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Genetic behavior of somatic hybrids in the genus *Nicotiana*: *N. otophora* + *N. tabacum* and *N. sylvestris* + *N. tabacum*

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Summary. Somatic hybrids have been produced between *N. tabacum* and two closely related species in the genus *Nicotiana*, *N. otophora* and *N. sylvestris*, to evaluate interclonal variation and genetic behavior of these hybrids. As with the previously reported *N. nesophila* + *N. tabacum* somatic hybrids, we have detected variation for morphological and isoenzyme characters between somatic hybrid clones, despite stability of chromosome number. One clone of *N. sylvestris* + *N. tabacum* was marked by variation in leaf spot frequency. The inheritance of this unstable trait was monitored through two sexual generations. Transmission of the Su gene marker was monitored in self-fertilized and backcrossed progeny of the *N. sylvestris* + *N. tabacum* somatic hybrids. Segregation ratios were similar to those previously reported for amphiploid *N. sylvestris* × *N. tabacum* sexual hybrids.

Key words: *Nicotiana* – Somatic hybrids – Protoplast fusion – Genetic variability – Variegation

Introduction

The genus *Nicotiana* contains 65 taxonomically characterized species. Several species of *Nicotiana* have been combined with cultivated tobacco using sexual hybridization to introduce useful agricultural traits into tobacco. This has resulted in the development of several cultivated tobacco varieties that contain genes derived from the wild species (Collins et al. 1978). Since routine techniques are available for plant regeneration from isolated leaf mesophyll protoplasts of numerous *Nicotiana* species (Takebe et al. 1971; Bourgin et al. 1979; Evans 1979), a great deal of effort has been directed toward production of interspecific *Nicotiana* somatic hybrids. Most protoplast fusion experiments have used *N. tabacum*, cultivated tobacco, as one source of protoplasts and combined this species with protoplasts derived from the wild species. Emphasis has been placed on the production of somatic hybrids between species that are difficult or impossible to produce sexually (Evans et al. 1981), and include numerous unsuccessful attempts to produce intergeneric hybrids using *Nicotiana* species (Zenkteler and Melchers 1978; Kao 1977). Intergeneric hybrids of *N. chinensis* + *Atropa belladonna* (Gleba et al. 1982) and *N. tabacum* + *Salpiglossis sinuata* (Nagao 1982) have recently been reported. However, both of these hybrids are sterile and, at least temporarily, cannot be incorporated into breeding programs.

Phenotypic variability has been reported in several somatic hybrids. While in most cases this variability can be ascribed to variation in chromosome number, we have previously reported phenotypic variability despite chromosome stability between clones of somatic hybrids between distantly related *Nicotiana* species (Evans et al. 1982). In order to understand the nature of this interclonal variation and to examine the genetic behavior of somatic hybrids, we produced somatic hybrids combining *N. tabacum* with *N. sylvestris*, a progenitor species, and with *N. otophora*, a sexually compatible species in the Tomentosa subsection of the genus.

Materials and methods

Plant material

Seeds of *N. sylvestris* and *N. otophora* were supplied by L. G. Burk (Oxford, NC). We produced reciprocal sexual hybrids between these two wild species and light green (Su/su)

N. tabacum (John Williams Broadleaf), the same variety of tobacco used in our earlier somatic hybridization experiments. All parental and hybrid plants were raised to maturity in the greenhouse. *N. otophora*, which normally requires a year to flower, could be induced to flower prematurely using short day conditions. Cell suspension cultures of albino (Su/Su) *N. tabacum* were maintained in liquid MS medium (Murashige and Skoog 1962) with 4.5 μM 2,4-D. These cell cultures were monitored for chromosome number (2n = 48) prior to protoplast fusion, using previously reported methods (Evans and Reed 1981). This cell line has remained chromosomally stable for over four years, and cells are capable, when transferred to appropriate culture medium, of regenerating plants with stable chromosome number (Evans and Gamborg 1982).

Protoplast isolation, culture, and fusion

N. otophora and *N. sylvestris* plants could be used for protoplast isolation until flowering. Plants were placed in the dark for 24 h prior to treatment. A young, mature leaf was selected, surface sterilized, and the lower leaf epidermis was peeled. Leaf tissue was incubated in enzyme solution prepared by dissolving 0.5% cellulysin (CalBiochem), 0.25% pectinase (Sigma), and 0.25% hemicellulase (Rohm and Haas, Phila.) in Kao and Michayluk's (1975) protoplast medium 8p. Leaves were incubated for 5–8 h at room temperature in the dark, then diluted, filtered, and centrifuged to remove enzymes and cell debris as previously described (Kao 1977). Su/Su protoplasts were isolated by mixing 2 ml of cell culture 3–4 days after subculture, with an enzyme solution as previously described (Gamborg et al. 1979). The PEG-fusion method used has been described (Kao 1976). During each fusion experiment, nine separate dishes were used with variable concentration of protoplasts and duration of exposure to PEG (5, 10, 15 min). To conservatively identify separate hybrid clones, only one putative somatic hybrid was isolated from each fusion dish. Putative hybrids were identified using our previously reported selection method (Evans et al. 1980), i.e. wild species protoplasts regenerate only dark green shoots, Su/Su protoplasts regenerate only albino shoots, and somatic hybrids can be distinguished as light green shoots. Following fusion, protoplasts were cultured in Kao and Michayluk (1975) medium 8p. Three weeks after fusion, cells were mixed with liquid regeneration medium (MS medium with 5 μM 6-benzyladenine) and immediately plated onto agar containing this medium. Shoots could be phenotypically distinguished 3–4 weeks later. Putative hybrid shoots were transferred to rooting medium (Evans et al. 1980) as soon as possible, to minimize the accumulation of chromosomal abnormalities associated with extended duration of culture. Once rooted, plantlets could be transferred to the greenhouse or shoots could be maintained in vitro on MS medium with 1–5 μM 6-benzyladenine.

Morphological analysis

Leaf and floral measurements were recorded from young, fully expanded leaves of all plants that appeared to be in the same stage of development and from flowers immediately following anthesis. Leaf spots were counted on mature leaves as previously described (Evans and Paddock 1976). To be consistent with earlier methods, only spots that were at least 0.5 mm² were counted. The frequency of genetic events per mitosis was calculated for somatic hybrids as described for Su/su *N. tabacum* (Evans and Paddock 1976). Cells of the polyploid somatic hybrids were larger than cells of *N. tabacum* so that a correction factor of 1.20 was used in calculation of rate of MCO. At the time of counting, plants were transferred from sunlight to shade in the greenhouse to maximize visibility of spots.

Cytology

Pollen viability of hybrids was measured using the aceto-carmine method routinely reported for tobacco and closely related species (Reed and Collins 1978). Chromosomes of root tip cells were counted in somatic hybrid plants as previously described (Evans and Reed 1981). Heterochromatic knobs and megachromosomes were previously reported for certain *N. otophora* × *N. tabacum* hybrids and backcrosses (Gerstel and Burns 1970). We examined root tips for cytological phenomena using carbol fuchsin stained cells.

Isoenzymes

Isoenzymes were analyzed using methods described earlier (Evans et al. 1982). Young, fully expanded leaves were used for all samples. Horizontal 12% hydrolysed starch gels were used to evaluate peroxidase. Alanylaminopeptidase was separated on 7.5% polyacrylamide gels containing 0.5% soluble starch.

Anther culture

Young flowers were harvested from the somatic hybrids when the corolla tip just extended beyond the calyx, a developmental characteristic used to identify the optimum stage for culture of *N. tabacum* anthers. Flowers were surface sterilized and anthers excised and placed onto MS medium devoid of hormones.

Results

Verification of hybridity

Several criteria could be used to verify hybridity of plants regenerated following protoplast fusion. Shoots of putative *N. otophora* + *N. tabacum* (*NO* + Su/Su) and *N. sylvestris* + *N. tabacum* (*NS* + Su/Su) somatic hybrids were selected based on light green pigmentation. While all isolated shoots for both hybrid combinations were light green, the recovered plants varied from pale yellow-green (Clone *NS* + Su/Su-D) to almost normal dark green (Clone *NO* + Su/Su-B). Both *N. tabacum* × *N. otophora* and *N. tabacum* × *N. sylvestris* interspecies sexual hybrids were uniformly pale yellow-green. Five clones of each hybrid were separated and maintained for further analysis (Table 1). Each of these 10 hybrid clones initially contained the summation chromosome number (2n = 72) of cultivated tobacco plus the wild species. Also, as a reflection of ploidy differences, the number of chloroplasts per guard cell (40–50) and the guard cell dimensions (40 × 30 μm) were significantly larger than in either parent species (15–20 chloroplasts and 30 × 24 μm, respectively). Mor-

Table 1. Floral morphology of *N. sylvestris* + *N. tabacum* and *N. otophora* + *N. tabacum* somatic hybrids

Species	Corolla length (cm)	Corolla diameter (cm)	Stigma length (cm)	Calyx length (cm)
Nicotiana otophora	3.36±0.07	2.17±0.09	3.97±0.03	2.50±0.25
N. tabacum × *N. otophora*	4.15±0.12	2.10±0.15	4.50±0.08	2.55±0.03
NO + Su/Su-A	3.88±0.08	3.30±0.06	4.13±0.05	2.05±0.03
NO + Su/Su-B	3.18±0.09	2.45±0.13	2.38±0.11	1.80±0.11
NO + Su/Su-C	3.20±0.12	3.03±0.03	3.50±0.04	2.15±0.06
NO + Su/Su-D	3.45±0.06	2.58±0.08	3.78±0.03	1.45±0.06
NO + Su/Su-E	3.13±0.13	3.48±0.06	3.98±0.03	1.63±0.05
N. sylvestris	9.45±0.03	2.98±0.17	7.38±0.17	1.48±0.03
N. tabacum × *N. sylvestris*	6.35±0.06	2.98±0.03	4.78±0.05	2.05±0.05
NS + Su/Su-A	5.50±0.04	3.08±0.05	4.95±0.06	1.95±0.05
NS + Su/Su-B	5.70±0.15	2.83±0.09	4.50±0.06	1.80±0.12
NS + Su/Su-C	3.68±0.06	2.08±0.05	3.80±0.12	1.55±0.03
NS + Su/Su-D	5.85±0.12	2.78±0.08	5.20±0.04	1.93±0.05
NS + Su/Su-E	5.58±0.09	2.95±0.10	4.68±0.05	1.90±0.04
N. tabacum	5.23±0.13	2.93±0.08	4.03±0.03	2.00±0.01

phological characters for each of the somatic hybrids were intermediate for most of the clones. Data for four floral characters are summarized in Table 1 while leaf shape, measured as the ratio of width over length, is summarized in Table 2. Most *NO* + Su/Su somatic hybrids contained both wild species and cultivated tobacco enzyme bands for both peroxidase and alanyl-aminopeptidase (AAP). The banding patterns of *N. sylvestris* and *N. tabacum* were identical for AAP, however, *NS* + Su/Su hybrids contained distinct peroxidase bands derived from each of the parent species. As expected, pollen viability was reduced for each hybrid line and was quite variable between clones.

Table 2. Pollen viability and leaf shape of *N. sylvestris* + *N. tabacum* and *N. otophora* + *N. tabacum* somatic hybrids

Plants	Pollen viability[a]	Leaf width/length[b]
Nicotiana otophora	100.0	0.452±0.006
N. tabacum × *N. otophora*	0.0	0.416±0.003
NO + Su/Su-A	53.1	0.582±0.034
NO + Su/Su-B	23.2	0.479±0.004
NO + Su/Su-C	44.2	0.665±0.031
NO + Su/Su-D	53.2	0.550±0.010
NO + Su/Su-E	24.2	0.533±0.028
N. sylvestris	100.0	0.449±0.011
N. tabacum × *N. sylvestris*	84.9	0.571±0.033
NS + Su/Su-A	92.6	0.638±0.015
NS + Su/Su-B	none[c]	0.587±0.015
NS + Su/Su-C	49.8	0.495±0.032
NS + Su/Su-D	90.6	0.499±0.034
NS + Su/Su-E	74.2	0.502±0.034
N. tabacum	99.0	0.239±0.004

[a] Based on 300 – 1500 pollen grains per plant
[b] Mean of four leaves per plant
[c] Clone with petaloid anthers

As *N. otophora* and *N. tabacum* are more distantly related than *N. sylvestris* and *N. tabacum,* it is not surprising that more unique morphological characteristics could be identified in *NO* + Su/Su somatic hybrids. Several *N. otophora*-derived characters were expressed in *NO* + Su/Su plants. Both *N. otophora* and *NO* + Su/Su plants have branched trichomes, while *N. tabacum* has only simple trichomes. The number of trichomes per mm² is less in *N. otophora* and *NO* + Su/Su plants (15–16/mm²) than in *N. tabacum* plants (25–30/mm²). Following a successful pollination, the seed capsules of both *N. otophora* and *NO* + Su/Su plants turn pink, another trait unique to *N. otophora.* Cells of *N. otophora* contain distinct heterochromatic knobs. This character is also expressed in *NO* + Su/Su somatic hybrids (Fig. 1). The *NO* + Su/Su-A and *NO* + Su/Su-D clones were previously reported to be tentoxin sensitive suggesting that both clones have cytoplasm derived from *N. otophora* (Flick and Evans 1982).

Phenotypic variability

Nearly all the morphological characters described above are quite variable between clones of somatic hybrids (Table 1 and 2). Leaf color was quite variable. For example, *NS* + Su/Su-C, the clone with highest pigment content, was almost dark green in color. On

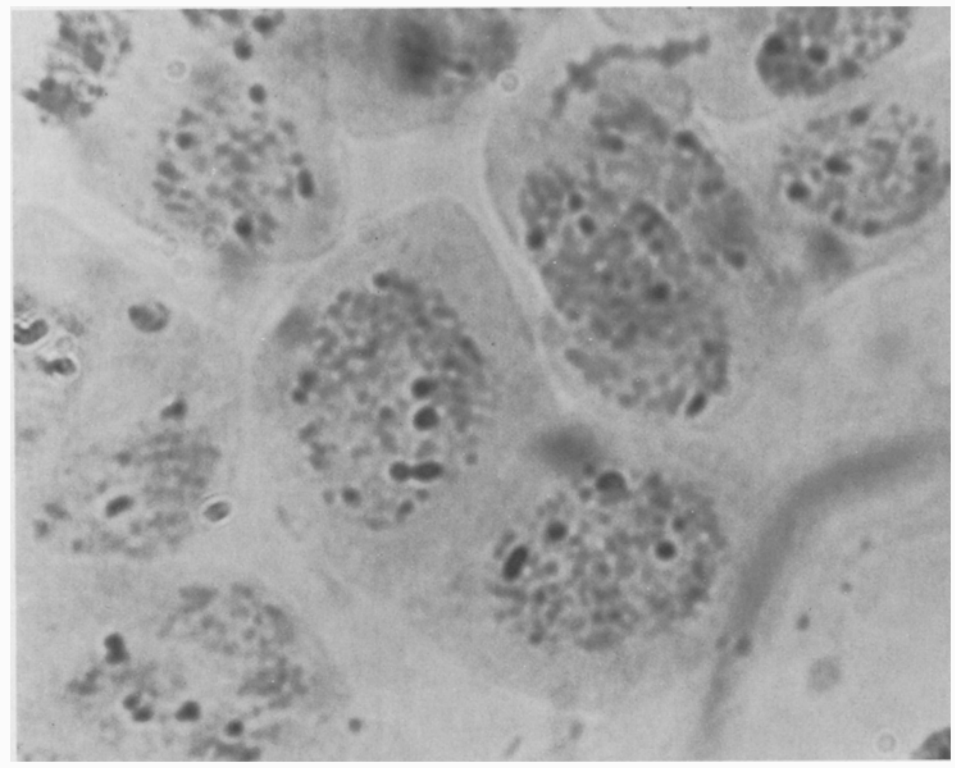

Fig. 1. Heterochromatic knobs in root tip cells of *N. otophora* + *N. tabacum* (*NO* + Su/Su) somatic hybrid. This represents expression of an *N. otophora*-specific trait

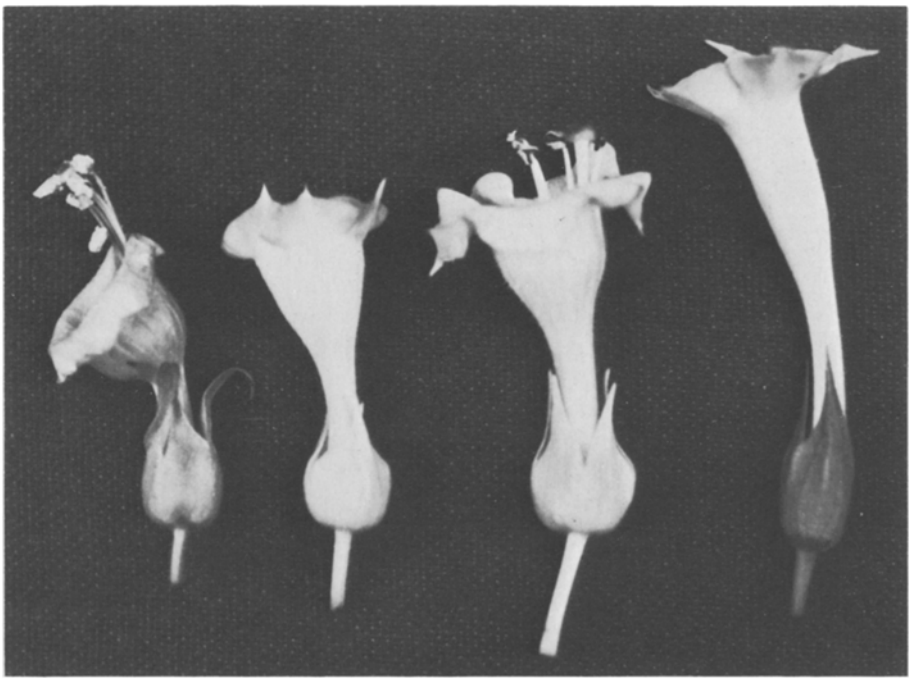

Fig. 2. (*Left to right*) Flowers of *N. otophora*, *NO* + Su/Su-A, *NO* + Su/Su-B somatic hybrids, and *N. tabacum*. The *NO* + Su/Su-B clone has inserted stigmas, while the remaining clones of *NO* + Su/Su have exerted stigmas

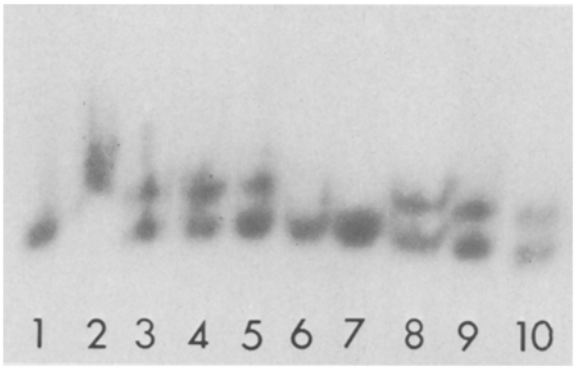

Fig. 3. Gel electrophoresis of alanylaminopeptidase: (*1*) *N. tabacum*, (*2*) *N. otophora*, (*3*) *N. tabacum* × *N. otophora* sexual hybrid, (*4*) *NO* + Su/Su-A somatic hybrid, (*5*) *NO* + Su/Su-B, (*6*) and (*7*) two regenerated plants from leaf explants of *NO* + Su/Su-C clone, (*8*) *NO* + Su/Su-D, (*9*) and (*10*) two haploid (2n = 36) plants derived from anther culture of the *NO* + Su/Su somatic hybrid. Clone *NO* + Su/Su-C is missing the band derived from *N. otophora* despite the presence of 72 chromosomes

the other hand, clone *NS* + Su/Su-D, the lightest clone, was almost yellow in color. In general, the *NS* + Su/Su and *NO* + Su/Su somatic hybrids and the *N. tabacum* × *N. sylvestris* and *N. tabacum* × *N. otophora* sexual hybrids had lighter leaf color than our previously reported *NN* + Su/Su, *NSt* + Su/Su, and *NG* + Su/Su somatic hybrids (Evans et al. 1980, 1981). As both *N. sylvestris* and *N. otophora* are closely related to *N. tabacum* and contain only 24 chromosomes, this lighter leaf color, i.e. greater influence of the Su gene, may be the result of gene dosage. Pollen viability of most *NS* + Su/Su clones is comparable to amphiploid sexual hybrids between these two closely related species. However, one clone, *NS* + Su/Su-B, was male sterile with petaloid anthers and no pollen. Sexual hybrids between *N. otophora* and *N. tabacum* (2n = 36) are sterile. All the *NO* + Su/Su somatic hybrids are at least partially fertile. However, pollen viability is variable between these clones (Table 2). Flower morphology was also variable both within and between clones of these somatic hybrids. Some plants had flowers with split petals. One clone, *NO* + Su/Su-B, had inserted stigmas, an *N. tabacum*-derived trait, while the remaining clones had exerted stigmas, typical of *N. otophora* (Fig. 2). The *NS* + Su/Su-B clone had exerted stigmas, a trait uncommon in either parent, while all other *NS* + Su/Su clones had inserted stigmas. While nearly all clones contained a sum of parental bands for peroxidase and alanylaminopeptidase (AAP), clone *NO* + Su/Su-C had only the *tabacum*-derived band for AAP (Fig. 3). *NO* + Su/Su-C had both the *tabacum* and *otophora* peroxidase bands. The *otophora* AAP band was also absent in two plants regenerated from different leaf explants of the *NO* + Su/Su-C plant.

Spots, similar to those observed on the leaves of Su/Su *N. tabacum*, were observed on the leaves of *NO* + Su/Su and *NS* + Su/Su somatic hybrids. The frequency of spot formation and the ratio of spot types was variable between clones of these somatic hybrids. To a large degree, the ratio of dark green to aurea spots is dependent on the visibility of the spots, i.e. with darker leaves aurea spots are more visible, while with lighter leaves, dark green spots are more visible. This is reflected in the dark green : aurea spot ratio (Table 3). The two clones with

Table 3. Spot frequency of leaves of *N. otophora* + *N. tabacum* and *N. sylvestris* + *N. tabacum* somatic hybrids

Plant	Mean spots per square decimeter of leaf surface				Ratio DG/A
	Dark green	Aurea	Double	Total	
Nicotiana tabacum (Su/su)	1.38	–	0.17	1.55	–
N. otophora	–	–	–	–	–
N. tabacum × *N. otophora*	1.21	1.38	–	2.60	0.9
NO + Su/Su-A	10.9	0.52	–	11.46	21.0
NO + Su/Su-B	6.1	30.9	0.28	37.25	0.2
NO + Su/Su-C	17.6	0.83	0.12	18.53	21.2
NO + Su/Su-D	7.6	4.2	–	11.79	1.8
N. sylvestris	–	–	–	–	–
N. tabacum × *N. sylvestris*	3.32	0.14	–	3.46	23.7
NS + Su/Su-A	14.5	0.48	0.32	15.34	30.2
NS + Su/Su-B	4.3	0.51	–	4.76	8.4
NS + Su/Su-C	0.39	3.33	–	3.73	0.1
NS + Su/Su-D	16.0	0.13	–	16.17	123.1
NS + Su/Su-E	578.7	10.37	12.33	601.40	55.8
cutting of *NS* + Su/Su-E	231.9	0.86	8.92	241.67	269.6
NS + Su/Su-E × self[a]	285.0	1.24	6.67	292.94	229.8

[a] Single plant in R_1 generation with high spot frequency

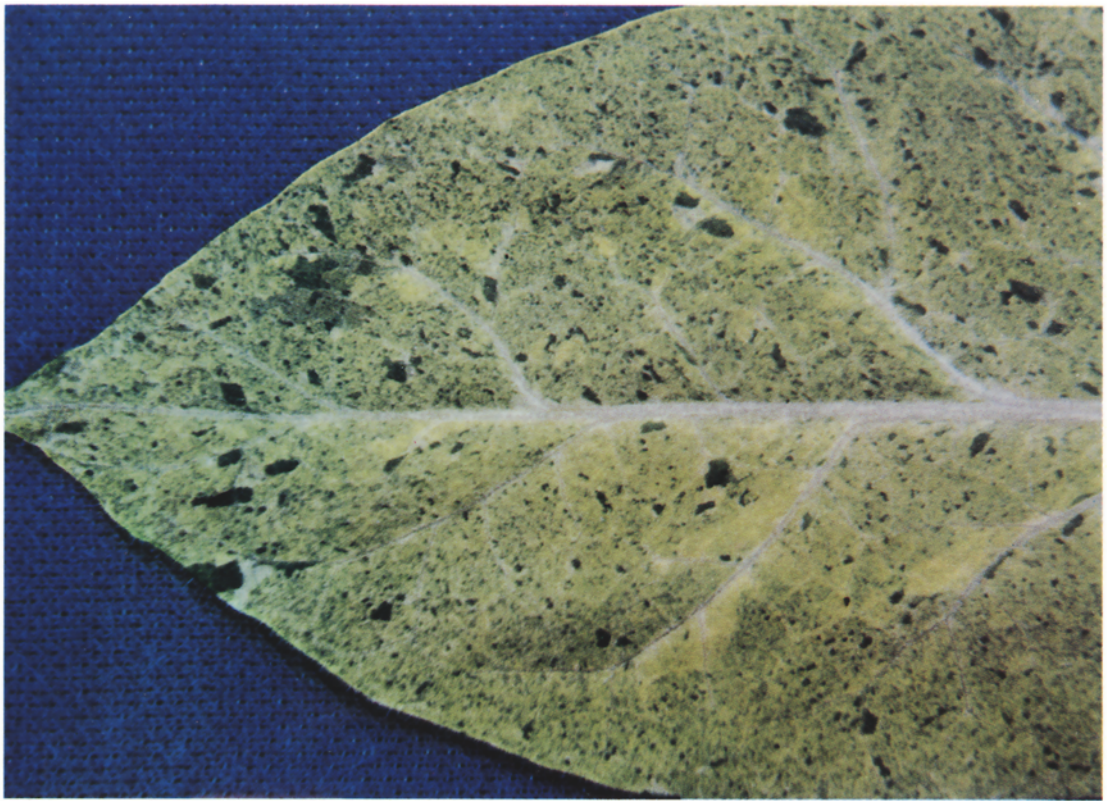

Fig. 4. Clone of *N. sylvestris* + *N. tabacum* somatic hybrid (*NS* + Su/Su-E) with high spot frequency

darkest leaves (*NO* + Su/Su-B and *NS* + Su/Su-C) have DG/A ratios less than 1, while three of the lightest clones (*NO* + Su/Su-A, *NO* + Su/Su-C, and *NS* + Su/Su-D) all have DG/A ratios greater than 20. However, total spot frequency is independent of background leaf pigment and reflects consistent significant variation between these clones. One clone, *NS* + Su/Su-E (designated 'Superspot'), had a much higher spot frequency than any other somatic hybrid clone (Fig. 4). The high spot frequency of Superspot was maintained in two separate cuttings of this clone and in sexual progeny of this clone (see below and Table 3). Plants with variable pigment have been previously reported for interspecific hybrids in the genus *Nicotiana* and in some cases represent instability resulting from intergenomic recombination (Gerstel and Burns 1970).

Genetic analysis

Several conventional genetic methods were used to analyze these somatic hybrids. Whenever possible, the somatic hybrids were both self-fertilized and backcrossed to each parent. In those somatic hybrids with low pollen viability, the range of crosses was limited. For example, the male sterile *NS* + Su/Su-B plant could be used as a female in backcrosses to each parent, but could, of course, not be self-fertilized. In general, *NS* + Su/Su plants were much more fertile than *NO* + Su/Su plants, and were therefore chosen for subsequent genetic analysis. Nonetheless, the *NS* + Su/Su × *N. sylvestris* cross was difficult to obtain and was only successful with some clones of *NS* + Su/Su. The two most distinct traits, leaf color and spot formation, were analyzed in detail. The Su leaf color gene segregated in the progeny of somatic hybrids. Data for self-fertilized and backcrossed *NS* + Su/Su progeny are summarized in Table 4. The aurea trait is transmitted in all crosses.

Table 4. Segregation data for Su phenotype in R_1 and BC, generation of *Nicotiana sylvestris* + *N. tabacum* (NS + Su/Su)[a] somatic hybrids

Cross[a]	Dark green[b]	Light green[b]	Aurea[b]
NS + Su/Su × self	30	19	6
NS + Su/Su × *N. tabacum* (Su/su)	86	45	16
NS + Su/Su × *N. sylvestris*	75	39	14

[a] Mixed progeny from several clones
[b] Classes are based on standard *N. tabacum* phenotypes. It should be recognized that there is a great deal of variation in pigment content between *NS* + Su/Su clones

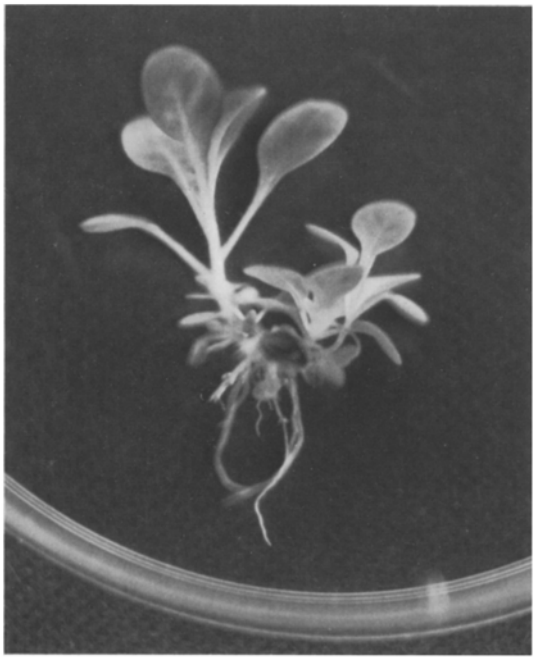

Fig. 5. Plants recovered from cultured anthers of an R_1 plant of the NS + Su/Su-E clone

The single plant with high spot frequency (Superspot) was self-fertilized and backcrossed to each parent. Only one of 10 light green plants in the R_1 generation had a similar high spot frequency (Table 3). None of the light green backcrossed (BC_1) progeny to either *N. sylvestris* or *N. tabacum* had an elevated spot frequency. This low transmission frequency precludes control by a simple Mendelian mutation, but is not unexpected for an unstable trait. The single R_1 Superspot plant was self-fertilized and the transmission of this trait to the R_2 generation was much higher. Of 37 light green R_2 plants, 27 plants were Superspot. In each instance, the spot frequency of R_2 plants was comparable to the spot frequency on leaves of the R_1 plant.

Plants were recovered from cultured anthers of an *NO* + Su/Su plant. All plants recovered were the same leaf color as the *NO* + Su/Su parent clone. In general, the plants contained fewer than the haploid chromosome number with a range of 33–36 chromosomes in three haploid *NO* + Su/Su plants examined in detail. These haploids all have abnormal leaf shape and shoot development and have not flowered even after 8 months in the greenhouse. However, these haploid plants contain all tabacum and otophora bands for both peroxidase and AAP (Fig. 3). Anthers of R_1 and BC_1 plants of *NS* + Su/Su were also used to recover haploid plants (Fig. 5). As with the *NO* + Su/Su plants, nearly all plants were light green in color, although, one very light green plant was obtained. This plant, that appeared in a culture from a self-fertilized *NS* + Su/Su R_1 plant, may represent a recombinant between paired S and S' chromosomes (Gerstel 1963). Chromosome number was less than the expected haploid number for all anther-derived plants. However, the haploid *NS* + Su/Su-derived plants appear to have more normal morphology than the *NO* + Su/Su-derived haploid plants. These anther-derived plants are being raised to maturity in the greenhouse.

Discussion

Chromosome abnormalities have been observed in anaphase and metaphase of certain plants derived from *N. tabacum* × *N. otophora* sexual hybrids. These aberrations were found in plants that were variegated for flower color. The most striking and extensively characterized cytological abnormality was the formation of megachromosomes. Megachromosomes have been shown to be the result of extensive duplication of heterochromatic blocks that have been transferred from *N. otophora* into the *N. tabacum* genomes via recombination. While we have observed distinct heterochromatic blocks in *NO* + Su/Su somatic hybrids (Fig. 1), even after examination of over 3,000 cells from all 5 clones, we have not observed a single megachromosome. This is not surprising though as Burns and Gerstel (1971) demonstrated that the presence of an intact genome of *N. otophora* inhibits megachromosome formation. In addition we have detected no variegation in these *NO* + Su/Su hybrids other than spot formation commonly associated with the Su locus. As with most *N. tabacum* × *N. otophora* sexual hybrids (Gerstel and Burns 1970), megachromosomes were also not observed in root tips of the haploid *NO* + Su/Su plants obtained by anther culture.

Classification of leaf pigment in these hybrids was complicated as the three distinct phenotypes commonly associated with the Su locus were not the only phenotypes observed. The hybrids included intermediate colors (between dark green and light green and between light green and aurea). The progeny analysis (Table 4) was also therefore complicated by the appearance of these intermediate phenotypes. It is possible that some of the plants in the arbitrary classes in Table 4 may be misclassified. However, with these data we have demonstrated that it is possible to monitor the fate of single gene traits in the progeny of interspecific somatic hybrids. Even using this arbitrary classification, it is evident that the ratios of green (light green + dark green) to aurea are comparable to previously reported discordant ratios for dominant to recessive traits in *N. tabacum* × *N. sylvestris* sexual hybrids. The approximate 8 : 1 ratio for the backcross of *NS* + Su/Su to *N. tabacum* obtained for Su is comparable to the ratios

obtained by Gerstel (1963) for the Rf locus in backcrosses of 6x (*N. sylvestris* × *N. tabacum*) to *N. tabacum*. These markers are on chromosomes R (Su locus) and M (Rf locus), each in the *sylvestris* genome. As with the ratio variation for the Rf locus (Gerstel 1963), the low frequency of light green plants in backcrosses to *N. sylvestris* may reflect incomplete pairing between the R chromosomes of the S and S' genomes. The general agreement of segregation results for our somatic hybrids with Gerstel's synthetic amphiploid sexual hybrids is reassuring. As expected, the albino trait was transmitted at a higher frequency in these somatic hybrids between closely related species than in the more distantly related *NN*+Su/Su somatic hybrids (Evans et al. 1981). This higher transmission frequency is consistent with the high degree of homology and pairing between *N. sylvestris* and *N. tabacum* chromosomes.

The genetic information for the occurrence and transmission of Superspot is more difficult to explain. Two genetic phenomena must be addressed: (1) the mechanism that controls the high frequency of spot formation on leaves of Superspot, and (2) the transmission of the trait in the R_1 and R_2 generation, i.e. the mode of inheritance of Superspot. Double spots, associated with the Su locus in *N. tabacum* occur primarily as a result of mitotic crossing over. As there is a great deal of homology between some S and S' chromosomes, it is likely that at least some of the double spots on *NS*+Su/Su leaves are the result of intergenomic recombination. We have previously reported the presence of double spots on other interspecific somatic hybrids (Evans et al. 1980, 1981). The frequency of double spot formation on leaves of Superspot can be estimated as 1.48×10^{-3} mitotic crossovers (MCO) per cell. This is much higher than our previously reported frequency of 7.70×10^{-6} MCO per cell of *N. tabacum* (Evans and Paddock 1976). The very high frequency of single spots suggests that other mechanisms besides mitotic crossing over are no doubt responsible for the high spot frequency on leaves of Superspot. Any genetic change that interferes with physical presence or expression of the Su gene can result in formation of a dark green single spot. Several hypotheses have been suggested to account for formation of single spots for this locus and a comparable locus in soybean (Vig 1978). These mechanisms include mutation, non-disjunction, transposons, gene conversion, deletion, etc. The pattern of inheritance in the R_1 (low frequency of transmission) and the R_2 (high frequency of transmission) generations preclude control of Superspot by recessive or dominant nuclear, multiple nuclear, or cytoplasmic genes. Restriction enzyme analysis showed no differences between Superspot, *N. tabacum*, or *N. sylvestris* in banding patterns for the enzymes Xba I, Kpn I, Sal I, Sma I, Eco RI, Bam H1, and Hind III (Flick and Evans, unpublished) suggesting that chloroplast DNA is not involved in control of Superspot. The transition from low transmission to high transmission frequency underscores the instability of this trait. Unstable traits resulting in variegated plants with similarities to Superspot have been described in several other higher plants. These include variation in maize kernel color (Fincham and Sastry 1974) and *Antirrhinum* and *Impatiens* flower color (Sastry et al. 1981). In most cases, these instabilities have been explained by transposable elements. As with our segregation data for Superspot, abnormal segregation ratios were also reported for the *Antirrhinum* system (Sastry et al. 1981). It should be noted that variation has previously been reported following interspecies sexual hybridization for other *Nicotiana* species. Flower color variation was observed in plants derived from *N. langsdorfii* × *N. sanderae* (Smith and Sand 1957) and *N. tabacum* × *N. otophora* (Gersel and Burns 1970) sexual hybrids. Hence, several characteristics of the Superspot system suggest comparisons to transposons.

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