983).

Rhodamine 6G (R6G) and iodoacetate treatments: Recipient protoplasts were either treated with 0.4 mM iodoacetate for 30 min. before fusion or were isolated in the presence of R6G (Fig. 1). In the latter case, the leaves were incubated in maceration-enzyme solution containing R6G (Eastman Organic) at 15ug/ml (33µM) for 13h.

Analysis of cybrid plants: After fusion, green calli were regenerated into plants which were

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transferred to the greenhouse. Flower morphology and male fertility/sterility were recorded. In two experiments the leaves of individual cybrid plants were harvested, the mitochondria were isolated and the mtDNA was analysed by Southern blot hybridization as previously described (Aviv et al.,1984).

RESULTS AND DISCUSSION

To assess the effect of R6G on Nicotiana protoplasts, we exposed VBW protoplasts to various concentrations of this compound. We found that when mesophyll tissue was maintained for 18h in maceration fluid containing 15 ug/ml R6G the resulting protoplasts did not divide but heterofusion products, which resulted from fusion of R6G treated protoplasts with non-treated ones, did divide and produced cybrid calli (Chen, 1985). This R6G treatment of recipient protoplasts, combined with X-irradiation of donor protoplasts was thus instrumental in retaining only heterofusion-derived cybrid plants.

Three donor-recipient protoplast-fusion combinations were performed in which protoplasts from albino plants were treated with R6G and served as recipients (Table 1, A). Only green plants were regenerated and all were cybrids. Thus, R6G is an efficient tool in cybrid production and can substitute iodoacetate treatment of recipient protoplasts, a treatment which was shown previously to effectively eliminate non-fused fusion partners (Medgyesy et al.,1980). The fusion combinations resulted in both fertile and sterile cybrid plants. To assess the correlation of sterility/fertility to mitochondrial compositions we analyzed the mtDNA of cybrids. Fig. 2 is an example of a Southern-blot hybridization of mtDNA from cybrid plants, regenerated from the fusion of albino N. tabacum (SR1 V35) protoplasts, treated with R6G, with X-irradiated protoplasts of N. rustica. Table 2 provides the data from 18 cybrid plants. It is noteworthy that contrary to all previous data on mitochondrial composition in cybrids and somatic hybrids (e.g. Nagy et al., 1981; Galun et al., 1982; Boeshore et al., 1983) only pure parental-type mtDNA restriction patterns were detected in cybrids derived from the present donor-recipient fusion-combination. Table 2 also demonstrates the consistancy of results concerning the three probes: whenever one probe showed a N. tabacum mtDNA pattern, this pattern was also exhibited by the other probes; the same was true in respect to probes showing N. rustica patterns. These results are also different from those obtained in a similar donor-recipient fusion, where N. rustica protoplasts also served as organelle donors but the recipient protoplasts were not treated with R6G (Aviv et al.,1984). In the latter combination no cybrids with donors mtDNA were obtained; all the analyzed cybrids had mtDNA which showed restriction patterns which were either identical or almost identical to the recipients (N. sylvestris) mtDNA. Our present result also indicate that the combination of N. tabacum nuclei with both plastomes and chondriomes of N. rustica can lead to functional cybrid plants.

The analysis of another donor-recipient combination (Exp 3, Table 1) showed (Fig. 3) that some of the cybrids contained parental-type mtDNA while other cybrids had novel restriction patterns - indicative of chondriome recombinations in the fused protoplasts. Only a sample of the mtDNA analyses, with the pmSylSa-3 probe, are shown in Fig. 3. More information on the chondriome composition of cybrids resulting from Exp. 3 is provided by Table 3. Thus, it seems possible that the process of chondriome recombination, in fused protoplasts, is dependent on the specific nuclear-genome/alien-cytoplasm combination. The question whether or not recipient mtDNA can be eliminated by R6G is therefore still open. Further studies, with higher concentrations of R6G, with reciprocal treatments of donors and

Table 1. Fertile and sterile cybrid plants regenerated from donor-recipient fusion-combinations. Recipient protoplasts were from albino plants and were treated with either rhodamine6-G or iodoacetate.

Fusion-combinations		Number of calli which differentiated into cybrid plants		
Recipients[a] (albino)	Donors (green)	Fertile plants	Male-sterile plants	Fertile + sterile plants
A. Prefusion treatment of recipients with Rhodamine 6 G				
1. VBW	I-2-4(*tbc/big/big*)	3	4	2
2. SR1 V35	*N.rustica* (*rus/rus/rus*)	1[d]	6	0
3. SR1 V35	92 *lin*^{R}-17(*tbc/und/und*)	0	5	4
	total	4	15	6
B. Prefusion treatment of recipients with iodoacetate				
4. VBW[b]	92 *str*^{R}-7(*tbc/und/und*)	6	0	1
5. VBW	92 *lin*^{R}-17(*tbc/und/und*)	1	2	1
6. VBW	92 *str*^{R}-7,*spec*^{R}-1(*tbc/und/und*)	3	1	0
7. VBW[c]	*N.bigelovii*(*big/big/big*)	2	1	0
8. VBW[c]	*N.undulata*(*und/und/und*)	0	7	1
	Total	12	11	3

[a]-all recipients were *tbc/tbc/tbc*; [b]-data from Fluhr et al.(1985); [c]-data from Aviv et al.(1984); [d]-additional 11 calli resulted in semi self-fertile plants.

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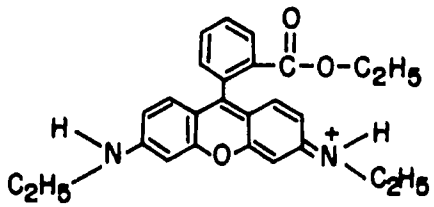

Fig. 1. Rhodamine 6G

Table 2. Southern-blot analysis of mitochondrial DNA in cybrid plants derived from fusing X-irradiated *N.rustica* protoplasts with rhodamine 6-G treated recipient protoplasts of albino *N.tabacum*(SR1 V35)

Cybrid designations	self fertility[1]	Southern hybridization patterns with three mtDNA probes		
		pmSylSa-1	pmSylSa-2	pmSylSa-8
A2	PSF	n.t.[3]	*rus*	*rus*
B2	PSF	n.t.	*rus*	*rus*
D2	PSF	n.t.	*rus*	*rus*
D3	PSF	*rus*	*rus*	*rus*
H2	PSF	*rus*	*rus*	*rus*
I2	PSF	n.t.	*rus*	*rus*
I4	NSF	n.t.	*rus*	*rus*
K3	NSF	n.t.	n.t.	*tbc*
L3	PSF	*rus*	*rus*	*rus*
L4	NSF	*rus*	*rus*	*rus*
M4	PSF	*tbc*	*tbc*	*tbc*
N1	NSF	n.t.	*tbc*	n.t.
R2	SF	n.t.	*tbc*	*tbc*
R3	SF	*tbc*	*tbc*	*tbc*
T5	PSF	n.t.	*tbc*	*tbc*
V3	NSF	*tbc*	*tbc*	*tbc*
W1	NSF	n.t.	*tbc*	*tbc*
Z3	NSF	n.t.	*tbc*	*tbc*

[1]Normal amount of seeds per pod is self-fertile (SF); few seeds per pod is partially self-fertile (PSF); no seeds is not self-fertile (NSF)

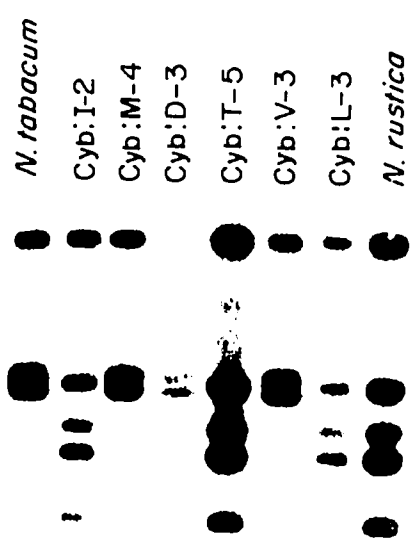

Fig. 2. Southern-blot hybridization of mtDNA from cybrid plants derived from the fusion between X-irradiated (donor) N. rustica protoplasts and R6G treated (recipient) N. tabacum (albino SR1 V35) protoplasts. Nick-translated pmtSylSa-8 was used as probe.

Table 3. Southern-blot analysis of mitochondrial DNA in cybrids derived from fusing X-irradiated 92 lin^R -17 protoplasts (having *N.undulata* mtDNA) with rhodamine 6-G treated protoplasts of albino *N.tabacum* (SR1 V35)

Cybrid designations	Male sterility[1] or fertility	Southern-blot hybridization patterns with 3 mtDNA probes		
		pmSylSa-1	pmSylSa-2	pmSylSa-8
A1	Mixed[2]	*tbc & und*	*tbc*	Novel
A2	Fertile	*tbc & und*	*tbc*	*tbc*
B5	Fertile	*tbc & und*	*tbc(?)*	Novel
C2	Fertile	*tbc & und*	*n.t.*	*tbc*
C4	Fertile	*tbc & und*	*tbc*	Novel
C6	Sterile	*tbc & und*	*tbc*	*tbc*
D1	Sterile	*tbc & und*	*tbc*	*tbc*
H2	Sterile	*und*	*und*	Novel
H3	Sterile	*und*	*und*	*und*
I1	Sterile	*tbc & und*	*tbc & und*	*und (?)*
I2	Sterile	*tbc & und*	*tbc (& und?)*	*tbc*
I3	Sterile	*tbc & und*	*tbc & und*	*und*

[1]Male-sterility is expressed as floral-member malformation (petaloid)

[2]This cybrid had both male-sterile and fertile flowers

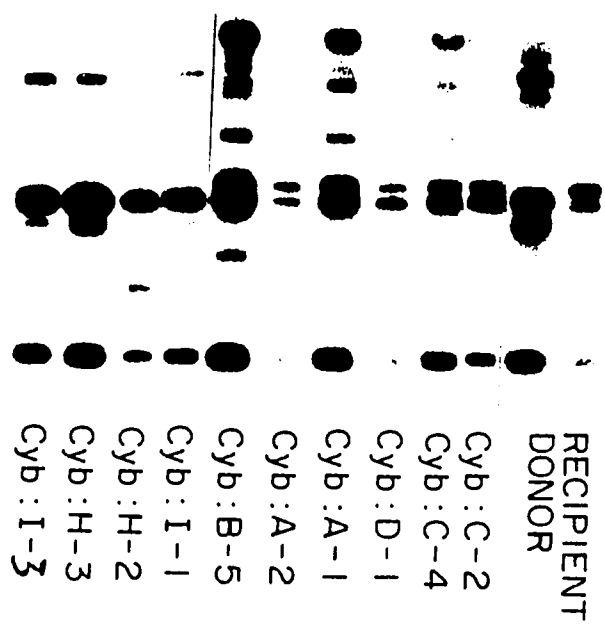

Fig. 3. Southern-blot hybridization of mtDNA from cybrid plants derived from the fusion between X-irradiated donor protoplasts (92lin^R-17) having N. undulata mtDNA and R6G treated (recipeint) N. tabacum (albino, SR1 V35) protoplasts. Probe - as in Fig. 2.

recipients and with additional fusion combinations are required for an affirmative answer.

Several previous cybridization studies (reviewed in Galun and Aviv, 1986) implicated the chondriome in Nicotiana with the control of male-sterility/fertility. The results of the present study confirm previous reports. However, novel mtDNA restriction pattern in cybrids are not necessarily leading to floral malformation. This malformation is therefore not a reliable indication of the mitochondrial composition of cybrids.

Iodoacetate treatment of recipient protoplasts also resulted in both male-sterile and male-fertile cybrids (Table 1B). Due to the relatively small number of calli from which cybrids were derived, a comparison between RGG and iodoacetate treatments is presently not feasible. On the other hand, the results of both treatments showed that either of these compounds is a useful tool in cybrid selection and in the induction of male-sterility by inter-species cybridization.

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REFERENCES

1. Aviv D, Arzee-Gonen P, Bleichman S, Galun E (1984) Mol Gen Genet 196: 244-253.

2. Aviv D, Galun E (1985) J. Heredity 76: 135-136.

3. Boeshore ML, Lifsnitz I, Hanson MR, Iznar L (1983) Mol Gen Genet 190:459-467.

4. Burk R, Stewart N, Dermen H (1964) Amer J Bot 51: 713-724.

5. Chen R (1985) M.Sc Thesis, Feinberg Graduate School, The Weizmann Institute of Science, Rehovot, Israel.

6. Fluhr R, Aviv D, Edelman M, Galun E (1983) Theor Appl Genet 65: 289-294.

7. Fluhr R, Aviv D, Edelman M, Galun E (1984) Theor Appl Genet 66: 491-497.

8. Fluhr R, Aviv D, Galun E, Edelman M (1985) Proc Nat Acad Sci (Wash) :82 1485-1489.

9. Galun E, Arzee-Gonen P, Fluhr R, Edelman M, Aviv D (1982) Mol Gen Genet 186: 50-56.

10. Galun E, Aviv D 1983. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y.(eds) Handbook of Plant Cell Culture Vol I, Macmillan, New York, pp.358-392.

11. Galun E, Aviv D (1986) Methods in Enzymology 118: 595-611.

12. Gear ARL (1974) J Biol Chem 249: 3628-3637.

13. Medgyesy P, Menczel L, Maliga P (1980) Mol Gen Genet 179: 693-698.

14. Murashige T, Skoog F (1962) Physiol Plant 15: 473-497.

15. Nagy F, Torok I, Maliga P (1981) Mol Gen Genet 183: 437-439.

16. Nitsch JP (1969) Phytomorphology 19: 389-404.

17. Smith R, Huston MM, Jenkins RN, Huston DP, Rich RR (1983) Nature 306: 599-601.

18. Ziegler ML, Davidson R L (1981) Somatic Cell Genetics 7: 73-88.