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Restoration of normal stamen development and pollen formation by fusion of different cytoplasmic male-sterile cultivars of *Nicotiana tabacum*

Waltraud Kofer[1,2], **Kristina Glimelius**[2,*], **and Howard T. Bonnett**[1]

[1] Department of Biology, University of Oregon, Eugene, OR 97403, USA
[2] Department of Plant Breeding, Swedish University of Agricultural Sciences, S-75007 Uppsala, Sweden

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Summary. Fusion of two cytoplasmic male-sterile cultivars of *Nicotiana tabacum*, one with *N. bigelovii* cytoplasm and one with *N. undulata* cytoplasm, resulted in the restoration of male fertility in cybrid plants. All male-fertile cybrids exhibited fused corollas, which is characteristic for the cultivar with *N. undulata* cytoplasm, while their stamen structures varied from cybrid to cybrid, some producing stamens with anthers fused to petal-like appendages and one producing stamens of a normal appearance for *N. tabacum*. Restriction enzyme digestion and agarose gel electrophoresis of mitochondrial DNA showed that mitochondrial DNA of the fertile cybrids was more similar to the male-sterile cultivar with the cytoplasm of *N. undulata* than to the cultivar with *N. bigelovii* cytoplasm. Some restriction fragments were unique to the male-fertile cybrids. Comparisons between stamen structure and mitochondrial DNA for eight fertile progeny from one cybrid plant led to the identification of several restriction fragments that appeared at enhanced levels in connection with normal stamen development.

Key words: Cybrid – Cytoplasmic male sterility – Mitochondrial DNA – *Nicotiana tabacum*

Introduction

Cytoplasmic male sterility (CMS) in *Nicotiana tabacum* frequently results when interspecific hybrids are backcrossed several times with *N. tabacum* as the pollen parent. The large number of *Nicotiana* cytoplasms which cause male sterility in *N. tabacum* is extraordinary; comparable numbers of male-sterile cultivars are found in only one other genus, *Solanum* (Kaul 1988). Most male-sterile cultivars of *N. tabacum* can be distinguished from each other by their distinctive patterns of floral development (Chaplin 1964; Kaul 1988). The availability of these distinctive cultivars renders *N. tabacum* a favorable material to study cytoplasmic control of floral development.

Somatic cell fusion is an ideal method to study CMS since cell fusion permits organellar interactions to occur, including the recombination and reassortment of cytoplasmic genes (Galun et al. 1982; Boeshore et al. 1983; Chetrit et al. 1985; Morgan and Maliga 1987; Ozias-Akins et al. 1987). Fusion experiments between male-sterile and male-fertile protoplasts of *N. tabacum* and also of other *Nicotiana* species have demonstrated that CMS can be transferred from one plant to another (Belliard et al. 1978; Fluhr et al. 1983; Pelletier et al. 1983). Furthermore, cell fusion can lead to floral phenotypes intermediate between the parental ones (Belliard et al. 1979; Asahi et al. 1988). Molecular analysis of those hybrids and cybrids which exhibited floral characteristics different from their parents revealed mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs), when compared to the parental patterns. Besides fragments from either parent, novel fragments were also found. Mapping experiments with *Petunia* and *Brassica* mtDNA showed that novel mtDNA fragments, present in the mtDNA pattern of somatic hybrids, resulted from recombination events between the parental mitochondrial genomes (Rothenberg et al. 1985; Vedel et al. 1987).

In a previous publication (Kofer et al. 1990) we described experiments in which protoplasts of phenotypically different cytoplasmic male-sterile cultivars of *N. tabacum* were fused. Several traits associated with male sterility, such as corolla length, corolla adnation, and

* To whom correspondence should be addressed

stamen morphology, were found to be inherited independently from each other, from which we concluded that two or more mitochondrial genes regulate floral development. The work described here is consistent with this conclusion, since fusion of appropriate male-sterile *N. tabacum* cultivars gives rise to fertile cybrids, presumably through the acquisition by their mitochondria of functional genes from both parental mitochondrial genomes.

Materials and methods

Plant material

Sexually produced cytoplasmic male-sterile cultivars of *Nicotiana tabacum* L. with cytoplasms of *Nicotiana bigelovii* L. or *Nicotiana undulata* Vent. were utilized in the fusion experiments. Seeds of the cultivar with *N. bigelovii* cytoplasm were donated by E. A. Wernsmann, North Carolina State University, Raleigh, NC, USA. The origin of the cultivar with *N. undulata* cytoplasm has been described in Bonnett and Glimelius (1983). These cultivars are referred to as 'Nta(big)S' and 'Nta(und)S' (Kofer et al. 1990).

Shoots were grown at 25 °C under continuous light on MS salts and vitamins (Murashige and Skoog 1962) supplemented with 1% sucrose and 0.9% Noble Agar. Shoot cuttings were transferred to fresh medium every 4th week.

Cell suspensions were established from leaves of shoot cultures. The suspension cultures were maintained on a rotary shaker (100 rpm) on a medium modified from Müller and Grafe (1978) as described in Kofer et al. (1990). The cells were subcultured every 4th day.

Protoplast isolation, fusion and plant regeneration

The procedures were identical to the ones previously reported (Kofer et al. 1990). Fusions were carried out between irradiated mesophyll cell protoplasts of 'Nta(big)S' and suspension cell protoplasts from 'Nta(und)S'. The fusion products were isolated with a micropipette controlled by a micromanipulator and cultured separately from the parental cells.

Mitochondrial DNA analysis

For the isolation, digestion, and electrophoresis of mtDNA the procedures of Bland et al. (1985) were followed as modified by Håkansson et al. (1988).

Results

Morphology of the male-sterile cultivars

Male-sterile *N. tabacum* cvs 'Nta(big)S' and 'Nta(und)S' vary considerably from each other in stamen and corolla features. 'Nta(big)S' possesses stamen filaments, but instead of an anther it produces a flat, light-pink structure with fringed tips. The petals of the 'Nta(big)S' corolla are only fused at the base; corolla and pistil are of approximately the same length. In contrast, 'Nta(und)S' does not develop normal stamen filaments; instead of stamens it has pink petalodes, narrow at the base and broadened at the tip. Anther sacs are occasionally attached to the

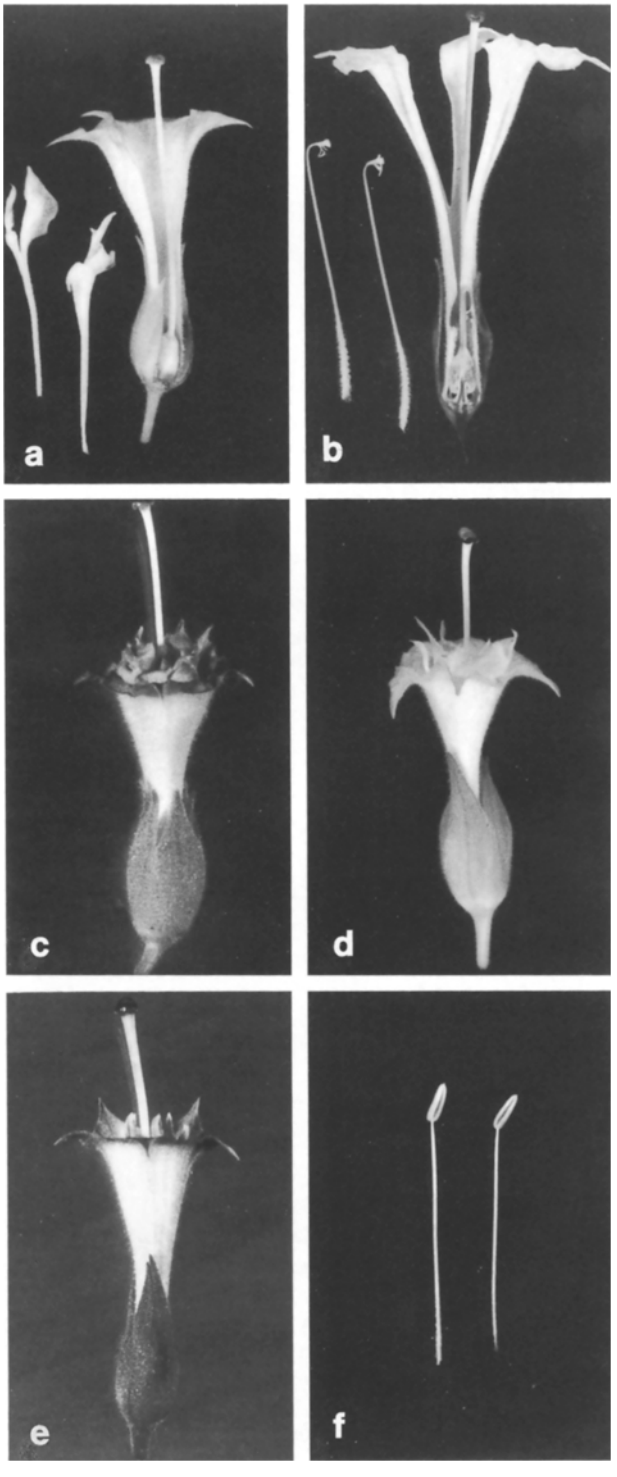

Fig. 1 a–f. Fusion parents and cybrids from callus 29. **a** Corolla and petaloid stamens of 'Nta(und)S'. The flower has been bisected longitudinally and the stamens removed. Note that the petals are fused but shortened relative to the style. **b** Corolla and stamens of 'Nta(big)S'. The flower has been bisected longitudinally and stamens removed. Note that the corolla is split and the stamens have feathery appendages on the tops of the filaments. **c** Flower of cybrid plant 29–3. **d** Flower of cybrid plant 29-1. **e** Intact flower of fertile progeny of cybrid 29-3. **f** Stamens of fertile flower shown in **e**

Fig. 2. A pair of stamens from cybrid 64. *Arrows* indicate anthers

Fig. 3. Stamen of cybrid 94. In this cybrid the anthers were attached in the middle with both ends folded backwards

Table 1. Stamen characteristics of fusion parents and cybrids

Fusion parents/cybrids	Filaments free from petalodes	Petaloid stamens	Anthers with pollen
Nta(big)S	+	−	−
Nta(und)S	−	+	−
64/3	−	+	Cybrid
94-3	+	−	Cybrid
29-1	−	+	Cybrid progeny
29-3	+	−/+	Cybrid progeny
31-3	−	+	Cybrid

−/+ indicates variations among individual flowers

petalodes, but they do not contain pollen. 'Nta(und)S' corollas are sympetallous, bell-shaped, and short, with the style protruding up to 1.5 cm from the corolla. Flowers of 'Nta(und)S' and 'Nta(big)S' are shown in Fig. 1 a, b.

Morphology of the fertile cybrids

Cybrids grown from calli exhibited a variety of phenotypes that were all categorized according to their stamen and corolla features into three different groups. These were parental, novel male-sterile, and male-fertile cybrids. Parental cybrids exhibited the stamen and corolla features of the 'Nta(und)S' parent; novel male-sterile cybrids showed the corolla features of either parent and stamen morphologies different from both parents, while

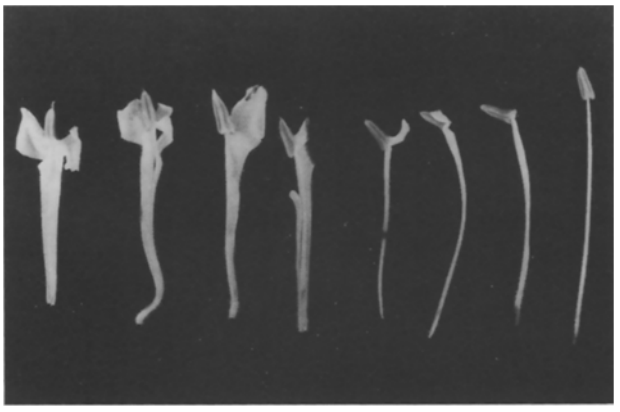

Fig. 4. Stamens produced by progeny from 29-1 and 29-3 cybrids, representing a continuum from anthers fused to a large petaloid structure (stamens to left) to anthers free from petaloid tissue and attached to a normal filament (last stamen to the right)

all male-fertile cybrids had fused petals similar to 'Nta(und)S' and stamens with anthers containing pollen. Of 26 calli, 22 gave male-sterile plants, 18 were of the parental phenotype and 4 were novel phenotypes. Four calli gave male-fertile plants; 1 of the 4 gave both fertile plants and novel male-sterile plants. This report focuses on the male-fertile cybrids obtained from the 4 calli, 29, 31, 64, and 94. Table 1 lists the stamen characteristics of five male-fertile cybrids obtained from these 4 calli. All cybrids produced flowers with pollen-bearing stamens and fused petals. Stamens of the different cybrid flowers are shown in Fig. 1 f, Fig. 2, and Fig. 3.

Callus 29 developed two sterile plants (cybrids 29-1 and 29-3, Fig. 1 c, d) that resembled 'Nta(und)S' except that their petaloid stamens were more voluminous, imparting a roseate appearance to the flowers. These cybrids were pollinated with *N. tabacum* pollen, seeds were collected, and 15 progeny plants from each cybrid were grown to flowering. All progeny of both cybrids produced stamens with anthers containing pollen. Although the anthers did not dehisce, self-pollination of 29-3 progeny was possible by removing pollen from the anthers and applying it to the stigma. All the progeny of cybrid 29-1 had some petalodes fused to the pollen-bearing anthers, whereas, among the progeny of cybrid 29-3, 3 plants had flowers in which all five stamens lacked petalodes (Fig. 1 e, f). These male-fertile stamens appeared identical to those found in *N. tabacum*. The various types of stamens found among the progeny of 29-1 and 29-3 cybrids are shown in Fig. 4. In general, stamens of 29-1 progeny plants had more voluminous petalodes fused to the anthers than stamens of 29-3 progeny plants.

Calli 31, 64, and 94 produced cybrids which, upon flowering, developed stamens with petalodes and anthers with pollen. Thus, these cybrids did not require a second generation to produce pollen.

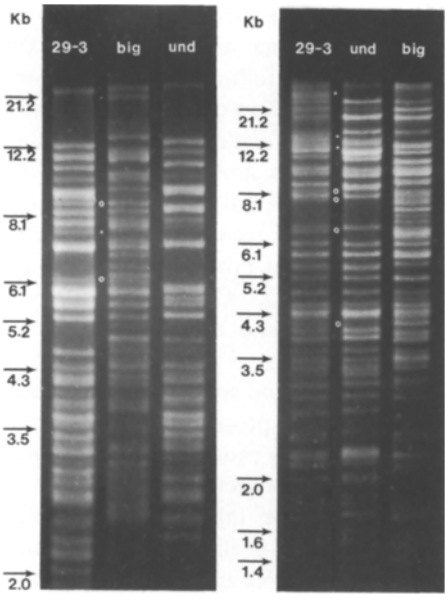

Fig. 5. MtDNA restriction fragments of 'Nta(big)S', labeled *big*, 'Nta(und)S', labeled *und*, and progeny of cybrid 29-3. MtDNA was restricted with HindIII (left) and PvuII (right). Both enzymes clearly distinguish between the mtDNA of 'Nta(big)S' and 'Nta(und)S'. The 29-3 mtDNA shares more fragments with 'Nta(und)S' than 'Nta(big)S'. The symbol o represents a fragment comigrating with 'Nta(big)S' only; the * represents a fragment that is novel (not visible in either parental mtDNA)

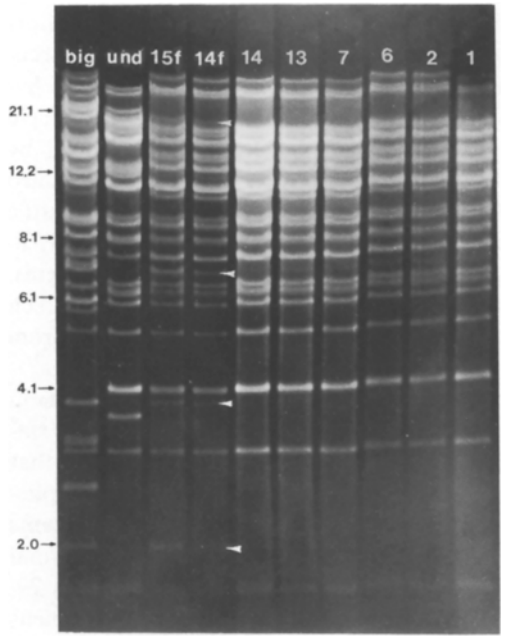

Fig. 6. MtDNA from 'Nta(big)S', 'Nta(und)S', and progeny of cybrid 29-3, digested with BglI. The lanes are designated as follows (left to right): *'Nta(big)S'*, *Nta(und)S'*, *15f* and *14f* (plants with normal-appearing stamens), *14, 13, 7, 6, 2,* and *1* (plants with petaloid stamens). The four fragments marked by *arrows* are present in higher quantity in cybrids with normal-appearing stamens

Analysis of mtDNA

MtDNA was isolated from 'Nta(big)S', 'Nta(und)S', and progeny from cybrids 29-1 and 29-3. MtDNA from the two male-sterile cultivars had restriction enzyme fragment sizes which were sufficiently different (Figs. 5 and 6) that RFLPs of cybrid mtDNA could be used to indicate the relationship of cybrid mtDNA to the parental cultivars. The progeny of cybrids 29-1 and 29-3 shared a majority of restriction fragments with the 'Nta(und)S' cultivar. However, some restriction fragments comigrated with fragments that were present in 'Nta(big)S' and absent in 'Nta(und)S', including at least four fragments in the PvuII digest and two in the HindIII digest for 29-3 progeny (Fig. 5). Novel fragments were found in both cybrid progeny with most of the restriction enzymes tested.

Five progeny of the 29-1 cybrid were compared, and all of them gave identical patterns of mtDNA using three different restriction enzymes. In contrast, differences were detected among progeny of the 29-3 cybrid. To further characterize these differences, mtDNA was isolated from plants obtained by pollinating flowers with normal-appearing stamens and flowers with petaloid stamens. MtDNA comparisons were of three types: (1) a plant with flowers, all of which had normal-appearing stamens, was compared to plants with flowers, all of which had petaloid stamens; (2) inflorescence branches bearing flowers, all of which had normal-appearing stamens, were compared to inflorescence branches on the same plant bearing flowers, all of which had petaloid stamens; and (3) a plant which first produced flowers, all of which had petaloid stamens, was compared to the same plant which later produced flowers, all of which had normal-appearing stamens.

The mtDNA from plants derived from flowers which had normal-appearing stamens showed four fragments when digested with BglI, which were present in much lower levels in plants derived from flowers that had petalodes attached to the anthers (Fig. 6, arrows). Figure 6 includes examples of the first (15 contrasted with 13, 7, 6, 2, and 1) and second comparison (14f contrasted with 14). The increase in the levels of these four fragments was consistently correlated with the absence of petalodes in all three types of comparisons. Digests with PstI and PvuII confirmed the results obtained with BglI in that the progeny of cybrid 29-3 differed from each other depending on whether their flowers had normal-appearing stamens or petaloid stamens.

Discussion

Plants were regenerated from 26 cybrid calli obtained from the fusion of 'Nta(big)S' and 'Nta(und)S'. Four of

the calli yielded cybrids capable of more normal stamen development than either male-sterile cultivar, including the ability to form pollen. We believe that the restoration of normal patterns of stamen development and fertility in the cybrids was a result of changes in mtDNA, and not due to nuclear restoring factors. Both 'Nta(big)S' and 'Nta(und)S' were derived from sexual crosses and have *N. tabacum* nuclear chromosomes with *N. undulata* or *N. bigelovii* cytoplasmic organelles. Conceivably, some *N. bigelovii* or *N. undulata* chromosomal material remains from the alloplasmic crosses that led to 'Nta(big)S' or 'Nta(und)S'. If small amounts of *N. undulata* nuclear material could restore 'Nta(big)S', or vice versa, this could account for the restoration of fertility observed in our cybrid materials. However, this mode of restoration is contradicted by the investigations of Reed and Burns (1986), who were unable to restore either 'Nta(und)S' with *N. bigelovii* chromosomes or 'Nta(big)S' with *N. undulata* chromosomes. Moreover, when 'Nta(big)S' was pollinated with pollen from 29-3 progeny, and the plants grown to maturity, the flowers of these plants were indistinguishable from the 'Nta(big)S' parent. No degree of restoration was observable. Furthermore, if there were a nuclear restorer present in the parental male-sterile cultivars, it would be heterozygous following pollination of the 29-3 cybrid plants with *N. tabacum* pollen. The male-sterility phenotype should then reappear in some plants of the next generation, however no parental male-sterile phenotypes were observed in the progeny of the fertile plants. Therefore, we conclude that the restoration to male fertility did not result from nuclear restoring factors.

Progeny of callus 29 which were fertile had fused petals of the 'Nta(und)S' type, whereas progeny which were male-sterile had split corollas. Belliard et al. (1978) conducted an experiment in which they fused male-sterile cv 'Nta(big)S' with male-fertile *N. tabacum*. All of the hybrid plants that inherited the split corolla were male sterile; all of the fertile plants recovered from the same fusion combination exhibited fused petals. In a subsequent analysis of progeny from Belliard et al.'s experiments, a correlation was found between the degree of fusion of the petals and the extent of normal stamen development (Pelletier 1986). Plants which produced flowers with more deeply split corollas produced distorted anthers containing stigmatoid tissues. In fusion experiments between irradiated *N. debneyi* and *N. tabacum* (Asahi et al. 1988), cybrids which produced a limited amount of fertile pollen had fused corollas and cybrids which produced no pollen had split corollas.

Reed and Burns (1986) showed a correlation between corolla fusion and fertility based on their results from sexual hybridization experiments. When *N. tabacum* was crossed with *N. debneyi*, then backcrossed with a restored line with *N. bigelovii* cytoplasm, the restoration of fertility coincided with fusion of the petals. The same phenomenon could be observed when *N. tabacum* was sexually crossed with *N. bigelovii* followed by backcrosses with a restored line with *N. bigelovii* cytoplasm. Sand and Christoff (1973) obtained four distinct male-sterile phenotypes from interspecific sexual crosses between *N. debneyi* and *N. tabacum* (pollen parent) followed by backcrosses with *N. tabacum* as the recurrent pollen parent. Those male-sterile progeny that produced the most abnormal stamens had split corollas. One male-sterile type with nearly normal stamens had a normal fusion of petals.

The results of both sexual and somatic hybridization studies point to a link between production of fertile stamens and fusion of petals. It is most likely that critical events in stamen morphogenesis occur at the same developmental time that petal fusion takes place. However, petal fusion is not sufficient of itself for normal stamen development, since several cytoplasmic male-sterile cultivars of *N. tabacum* have fused petals. In those cytoplasmic male-sterile cultivars displaying fused petals, stamen development may already be abnormal by the time of petal fusion, such as was found for the cultivars studied by Rosenberg and Bonnett (1983).

Fusion experiments conducted by Belliard et al. (1979) between 'Nta(big)S' and male-fertile *N. tabacum* protoplasts led to cybrids with floral morphologies and mtDNA restriction patterns different from both parents. From the fact that novel fragments appeared in all cybrids with non-parental floral morphologies, the authors concluded that mtDNA recombination was the molecular mechanism leading to the rearrangements in the hybrids' RFLPs. Similar findings and conclusions have been reported from fusion experiments conducted by Aviv et al. (1984), Chetrit et al. (1985), and Kumashiro et al. (1989). Aviv and Galun (1986) described a fertile cybrid plant resulting from fusion of male-sterile *Nicotiana* hybrids of various cytoplasmic and nuclear origins. Rearranged mtDNA was found in this plant, from which they concluded that restoration to fertility resulted from mtDNA recombination.

The four male-fertile cybrids recovered from fusion of 'Nta(und)S' and 'Nta(big)S' displayed different stamen morphologies. Calli 31 and 64 yielded cybrids that had anthers attached to petaloid stamens, and thus phenotypically similar to 'Nta(und)S', while cybrid plants obtained from callus 94 had anthers on top of the normal filaments. Finally, plants were derived from callus 29, some of which developed flowers with petaloid stamens and some of which developed flowers with normal-appearing stamens. The appearance of these male-fertile cybrids indicated that mtDNA interaction did occur. The appearance of different phenotypes among the male-fertile cybrids may indicate that parental mitochondrial genes occurred in different combinations in the cybrids.

MtDNA analysis of progeny from cybrids 29-1 and 29-3 revealed differences in their restriction fragment patterns when compared to those of 'Nta(big)S' and 'Nta(und)S'. These differences were not the result of mixtures of mitochondrial genomes of both parents or of mixtures of parental mitochondria, since some fragments from one parent were present, while others from the same parent were missing. MtDNA fragments from both parents were present and, in addition, novel fragments were found.

The progeny of cybrid 29-3 could be divided into three groups according to stamen morphology. One group of progeny had normal-appearing stamens, another had petaloid stamens, and a third consisted of plants that first produced petaloid stamens, but later developed branches with normal-appearing stamens. Floral phenotype and mitochondrial restriction fragments could be correlated by comparisons between these groups, from which some fragments were found to be quantitatively related to normal stamen development. Therefore, mitochondrial sequence amplification might be responsible for the genetic changes promoting the normal development of stamens. A role for differential amplification of specific mtDNA fragments in stamen development has been discussed by Small et al. (1987) and Leaver et al. (1988), who compared mtDNA restriction fragment patterns and Southern hybridization patterns of male-sterile and male-fertile cytoplasms of *Zea mays*. These authors found that restriction fragments which were previously thought to be unique to male-sterile cytoplasms were also present at low levels in male-fertile cytoplasms.

To summarize, fertile cybrids were obtained by the fusion of two cytoplasmic male-sterile cultivars of *N. tabacum*. The mtDNA of the fertile cybrids differed from the mtDNA of both male-sterile cultivars, including the appearance of restriction fragments of a size not present in either cultivar. In addition, some restriction fragments appeared to be present in enhanced copy number in those fertile cybrids with the most normal-appearing stamens. Thus, restoration to fertility and normal-appearing stamens were accompanied by mtDNA changes that were qualitative and quantitative in nature. The genetic mechanisms behind these changes are proposed to be mitochondrial recombination and differential amplification of the genes involved in the regulation of stamen development.

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