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Somatic hybridization in *Nicotiana*: behavior of organelles after fusion of protoplasts from male-fertile and male-sterile cultivars

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Summary. Protoplasts from a nitrate reductase-deficient mutant of *Nicotiana tabacum* (cnx-68) were fused with protoplasts of 3 different cytoplasmically male-sterile cultivars of tobacco. Two cultivars had no stamens in the mature flowers and the third had petaloid structures in place of the stamens. Plants were regenerated from the fused protoplasts and characterized with respect to stamen development, chromosome number, and two chloroplast-coded traits. Nearly all hybrid plants displayed the chloroplast traits of only one parent, indicating that chloroplast segregation had occurred. The frequency of appearance of each chloroplast type differed according to the species origin of the chloroplasts. Chloroplasts from *N. undulata* competed much better than those from *N. tabacum; N. suaveolens* somewhat better than *N. tabacum;* and *N. glauca* about equally with *N. tabacum.* These results are compatible with an interpretation that equal frequency of appearance of chloroplast type among the regenerated plants occurs if the chloroplast DNAs of the parents are similar, whereas a bias of chloroplast type appears among the regenerated plants when the chloroplast DNAs are different. The appearance of aberrations in stamen development resembling the cytoplasmic male-sterile parental types was infrequent among the hybrid plants in all three crosses. Thus sterility factors were generally overcome by fertility factors following somatic hybridization.

Key words: Somatic hybridization – Organelle segregation – *Nicotiana* – Male sterility – Nitrate reductase – Deficient mutant

Introduction

Following formation of hybrid cells by protoplast fusion, chloroplasts and mitochondria with different genetic traits are combined within a single cell. As a consequence of cell division during callus formation and plant regeneration, changes in frequency of each organelle type occur. Such changes will be expressed in plants with traits which differ according to the genetic determinants in their organelles.

The majority of studies of organelle behavior following somatic hybridization have used *Nicotiana* species as the experimental materials. The popularity of this genus reflects the ease of plant regeneration following protoplast fusion, the availability of cultivars of one species with organelles from another species, and the genetic variation which exists in chloroplast and mitochondrial DNA within the genus.

In the study described here, the disposition of organelles is described for three somatic hybridization experiments, each involving the same male-fertile cultivar of tobacco fused with a different male-sterile cultivar. The identity of chloroplast type in regenerated plants was determined by examinating the electrophoretic mobility of the enzyme, ribulose-bisphosphate carboxylase-oxygenase, and by assessing the response of seedlings from regenerated plants to the fungal toxin, tentoxin. The appearance of either normal or aberrant stamen development among regenerated plants served as an indicator of mitochondrial behavior, since such cytoplasmically inherited aberrations in stamen development are known to involve mitochondrial rather than chloroplast genetic material in *Nicotiana* (Gleba 1978; Belliard et al. 1978; Aviv and Galun 1980; Glimelius et al. 1981).

Materials and methods

Four different cultivars of *Nicotiana tabacum* L. were used as fusion partners in three separate combinations. One of them, a

mutant cell line derived from variety 'Gatersleben' (cnx-68, n=24) and deficient in nitrate reductase (Müller and Grafe 1978), was used in all three. It contained the organelles of *N. tabacum*. The other fusion partners were 3 different male-sterile cultivars of *N. tabacum* with the chromosomes of *N. tabacum* (2n=48) and the cytoplasmic organelles of either *N. suaveolens* Lehm., *N. glauca* Grah., or *N. undulata* Vent. These three cultivars will be identified as *(sua)tbc*, *(gla)tbc*, and *(und)tbc* after the system introduced by Gerstel (1980), in which the donor species of the cytoplasm is placed in parentheses followed by the symbol for the nuclear parent. Thus, the cnx-68 cultivar is designated *(tbc)tbc*. *(Sua)tbc* and *(und)tbc* were both in a 'Burley 21' nuclear background and *(gla)tbc* was in a BP 210 nuclear background described by Berbeć and Berbeć (1976). Seeds of the male-sterile cultivars were obtained from K. Chen (Chen et al. 1977a).

The cultivar identified as *(und)tbc* is the one originally described by Burk (1960), reported to result from an interspecific cross between *N. tabacum* and *N. plumbaginifolia*. However, we believe this to be an incorrect origin since it has an isoelectric focusing pattern for ribulose-bisphosphate carboxylase-oxygenase (RuBPCO) consistent with a derivation from *N. undulata* rather than *N. plumbaginifolia* (Chen et al. 1977a); it has an incorrect floral morphology for a male-sterile *(pbg)tbc* cultivar but a correct morphology for a male-sterile *(und)tbc* (Chaplin 1964); and it has a very dissimilar DNA restriction pattern for its chloroplast DNA from the pattern of chloroplasts isolated from *N. plumbaginifolia* (Kung et al. 1981). Its chloroplast DNA restriction pattern matches that of chloroplasts from *N. undulata* (K. Chen, pers. commun.). For these reasons, we have designated this male-sterile cultivar as *(und)tbc*.

Protoplasts isolated from a cell suspension of the cnx-68 mutant were fused with mesophyll protoplasts from one of the male-sterile cultivars. Methods for fusion, culture conditions, and selection were as previously described (Glimelius and Bonnett 1981). Regenerated plants were analyzed for chromosome number as described in Glimelius and Bonnett (1981); for the chloroplast-coded proteins RuBPCO, by isoelectric focusing, and ATPase, by resistance to the fungal toxin, tentoxin; and for one mitochondrial trait, aberrations in stamen development associated with cytoplasmic male sterility. These analyses were carried out as described in Glimelius et al. (1981). Each plant originated from a separate callus.

Results

Only plants with a chromosome number of about 72 were included in this investigation. This value is expected in fusion products between protoplasts isolated from the male-sterile cultivars (48 chromosomes) and protoplasts isolated from the cnx-68 cell line (24 chromosomes). Thus, these plants probably developed from fusion products where the cytoplasm as well as the nucleus of each of the two parental types were joined into one cell.

The frequency of the chloroplast types, evaluated by electrophoresis of RuBPCO and resistance to tentoxin, is shown in Table 1. In the hybrids in which a mixture of chloroplasts had been formed by a cross of *(tbc)tbc* (×) *(und)tbc* protoplasts, no plants with *(tbc)* chloroplasts were obtained[1]. When chloroplasts of *(gla)tbc* and *(tbc)tbc* were combined, each chloroplast type appeared with equal frequency among the hybrid plants. Combinations of chloroplasts of *(sua)tbc* and *(tbc)tbc* resulted in an intermediate result with 75% of the plants containing chloroplasts of the *(sua)* type. Mixtures of chloroplast types were not observed with the isoelectric focusing technique used to evaluate RuBPCO.

Table 1. Segregation of chloroplasts in plants regenerated from hybrid cells produced by fusing protoplasts isolated from male-fertile with those from male-sterile cultivars of *N. tabacum*

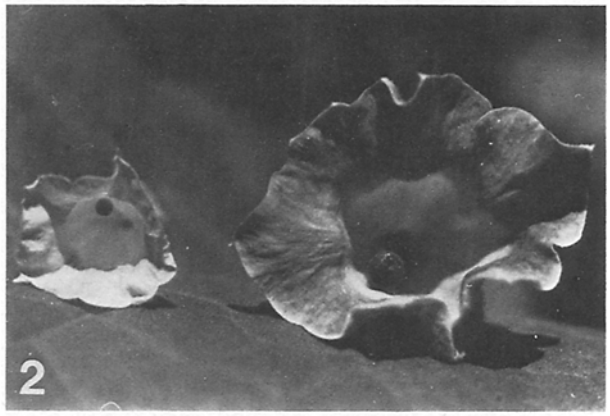
Cross	No. of hybrid plants	Chloroplast type	
		tbc	*gla/sua/und*
(tbc)tbc (×) *(gla)tbc*	21	11	10
(tbc)tbc (×) *(sua)tbc*	20	5	15
(tbc)tbc (×) *(und)tbc*	22	0	22

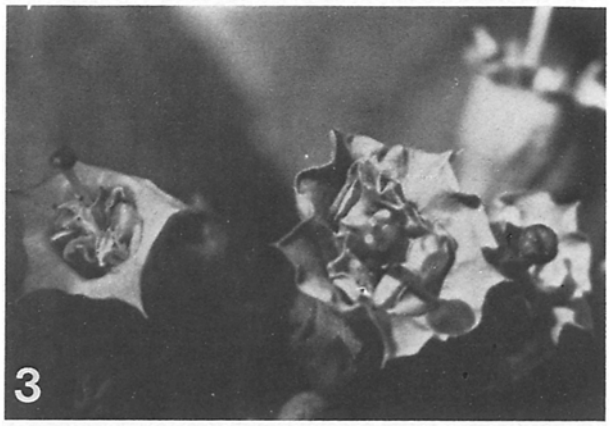
Table 2. The frequency of normal or aberrant stamen development in plants regenerated from hybrid cells produced by fusing protoplasts isolated from male-fertile with those from male-sterile cultivars of *N. tabacum*

Cross	No. of hybrid plants which flowered	Normal stamens	Aberrant stamens
(tbc)tbc (×) *(gla)tbc*	18	15	3
(tbc)tbc (×) *(sua)tbc*	21	20	1
(tbc)tbc (×) *(und)tbc*	21	15	6

Most plants in all three crosses produced flowers with stamens and pollen (Fig. 1), although pollen number and viability were reduced in all of the plants compared to male-fertile tobacco. The male-sterile morphological traits of the parental cultivar appeared in only a few hybrids (Figs. 2 and 3). One plant in the *(tbc)tbc* (×) *(gla)tbc* cross showed segregation of morphological traits. The plant produced 3 branches, one of which produced flowers exclusively of the phenotype of the *(gla)tbc* parent; the second branch produced flowers with normal stamens, while the third produced flowers with anthers on shortened filaments (Fig. 4). This plant was the only one in which the aberrant stamen development associated with the male-sterile trait segregated after plant regeneration. These results, summarized in Table 2, show that 84% of the

1 The designation proposed by Hoffmann (1983) has been used, according to which a somatic hybrid between A and B is A (×) B





hybrid plants developed anthers, whereas only 16% showed abberrations in stamen development of the type exhibited by the parental male-sterile cultivars.

Discussion

The production of somatic hybrids combining organelles in interspecific combinations has been reported by several workers within the genus, *Nicotiana.* The earliest work (Chen et al. 1977 b) supported a dual hypothesis of chloroplast behavior as follows: (1) most somatic hybrid plants do not contain mixtures of chloroplast types, but rather contain one or the other type; and (2) the appearance of one or the other chloroplast type is random with approximately half the plants containing chloroplasts of one species and half chloroplasts of other.

The first part of this hypothesis has been substantiated in numerous subsequent publications describing the chloroplast properties of somatic hybrids in *Nicotiana,* including the work described here. The second part of the hypothesis may require modification based on more recent work. The results of Chen et al. (1977 b) have been confirmed in two cases. Sidorov et al. (1981) reported a segregation of chloroplast types among hybrid plants very close to 1 : 1 for a cross of *N. tabacum* (×) *N. plumbaginifolia;* secondly, in the hybrids described here, where chloroplasts of *N. glauca* were combined with chloroplast of *N. tabacum,* a ratio of 10 : 11 was obtained with respect to chloroplast type. In these experiments no selection on the basis of organellar traits was used; nor were agents such as X-irradiation or metabolic poisons employed. According to Sidorov et al. (1981) these agents can modify the pattern of segregation of chloroplasts among the regenerated plants.

Fig. 1. Flower of the (*sua*)*tbc* cultivar (right) showing absence of stamens. Flower of somatic hybrid from (*sua*)*tbc* (×) (*tbc*)*tbc* cross showing stamen development. × 1

Fig. 2. Flower of the (*gla*)*tbc* cultivar (left) showing absence of stamens. Flower of somatic hybrid from (*gla*)*tbc* (×) (*tbc*)*tbc* cross showing the same male-sterile phenotype (no stamens). × 1

Fig. 3. Flower of the (*und*)*tbc* cultivar (left) showing the appearance of petaloids instead of stamens. Flowers of the somatic hybrid from (*und*)*tbc* (×) (*tbc*)*tbc* cross showing the same male-sterile phenotype (petaloidy). × 1

Fig. 4. Flowers of somatic hybrid from (*gla*)*tbc* (×) (*tbc*)*tbc* cross in which different shoots of the same plant exhibited different anther development. Shoot represented by flower on right produced no stamens; that in middle produced stamens on shortened filaments; that on left produced anthers on filaments of near normal length. × 0.7

In all three cases cited, the chloroplasts have developed from the same or closely similar ancestral stock. The chloroplasts of *N. tabacum* are indistinguishable from those of its ancestral female, *N. sylvestris*, when compared by restriction endonucleases and other chloroplast traits (Aviv et al. 1980). Moreover, the chloroplast DNAs of *N. tabacum*, *N. glauca*, and *N. plumbaginifolia* are very similar based on restriction endonuclease analyses (Kung et al. 1982). Thus, equal frequency of appearance of chloroplast type among the regenerated plants occurred when the hybrids were produced between parental types having very similar chloroplast DNA.

In contrast to an apparently random pattern of segregation, three reports exist of a biased pattern of segregation. Glimelius et al. (1981) reported that somatic hybrid plants regenerated from a cross combining chloroplasts of *N. tabacum* with those of *N. suaveolens* yielded a significant majority of plants with chloroplasts of *N. suaveolens*. *N. suaveolens* belongs to the Australian group of *Nicotiana* species which is not closely related to and which differs in DNA restriction profiles from *N. tabacum* (Kung et al. 1982). Douglas et al. (1981) reported that 79% of somatic hybrid plants (11 of 14) from a cross of *N. tabacum* (×) *N. rustica* contained chloroplasts of *N. rustica*. In the work described here, hybrids regenerated from a cross combining chloroplasts of *N. undulata* with those of *N. tabacum* all contained chloroplasts of *N. undulata*. *N. rustica* and *N. undulata* are closely related to each other; Goodspeed (1954) concluded that *N. undulata* or its progenitors contributed to the amphiploid origin of *N. rustica*. In addition, the chloroplast DNAs of the two species contain numerous differences in restriction sites from *N. tabacum* chloroplast DNA (Kung et al. 1982).

Thus, evidence for random and non-random segregation of chloroplasts now exists in *Nicotiana* which can be correlated with chloroplast DNA and phylogeny among the *Nicotiana* species. We propose that the behavior of mixtures of chloroplasts depends on whether their genotypes result in selective or non-selective replication within the nuclear environment they are placed. In either case, the population of chloroplasts in plants produced by somatic hybridization results from random partitioning of chloroplasts which usually culminates in complete segregation by the time chloroplast type is analyzed; i.e., only one chloroplast type is found. That this segregation is frequently completed in what appears to be too few cell generations (Chen et al. 1977b) could be due to the occurrence of unequal divisions early in the regeneration of protoplasts into small cell clusters. The reduction of chloroplast numbers which would occur in the smaller cell, if it were a progenitor cell of the shoot system, would result in a dramatic reduction in the number of cell generations necessary to achieve complete segregation. Coupled with this random partitioning is the relative replication behavior of the two chloroplast types. A selective replication would introduce a bias on behalf of one chloroplast type.

On the other hand, chloroplast segregation may appear complete only because methods used to analyze chloroplast types were not sensitive enough to detect low levels of chloroplast heterogeneity. For example, Iwai et al. (1981) grew anther plantlets from a plant regenerated after fusing *N. tabacum* and *N. rustica* protoplasts. The plant contained chloroplasts of *N. tabacum* based on isoelectric focusing of RuBPCO, but 2 of 9 androgenic plants contained chloroplasts of the *N. rustica* type. Furthermore, in other experiments, mixtures of plastid types have been detected among regenerated plants in all cases in which a plastid chlorophyll deficiency was used as one of the markers (Gleba et al. 1975; Gleba 1978; Glimelius and Bonnett 1981; Sidorov et al. 1981; Nakata and Oshima 1982).

Morphological aberrations in stamen development associated with the cytoplasmic male-sterile parental types frequently segregated independently of the chloroplast type, producing new combinations of organellar traits. Among the hybrid plants with normal anthers described here, many contained the chloroplasts of the male-sterile cultivar; of the few hybrid plants with aberrant stamen development, one had tobacco chloroplasts. Thus, the morphological traits associated with cytoplasmic male sterility and chloroplast type do not segregate together, as previously found by Belliard et al. (1978); Gleba (1978); Aviv and Galun (1980); and Glimelius et al. (1981).

Of the 60 hybrid plants from the three crosses, only 10 displayed aberrant development of stamens resembling the parental cultivars. Thus, sterility factors were generally overcome by fertility factors. In contrast to these results are those of Gleba (1978), in which somatic hybridization of a male-sterile and male-fertile cultivar gave plants which were entirely male sterile, and Belliard et al. (1978), in which different degrees of expression of male sterility appeared among plants regenerated in a similar somatic hybridization experiment. In addition, both Belliard et al. (1978) and Aviv et al. (1980) recovered male-sterile cybrids among regenerated plants which proved that cytoplasmic male sterility could be transmitted from one cultivar to another in a single-step process by protoplast fusion (Galun et al. 1982).

One interpretation of the results described here is that the "dominance" of fertility among the hybrids occurs because protoplasts contain many more mitochondria than chloroplasts. If mitochondrial-encoded fertility factors are dominant over sterility factors, a preponderance of hybrids with normal stamen development would result from an insufficient number of cell divisions to permit segregation of mitochondrial DNA by the time of flowering. More cell divisions, which may be realized by rearing seeds from male-fertile hybrids, may permit segregation of the fertility and sterility traits to occur, resulting in the reappearance of male sterility in subsequent generations. Experiments to test this interpretation in our *Nicotiana* somatic hybrids are currently under way.

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