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Modifications of Mitochondrial DNA Cause Changes in Floral Development in Homeotic-like Mutants of Tobacco

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To investigate the influence of mitochondrial genes on stamen development of higher plants, protoplasts from three different, male-sterile tobacco cultivars were fused. The fused cells were cultured individually into calli, from which plants were regenerated. Cybrid plants were obtained that exhibited flowers with recombined biparental male-sterile morphology and with novel male-sterile stamens that differed from any types from sexual or somatic hybridizations described previously. The male-sterile morphologies of these cybrids and their parents support the hypothesis that nuclear-mitochondrial interaction occurs at several stages in tobacco floral development and that several mitochondrial genes are necessary for normal stamen and corolla development. Analysis by restriction endonuclease digestion of mitochondrial DNA of male-sterile cybrids and their parents revealed that the mitochondrial DNA of male-sterile cybrids with parental floral morphology was unchanged when compared with parental mitochondrial DNA. Cybrids that were morphologically similar to one parent's male-sterile phenotype had mitochondrial DNA almost identical to that parent, whereas cybrids with recombined biparental or novel male-sterile phenotypes contained mitochondrial DNA different from both male-sterile parents and from each other. A set of mitochondrial DNA fragments could be correlated with split corollas, a feature found in several tobacco male-sterile cultivars. DNA gel blot analysis using a number of mitochondrial genes confirmed the conclusions based on ethidium bromide staining of mitochondrial DNA restriction digests.

INTRODUCTION

Male sterility was defined by Edwardson (1970) as the inability of a plant to produce functional pollen. Cytoplasmic male sterility (CMS) often occurs when cytoplasmic and nuclear genetic material from two different species are combined. In the genus *Nicotiana*, structural abnormalities in both stamen and corolla frequently accompany the failure to produce pollen (Chaplin, 1964; Gerstel, 1980). The nature of the stamen and corolla abnormalities is determined by the species of cytoplasm that is combined with the tobacco nucleus. Thus, CMS in tobacco is well-suited to study the coevolutionary regulation of floral organs by the nucleus and mitochondria. That the mitochondrial genome influences floral development in plants is supported by the association of specific mitochondrial DNA (mtDNA) sequences with CMS in petunia (Boeshore et al., 1985) and maize (Dewey et al., 1986) for example. Although the mechanism of CMS has not been elucidated for any species, its occurrence is widespread among flowering plants.

The influence of mitochondrial genes on CMS in tobacco has been investigated by protoplast fusion, whereby mitochondrial genotypes can be combined and the effect of mitochondrial composition on floral phenotype can be studied. All CMS cultivars were tobacco to assure that changes in floral phenotype resulted from changes in mitochondrial rather than nuclear composition. CMS cultivars that exhibited clear differences in their floral phenotype and that allowed floral structures of the cybrids to be related to those of the male-sterile cultivars were chosen.

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RESULTS

Fusions were carried out between protoplasts of the male-sterile cultivars of tobacco Nta(big)S and Nta(und)S and between Nta(big)S and Nta(sua)S (for detailed explanations of these abbreviations, see Kofer et al., 1990). The three male-sterile tobacco cultivars varied in their cytoplasmic composition with Nta(big)S carrying the cytoplasm of *Nicotiana bigelovii*, Nta(und)S of *N. undulata*, and Nta(sua)S of *N. suaveolens*. These male-sterile cultivars resulted from the introgression of *N. tabacum* nucleus to the cytoplasm of respective donor species. All three cultivars have been backcrossed for 12 to 16 generations. Thus, all the parental materials represent highly inbred

lines. The different CMS cultivars are very stable. No variation in phenotypes related to male sterility has been detected after growing several hundred plants over the years in our greenhouses.

As shown in Figure 1, each of the three male-sterile cultivars used in the fusion experiments possesses specific features associated with the male-sterility trait. In Nta(und)S, the stamen filaments are replaced by pink petalodes, to which empty anther sacs are attached occasionally; the corollas are sympetalous and short, with the style protruding from the corolla. In Nta(sua)S, stamen development is arrested at the primordial stage, and the corolla is normal when compared with fertile tobacco. Nta(big)S stamens exhibit well-developed filaments, whereas the anthers are replaced by feathery appendages with fringed tips; the petals of the corolla are only fused at the base.

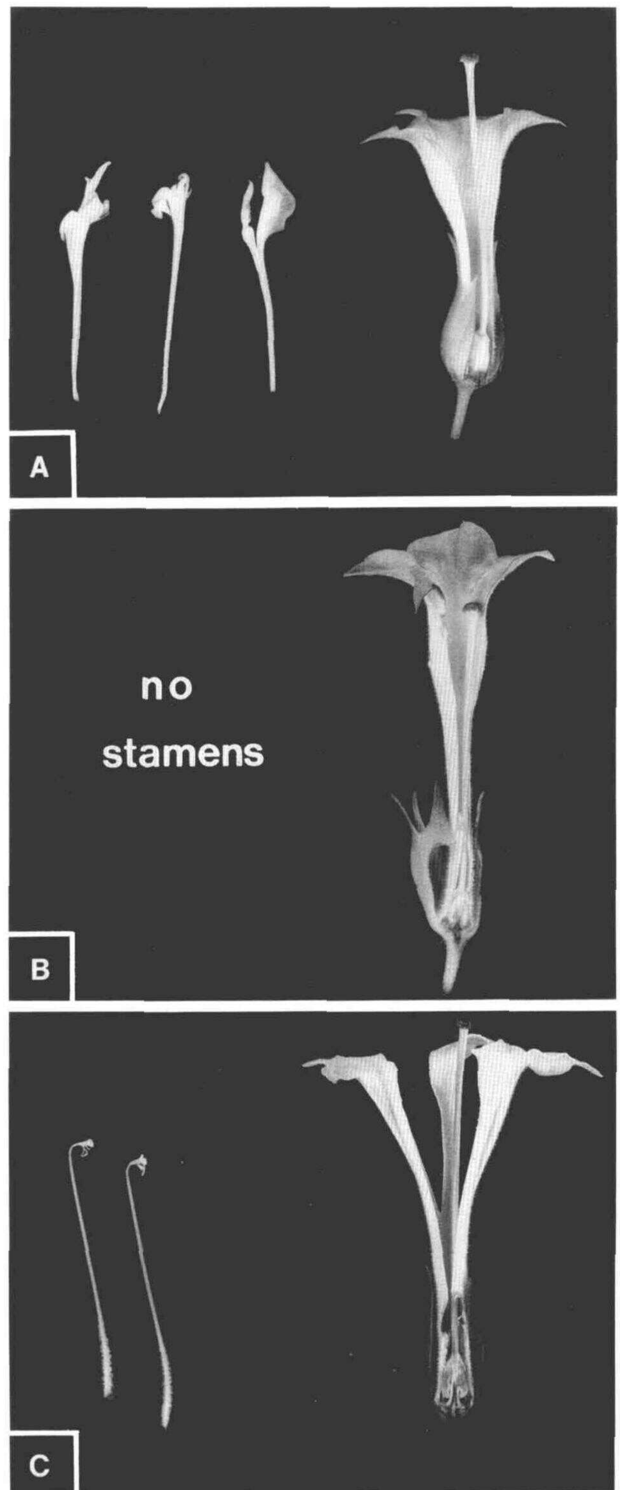

Figure 1. Dissected Flowers of Male-Sterile Fusion Parents.

(A) Nta(und)S.
(B) Nta(sua)S.
(C) Nta(big)S.

Fusion between Nta(big)S and Nta(und)S

The majority of the cybrid calli (18 of 25) carried the male-sterile traits characteristic for Nta(und)S. Some cybrids (four plants from three calli) were restored to fertility, whereas others were characterized as novel male-sterile cybrids because they differed from any phenotypes from sexual or somatic hybridizations described previously.

Novel male-sterile cybrids were obtained from five calli, of which two (calli 28 and 29) are described in this study. From callus 28, 21 male-sterile cybrids were regenerated. From callus 29, four cybrids were regenerated, two of which (29-1 and 29-3) were male-fertile and were described in a previous publication (Kofer et al., 1991). The other two (29-2 and 29-4) were of the novel male-sterile type.

Novel Male-Sterile Cybrids from Callus 29

As illustrated in Figure 2, cybrids 29-2 and 29-4 had narrow petals that were fused only at the base, a characteristic of Nta(big)S. The cybrids' stamens were neither petaloid, as found in Nta(und)S, nor filamentous with flat, feathery tips, as found in Nta(big)S. Cybrid 29-2 (Figures 2A and 2B) had narrow, pink stamens (Figure 2A, left) resembling its petals (Figure 2A, right), and 29-4 (Figures 2C and 2D) had stamens with branched tips (Figure 2C, left). Both 29-2 and 29-4 stamens frequently were tipped by stigmatoids that are also common on the petalodes of Nta(und)S.

Morphology of R_1 and R_2 Generation Plants Raised from 29-2 and 29-4

Cybrids 29-2 and 29-4 were pollinated with tobacco and the seeds were germinated. According to the scheme in Figure 3, 15 R_1 plants and 120 R_2 plants were grown from

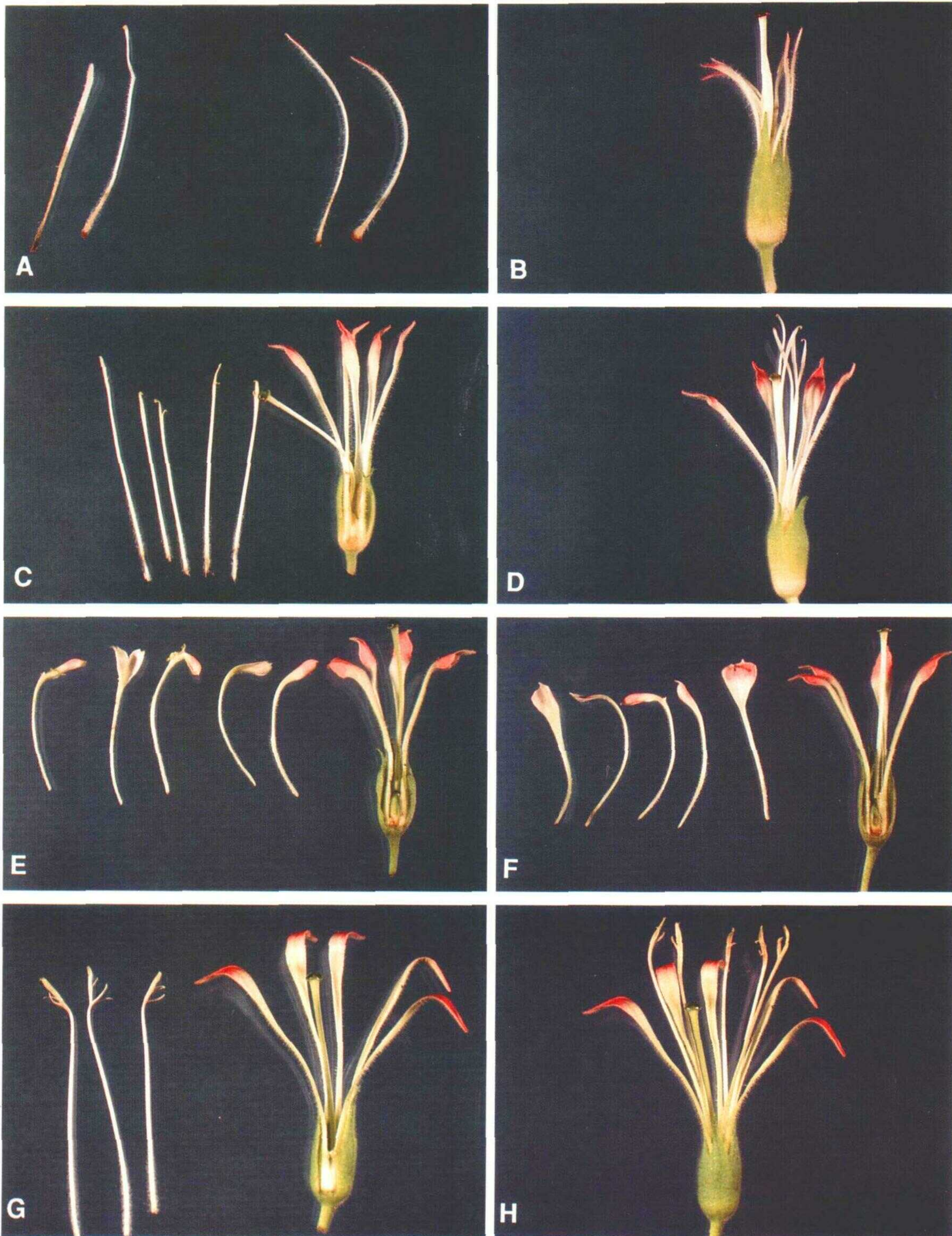

Figure 2. Corollas and Stamens of 29-2 and 29-4 R_0 and R_1 Generations.

(A) Left, stamens of novel male-sterile cybrid 29-2; right, petals.
(B) Intact flower of novel male-sterile cybrid 29-2.
(C) Novel male-sterile cybrid 29-4: left, stamens; right, dissected corolla.
(D) Intact flower of novel male-sterile cybrid 29-4.
(E) and **(F)** R_1 progeny of 29-2: left, stamens; right, dissected corolla.
(G) R_1 progeny of 29-4: left, stamens; right, dissected corolla.
(H) Intact flower of R_1 progeny of 29-4.

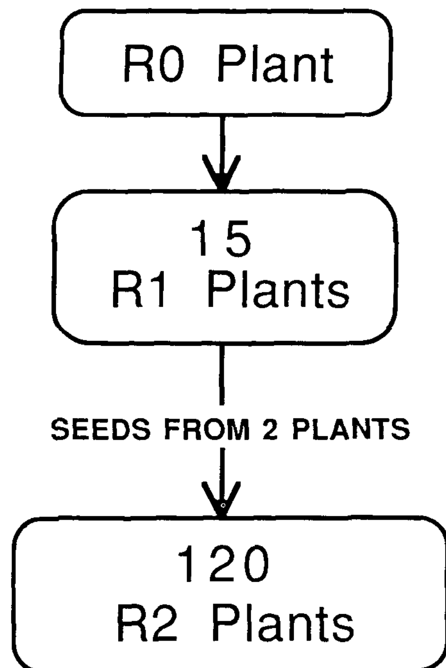

Figure 3. Diagram Showing Numbers of Plants Raised in the Cybrid R_1 and R_2 Generations.

From each cybrid callus several R_0 plants were grown. Each R_0 plant was pollinated with tobacco pollen. Seeds were collected and 15 R_1 plants grown from each cybrid. From the R_1 plants two were selected and pollinated with tobacco pollen. Seeds were collected and 120 R_2 plants grown to maturity.

each cybrid. All R_1 plants had novel male-sterile stamens combined with split, shortened corollas. The 29-2 R_1 plants displayed pink, petaloid stamens that varied in shape and were tipped with stigmatoids. A variety of petaloid stamen types was found (Figures 2E and 2F) on most of the R_1 plants. The petaloid stamens were either flat and shieldlike or inwardly rolled along their axis, thus creating a three-dimensional structure that failed to fuse or only partially fused. The 29-2 R_2 generation showed the same range of traits found for the 29-2 R_1 generation. All flowers of the R_1 and R_2 generations of 29-4 displayed stamens that were filamentous and forked at the tips with occasional stigmatoids. The corolla was split and the petals were shortened to the length of the Nta(und)S cultivar (Figures 2G and 2H).

Novel Male-Sterile Cybrids from Callus 28 and Their Progenies

The cybrid plants from callus 28 exhibited a wide range of novel stamen morphologies. Altogether, 21 cybrid plants were grown to flowering from this callus. All exhibited a split corolla as found in the Nta(big)S parent; however, petal length, petal width, and stamen morphology were highly variable. Figure 4 illustrates nine distinct stamen types. Of the cybrid plants, three exhibited stamen morphologies quite similar to Nta(big)S (Figure 4H). The remaining 18 plants had stamens very different from both parental cultivars, yet each novel stamen type incorporated elements of both parents in a novel arrangement. For example, cybrids of the type shown in Figure 4C had stamens with a filamentous basal portion, as found in Nta(big)S, broadening into petalodes, which are characteristic of Nta(und)S stamens. The stigmatoids on top of the petalodes of many of the novel stamen types are frequently found on Nta(und)S stamens.

R_1 and R_2 progenies were grown according to the scheme in Figure 3 from cybrids 28-1, 28-4, 28-6, 28-11,

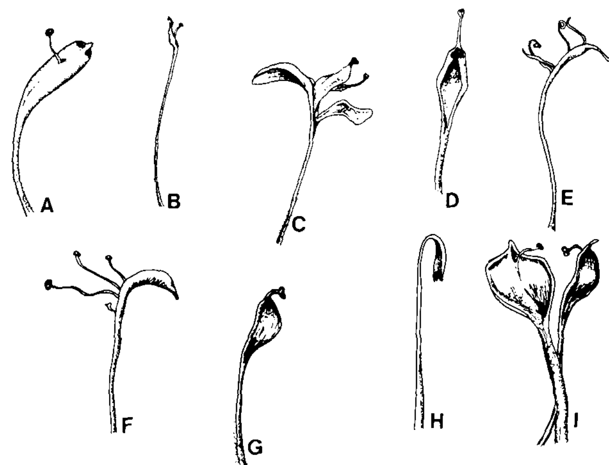

Figure 4. Sketches of Novel Male-Sterile Stamens of Cybrid Plants Grown from Callus 28.

(A) Petaloid type stamen, fused along its longitudinal axis with stigmatoid outgrowth.
(B) Filamentous stamen tipped by a small petalode with a stigmatoid outgrowth.
(C) Filamentous stamen branching into petalodes that are frequently tipped by stigmatoids.
(D) Petaloid stamen that is inwardly rolled along its longitudinal axis fused only in the basal region. The stamen is tipped by a stigmatoid.
(E) Filamentous stamen almost entirely fused along its axis with filamentous appendages sometimes tipped by stigmatoids.
(F) Filamentous stamen, with partially fused petalode at top, having long appendages that are usually tipped by stigmatoids.
(G) Filamentous stamen (side view) broadening into a cup-shaped petalode tipped by a stigmatoid.
(H) Stamen very similar to Nta(big)S-type stamen.
(I) Filamentous stamens (side and front view) broadening into a cup-shaped petalode with stigmatoid appendages protruding from the cup.

28-14, and 28-16. The cybrids were true breeding with respect to their flower morphologies; i.e., if a particular R_0 plant exhibited three different types of novel stamens, the R_1 and R_2 generation plants derived from it did so, too. None of the novel male-sterile cybrids or their progeny reverted to the male-sterility type of either Nta(big)S or Nta(und)S.

To summarize, all the novel male-sterile cybrids derived from calli 28 and 29 formed a filament, a feature of Nta(big)S, combined with a pigmented petal-like structure, a feature of Nta(und)S. Frequently, stigmatoids appeared that are also found in Nta(und)S. The corollas were always split as in Nta(big)S, but petal length could be either normal, as in Nta(big)S, or shortened, as in Nta(und)S. The alterations that were represented among the novel phenotypes were transmitted stably to subsequent generations.

Mitochondrial Analysis of Cybrids from Calli 28 and 29

For comparison of mtDNA between cybrids and male-sterile parents, R_2 generation plants of the cybrids were used. A comparison of mtDNA from Nta(big)S, Nta(und)S, and R_2 generation plants from cybrid 29-2 is shown in Figure 5. The mtDNA restriction patterns of Nta(und)S and Nta(big)S are readily distinguished from each other. The restriction digest patterns of the 29-2 plants show a number of fragments in common with one or the other parent and also novel fragments, when restricted with PvuII, PstI, XhoI, or BglI.

mtDNA analyses were carried out on 28-1, 28-4, 28-6, 28-11, 28-14, and 28-16. Of these, 28-1 and 28-16 had flower morphologies quite similar to Nta(big)S, whereas 28-4, 28-6, 28-11, and 28-14 were of different novel male-sterile forms. mtDNA digests with PvuII, as shown in Figure 6, lane 4, BglI, XhoI, and PstI gave restriction patterns for 28-16 that were virtually identical to Nta(big)S. The individual novel male-sterile plants had mtDNA patterns that were different from each other and from either parent (Figure 6, lanes 1 to 3). However, all had more fragments in common with Nta(big)S than with Nta(und)S. All of the mitochondrial restriction patterns from novel male-sterile plants contained novel fragments.

As given in Figure 7, the results of analysis by DNA gel blot hybridization of the mitochondrial genes *atpA*, *nad1*, *orf25*, and *atp6* confirmed the results of ethidium bromide-stained gels. Cybrid 28-1, with mtDNA that appeared most like Nta(big)S, hybridized to the mitochondrial genes in a manner identical to Nta(big)S. The mtDNA of cybrids 28-4, 28-6, and 28-11 hybridized to all four genes in the same manner as Nta(big)S, but also hybridized to *atpA* in the manner of Nta(und)S. mtDNA of cybrid 28-14 had all fragments representing both parents for all four genes and, in addition, novel fragments that hybridized to *atpA*, *nad1*, and *atp6*.

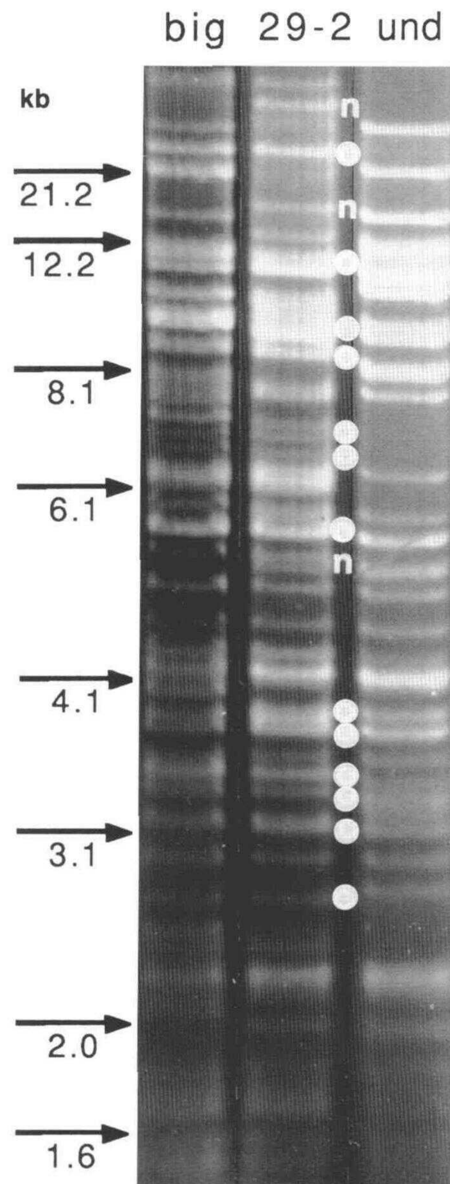

Figure 5. Comparison of mtDNA from 29-2 with Parents Nta(big)S and Nta(und)S Digested with PvuII.

White circles indicate fragments of 29-2 comigrating with Nta(big)S; n's indicate novel fragments not occurring in either parent. The positions of the DNA size markers are indicated at left in kilobases.

The mitochondrial restriction patterns of novel male-sterile cybrids obtained from calli 28 and 29 were compared with two fertile cybrids that were also derived from callus 29 (Kofer et al., 1991). All male-sterile plants from calli 28 and 29 had split corollas as does Nta(big)S,

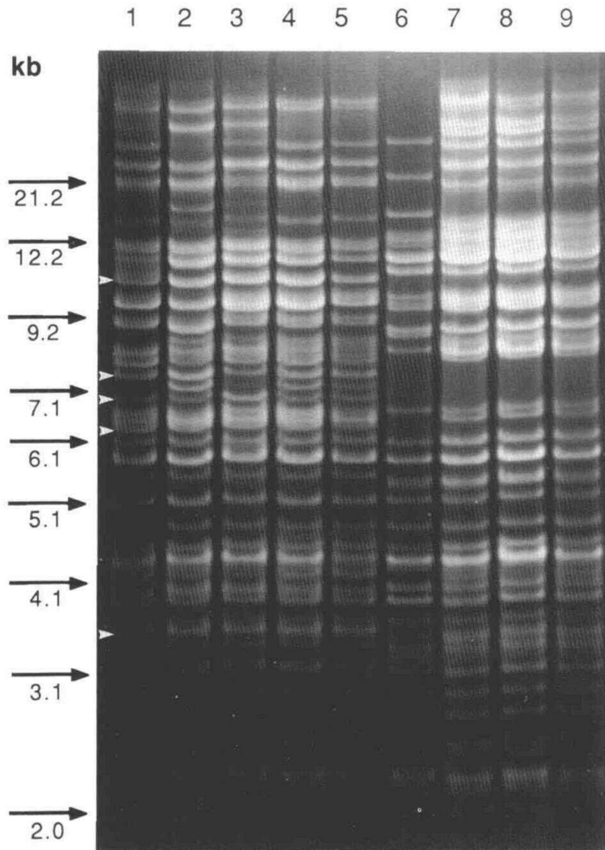

Figure 6. Comparison of mtDNA That Was Digested with PvuII, of Cybrid Plants Grown from Calli 28 and 29.

Lanes 1 to 4 contain mtDNA from cybrids from callus 28: 28-6, 28-11, 28-14, and 28-16. Cybrids 28-6, 28-11, and 28-14 were of novel male-sterile stamen morphologies, and 28-16 resembled Nta(big)S. Lane 5 contains mtDNA from Nta(big)S; lane 6, Nta(und)S; lanes 7 to 9, male-fertile cybrids from callus 29. Lanes 1 to 5 represent mtDNA from plants with petals that failed to fuse. Lanes 6 to 9 represent mtDNA from plants with fused petals. Arrows indicate fragments that are absent in cybrids with fused corollas. The positions of the DNA size markers are indicated at left in kilobases.

whereas the two male-fertile plants (29-1 and 29-3) and parent Nta(und)S had fused corollas. By comparing restriction digest patterns between the plants with split corollas and the plants with fused corollas, five fragments were detected that were present in all plants with split corollas and absent in all plants with fused corollas (Figure 6).

Fusion between Nta(big)S and Nta(sua)S

Of 168 cybrid calli obtained from this fusion combination, 64.5% resulted in cybrid plants with male-sterile characteristics identical to Nta(sua)S and 16.5% in plants of Nta(big)S male-sterile morphology. The remainder of the calli (19%) resulted in plants that were categorized as recombined biparental because their male-sterile flower morphologies included floral features from both parents. As shown in Figure 8, the petals were split as in Nta(big)S, whereas the stamens were absent or reduced to stigmatoid remnants that were curled up at the bottom of the corolla, as occasionally found in Nta(sua)S. The floral phenotypes of both parental and recombined biparental cybrids were followed through two subsequent generations according to the scheme in Figure 3 and no changes in floral phenotype were observed.

mtDNA Analysis of Male-Sterile Cybrids with Parental Morphology

Eight cybrids with floral morphologies like Nta(big)S and five cybrids like Nta(sua)S were investigated. Cybrids with a floral morphology like the Nta(big)S parent had an identical pattern to Nta(big)S with the restriction enzymes

Enzyme	Probe	CYBRIDS				
		28-1	28-4	28-6	28-11	28-14
BglI	atpA	b	u+b	u+b	u+b	u+b+n
	BS nad	b	b	b	b	u+b+n
	orf 25	b	b	b	b	u+b
XhoI	atpA	b	u+b	u+b	u+b	u+b
	atp6	b	b	b	b	u+b+n
	orf 25	b	b	b	b	u+b

Figure 7. Schematic Presentation of DNA Gel Blot Hybridizations of mtDNA from a Cybrid (28-1) with a Male-Sterile Morphology Resembling Nta(big)S and Four Novel Male-Sterile Cybrids (28-4, 28-6, 28-11, and 28-14).

The mtDNA was digested with BglI or XhoI. The b and u indicate that the probe hybridized to fragments of the same length as Nta(big)S or Nta(und)S, respectively; n indicates that the probe hybridized to a fragment of novel length, specific to the cybrid.

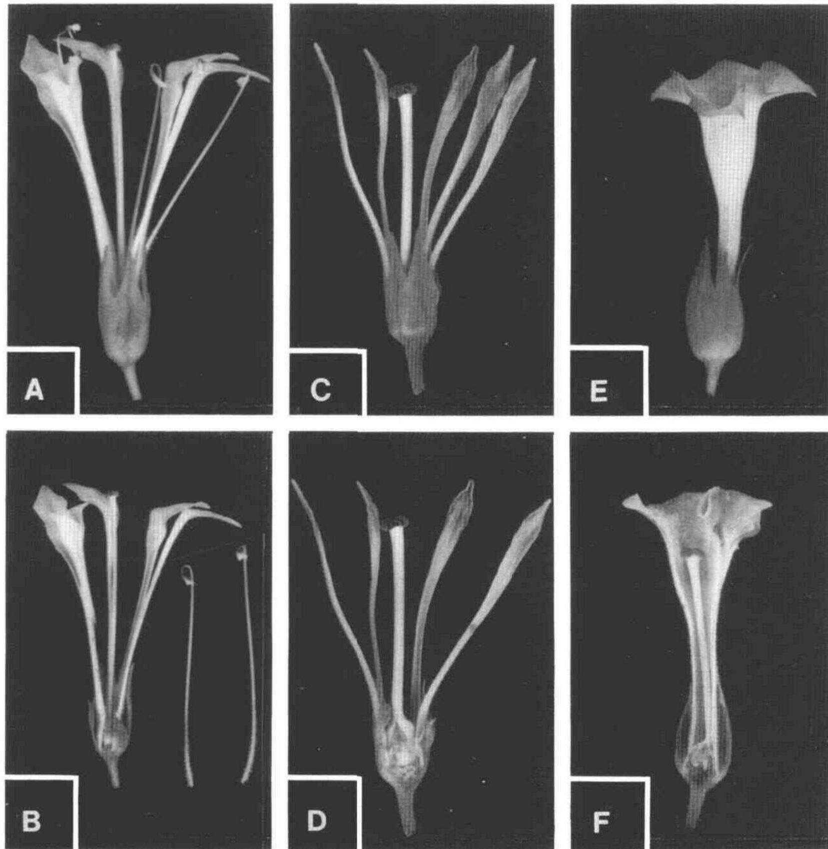

Figure 8. Cybrid Flowers (Intact and Dissected) from Fusion Combination Nta(sua)S (+) Nta(big)S.

(A) and **(B)** Nta(big)S type cybrid.
(C) and **(D)** Recombined biparental cybrid.
(E) and **(F)** Nta(sua)S type cybrid.

BamHI, PvuII, SalI, BglI, or XhoI. All Nta(sua)S-type cybrids had mtDNA patterns identical to Nta(sua)S when digested with BamHI, XhoI, PvuII, or BglI.

mtDNA Analysis of Male-Sterile Cybrids with Recombined Biparental Morphology

After digestion with PvuII, BglI, PstI, and XhoI, mtDNA from four cybrids with recombined biparental morphology showed an incomplete mixture of parental fragments. Figure 9 demonstrates that although each cybrid had more fragments in common with Nta(big)S than Nta(sua)S, one or more fragments of each parent were absent from all the cybrids. Cybrids 101-1, 101-2, and 101-4, from the same callus, exhibited only minor differences from each other (see arrows in Figure 9). These three cybrids differed in more fragments from 101-5, which was from another callus.

Figure 10 illustrates DNA gel blot hybridization experiments with probes of mitochondrial genes, showing that the mtDNA of the cybrids contained fragments of lengths characteristic for either or both parents. In some cases, hybridization to one of several fragments in length characteristic of Nta(big)S was missing in a cybrid. XhoI fragments of novel size were found in cybrids 101-1, 101-2, 101-4, and 101-5 when hybridized to *atp6*. Thus, the results of the DNA gel blot analysis confirmed the conclusion, based on ethidium bromide staining, that the recombined biparental phenotype resulted from the combination of an incomplete set of mtDNA fragments from each parent.

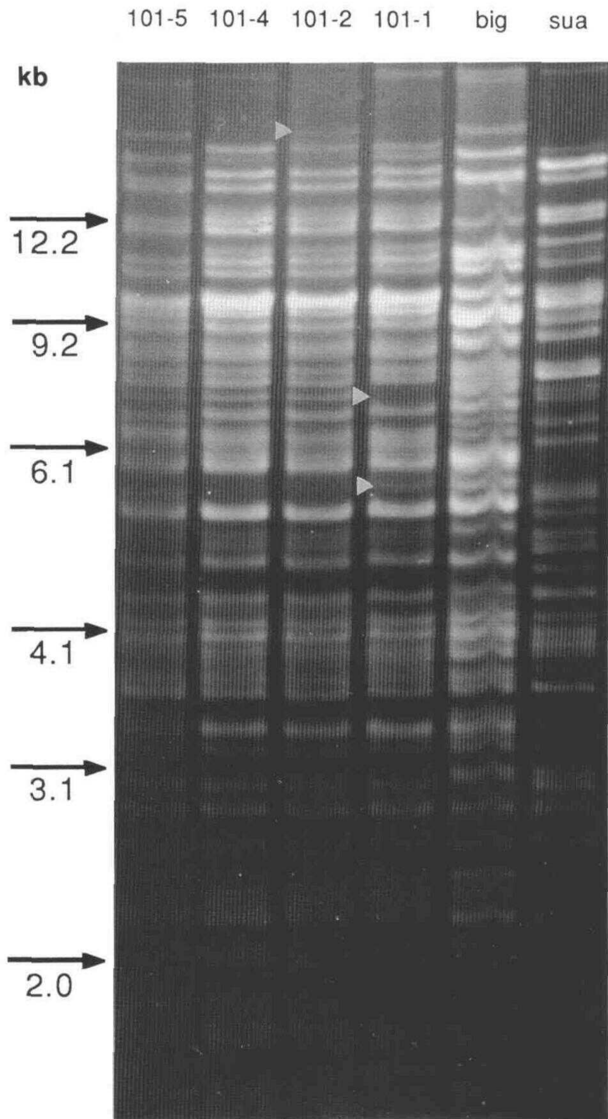

Figure 9. Comparison of mtDNA of Recombined Biparental Cybrids, Nta(big)S and Nta(sua)S Restricted with PvuII.

Arrows indicate differences between plants 101-1, 101-2, and 101-4 that originated from the same cybrid callus. The positions of the DNA size markers are indicated to the left in kilobases.

DISCUSSION

Somatic hybridization experiments fusing Nta(sua)S (+) Nta(big)S and Nta(big)S (+) Nta(und)S resulted in cybrids with parental, recombined biparental, novel male-sterile, and fertile floral morphologies. The occurrence of the recombined biparental, novel male-sterile, and fertile cybrids demonstrated that cell fusion evoked modifications in mtDNA that resulted in changes in male-sterile floral morphologies. The participation of several mitochondrial genes in stamen and petal development can explain the results of these experiments. That several mitochondrial genes could regulate floral morphology was hypothesized by Rosenberg and Bonnett (1983), based on their developmental study of different tobacco CMS cultivars. Pelletier (1986) also concluded that CMS is a consequence of two or more differences between male-fertile and male-sterile mitochondrial genomes, based on an analysis of floral phenotypes of hybrids and cybrids derived from fusion of a male-fertile and CMS cultivar of tobacco.

Floral features used as morphological markers in support of this hypothesis were the presence or absence of petal fusion, the length of petals, and the structure of the

Enzyme	Probe	Recombined Biparental Cybrids 101-5	101-4	101-2	101-1
PvuII	cyt b	s	s	s	s
	orf 25	b + s	b + s	b + s	b + s
	BS nad	b	s	s	s
XhoI	atp6	b + n	b + n	b + n	b + n
	26S	b	b[1]	b[1]	b[1]
	orf 25	b	b + s	b + s	b + s[2]
	5S	s/b	s/b	s/b	s/b[1,2]

Figure 10. Schematic Presentation of DNA Gel Blot Hybridizations of mtDNA Digested with PvuII and XhoI from Recombined Biparental Cybrids.

The b and s indicate that the probe hybridized to fragments of the same length as Nta(big)S or Nta(sua)S, respectively; n indicates that the probe hybridized to a fragment of novel length, specific to the cybrid; s/b indicates that the DNA gel blot hybridization patterns of Nta(big)S and Nta(sua)S were identical. The [1] indicates that one of the parent-specific fragments is absent in the cybrid; the [2] indicates that one of the parent-specific fragments is much stronger hybridizing to the probe than that parent.

stamens. The initiation of both stamens and petals is likely to be independent of mitochondrial genes because primordia of both organs form in the male-sterile cultivars and in all cybrids. Alternatively, initiation of stamens and petals could be dependent upon mitochondrial genes, with these genes completely conserved across different species' organelles. Consequently, there would not be genetic variation for these genes in mitochondria of *N. tabacum*, *N. bigelovii*, *N. suaveolens*, and possibly other *Nicotiana* species. Thus, these traits remain unchanged when the tobacco nucleus is combined with the cytoplasm of another species. Evidence for the participation of a mitochondrial gene in petal development occurs as petal primordia continue to grow to the length of petals in male-fertile tobacco. Petal length is shortened in Nta(und)S, cybrids from callus 29, and some novel cybrids from callus 28. Fusion of petal primordia does not occur in Nta(big)S, the recombined biparental cybrids, and novel male-sterile cybrids from calli 28 and 29. That mitochondrial regulation of petal fusion is genetically independent of petal elongation can be deduced from the fact that novel male-sterile cybrids with a split corolla had either shortened petals typical in length of Nta(und)S or petals of normal length, typical of Nta(big)S. The recombined biparental cybrids illustrate that mitochondrial regulation of stamen development is genetically independent of petal fusion. In these cybrids, the failure of stamen development to proceed past the stage of stamen initiation, a parental trait from Nta(sua)S, appeared together with the failure of petals to fuse, a parental trait from Nta(big)S. Thus, evidence was obtained for the participation of two separate mitochondrial genes for petal development and one mitochondrial gene for stamen development. Moreover, the large variety of stamen structures detected among the cybrids obtained from only three different CMS cultivars suggests that several more mitochondrial genes participate in stamen development.

The fusion of Nta(big)S and Nta(und)S produced cybrids that exhibited an array of morphological variations in stamen structure. These variations were inherited stably over two generations examined in this study. The specific structures frequently associated with these stamens included petalodes of varying widths and pigmentation, as well as filamentous outgrowths lacking in pigmentation but frequently tipped with green stigmatoids. These structures are readily interpreted in terms of the floral structure of petals and carpels.

The clear morphological distinctions between floral organs is less precise in flowers of more primitive plant species, an observation explained by the hypothesis that all floral organs evolved from leaves. For example, shield-like stamens are present in primitive genera, such as *Degeneria* (Canright, 1952), and vividly colored staminoids are found in members of the Zingiberales (Strasburger et al., 1983). Thus, the progression from primitive to advanced stamen phenotypes has included the loss of petaloid structures and pigmentation found in stamens of primitive genera. Mixing mitochondria from two different species by protoplast fusion created novel stamen structures in the cybrid plants that resembled primitive stamens. That these primitive features appear in male-sterile cultivars and cybrids indicates that mitochondrial-nuclear coevolution is important in defining the transition of primitive stamens into modern stamens that are characterized by nonpigmented filaments and petaloid-free anthers.

Recently, several homeotic mutants have been described in organisms such as Arabidopsis (Haughn and Somerville, 1988; Bowman et al., 1989) and tobacco (Malmberg and McIndoo, 1983; Evans and Malmberg, 1989) that exhibit phenotypes similar to those found in tobacco CMS cultivars and the cybrids described in this study. Stigmatoids, ovules, and petals were found in conjunction with, or in place of, stamens. The finding that nuclear and cytoplasmic mutations result in similar developmental defects is consistent with the hypothesis that floral development requires the cooperative interaction of both genomes. Communication between the two genomes could be achieved by assembly of a protein from subunits coded by nuclear and mitochondrial genes or, for example, by the processing of mitochondrial transcripts by proteins encoded by the nuclear genome. Thus, nuclear homeotic mutations could mark genes encoding polypeptides that function in mitochondria.

mtDNA analyses were conducted with respect to the floral male-sterile features of the cybrids. The cybrids were categorized as parental, novel, or recombined biparental phenotypes. Cybrids identical to their parents showed essentially no alterations of mtDNA in the restriction patterns. Likewise, cybrids with close phenotypic similarities to one parent exhibited mtDNA patterns that resembled that parent. Finally, cybrids with floral features very different from both parents had restriction digest patterns with many differences, including novel fragments, visible both by ethidium bromide staining and by DNA gel blot hybridization analysis. A correlation between cybrid floral morphology and mtDNA patterns was also observed by Belliard et al. (1979) and Kumashiro et al. (1989). Belliard et al. (1979) fused male-fertile *N. tabacum* with male-sterile *N. tabacum* with the cytoplasmic organelles of *N. debneyi.* mtDNA comparisons between cybrids with different degrees of stamen and corolla abnormalities revealed a positive correlation between more normal development and a higher proportion of mtDNA fragments from the male-fertile cultivar. In the experiments of Kumashiro et al. (1989), CMS was induced in cybrids by the fusion of *N. repanda* and *N. tabacum* protoplasts. Male-sterile cybrids with identical floral morphologies exhibited identical mtDNA restriction patterns. The cybrids' restriction patterns were different from both parents and contained *N. repanda* as well as *N. tabacum* mtDNA fragments.

Our results showed that mtDNA changes always accompanied changes in floral phenotype, and changes in floral phenotype were always predictive of changes in mtDNA.

Surprisingly, no mitochondrial rearrangements were observed in cybrids with parental floral morphology. Either one of two suggestions by Pelletier (1986) could explain this observation. Pelletier proposed that either the mtDNA that regulates floral development is scattered over the mitochondrial genome so diffusely that any rearrangement affects floral phenotype, or it is arranged in a continuous sequence within which protoplast-induced intergenomic recombination occurs preferentially. Currently, knowledge of the mtDNA of these tobacco species is insufficient to distinguish between the two proposals.

In summary, fusion of several male-sterile cultivars of tobacco has provided evidence that mitochondrial genes regulate several stages of stamen and petal development. Stamens of novel male-sterile cybrids revealed potentials for development that mirrored those expressed in stamens of primitive genera of flowering plants. All alterations in floral phenotype were accompanied by changes in mtDNA, as demonstrated by ethidium bromide staining and DNA gel blot hybridization of mtDNA.

METHODS

Plant Material

Three alloplasmic male-sterile tobacco cultivars were used in the fusion experiments: one with cytoplasmic organelles of *Nicotiana suaveolens*, Nta(sua)S (Schweppenhauser and Mann, 1968; Hosfield and Wernsman, 1974); one with cytoplasmic organelles of *N. bigelovii*, Nta(big)S (obtained by E.A. Wernsman, North Carolina State University at Raleigh); and one with cytoplasmic organelles of *N. undulata*, Nta(und)S (Burk, 1967; Bonnett and Glimelius, 1983). Because we had concerns about the cytoplasmic origin of the cultivar with cytoplasm from *N. undulata*, we confirmed its origin by comparing the mtDNA of the cytoplasmic male-sterile cultivar with *N. undulata* maintained by the Tobacco Research Center in Oxford, NC. The male-sterile cultivars were fused in two combinations (Kofer et al., 1990). In the first combination, protoplasts derived from leaf tissue of Nta(big)S were fused with protoplasts derived from suspension cells of Nta(und)S. In the second fusion combination, protoplasts derived from leaf tissue of Nta(sua)S were fused with protoplasts from cell suspensions of Nta(big)S.

The plants were grown as in vitro shoot cultures at 25°C under continuous light on MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.9% Noble agar and without hormones. The shoot cultures were transferred to fresh medium every 4 weeks.

Cell suspensions were established from callus culture derived from leaf explants of the in vitro grown shoot cultures. The cell suspensions were maintained in continuous darkness on a rotary shaker (100 rpm) at 27°C in a medium modified from Müller and Grafe (1978) as described in Kofer et al. (1990). The suspensions were subcultured every fourth day.

Protoplast Isolation, Irradiation, Fusion, and Plant Regeneration

The isolation of the protoplasts, the irradiation of mesophyll-derived protoplasts with X-rays before fusion, the fusion of protoplasts, the isolation of fusion products, and the regeneration of cybrid plants were carried out as reported previously (Kofer et al., 1990).

mtDNA and DNA Gel Blot Hybridization Analyses

For the isolation, digestion, and electrophoresis of mtDNA, the procedure of Bland et al. (1985) as modified by Håkansson et al. (1988) was used. mtDNA was transferred to nylon filters (Pall Biodyne membrane, Ilfracombe, England) by the DNA hybridization procedure described by Maniatis et al. (1982). Clones of mitochondrial genes, kindly supplied by C.S. Levings III and M.R. Hanson, were random labeled with ^{32}P. Hybridization was performed for 15 to 18 hr at 42°C. After hybridization, the nylon filters were washed twice at 58°C.

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REFERENCES

Belliard, G., Vedel, F., and Pelletier, G. (1979). Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature **281,** 401–403.

Bland, M.M., Matzinger, D.F., and Levings, C.S., III (1985). Comparison of the mitochondrial genome of *Nicotiana tabacum* with its progenitor species. Theor. Appl. Genet. **69,** 535–541.

Boeshore, M.L., Hanson, M.R., and Izhar, S. (1985). A variant mitochondrial DNA arrangement specific to *Petunia* stable sterile somatic hybrids. Plant Mol. Biol. **4,** 125–132.

Bonnett, H.T., and Glimelius, K. (1983). Somatic hybridization in *Nicotiana:* Behavior of organelles after fusion of protoplasts from male-fertile and male-sterile cultivars. Theor. Appl. Genet. **65,** 213–217.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in *Arabidopsis.* Plant Cell **1,** 37–52.

Burk, L.G. (1967). An interspecific bridge-cross: *Nicotiana repanda* through *N. sylvestris* to *N. tabacum.* J. Hered. **58,** 215–218.

Canright, J.E. (1952). The comparative morphology and relationships of the *Magnoliaceae.* I. Trends of specialization in the stamens. Am. J. Bot. **39,** 484–497.

Chaplin, J.F. (1964). Use of male-sterile tobacco in the production of hybrid seed. Tob. Sci. **8,** 105–109.

Dewey, R.E., Levings, C.S., III, and Timothy, D.H. (1986). Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44,** 439–449.

Edwardson, J.R. (1970). Cytoplasmic male-sterility. Bot. Rev. **36,** 341–420.

Evans, P.T., and Malmberg, R.L. (1989). Do polyamines have roles in plant development? Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 235–269.

Gerstel, D.U. (1980). Cytoplasmic male sterility in *Nicotiana* (a review). N.C. Agric. Res. Serv. Tech. Bull. **263,** 1–31.

Håkansson, G., van der Mark, F., Bonnett, H.T., and Glimelius, K. (1988). Variant mitochondrial protein and DNA patterns associated with cytoplasmic male-sterile lines of *Nicotiana.* Theor. Appl. Genet. **76,** 431–437.

Haughn, G.W., and Somerville, C.R. (1988). Genetic control of morphogenesis in *Arabidopsis.* Dev. Genet. **9,** 73–89.

Hosfield, G.L., and Wernsman, E.A. (1974). Effect of an alien cytoplasm and fertility restoring factor on growth, agronomic characters, and chemical constituents in a male-sterile variety of flue-cured tobacco. Crop. Sci. **14,** 575–577.

Kofer, W., Glimelius, K., and Bonnett, H.T. (1990). Modifications of floral development in tobacco induced by fusion of protoplasts of different male-sterile cultivars. Theor. Appl. Genet. **79,** 97–102.

Kofer, W., Glimelius, K., and Bonnett, H.T. (1991). Restoration of normal stamen development and pollen formation by fusion of different male-sterile *Nicotiana tabacum* cultivars. Theor. Appl. Genet. **81,** 390–396.

Kumashiro, T., Asahi, T., and Nakakido, F. (1989). Transfer of cytoplasmic factors by asymmetric fusion from a cross-incompatible species, *Nicotiana repanda* to *N. tabacum*, and characterization of cytoplasmic genomes. Plant Sci. **61,** 137–144.

Malmberg, R.L., and McIndoo, J. (1983). Abnormal floral development of a tobacco mutant with elevated polyamine levels. Nature **305,** 623–625.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Müller, A.J., and Grafe, R. (1978). Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol. Gen. Genet. **161,** 67–76.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Pelletier, G.R. (1986). Plant organelle genetics through somatic hybridization. Oxf. Surv. Plant Mol. Cell Biol. **3,** 97–121.

Rosenberg, S.M., and Bonnett, H.T. (1983). Floral organogenesis in *Nicotiana tabacum:* A comparison of two cytoplasmic male-sterile cultivars with a male-fertile cultivar. Am. J. Bot. **70,** 266–275.

Schweppenhauser, M.A., and Mann, T.J. (1968). Restoration of staminal fertility in *Nicotiana* by introgression. Can. J. Genet. Cytol. **10,** 401–411.

Strasburger, E., Noll, F., Schenck, H., and Schimper, A.F.N. (1983). Lehrbuch der Botanik für Hochschulen, 32. Auflage. (Gustav Fischer Verlag, Stuttgart, Germany), p. 809, pp. 902–903.