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Modifications of floral development in tobacco induced by fusion of protoplasts of different male-sterile cultivars

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Summary. Protoplasts derived from different cytoplasmic male-sterile cultivars of *Nicotiana tabacum* were fused. Nearly 200 cybrid calli were regenerated into plants and their flower morphologies were examined. Most cybrids exhibited parental-type male-sterile morphologies. Some, however, showed novel male-sterile phenotypes or phenotypes which combined traits of both male-sterile parents in a new combination. Others were restored to fertility, with stamens which produced functional pollen.

Key words: Male sterility – Cybrid – Tobacco – Somatic hybridization

Introduction

Male-sterile cultivars of tobacco are obtained through interspecific crosses within the genus *Nicotiana* (Gerstel 1980). Backcrossing the hybrid plant with tobacco as the pollen parent and repeating the backcross for several generations yields progeny in which the cells contain nuclei with genetic material of tobacco and cytoplasmic organelles of the maternal species. Male sterility results from the interaction of the tobacco chromosomes with a foreign cytoplasm and is maternally inherited. Correlative evidence suggests that the male sterility trait in *Nicotiana* is associated with the mitochondrial genome (Belliard et al. 1978; Gleba 1978; Aviv and Galun 1980; Glimelius et al. 1981) rather than the chloroplast genome.

There are several different male-sterile tobacco cultivars which differ in the species origin of the cytoplasmic organelles. Each male-sterile cultivar has a flower morphology that is unique from other male-sterile cultivars and different from the parental species from which it originated. Both stamens and petals may be affected in a specific male sterility type. For example, stamens may be absent, arrested at a particular stage of development, or may form structures resembling petals or stigmas. The petals may not fuse normally or may fuse but be shortened so that the pistil protrudes from the petals. Because of the variety of developmental abnormalities which appears in the different male-sterile tobacco cultivars, Rosenberg and Bonnett (1983) hypothesized that the different morphological manifestations of male sterility in these cultivars reflect developmental dysfunctions at one of several specific stages in stamen development and that two or more cytoplasmic genes are involved.

Somatic cell fusion makes possible the transfer of cytoplasmic male sterility from one species to another (Aviv et al. 1980) and even from one genus to another (Pelletier et al. 1983). Since somatic cell fusion allows the combination of cytoplasms of two species in one cell, it provides an opportunity for reassortment and recombination of the cytoplasmic genes associated with cytoplasmic male sterility (Belliard et al. 1978; Pelletier 1986).

Be using somatic cell fusion of male-sterile cultivars of tobacco, we have investigated the cytoplasmic control of male sterility and the interaction of cytoplasmic genes involved in it. We describe the morphologies of the cybrid plants and compare them to their male-sterile parents. The parents were selected to show distinctly different alterations in stamen and petal morphology.

Materials and methods

Plant material. For the fusion experiments we used male-sterile cultivars of *Nicotiana tabacum* L. with cytoplasms from *Nicotiana sauveolens* Lehm [referred to as Nta(sua)S, according to the nomenclature proposed by Gerstel (1980) and Lonsdale and

Leaver (1988), in which 'Nta' designates genus and species of the nucleus, 'sua' designates cytoplasmic origin, and 'S' indicates male sterile], from *Nicotiana bigelovii* L. (Nta(big)S) and *Nicotiana undulata* Vent. (Nta(und)S).

Shoot cultures. Seeds from Nta(sua)S, Nta(big)S, and Nta(und)S were germinated and grown *in vitro* on MS medium (Murashige and Skoog 1962) with 1% sucrose 0.9% Noble Agar, no hormones, and under continuous light at 25 °C. The seedlings were grown into plants, and shoot cultures were established by excising the apical 1 cm of a shoot and transferring it to fresh medium. Every 4 weeks shoot apices were transferred to fresh medium.

Cell suspensions. Suspension cultures were established from callus derived from leaf explants taken from aseptic shoot cultures. The cell suspensions were grown at 27 °C on a rotary shaker (100 rpm) in continuous darkness in a modified ammonium nitrate medium (Müller and Grafe 1978) containing MS salts (Murashige and Skoog 1962) with 30 m*M* NH_4NO_3 and 2.5 m*M* KH_2PO_4. The cells were subcultured every 4 days.

Protoplast isolation. Mesophyll protoplasts were prepared from leaf tissue of aseptic shoot cultures 3–4 weeks after transfer. The leaves were cut, pretreated with 0.3 *M* sorbitol plus 0.05 *M* $CaCl_2 \cdot 2H_2O$ (Glimelius et al. 1978) for 1 h, and digested in K_3 medium (Nagy and Maliga 1976) containing 0.4 *M* sucrose, 1 mg/l NAA, 0.1 mg/l 2,4-D, 0.2 mg/l BAP, 1.2% cellulysin (Calbiochem), and 0.12% macerase (Calbiochem). Suspension cells were pretreated in the same solution used for mesophyll cells for 20 min, filtered, and treated for 2.5 h with 0.8% driselase (Kyowa Hakko Kogyo Co. Ltd.), 1.6% cellulysin, and 0.16% macerase dissolved in K_3 medium as for the isolation of mesophyll protoplasts. The isolation of cell suspension protoplasts was facilitated by rocking the cells gently (about 2 rocks per min).

Protoplast fusion. Protoplasts were separated from debris as described by Glimelius et al. (1978) and collected by adding an equal volume of a solution containing 50% Percoll (v/v) and 50% 0.6 *M* sorbitol plus 0.1 *M* $CaCl_2 \cdot 2H_2O$. After centrifugation (50 g for 5 min), the protoplasts were resuspended in W_5 (Menczel et al. 1981). Mesophyll protoplasts were irradiated with X-rays for 25 min (total dosage of 125 gy) with a Siemens stabilipan 200 apparatus. The tube Tr 200f was operated at 180 kV, 10 mA, and the radiation was filtered through 4 mm Al. The irradiated protoplasts were treated for 30 min at 8 °C. Then both the suspension and the mesophyll protoplasts were centrifuged at 100 g, suspended in a few drops of W_5, and combined in a proportion of 1:2, respectively. Protoplasts were fused with polyethylene glycol (Sundberg and Glimelius 1986) according to a slightly modified procedure of Hein et al. (1983). After a 6-min incubation time in polyethylene glycol, 3 ml $CaCl_2$ solution (0.1 *M* $CaCl_2$, 0.1 *M* sorbitol, pH 7.0) were added over a period of 20 min and the fusion sites were left undisturbed for another 10 min. The protoplasts were cultured in T_0 medium (Caboche 1980) containing 1 mg/l NAA and 0.5 mg/l BAP in darkness at 25 °C until cybrid selection.

Cybrid selection. The cybrids were selected during the period of 16–40 h after fusion. They were isolated with a micropipette operated with a Leitz micromanipulator; we chose only the cells that unambiguously displayed chloroplasts, originating from the mesophyll cell protoplasts, and transvacuolar strands, originating from the suspension cell protoplasts.

Cybrid culture and plant regeneration. Approximately 20 cybrid cells were cultured together in a microwell (Falcon 3072) with 20 µl 8 PM medium (Kao and Michayluk 1975, modified by Glimelius et al. 1986) containing 0.1 mg/l NAA and 0.5 mg/l BAP in darkness at 27 °C. After 3 days they were fed with 12 µl 8 PM medium and then fed with additional 20 µl at weekly intervals. When small cell colonies were obtained, they were separated from each other and plated into bigger microwells (Costar 3548) on K_3 medium containing 0.1 *M* sucrose, 1 mg/l NAA, 0.2 mg/l BAP, and 0.13% Agarose. When the colonies had grown into small calli, they were transferred to the modified ammonium nitrate medium containing 7 g/l Noble Agar. From then on procedures described in Glimelius and Bonnett (1981) were followed to regenerate plants to the reproductive stage in the greenhouse.

Anther culture. Anther cultures from buds of the male-fertile plants were established according to the procedures described by Johansson et al. (1982) using Medium H (Nitsch and Nitsch 1969).

Results

As fusion parents three cytoplasmic male-sterile (CMS) tobacco cultivars were chosen. The morphologies of these cultivars are summarized in Table 1 and shown in Figs. 1a and e, and 2a. They were fused in two different pairwise combinations. In the first combination, protoplasts of Nta(big)S were fused with protoplasts from Nta(sua)S. In the second combination, protoplasts from Nta(big)S were fused with protoplasts from Nta(und)S.

Table 1. Description of floral structures in the parental CMS cultivars

CMS cultivar	Corolla		Stamens	Pistil length
	Length[a]	Form		
Nta(big)S	normal	split	filaments with sterile, flattened, fringed ends	normal
Nta(sua)S	normal	fused	absent or reduced to remnant	variable, frequently up to 1 cm shorter
Nta(und)S	shortened by 1–1.5 cm	fused	petalodes, no filaments, occasionally tipped with stigmatoids	normal (protruding from corolla 1–1.5 cm)

[a] In comparison to male-fertile *N. tabacum*

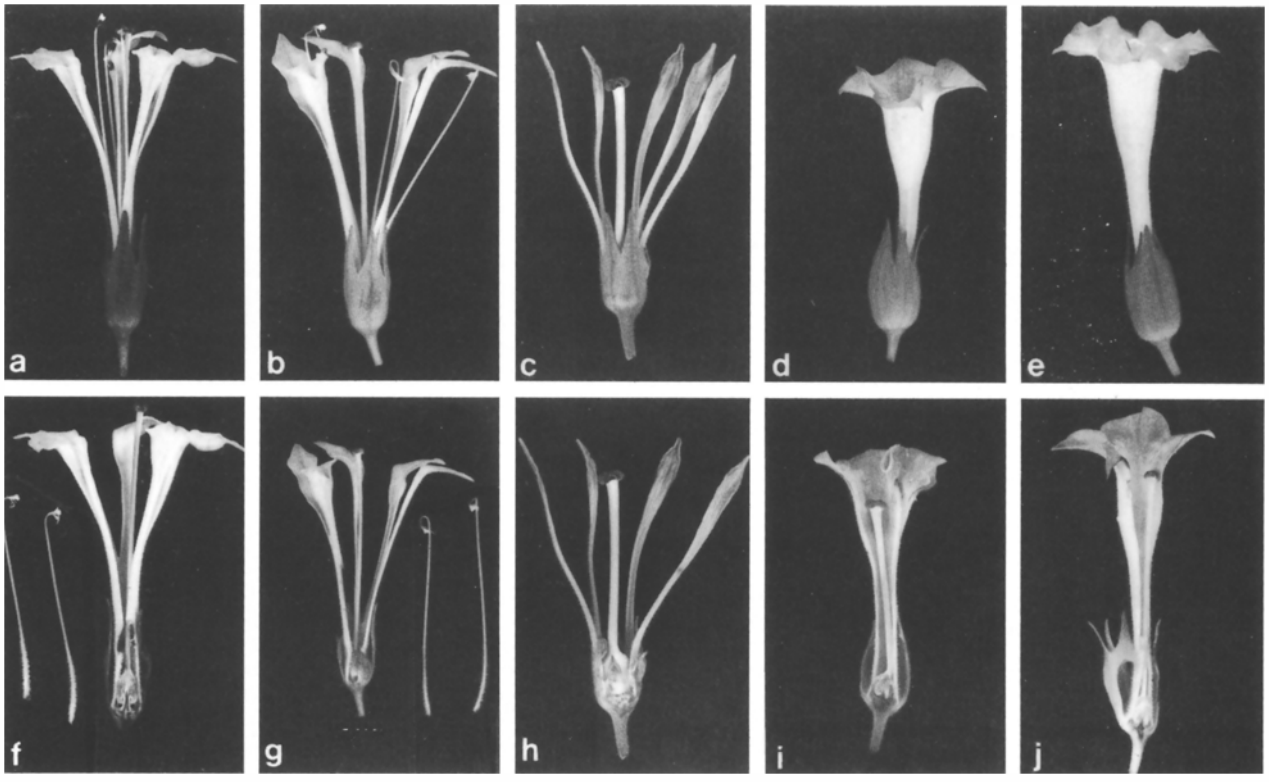

Fig. 1 a–j. Parental and cybrid flowers belonging to Groups I and II. **a** Flower of Nta(big)S. The corolla is tubular only at the base, distally split to form five narrow petals. **b** Parental-type cybrid flower resembling parent Nta(big)S (Group I). **c** Cybrid flower (Group II) displaying a recombined biparental phenotype. **d** Parental-type cybrid flower of Nta(sua)S (Group I). **e** Flower of the Nta(sua)S.The corolla is sympetalous, tubular, with five petal lobes. **f** Dissected flower of Nta(big)S. Stamens have phenotypically normal filaments, however, the anther is altered to a sterile flat structure with a fringed tip. **g** Dissected parental-type cybrid flower. The corolla and stamens are identical to parent Nta(big)S. **h** Dissected recombined biparental cybrid flower. The corolla is split and narrow as in Nta(big)S parent. Stamens are absent as in the Nta(sua)S parent. **i** Dissected parental-type cybrid flower. The corolla is identical to parent Nta(sua)S. Stamens are absent as in Nta(sua)S. **j** Dissected flower of Nta(sua)S, showing the absence of stamens

Table 2. Distribution of cybrids among the four phenotypic classes

Somatic cross			Cybrid groups			
			I Parental	II Recombined biparental	III Novel male-sterile	IV Fertile
Nta(sua)S	(+)	Nta(big)S	137	32	(−)	(−)
Nta(big)S	(+)	Nta(und)S	18	(−)	5	4

The plant material used in these fusions is subsequently designated as parental.

Several morphological features were examined to characterize the floral phenotype of the mature cybrids. These were stamen morphology, i.e., the presence and structure of anther and filament; and corolla morphology, i.e., the degree to which the corolla was split, its shape and length, and its length in comparison to style length. On the basis of these traits, the cybrids were classified into four groups (Table 2). Group I consisted of male-sterile phenotypes in which the stamen and corolla of the cybrids were identical to those of one or the other parent. Of all cybrids, 78% belonged to this group. Group II cybrids were classified as recombined biparental phenotypes, i.e., the stamens were identical to one parent, whereas the corolla was identical to the other parent. Of the cybrid plants recovered from the two fusion combinations, 17% exhibited this morphology. Group III was composed of novel male-sterile phenotypes, i.e., the cybrids had stamens which were completely different from

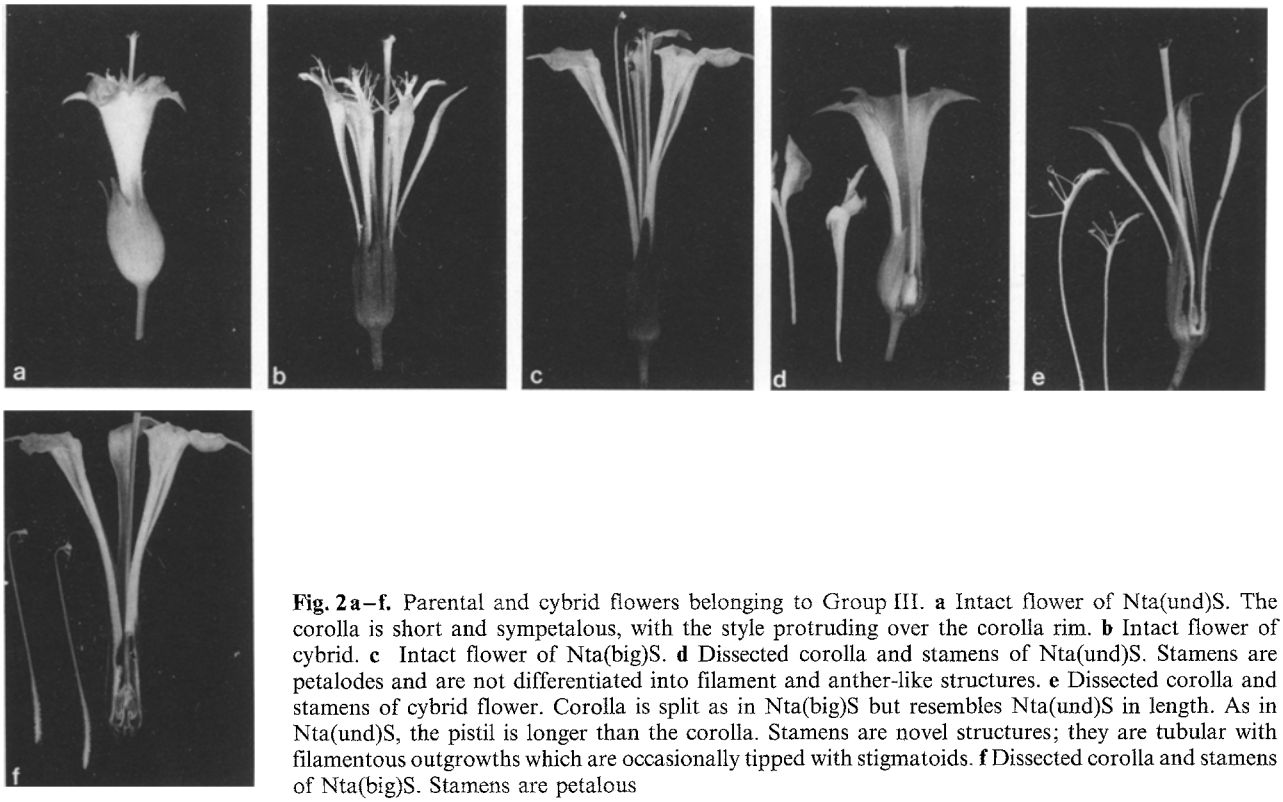

Fig. 2a–f. Parental and cybrid flowers belonging to Group III. **a** Intact flower of Nta(und)S. The corolla is short and sympetalous, with the style protruding over the corolla rim. **b** Intact flower of cybrid. **c** Intact flower of Nta(big)S. **d** Dissected corolla and stamens of Nta(und)S. Stamens are petalodes and are not differentiated into filament and anther-like structures. **e** Dissected corolla and stamens of cybrid flower. Corolla is split as in Nta(big)S but resembles Nta(und)S in length. As in Nta(und)S, the pistil is longer than the corolla. Stamens are novel structures; they are tubular with filamentous outgrowths which are occasionally tipped with stigmatoids. **f** Dissected corolla and stamens of Nta(big)S. Stamens are petalous

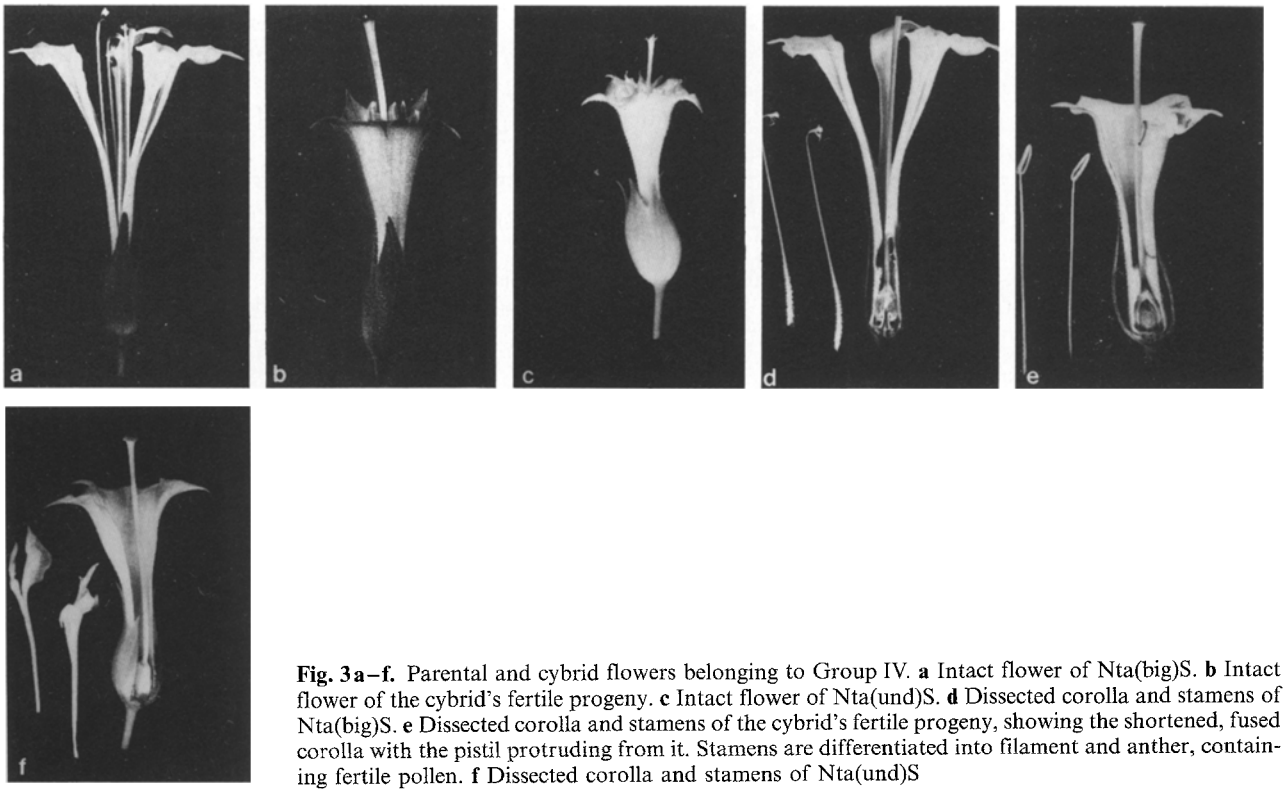

Fig. 3a–f. Parental and cybrid flowers belonging to Group IV. **a** Intact flower of Nta(big)S. **b** Intact flower of the cybrid's fertile progeny. **c** Intact flower of Nta(und)S. **d** Dissected corolla and stamens of Nta(big)S. **e** Dissected corolla and stamens of the cybrid's fertile progeny, showing the shortened, fused corolla with the pistil protruding from it. Stamens are differentiated into filament and anther, containing fertile pollen. **f** Dissected corolla and stamens of Nta(und)S

either parent and a corolla which was biparental. About 3% of the cybrids belonged to this group. Cybrid plants representing Group IV produced stamens with fertile pollen and a corolla which was of parental type. Of the cybrid plants, 2% produced stamens containing fertile pollen.

Figure 1 illustrates the flowers of Nta(sua)S, Nta(big)S, and three cybrids, classified as members of the first and the second group. The cybrids illustrated as representative of Group I were identical to parent Nta(big)S (Fig. 1 b and g) or Nta(sua)S (Fig. 1 d and i), respectively. The cybrid illustrated as a representative of Group II had a corolla that was split as is found in Nta(big)S, yet the stamens were missing as is found in Nta(sua)S (Fig. 1 c and h).

Cybrids in Group III, the novel male-sterile phenotypes, exhibited features not found in either parent. The cybrid shown in Fig. 2 b originated from a fusion between Nta(und)S (Fig. 2 a) and Nta(big)S (Fig. 2 c). The stamens of the cybrid, shown in Fig. 2 e, resembled neither the flat-feathery structure of Nta(big)S (Fig. 2 f) nor the petalode structure of Nta(und)S (Fig. 2 d). The stamens were filamentous at the base and tubular towards the tip with filamentous outgrowths from the adaxial surface. These outgrowths were occasionally tipped with green stigmatoids. The corolla of this cybrid was biparental. In length it resembled Nta(und)S, with the style protruding from the corolla rim. However, the corolla was split to the base of the tube, resembling the Nta(big)S parent.

A Group IV flower is shown in Fig. 3 b and e. Among the cybrids, four plants were placed in Group IV. Two cybrids produced anthers with fertile pollen; the other two were not fertile but produced progeny which were fertile. All four originated from the parental combination of Nta(big)S (+) Nta(und)S. Pollen production by these plants was less than normal and the anthers did not dehisce. By hand pollination, pollen from these plants successfully fertilized Nta(big)S. Seeds were germinated into plants which had the CMS phenotype typical for Nta(big)S. Haploid anther plantlets were also obtained from several of the cybrid's progeny. Although anthers with fertile pollen were present, the corolla characteristics of the cybrid's progeny were those of Nta(und)S with a shortened, fused corolla, shallow lobes, and pistil protruding from the corolla rim.

Discussion

By fusing different CMS cultivars of *Nicotiana tabacum*, new floral phenotypes have been obtained. These include male-fertile types, floral types with novel stamen structures, and flowers characterized as recombined biparental phenotypes. However, most of the cybrid plants exhibited a parental male-sterile phenotype. Among these cybrids, no evidence was found that sterility factors were present in a mixed state after the early stages of colony formation, since all flowers on a cybrid plant exhibited the same phenotype, and since no variation in phenotype was found among as many as 10–15 plants derived from the same cybrid callus. Furthermore, no variation in phenotype was found among the progeny of the subsequent sexual generations.

When Nta(big)S protoplasts were fused with Nta (sua)S protoplasts, cybrids were produced in which the corolla type of Nta(big)S was combined with the stamen type of Nta(sua)S. This recombined biparental phenotype continued to be expressed after two subsequent sexual generations. To achieve this phenotpye, genetic information from both sterility phenotypes must be present in the cybrid plants and their progeny.

The appearance of cybrids with new flower types agrees with the experiments of Belliard et al. (1978) in which they fused male-fertile and male-sterile cultivars of tobacco. The new flower types they described were also inherited in a maternal fashion (Belliard et al. 1979). On the basis of their studies, Pelletier (1986) concluded that there is more than one difference between the fertile and sterile mitochondrial genomes affecting male sterility. The recombined biparental cybrids resulting from the fusion of two CMS tobacco cultivars clearly demonstrate that two independent mitochondrial genes affect flower development, since stamen and petal features were expressed independently of each other in these cybrids.

When Nta(big)S and Nta(und)S were fused, some of the male-sterile cybrids exhibited stamen structures which were not found in either parent. In these cybrids the corolla showed biparental inheritance, exhibiting the split trait of Nta(big)S and the shortened trait of Nta (und)S. Thus, the corolla traits, length and adnation, appear to be regulated by different mitochondrial genes.

Both the novel male-sterile cybrids and the recombined biparental cybrids support the conclusion that the mitochondria brought together by cell fusion interacted. This interaction was particularly apparent in Group IV cybrids, in which male fertility was restored. Aviv and Galun (1986) described a fusion experiment in which a single fertile cybrid was obtained after fusion of CMS *N. tabacum* with CMS *N. sylvestris* cells. Rearranged mtDNA was found in this plant, suggesting to the authors that restoration to fertility was the result of recombination of mtDNA. In our experiments, fertility was only restored in cybrids when Nta(und)S protoplasts were fused with Nta(big)S protoplasts. These plants continued to produce anthers with pollen in subsequent sexual generations. No fertile cybrids were obtained when Nta (sua)S protoplasts were fused with Nta(big)S protoplasts. The finding of several cybrids which produce anthers with functional pollen provides further evidence

that the mitochondrial genomes of male-sterile cultivars can interact to achieve restoration of fertility.

These experiments indicate that the genetic traits responsible for each particular alloplasmic CMS phenotype in tobacco can be brought together to produce different combinations of sterility traits and, more strikingly, they can be reassociated to create new, heritable CMS phenotypes. Thus, these experiments indicate a strong mitochondrial involvement in floral development in tobacco. Stamens produce filaments and anthers if they develop in a compatible nuclear-mitochondrial environment. By altering the parental mitochondrial constitution through cybridization, a variety of floral structures was created, including structures not previously seen in CMS lines of tobacco.

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