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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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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 coexistence 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

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be produced, e.g. recipients chloroplasts with donor's mitochondria, recipients mitochondria with alien chloroplasts. Moreover, the initial cytoplasmic coexistence 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.

Materials 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 μg/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

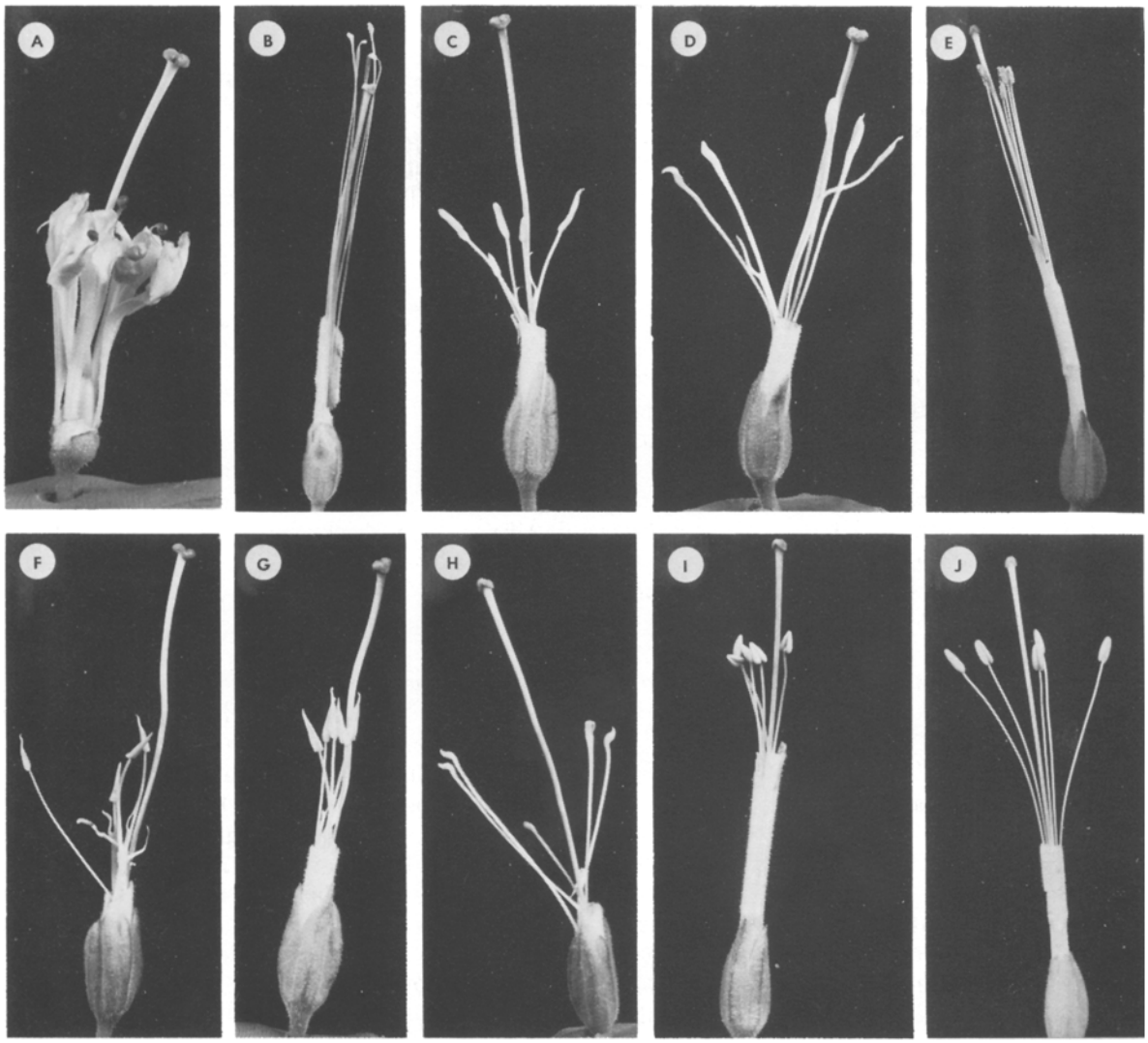

Fig. 1. Floral morphologies of parental fusion-partners and their cybrids; A. CMS fusion-partner (donor) B-C-1-2; B. CMS fusion-partner (recipient) J-5-5-2; C. first-generation cybrid G-1-6-2; D. first-generation cybrid G-3-1-1; E. third-generation cybrid G-2-1-1 (c-3) at anthesis; F. first-generation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

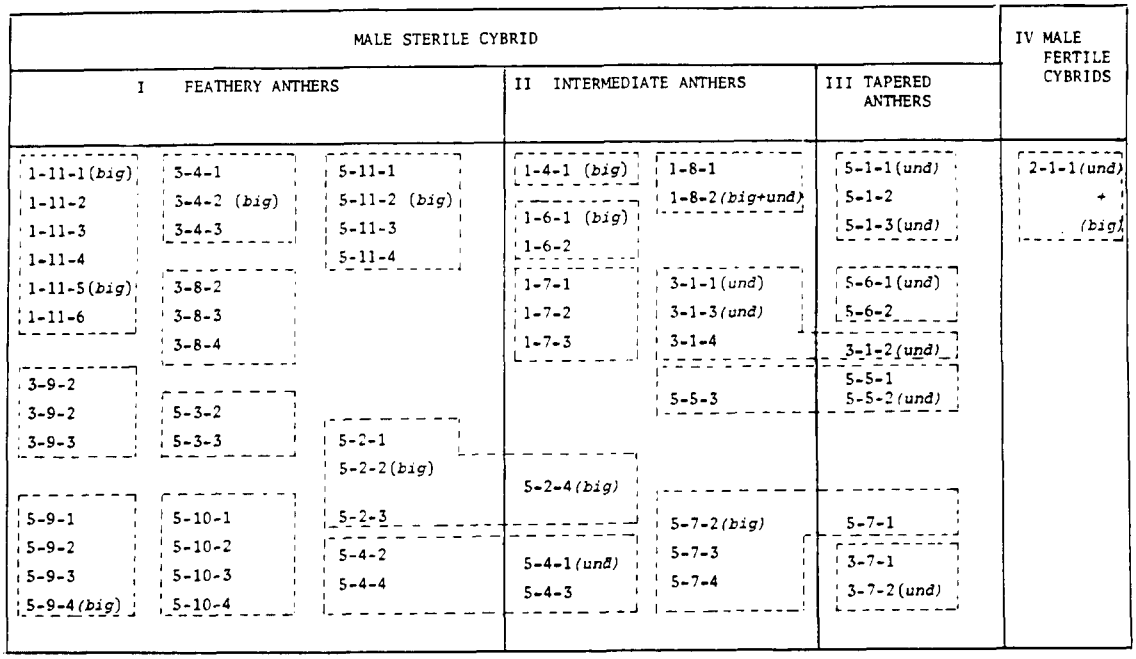

Fig. 2. Male-sterile and male-fertile cybrid plants which resulted from a donor-recipient protoplast-fusion between two male-sterile plants: X-irradiated B-C-1-2 (*tbc/und/und-tbc*) and iodoacetate-treated J-5-5-2 (*syl/big/big-syl*). The cpDNA constitution of the analysed cybrids are presented in parentheses, e.g. (*big*), (*und*) 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* chloroplasts (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 progenies 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 the 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) &

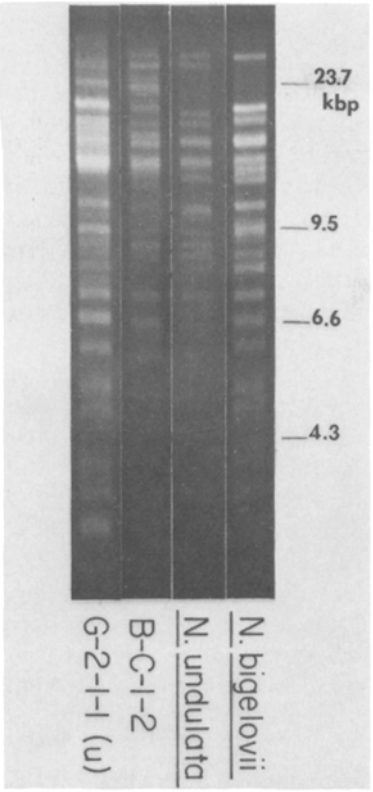

Fig. 3. Restriction-patterns of mtDNAs from *N. bigelovii, N. undulata*, one of the parent-lines (B-C-1-2) and a second-generation fertile cybrid (G-2-1-1 (u)). MtDNA was digested with *Sal*I and stained by ethidium bromide.

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. 4,B shows part of this data, other data not shown).

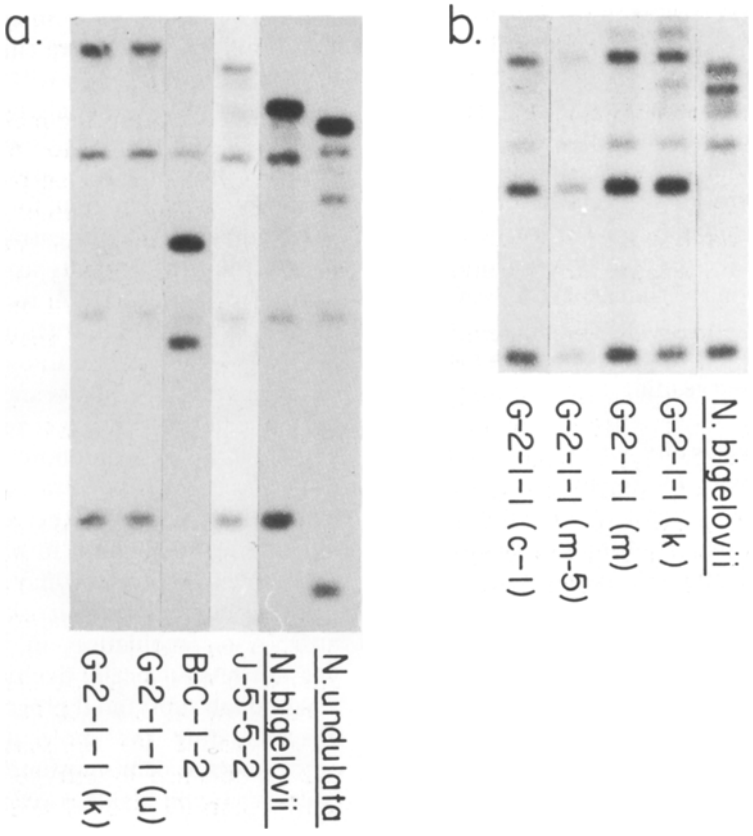

Fig. 4. Southern-blot-hybridization of mtDNAs. a. *N. undulata* and *N. bigelovii*, the two CMS parental lines J-5-5-2 and B-C-1-2 as well as two second-generation fertile cybrids G-2-1-1 (u) and G-2-1-1 (k); mtDNA was digested with *pst*I and hybridized with *pmtSylSa*-2. b. *N. bigelovii*, two second-generation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

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. 4,B). 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 leads to restoration of male fertility.

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