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Non-random chloroplast segregation in *Nicotiana tabacum* (+) *N. rustica* somatic hybrids selected by dual nuclear-encoded resistance

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Abstract. *Nicotiana tabacum* (+) *N. rustica* interspecific somatic hybrids were produced by fusion of leaf mesophyll protoplasts of transgenic methotrexate-resistant *Nicotiana tabacum* L. with leaf mesophyll protoplasts of transgenic kanamycin-resistant *N. rustica* L. Somatic hybrids were selected on the basis of resistance to both methotrexate and kanamycin. Evidence for nuclear hybridization was obtained for 21 hybrids by restriction-fragment-length-polymorphism (RFLP) analysis using a heterologous wheat nuclear ribosomal-DNA (rDNA) probe and by analysis of glutamate-oxaloacetate transaminase (GOT) isoenzymes. Chloroplasts segregated non-randomly as 20 of the somatic hybrids possessed *N. rustica* chloroplasts and only one had *N. tabacum* chloroplasts. Patterns of mitochondrial inheritance were examined by hybridization of a heterologous wheat cytochrome oxidase subunit II (*coxII*) gene with genomic DNA of the somatic hybrids. Four somatic hybrids with hybridization patterns similar to *N. rustica* and 17 with hybridization patterns consistent with mitochondrial DNA (mtDNA) rearrangement or recombination were obtained. None of the somatic hybrids had patterns of *coxII* hybridization identical with *N. tabacum*. Male-fertility levels in the hybrids ranged from undetectable to 87% and only nine hybrids produced a limited amount of viable seed. There was no apparent correlation between the patterns of organelle inheritance in the somatic hybrids and the relative degree of fertility.

Key words: Antibiotic selection – Somatic hybrid – *Nicotiana tabacum* (+) *N. rustica* – Chloroplast segregation – Mitochondrial inheritance

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Introduction

Somatic hybridization facilitates genetic exchange between plant species which are sexually incompatible. In the genus *Nicotiana*, a variety of agronomically important traits which are present in reproductively-isolated wild species may be transferred, using somatic hybridization, to the cultivated species *N. tabacum* L. (tobacco). In the past, lack of universal hybrid selection systems often restricted the parental species used for somatic hybridization to only a few select mutant genotypes. For example, an improved tobacco variety recently registered in Canada, has in its pedigree an interspecific *N. tabacum* (+) *N. rustica* somatic hybrid (Pandeya et al. 1986, 1991). This somatic hybrid was selected by complementation of parental chlorotic mutant genotypes *N. tabacum* L. cv WS and *N. rustica* L. cv chlorotica (Douglas et al. 1981a). Future use of somatic hybridization for crop improvement, in *Nicotiana* and other genera, will benefit from the development of efficient hybrid selection systems and the recovery of fertile somatic hybrids which can be backcrossed with the crop species. Recently we recovered fertile *N. tabacum* (+) *N debneyi* somatic hybrids following fusion of leaf mesophyll protoplasts of transgenic methotrexate-resistant *N. tabacum* and kanamycin-resistant *N. debneyi* (Sproule et al. 1991). Fusion products were selected on the basis of resistance to both methotrexate and kanamycin. We use the same approach here in attempts to recover fertile *N. tabacum* (+) *N. rustica* somatic hybrids using elite *N. tabacum* L. cultivars Delgold and Candel and the wild species *N. rustica* var. NRT.

Sexual hybridization of *N. tabacum* and *N. rustica* is incompletely blocked by post-zygotic incompatibilities (Brink and Cooper 1941). Recovery of sexual hybrids has only been possible using *N. rustica* as the maternal parent

(Brink and Cooper 1941; Swaminathan and Murty 1957; Douglas et al. 1983). Novel alloplasmic combinations may thus be obtained by somatic hybridization of these species. Chloroplasts of somatic hybrids are often derived at random from one or the other parental species (Chen et al. 1977; Sundberg and Glimelius 1991) although hybrids, or cybrids, obtained from fusions of partially or completely incompatible species, may show non-random patterns of chloroplast segregation (Flick and Evans 1982; Bonnett and Glimelius 1983; Kushnir et al. 1991; Sundberg and Glimelius 1991). A survey of chloroplast segregation patterns reported for *N. tabacum* (+) *N. rustica* somatic hybrids (Nagao 1978; Iwai et al. 1980; Douglas et al. 1981a, c; Hamill et al. 1984; Pental et al. 1984; Toki and Kameya 1988) reveals, in several instances, a bias towards the inheritance of *N. rustica* chloroplasts. Extenuating factors, including the cell type used for fusion (Hamill et al. 1984) or the heterokaryon selection strategy used (Douglas et al. 1981 a, c), were proposed to be the cause. However, other factors such as alloplasmic incompatibility or plastid input bias are also known to influence chloroplast segregation between incompatible and/or distantly related species (Kushnir et al. 1991; Sundberg et al. 1991). We investigated patterns of chloroplast inheritance in the hybrids we obtained, to determined whether a random pattern of chloroplast segregation can be obtained for *N. tabacum* (+) *N. rustica* somatic hybrids recovered in the absence of extenuating protocol-related factors which might otherwise affect chloroplast segregation.

We also examine mitochondrial inheritance in the somatic hybrids since such data is not available for most *N. tabacum* (+) *N. rustica* somatic hybrids described previously (Nagao 1978; Iwai et al. 1980; Douglas et al. 1981 a; Hamill et al. 1984; Pental et al. 1984; Toki and Kameya 1988). Also, it remains unclear whether the unique patterns of mitochondrial segregation which were reported by Pental et al. (1989) for 22 *N. tabacum* (+) *N. rustica* gametosomatic hybrids (in which none inherited mtDNA identical to the parental tobacco mtDNA) are unique to the gametosomatic hybrids of these species.

Materials and methods

Plant material

Transgenic parental genotypes used for fusions were *Nicotiana tabacum* L. (cultivars Delgold and Candel) carrying a chimaeric dihydrofolate reductase (*dhfr*) gene and *N. rustica* L. (cultivar NRT) carrying a chimaeric neomycin phosphotransferase II (*npt*II) gene. These were produced via *Agrobacterium*-mediated transformation of leaf explants as described previously (Dijak et al. 1991). Selfed-seed (S1), or alternatively backcross seed (BC1) from crosses of the transgenic plants with control untransformed genotypes, was germinated on agar-solidified (0.8% w : v Difco agar) B5 medium (Gamborg et al. 1968) with 2% sucrose and supplemented with either 150 mg/l kanamycin sulphate or 10 mg/l methotrexate [(+)-amethopterin, Sigma A-6770]. Selected methotrexate- or kanamycin- resistant S1 or BC1 progeny were maintained in vitro in the absence of selective pressure as described previously (Sproule et al. 1991) and were used for protoplast isolations.

Isolation/fusion of protoplasts and recovery of double-resistant calli

Leaf mesophyll protoplasts were isolated from young in-vitro grown seedlings, essentially as described previously (Dijak et al. 1991) but, prior to enzyme digestion, leaves were cut into narrow strips and the abaxial epidermis was left intact. PEG-mediated fusion in glass tubes was performed according to Douglas et al. (1981 a) with some modifications described by Sproule et al. (1991). Fused protoplasts (5×10^4 of each species), or control unfused protoplasts (5×10^4) were suspended in 1 ml of NT medium and plated in 60 × 15 mm Falcon dishes over 2 ml of 'underlayer' medium composed of NT medium solidified with 0.4% w : v agarose (SeaKemLe) and supplemented with either 150 mg/l kanamycin, 10 mg/l methotrexate or no selective agent. NT medium was as described by Nagata and Takebe (1971) and modified by Douglas et al. (1981 a) containing 40 mg/l sequestrene Fe330 as a source of iron, and 0.45 M glucose. One week after fusion, 1 ml of NT medium with only 0.3 M glucose (and supplemented with the same selective agent present in the 'underlayer' of the respective culture) was added to each culture. Three to six weeks after fusion, the liquid medium with surviving microcolonies and cellular debris was transferred to regeneration medium. The regeneration medium consisted of agar-solidified Murashige and Skoog (1962) medium modified by the addition of B5 vitamins (Gamborg et al. 1968) and 40 mg/l sequestrene Fe330 with 2% sucrose, 2 mg/l kinetin and 0.1 mg/l NAA and supplemented with either 150 mg/l kanamycin, or 2 mg/l methotrexate. After 4 weeks, surviving microcolonies were transferred to fresh regeneration medium containing 150 mg/l kanamycin and 2 mg/l methotrexate. Shoot cultures were maintained on regeneration medium containing both kanamycin and methotrexate. Shoots were rooted in vitro and the plants were grown to maturity as described previously (Sproule et al. 1991).

RFLP, isoenzyme and organellar DNA analysis

Genomic DNA was isolated from fresh leaves of the parental species and the somatic hybrids by the SDS/proteinase K treatment, phenol/chloroform extraction method essentially as described (Draper and Scott 1988). Five micrograms of genomic DNA were digested with restriction endonucleases *Eco*R1, *Bgl*I, or *Pvu*II (Promega). DNA fragments were separated by electrophoresis in an 0.8% agarose gel, denatured, and transferred to nylon membranes (Nytran™, Schleicher and Schuell) using standard solutions and protocols as described by Maniatis et al. (1982). Membranes were prehybridized in 6 × SSC, 10 × Denhardt's reagent, 0.5% SDS, and 100 µg/ml sheared, denatured calf thymus DNA, and hybridizations were carried out at 65 °C in 6 × SSC, 0.5% SDS, and 50 µg/ml sheared, denatured calf thymus DNA. Following hybridization, final stringent washes were performed at 65 °C in 0.1 × SSC, 1% SDS. DNA probes used for analysis of the nuclear, chloroplast and mitochondrial genomes respectively were: pTA71 encoding the heterologous wheat nuclear ribosomal DNA repeat unit (Gerlach and Bedbrook 1979) pBa1–9 which consists of three *Pvu*II fragments of the *N. tabacum* chloroplast genome cloned in pBR322 (Aviv et al. 1984) (kindly provided by E. Galun), and a heterologous mtDNA probe consisting of a 5′ portion of the wheat cytochrome oxidase subunit II (*cox*II) gene (Bonen et al. 1984)

kindly provided by L. Bonen. DNA probes were radioactively labelled by the random-primer method, according to the manufacturer's specifications, using a DNA-labelling kit (Prime-a-Gene, Promega).

Leaf glutamate-oxaloacetate transaminase (GOT) isozymes were extracted in ice-cold 0.2 M Tris. HCl (pH 8.5) with 30 mM dithiothreitol (DTT) and subjected to nondenaturing polyacrylamide gel electrophoresis (5.0% acrylamide). GOT activity was detected by incubation of the gel as described (Thorpe et al. 1987).

Fertility and morphology

The percentage of pollen stainable with acetocarmine was determined as the average value for three individual flowers per somatic hybrid. One-thousand pollen grains from one anther per flower were scored. The frequency of in-vitro germination of selfed-seed of the fertile somatic hybrids was tested on agar-solidified B5 medium with 2% sucrose. For morphology analysis, flower length was measured as the distance from the sepal base at the pedicel to the tip of the corolla lobes.

Results

Protoplast fusion and recovery of double-resistant calli

Protoplast viability and division frequency, and the formation of protoplast-derived microcolonies was compared for control *N. tabacum* (*dhfr*) and *N. rustica* (*npt*II) protoplasts and for *N. tabacum* (*dhfr*) + *N. rustica* (*npt*II) fusions plated on either selective-agent-free medium, or medium with only kanamycin or only methotrexate. *N. tabacum* (*dhfr*) protoplasts had similar rates of viability, division, and microcolony formation on medium containing methotrexate compared with medium lacking this selective agent. *N. rustica* (*npt*II) protoplasts, in contrast, were unable to proliferate and form colonies on medium containing methotrexate. Similarly, growth of *N. tabacum* (*dhfr*) protoplasts in the presence of kanamycin was effectively inhibited. For *N. rustica* (*npt*II) protoplasts, growth was unexpectedly inhibited in the presence of kanamycin in comparison with growth on selective-agent-free medium. In most experiments, *N. rustica* (*npt*II) did not develop colonies in the presence of kanamycin. However *N. rustica* (*npt*II) protoplasts which were allowed to develop on control medium were capable of subsequent growth in the presence of kanamycin upon transfer to regeneration medium. In spite of the poor early expression of kanamycin resistance by the *N. rustica* (*npt*II) protoplasts, we recovered 22 independent *N. tabacum* (*dhfr*) (+) *N. rustica* (*npt*II) fusion-derived calli which were resistant to both selective agents. Eight double-resistant calli were recovered from cultures plated initially on 'underlayer' medium containing no selective agent and 14 were recovered from those initiated on 'underlayer' medium with methotrexate. However, none were obtained from fusions which were initiated on 'underlayer' medium containing kanamycin. In total 21 of the 22 calli eventually regenerated in the presence of both selective agents. Results of morphological evaluation, and RFLP and isoenzyme analysis, indicated all 21 of the resulting plants were hybrids.

Nuclear RFLP and isoenzyme analysis

Hybridization of heterologous wheat nuclear rDNA sequences (cloned in pTA71) with *Eco*R1-digested genomic DNA isolated from the parental species gave rise to a species-specific pattern of hybridization for each parent. The hybridization pattern obtained for the somatic hybrids provided evidence for the presence of nuclear DNA from both parents in all 21 hybrids. Data for selected somatic hybrids are shown in Fig. 1. Both *N. tabacum*-specific and *N. rustica*-specific hybridizing bands were present in the somatic hybrids. Additional evidence for nuclear hybridity was obtained by analysis of GOT isoenzymes in the parental species and the somatic hybrids. A distinct set of GOT isoenzymes was detected in leaf extracts of the parental species. However, while several bands are unique to the *N. rustica* (*npt*II) parent, all of the *N. tabacum* (*dhfr*) bands are also present in *N. rustica* (*npt*II) as shown in Fig. 2. Interestingly, in all of the somatic hybrids we detected a unique isozyme band of intermediate mobility to two of the parental isozyme bands. This may be a heterodimer formed from two of the common parental peptides. This isozyme band was not detected in a sample containing a 1 : 1 mixture of leaf extracts from *N. tabacum* and *N. rustica* (see Fig. 2, lane 3). In addition to the unique GOT isozyme, all 21 somatic hybrids also possessed *N. rustica*-specific GOT isoenzyme bands, as well as those common to both parental species.

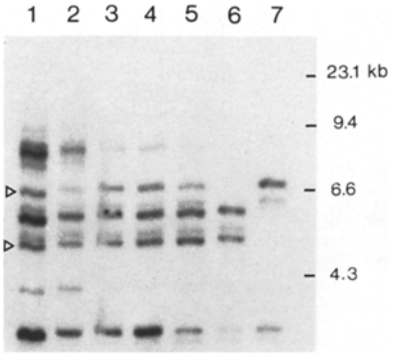

Fig. 1. Hybridization of heterologous nuclear ribosomal DNA (cloned in pTA71) with *Eco*R1-digested total cellular DNA isolated from *N. tabacum* (*dhfr*), *N. rustica* (*npt*II), and selected somatic hybrids. *Lanes* from left to right are: (*1*) somatic hybrid HDr-12, (*2*) HDr-11, (*3*) HDr-6, (*4*) HDr-5, (*5*) HDr-1, (*6*) *N. rustica* (*npt*II), and (*7*) *N. tabacum* (*dhfr*). Size markers in kilobases are given to the right side. The somatic hybrids possess species-specific hybridizing bands from both *N. tabacum* (one of these is marked by the *top arrow* at the left of the figure) and *N. rustica* (one of these marked by the *bottom arrow*)

Cytoplasmic inheritance

Hybridization of chloroplast DNA (cpDNA)-specific sequences (pBa1–9) with *Pvu*II-digested genomic DNA of the parental species resulted in a different pattern of hybridization for each species. The somatic hybrids all showed a pattern of hybridization which was identical with one of the parental patterns, as shown in Fig. 3a, b for selected hybrids. Data for all hybrids is shown in Table 1. There was a bias towards somatic hybrids with a hybridization pattern identical to that of *N. rustica*. Based on the hybridization results 20 somatic hybrids possessed chloroplasts of *N. rustica* while only one somatic hybrid (HDr-19) possessed chloroplasts of *N. tabacum*.

Results of the hybridization of heterologous wheat mitochondrial 5′-*cox*II sequences with genomic DNA of the parental species and selected somatic hybrids are shown in Fig. 4 and results for all hybrids are summarized in Table 1. Four somatic hybrids showed patterns of hybridization identical to *N. rustica* while none had a

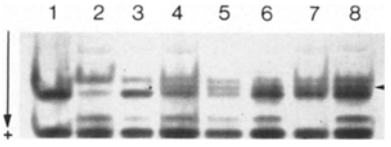

Fig. 2. Non-denaturing polyacrylamide-gel electrophoresis of leaf glutamate-oxaloacetate transaminases of *N. tabacum* (*dhfr*), *N. rustica* (*npt*II), and selected somatic hybrids. *Lanes* from the left to right are: (*1*) *N. tabacum* (*dhfr*), (*2*) *N. rustica* (*npt*II), (*3*) a 1 : 1 mixture of leaf extracts of *N. tabacum* (*dhfr*) and *N. rustica* (*npt*II), (*4*) somatic hybrid HDr-1, (*5*) HDr-2, (*6*) HDr-3, (*7*) HDr-4, and (*8*) HDr-5. The unique isozyme band which was detected in all of the somatic hybrids is indicated by the *arrow* at the right of the figure. The *arrow* at the left shows the direction of migration of samples towards the anode

Table 1. Somatic hybrid morphology, fertility and organelle inheritance

Plant	Flower length (mm)[a]	% Pollen viability[b]	Self-fertility[c]	CpDNA[d]	MtDNA[e]
N. tabacum	71±1.3	92± 8.0	+	T	T
N. rustica	21±0.8	94± 2.2	+	R	R
HDr-1	41±0.9	87±10.4	$+^{31/43}$	R	U1
HDr-2	42±1.4	63±12.7	$+^{22/36}$	R	U2
HDr-3	43±1.9	1± 0.5	−	R	U1
HDr-4	38±2.3	60± 4.0	$-^{s}$	R	R
HDr-5	44±3.7	57± 2.6	$-^{s}$	R	U1
HDr-6	38±1.0	65± 4.5	$+^{0/26}$	R	U1
HDr-7	36±1.0	38± 3.7	$-^{s}$	R	U1
HDr-8	41±2.5	10±12.3	$+^{0/50}$	R	U2
HDr-9	40±0.9	58± 5.0	$+^{3/15}$	R	U2
HDr-10	39±0.9	59± 7.0	$-^{s}$	R	U2
HDr-11	38±1.8	79± 9.5	$+^{0/14}$	R	U1
HDr-12	28±2.3	0	−	R	U1
HDr-13	40±1.7	74± 3.6	$+^{0/50}$	R	U1
HDr-14	40±0.7	62± 4.0	$+^{2/50}$	R	R
HDr-15	37±1.5	0.5± 0.4	−	R	U1
HDr-16	39±1.8	42±16.0	$+^{4/7}$	R	U1
HDr-17	43±1.6	6± 3.8	$+^{1/5}$	R	R
HDr-18	40±1.1	62±16.5	$+^{13/23}$	R	U1
HDr-19	36±2.1	74± 5.5	$+^{10/31}$	T	R
HDr-20	39±1.0	38±11.7	$+^{7/13}$	R	U2
HDr-21	29±2.6	26± 7.1	−	R	U2

[a] Value is mean ±SD for five individual flowers
[b] Value is mean ±SD for three pollen counts as described in Materials and methods
[c] +, selfed-seed was produced. The superscript value is fraction of seed which germinated in vitro; −, no seed set; $-^{s}$, only small shrivelled seed was produced
[d] Based on cpDNA (pBa1–9) hybridization data: R, *N. rustica* chloroplasts; T, *N. tabacum* chloroplasts
[e] Based on 5′-*cox*II hybridization data: R, pattern of hybridization identical with *N. rustica*, U1 and U2, a unique pattern of hybridization consistent with mitochondrial recombination or mtDNA rearrangement. These patterns of hybridization are shown for selected somatic hybrids in Fig. 4

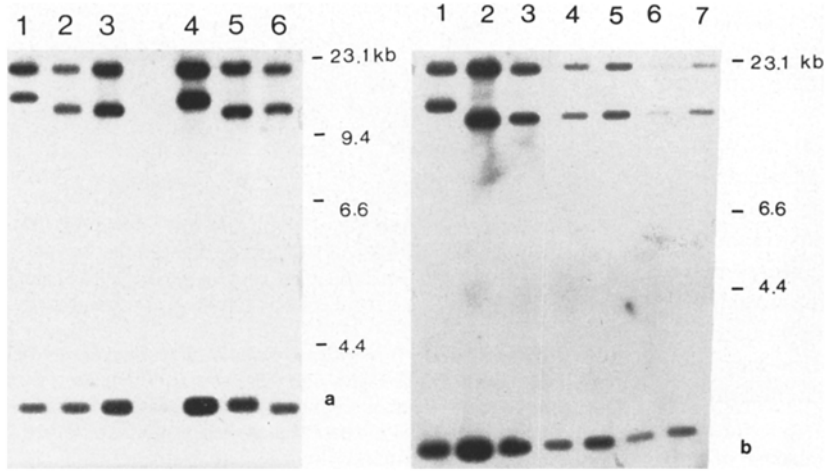

Fig. 3a, b. Chloroplast genome analysis: hybridization of cpDNA-specific sequences (cloned in pBa1–9) with total cellular DNA isolated from *N. tabacum* (*dhfr*), *N. rustica* (*npt*II) and selected somatic hybrids and digested with *Pvu*II. Size markers are given in kilobases on the right side of each figure. **a** *Lanes* from left to right are: (*1*) *N. tabacum* (*dhfr*), (*2*) *N. rustica* (*npt*II), (*3*) somatic hybrid HDr-17, (*4*) HDr-19, (*5*) HDr-1, and (*6*) HDr-2. **b** *Lanes* from left to right are (*1*) *N. tabacum* (*dhfr*), (*2*) *N. rustica* (*npt*II), (*3*) somatic hybrid HDr-18, (*4*) HDr-6, (*5*) HDr-8, (*6*) HDr-10 and (*7*) HDr-9

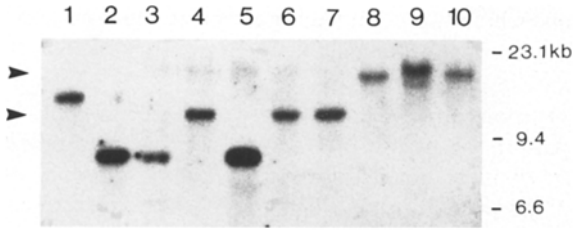

Fig. 4. Mitochondrial genome analysis: hybridization of a heterologous wheat 5′-*cox*II mtDNA probe with total cellular DNA isolated from *N. tabacum* (*dhfr*), *N. rustica* (*npt*II) and selected somatic hybrids and restricted with *Bgl*I. *Lanes* from left to right are (*1*) *N. tabacum* (*dhfr*), (*2*) *N. rustica* (*npt*II), (*3*) somatic hybrid HDr-17, (*4*) HDr-1, (*5*) HDr-4, (*6*) HDr-6, (*7*) HDr-7, (*8*) HDr-8, (*9*) HDr-9, and (*10*) HDr-10. The two *arrows* at the left of the figure indicate the relative positions of the two unique hybridizing bands which appear in some of the somatic hybrid lanes. The pattern of hybridization obtained for somatic hybrids in *lanes 4, 6, and 7* has been referred to as U1 and the pattern in *lanes 8, 9, and 10* as U2 in Table 1

hybridization pattern like *N. tabacum*. Eleven hybrids showed a novel pattern of hybridization (U1) unlike either parental pattern and consistent with mtDNA rearrangement or recombination, while six additional somatic hybrids shared a different novel hybridization pattern (U2) which was also consistent with mtDNA rearrangement or recombination.

Morphology and fertility

Growth habit varied amongst individual somatic hybrids with height and panicle type intermediate to the two parental types as shown in Fig. 5a. However, two somatic hybrids, HDr-11 and HDr-21, had short internodes and a stunted growth habit as shown in Fig. 5b for HDr-11. Leaf morphology is shown in Fig. 5c. Three somatic hybrids had nearly sessile leaves, similar to the *N. tabacum* parent. Most of the somatic hybrids, however,

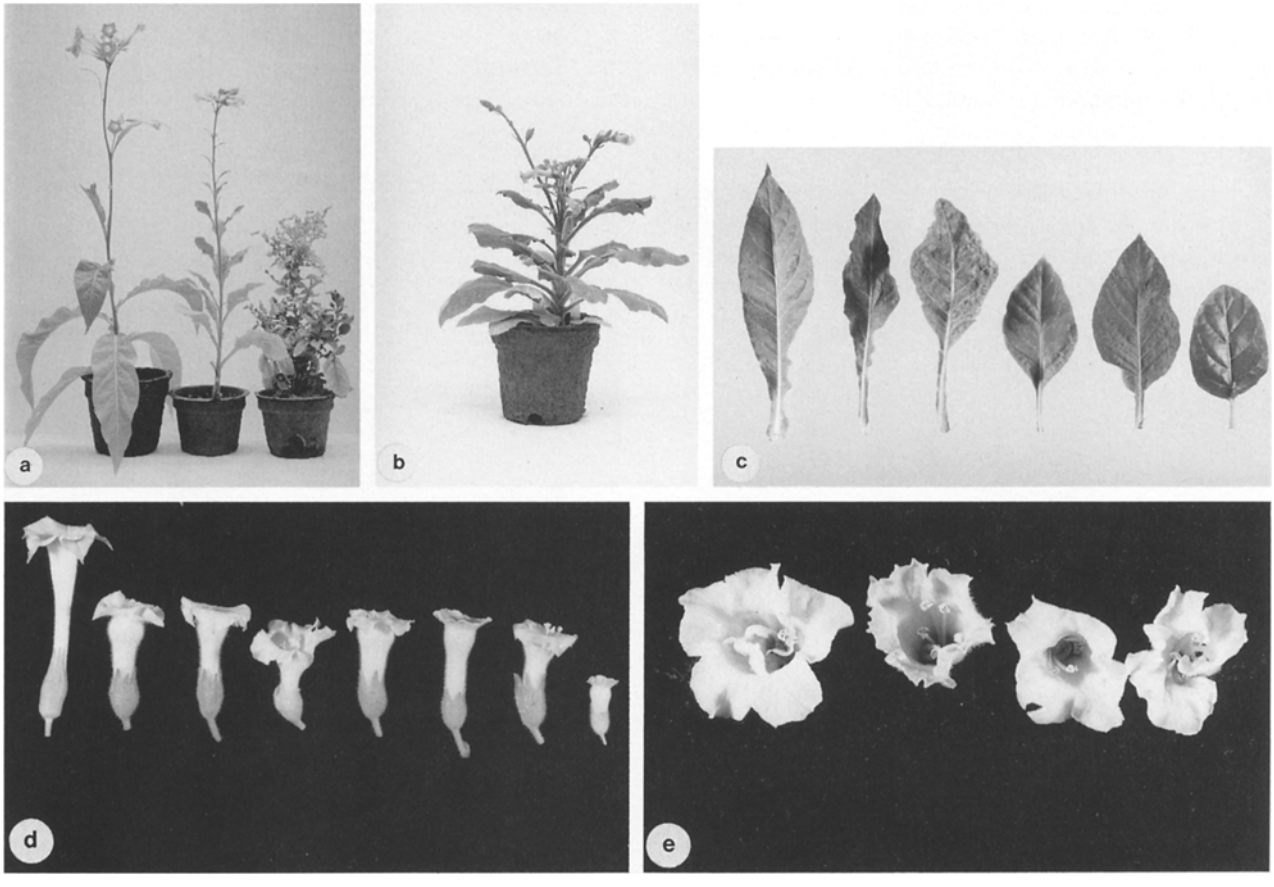

Fig. 5a–e. Somatic hybrid morphology: **a** Shown from left to right are *N. tabacum* (*dhfr*), somatic hybrid HDr-18, and *N. rustica* (*npt*II) **b** Somatic hybrid HDr-11 with short internodes and large leaves **c** Leaf morphology: *N. tabacum* (*dhfr*) (left), *N. rustica* (*npt*II) (right), and four selected somatic hybrids (centre). **d** Flower morphology: *N. tabacum* (*dhfr*) (left), *N. rustica* (*npt*II) (right), and six selected somatic hybrids (centre). The flower color also varied amongst individual somatic hybrids. **e** Flowers of somatic hybrid HDr-21 with irregularly-lobed and ruffled corolla and some petalloid and/or fused anthers

had petiolated leaves like the parental *N. rustica* but the petioles were narrowly winged unlike those of *N. rustica*. Leaf shape and size varied amongst somatic hybrids and extremely irregular deformed leaves, irregular leaf margins, and chlorotic sectors were occasionally observed. Only one somatic hybrid (HDr-9) displayed an overall chlorosis of the kind described by Douglas et al. (1981 b) for *N. tabacum* (+) *N. rustica* somatic hybrids. Somatic hybrid flowers, shown in Fig. 5 d, were consistently intermediate in length and color with respect to the parental species. Flower lengths are reported for all of the hybrids in Table 1. The corolla had five acute lobes like *N. tabacum*, or had irregular lobes and/or 'ruffled' margins as shown in Fig. 5 e for somatic hybrid HDr-21. Flower color also varied widely and included a pink (similar to *N. tabacum*), yellow (like *N. rustica*), and also pale-pink, beige, or white. In some cases one predominant color was interrupted by sectors of a different color. Seven hybrids bore some flowers with split corolla tubes. These were unlike those which have been described for certain male-sterile tobacco types (see Kofer et al. 1990). Fused and/or petalloid anthers, also shown in Fig. 5 e, were present in somatic hybrids HDr-8 and HDr-21.

The amount of normal pollen in the somatic hybrids, indicated in Table 1, varied from undetectable to nearly 90% depending on the somatic hybrid. No pollen could be detected in anthers of somatic hybrid HDr-12. The degree of self-fertility was very low with only nine somatic hybrids producing a small amount of germinating seed. Eight produced either shrivelled or apparently normal seed which would not germinate and the remaining four somatic hybrids, which displayed some of the lowest levels of male-fertility, did not set seed.

Discussion

Twenty-one methotrexate + kanamycin-resistant *N. tabacum* (+) *N. rustica* somatic hybrids were recovered. With the exception of one doubly-resistant putative hybrid callus which failed to regenerate, all of the doubly-resistant calli which were recovered gave rise to plants which were later confirmed as nuclear hybrids. Morphological evaluation, RFLP analysis with the rDNA probe, and isozyme analysis provided evidence for a hybrid nuclear genome in the plants. Interestingly, we did not recover double-resistant calli from fusions plated initially on 'underlayer' medium which contained kanamycin. This may correlate with our observation that *N. rustica* (*npt*II) protoplasts did not express kanamycin resistance effectively during early stages of protoplast culture. On the other hand, *N. glutinosa* (*npt*II) leaf mesophyll protoplasts do express kanamycin resistance in early stages of protoplast culture, and *N. tabacum* (*dhfr*) (+) *N. glutinosa* (*npt*II) somatic hybrids have been recovered from fusions which were plated directly on medium with kanamycin (P. A. Donaldson, E. Bevis, R. Pandeya, S. Gleddie, in preparation).

As reported previously by Aviv et al. (1984), a different pattern of hybridization with the cpDNA probe (pBa1–9) is obtained for the parental *N. tabacum* as compared with *N. rustica*. This probe was used to determine which chloroplast type was inherited in the somatic hybrids. Twenty of the twenty-one somatic hybrids were found to possess *N. rustica* chloroplasts based on the hybridization data, while only one possessed chloroplasts from *N. tabacum*. Thus, chloroplast segregation is apparently biased in favor of *N. rustica* chloroplasts. Typically, plastids are derived at random from either parent in somatic hybrids (Chen et al. 1977; Sundberg and Glimelius 1991) but fusions made between partially or completely incompatible species can lead to non-random patterns of chloroplast segregation (Bonnett and Glimelius 1983; Kushnir et al. 1991; Sundberg and Glimelius 1991). Amongst the *N. tabacum* (+) *N. rustica* somatic hybrids described previously, a variety of heteroplasmic conditions have been reported. A biased inheritance of *N. rustica* chloroplasts found in several instances was attributed to experimental factors such as the cell type used for fusion (Hamill et al. 1984) or to aspects of the hybrid selection system (Douglas et al. 1981 a, c).

The inheritance of *N. rustica* in 11 of 14 *N. tabacum* (+) *N. rustica* somatic hybrids selected by complementation of parental nuclear, chlorotic mutations was attributed to the early-greening trait associated with the *N. rustica* parent (Douglas et al. 1981 a, c). The predominance of *N. rustica* chloroplasts in 5/6 visually-selected somatic hybrids between leaf mesophyll protoplasts of *N. tabacum* cv Xanthi and cell suspension protoplasts of *N. rustica* V27 was attributed to a selective advantage of the latter because they had been grown heterotrophically (Hamill et al. 1984). For other *N. tabacum* (+) *N. rustica* somatic hybrids the patterns of chloroplast segregation were either not described (Nagao 1978; Toki and Kameya 1988), or only a few somatic hybrids were obtained (Iwai et al. 1980). In contrast, inheritance of only *N. tabacum* chloroplasts instead of *N. rustica* chloroplasts was reported for hybrids selected on the basis of streptomycin resistance which was encoded in the chloroplast genome of the *N. tabacum* genotype used in the fusion (Pental et al. 1984). In addition to somatic hybrids, all 22 gametosomatic hybrids derived from a fusion of *N. tabacum* microspore protoplasts with somatic cells of *N. rustica*, carried *N. rustica* chloroplasts. Lack of chloroplast transmission from the microspore parent was cited as the possible reason for the absence of *N. tabacum* chloroplasts in the hybrids. However, evidence for the transmission of mitochondrial sequences from the microspore parent was obtained (Pental et al. 1989).

The non-random pattern of chloroplast segregation we observed cannot easily be attributed to either the cell type or to the selection protocol used in our study. For each parental genotype we used leaf mesophyll protoplasts isolated from young seedlings and hybrid selection was based on expression of a dual-resistance phenotype. Therefore, it is likely that other factors known to influ-

ence chloroplast segregation contributed to the biased inheritance of *N. rustica* chloroplasts obtained in this study.

Firstly, a partial alloplasmic incompatibility may be the cause. While alloplasmic incompatibilities involving mitochondria are well known (see Håkansson and Glimelius 1991), incompatible chloroplast/nuclear genome interactions have been reported (Kushnir et al. 1991). Interestingly, sexual crosses of *N. tabacum* and *N. rustica*, which are limited by post-zygotic incompatibilities, have only been possible using *N. rustica* as the female parent (Brink and Cooper 1941; Swaminathan and Murty 1957). Thus, notwithstanding the low frequency paternal transmission which occurs in *Nicotiana* (Medgyesy et al. 1986; Horlow et al. 1990), *N. rustica* cytoplasmic genomes would be expected to predominate in the sexual hybrids. Secondly, differences in ploidy level also potentially lead to non-random patterns of chloroplast segregation because of plastid input bias (Landgren and Glimelius 1990; Sundberg et al. 1991). Input bias reflects the correlation between nuclear DNA content and relative cellular plastid number (Butterfass 1988). It is unlikely that ploidy level was a factor in our study since for *N. tabacum* (2n = 4x = 48) and *N. rustica* (2n = 4x = 48) ploidy levels are comparable and the cellular DNA contents of the two species are similar (Bennett and Smith 1976). However, recent reports indicate that with increasing genetic divergence between species there is an increased possibility of elimination of one or other of the species' chromosomes and this may favor retention of chloroplasts from the species whose nuclear genome dominates in the somatic hybrid (Sundberg and Glimelius 1991). With the exception of one somatic hybrid described by Douglas et al. (1981 b) all *N. tabacum* (+) *N. rustica* somatic (Nagao 1978; Iwai et al. 1980; Douglas et al. 1981 b) and gametosomatic hybrids (Pental et al. 1988) which have been examined were aneuploid, but there is no evidence that a preferential loss of *N. tabacum* chromosomes, instead of those of *N. rustica*, occurred. Finally, differential plastid replication rates could result in a non-random pattern of segregation. An example of this is in interspecific crosses within the genus *Oenothera* (Kirk and Tilney-Basset 1978). Interestingly, biased inheritance of *N. rustica* plastids over *N. sylvestris* plastids, which are indistinguishable from those of *N. tabacum* (Yang et al. 1992), also occurred amongst *N. rustica* (+) *N. sylvestris* somatic hybrids (Gleddie et al. 1983). This may be due to differential rates of replication or, as was mentioned previously, the hybrid identification procedure may have favored hybrids with *N. rustica* plastids.

Hybridization of a heterologous *cox*II probe with *Bgl*I digests of *N. tabacum* and *N. rustica* DNA was previously shown to produce distinctive patterns diagnostic of the respective mtDNA of these two species and, in addition, could be used to detect rearranged mtDNA in hybrids of these species (Pental et al. 1989). Similarly, we have used the results of *cox*II hybridization to assess mitochondrial inheritance patterns in our somatic hybrids. Of the 21 somatic hybrids obtained, four showed patterns of hybridization identical with the *N. rustica* parent and 17 possessed hybridization patterns consistent with mtDNA rearrangement. None showed a hybridization pattern similar to that of *N. tabacum*. These patterns of inheritance are similar to those described by Pental et al. (1989) for *N. tabacum* (+) *N. rustica* gametosomatic hybrids in which eight had hybridization patterns identical with the *N. rustica* parent, while the remaining 14 showed patterns consistent with mitochondrial recombination and none showed patterns of hybridization identical with *N. tabacum*. Our data suggest this mode of inheritance is not unique to gametosomatic hybrids of these species. This is in agreement with a recent report in which data for three individual somatic hybrids also indicated a similarity between patterns of mitochondrial inheritance for gametosomatic and somatic hybrids between *N. tabacum* and *N. rustica* (Mukhopadhyay et al. 1991).

The overall degree of self-fertility amongst the somatic hybrids was quite low. There was no apparent correlation between self-fertility or male-fertility levels and any particular pattern of organelle inheritance. Shrivelled seed was produced by many of the somatic hybrids and similarly, for *N. tabacum* × *N. rustica* sexual crosses, an embryo-endosperm incompatibility often leads to abortion of the developing seeds (Brink and Cooper 1941). Previous reports of *N. tabacum* (+) *N. rustica* somatic hybrids also indicate poor self-fertility (Iwai et al. 1980; Hamill et al. 1984, 1985; Pental et al. 1984). The relative lack of self-fertility in 14 *N. tabacum* (+) *N. rustica* somatic hybrids described by Douglas et al. (1981 b), where a total of only six seeds were obtained from two of the hybrids, was attributed to the use of protoplasts derived from long-term suspension cultures. In the present study, however, although protoplasts were isolated directly from young seedling leaves, the overall level of self-fertility amongst somatic hybrids was low. It is thus unlikely that donor-tissue source is a major cause of poor fertility of the *N. tabacum* (+) *N. rustica* somatic hybrids described here or previously. The variability in morphology and fertility amongst individual somatic hybrids is also typical of other *N. tabacum* (+) *N. rustica* somatic hybrids (Nagao 1978; Douglas et al. 1981 b; Hamill et al. 1984) and is in contrast to the reported uniform morphology and complete sterility of *N. tabacum* × *N. rustica* sexual hybrids (Chobanova 1977; Douglas et al. 1983). Corolla tubes which were only partially split were present in some of the hybrids obtained in the present study but their presence could not be correlated with a particular pattern of organelle inheritance.

Using the dual-selective-agent approach we obtained partially fertile interspecific somatic hybrids between partially incompatible species. Experimental factors which may influence the patterns of organelle inheritance amongst somatic hybrids were minimized since leaf mesophyll protoplasts from young plants were used and hybrid selection was based on dual expression of parental-specific resistance genes. In spite of this, a biased inheritance of *N. rustica* chloroplasts was obtained, and a relatively low level of fertility was present in the somatic hybrids. The degree of fertility was, however, sufficient to allow backcrosses to the tobacco parent. Incorporation of these backcross progeny in Canadian tobacco-breeding programs may result in variety improvements more significant than those obtained in the *N. tabacum* L. cultivar Delfield.

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