Theor Appl Genet (1994) 87:900–908

THEORETICAL AND APPLIED GENETICS

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Random chloroplast segregation and frequent mtDNA rearrangements in fertile somatic hybrids between *Nicotiana tabacum* L. and *N. glutinosa* L.

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Received: 1 June 1993 / Accepted: 23 July 1993

Abstract. Patterns of organelle inheritance were examined among fertile somatic hybrids between allotetraploid *Nicotiana tabacum* L. (2n = 4x = 48) and a diploid wild relative *N. glutinosa* L. (2n = 2x = 24). Seventy somatic hybrids resistant to methotrexate and kanamycin were recovered following fusion of leaf mesophyll protoplasts of transgenic methotrexate-resistant *N. tabacum* and kanamycin-resistant *N. glutinosa.* Evidence for hybridization of nuclear genomes was obtained by analysis of glutamate oxaloacetate transaminase and peroxidase isoenzymes and by restriction fragment length polymorphism (RFLP) analysis using a heterologous nuclear ribosomal DNA probe. Analysis of chloroplast genomes in a population of 41 hybrids revealed a random segregation of chloroplasts since 25 possessed *N. glutinosa* chloroplasts and 16 possessed *N. tabacum* chloroplasts. This contrasts with the markedly non-random segregation of plastids in *N. tabacum* (+) *N. rustica* and *N. tabacum* (+) *N. debneyi* somatic hybrids which we described previously and which were recovered using the same conditions for fusion and selection. The organization of the mitochondrial DNA (mtDNA) in 40 individuals was examined by RFLP analysis with a heterologous cytochrome B gene. Thirty-eight somatic hybrids possessed mitochondrial genomes which were rearranged with respect to the parental genomes, two carried mtDNA similar to *N. tabacum,* while none had mtDNA identical to *N. glutinosa.* The somatic hybrids were self-fertile and fertile in backcrosses with the tobacco parent.

Key words: *Nicotiana tabacum* – *N. glutinosa* – Fertile somatic hybrids – Random chloroplast segregation – Mitochondrial DNA rearrangement

Introduction

Somatic hybridization is used to combine the nuclear and cytoplasmic genomes of sexually-incompatible plants and may facilitate the transfer of agronomically-useful germplasm from wild plants to crop species. Since both parents of a somatic hybrid may contribute cytoplasmic genomes, novel nuclear-cytoplasmic interactions may arise which are not usually evident in sexual hybrids (due to maternal cytoplasm transmission). These interactions affect the expression of cytoplasmic determinants of agronomic fitness such as herbicide resistance, photosynthetic efficiency, and cytoplasmic male sterility (see Pelletier et al. 1988). Since somatic hybrids originate from heteroplasmic cells the nuclear-cytoplasmic combinations present depend upon mechanisms of organelle segregation and recombination.

Recombination between parental mitochondrial DNAs (mtDNA), as a consequence of somatic hybridization (Belliard et al. 1979; Nagy et al. 1983), is seen in many, but not all, somatic hybrids (see review by Rose et al. 1990). Recombination between parental chloroplast (cpDNA) genomes is only observed using strong selective pressure (Medgyesy et al. 1985; Thanh and Medgyesy 1989). Typically, segregation of chloroplasts results in hybrids with only one parental chloroplast DNA type. Segregation is often random, especially among somatic hybrids between close relatives (*Nicotiana*: Chen et al. 1977; *Brassica*: Landgren and

Communicated by P. Maliga
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Contribution No. 1487 Plant Research Centre
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Glimelius 1990; Sundberg and Glimelius 1991; *Citrus*: Kobayashi et al. 1991; *Solanum*: Pehu et al. 1989), but selection for chloroplast-encoded resistance can lead to unilateral chloroplast inheritance (Medgyesy et al. 1980; Moll et al. 1990; Malone et al. 1992). However, for hybridizations between distant relatives, non-random segregation often occurs in the absence of selection (*Nicotiana*: Douglas et al. 1981; Flick and Evans 1982; Donaldson et al. 1993; Solanum: Perl et al. 1991; *Brassica*: Landgren and Glimelius 1990; *Lycopersicon*: Bonnema et al. 1992). A direct link has been shown between nuclear-plastome incompatibility and non-random inheritance in some cases (Thanh et al. 1988; Kushnir et al. 1991) while in others extenuating factors, such as donor tissue type (Glimelius et al. 1981) or the selection system used (Douglas et al. 1981), were implicated. There is also evidence for the involvement of other genetic factors, including parental ploidy level (Sundberg and Glimelius 1991; Sundberg et al. 1991) and preferential elimination of parental species' chromosomes (Sundberg and Glimelius 1991; Derks et al. 1992). We previously reported biased chloroplast segregation among somatic hybrids between *N. tabacum* ($2n = 4x = 48$) and two different distantly-related wild species, *N. rustica* ($2n = 4x = 48$) (Donaldson et al. 1993) and *N. debneyi* ($2n = 4x = 48$) (Sproule et al. 1991), which were selected in the absence of extenuating factors which might influence segregation patterns. In the present study we use identical fusion and selection conditions to produce somatic hybrids between *N. tabacum* ($2n = 4x = 48$) and *N. glutinosa* ($2n = 2x = 24$), a diploid species and a member of the same subgenus as tobacco (subgenus: *Tabacum*). To determine correlations between chloroplast segregation and both the genetic relatedness and ploidy level of the parental species in *Nicotiana*, results are compared to our previous observations of non-random chloroplast segregation.

Mitochondrial inheritance is also examined since little data is available for interspecific hybridization in *Nicotiana* and none in the case of hybrids between *N. tabacum* and *N. glutinosa*. Also, since our protocols exclude the use of long-term suspension cultures implicated in reductions of fertility in somatic hybrids (Hamill et al. 1985), we compare levels of fertility obtained in the hybrids to those described previously (Nagao 1979; Uchimiya 1982; Horn et al. 1983). Fertile somatic hybrids between these species will be useful for renewed attempts to exploit *N. glutinosa* germplasm for the creation of improved tobacco breeding lines.

Materials and methods

Plant material

Parental genotypes used for fusion were transgenic *N. tabacum* cv Delgold, carrying a chimaeric dihydrofolate reductase gene (*dhfr*) as described by Dijak et al. (1991), and transgenic *N. glutinosa* (the race with yellow corolla was used) carrying a chimaeric neomycin phosphotransferase (*nptII*) gene under the control of the nopaline synthase gene promoter. *N. glutinosa* (*nptII*) was produced by *Agrobacterium*-mediated transformation of leaf discs with *A. tumefaciens* carrying the Ti plasmid pGV3850 and the binary vector pBCAT (Charest et al. 1988) essentially as described previously (Dijak et al. 1991). Heterozygous resistant progeny (BCI) from backcrosses of transgenic *N. glutinosa (nptII)* with a control, untransformed, *N. glutinosa* plant were germinated in vitro in the presence of 150 mg/l of kanamycin as described previously (Sproule et al. 1991). Resistant progeny comprised approximately 50% of the backcross progeny (i.e., 608 resistant: 573 susceptible). Similarly, heterozygous methotrexate-resistant BC1 progeny form transgenic *N. tabacum (dhfr)* were selected in vitro and BC1 progeny were maintained in vitro as leaf mesophyll protoplast donors.

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

Isolation of leaf mesophyll protoplasts from young back-cross progeny of *N. glutinosa* (*nptII*) and *N. tabacum* (*dhfr*) and PEG-mediated protoplast fusion were both performed as described (Dijak et al. 1991; Sproule et al. 1991). Hybrid calli resistant to methotrexate and kanamycin were selected in a step-wise manner on underlayer medium with a single selective agent followed by transfer to regeneration medium with both selective agents as described by Donaldson et al. (1993). Some fusions were also plated initially on control medium with hybrid calli subsequently selected on regeneration medium with one or other selective agent, followed by exposure to both. To verify stability of the dual-resistance phenotype, leaf explants from rooted hybrids were re-tested for the ability to regenerate shoots in the presence of both selective agents.

RFLP/isoenzyme analysis and organellar DNA analysis

Isolation of total cellular DNA, restriction enzyme digestions, and Southern-blot hybridizations were performed as described (Donaldson et al. 1993). Total cellular DNA of the parental species and the somatic hybrids was restricted with *Eco*RI and hybridized with heterologous wheat ribosomal DNA (rDNA) sequences (pTA71; Gerlach and Bedbrook 1979). The patterns of chloroplast inheritance were analysed with total cellular DNA restricted with either *Bgl*I or *Xho*I and hybridized with cloned fragments of *N. tabacum* cpDNA (pBal-9; Aviv et al. 1984) kindly provided by E. Galun. For analysis of the mitochondrial genome in the somatic hybrids, total cellular DNA was restricted with *Pvu*II, *Bam*HI, or *Pst*I and Southern blots were hybridized with a cloned 5.6-kb sequence containing the heterologous wheat cytochrome B gene (Boer et al. 1985) kindly provided by L. Bonen (U. of Ottawa).

Non-denaturing polyacrylamide gel electrophoresis of leaf glutamate oxaloacetate transaminases (GOT) and peroxidases and detection of GOT activity was as described (Donaldson et al. 1993). Peroxidase activity was detected following incubation of gels in a solution of 42 mM sodium acetate/0.5 mg/ml 4-chloro-1-naphthol/0.0375% H_2O_2.

Fertility

Pollen from freshly-dehisced anthers was stained in 1% acetocarmine. Percent viable pollen was determined by counting stained pollen from three individual flowers per somatic hybrid (1000 grains from one anther per flower were scored).

Results

Selection of methotrexate + kanamycin-resistant somatic hybrids

Heterofusions (but not control parental cultures or homofusions) gave rise to microcolonies on control medium on both types of selective underlayer medium and many of these produced calli resistant to both selective agents. In total, 98 independent double-resistant calli regenerated on double-selection. Fifty were derived from fusions plated initially on methotrexate-containing underlayer medium, 26 were from kanamycin underlayer plates, and 22 were from cultures plated initially on control medium. There was no apparent correlation between the order of exposure to the selective agent and the characteristics of the individual somatic hybrids obtained with respect to morphology, fertility, or organelle content. Sixteen regenerating shoots failed to reach maturity and 70 of the remaining 82 plantlets, were grown to maturity and confirmed as somatic hybrids.

Morphology and fertility

The majority of the somatic hybrids were morphologically similar to one another. They were generally intermediate to the parental species in height but with a growth habit and influoresence form more similar to *N. tabacum* (however a few hybrids were severely stunted and senesced quickly after flowering). Flowering time resembled that of the *N. tabacum* parent in contrast to the lengthy vegetative phase of *N. glutinosa*. Hybrid flower shape, size, and color were intermediate to the parental types (Fig. 1a), as were the hybrid leaves which were large and ovate with narrowly-winged petioles (Fig. 1b). Somatic hybrids HDg-9, HDg-102, and HDg-107 were stunted, slow to flower, and bore crinkled deformed leaves. Overall, levels of pollen stainability and self-fertility were high and the hybrids were fertile as female parents in backcrosses to tobacco (reciprocal backcrosses were not tested). Exceptions to the high male-fertility levels (shown in Table 1) were HDg-9, HDg-50 (not shown in Table 1 since organelle analysis was not done), and HDg-102 with 0.7, 11 and 32% viable pollen respectively; selfed-seed was nevertheless obtained from these three hybrids.

Nuclear RFLP and isoenzyme analysis

Hybridization of a heterologous wheat nuclear rDNA probe (in pTa71) with *Eco*R1-digested total cellular DNA of *N. tabacum* (*dhfr*) and *N. glutinosa* (*nptII*) produced species-specific patterns of hybridization. Hybridization to DNA isolated from the somatic hybrids revealed the presence of species-specific hybridizing bands from both parents (Fig. 2).

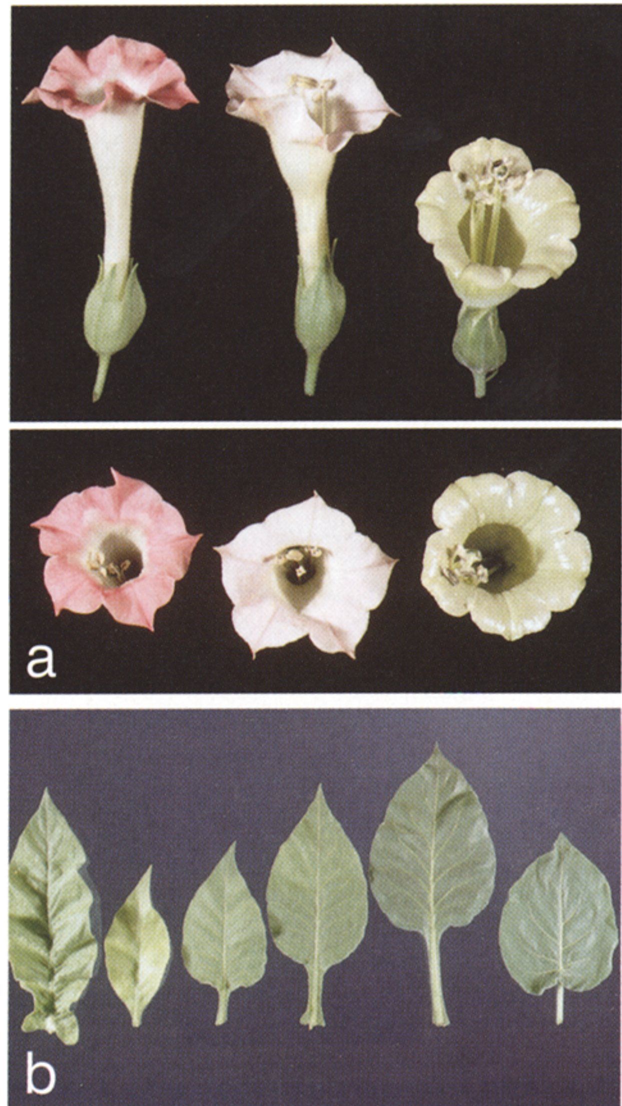

Fig. 1a, b. *N. tabacum* (+) *N. glutinosa* somatic hybrid morphology **a** upper and lower: flowers of *N. tabacum* (*dhfr*) (left), *N. glutinosa* (*nptII*) (right), and a somatic hybrid (centre). **b** Leaves of somatic hybrid (in centre) with parental *N. tabacum* (*dhfr*) (left) and *N. glutinosa* (*nptII*) (right)

Analysis of glutamate-oxaloacetate-transaminases (GOT) revealed two anodal GOT bands in extracts prepared from *N. tabacum* (*dhfr*), while three anodal bands, one of which was common to an *N. tabacum* GOT band, were detected in *N. glutinosa* (*nptII*) leaf extracts (Fig. 3). All the somatic hybrids possessed the common GOT band, one *N. glutinosa* (*nptII*)-specific band, and a unique band not present in either parental extract, or in a mixture of parental extracts (lane 3, Fig. 3). One *N. tabacum* (*dhfr*)-specific band and two *N. glutinosa* (*nptII*)-specific bands were apparently absent from all somatic hybrids examined. Peroxidase

Table 1. Male-fertility levels and organelle content of selected *N. tabacum* (*dhfr*) (+) *N. glutinosa* (*nptII*) somatic hybrids

Hybrid #	Male-fertility[a] %	Chloroplast genome[b]	Mitochondrial genome[c]
HDg-1	91 ± 7.1	T	R1
HDg-2	87 ± 5.0	G	R1
HDg-3	88 ± 2.5	T	R
HDg-4	79 ± 6.8	–	R
HDg-5	88 ± 1.5	G	R
HDg-6	84 ± 0.6	T	R1
HDg-7	85 ± 1.5	G	R1
HDg-9	0.7 ± 0.23	G	R
HDg-10	73 ± 9.6	T	R3
HDg-11	97 ± 0.6	G	R
HDg-22	93 ± 3.9	G	R1
HDg-24	76 ± 8.1	G	R
HDg-26	92 ± 1.5	T	–
HDg-34	94 ± 1.5	G	R1
HDg-37	83 ± 10.0	G	R
HDg-39	94 ± 1.5	T	R3
HDg-41	89 ± 3.0	T	T
HDg-46	85 ± 11.6	G	R1
HDg-48	87 ± 1.1	G	R1
HDg-53	nd	T	R1
HDg-57	89 ± 4.0	G	R
HDg-61	65 ± 4.6	T	R1
HDg-64	86 ± 6.4	G	R1
HDg-68	92 ± 4.2	T	R1
HDg-71	85 ± 7.9	T	R1
HDg-73	67 ± 9.5	G	R1
HDg-77	88 ± 1.1	G	R
HDg-83	88 ± 5.1	G	R2
HDg-84	95 ± 3.0	T	T
HDg-87	82 ± 6.8	G	R2
HDg-88	95 ± 2.6	G	R2
HDg-90	76 ± 7.8	G	R1
HDg-91	91 ± 4.9	T	R3
HDg-94	96 ± 0.6	G	R2
HDg-95	86 ± 7.3	G	R1
HDg-96	88 ± 1.7	T	R1
HDg-97	nd	G	R2
HDg-98	92 ± 2.9	G	R2
HDg-102	32 ± 19.7	T	–
HDg-104	95 ± 1.6	G	R1
HDg-105	64 ± 4.6	G	R1
HDg-106	90 ± 1.9	T	R1

[a] % pollen stained as described in Materials and methods. Values for parental *N. tabacum* and *N. glutinosa* were 92 ± 8.0 and 93 ± 5.3 respectively
[b] *N. tabacum* chloroplasts (T) or *N. glutinosa* chloroplasts (G)
[c] Rearranged mtDNA type (R, R1, R2, or R3) as described in the text or mtDNA similar to parental *N. tabacum* (T)

isozyme analysis was also performed but peroxidase activity could not be detected in leaf extracts from leaves of young, immature *N. glutinosa*. In contrast, peroxidases were readily detected in leaf extracts of young plants of *N. tabacum* and the somatic hybrids (data not shown). Of the four anodal bands detected in somatic hybrids, two corresponded with parental *N. tabacum* peroxidases, one appeared to be unique to the hybrids, and a faint band detected in some hybrids appeared to correspond with a peroxidase isozyme observed only in extracts of older *N. glutinosa* plants.

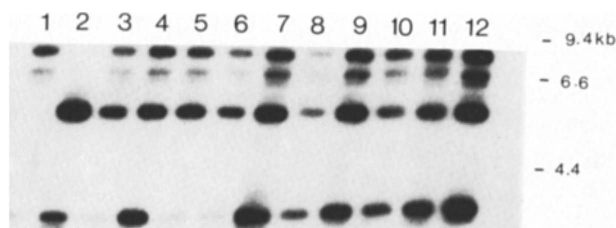

Fig. 2. Hybridization of a heterologous wheat nuclear ribosomal DNA (in pTa71) with total cellular DNA isolated from *N. tabacum* (*dhfr*), *N. glutinosa* (*nptII*), and selected somatic hybrids, digested with *Eco*R1. *Lanes* from left to right are: (*1*) *N. tabacum* (*dhfr*) (*2*) *N. glutinosa* (*nptII*), (*3*) HDg-84, (*4*) HDg-68, (*5*) HDg-91, (*6*) HDg-105, (*7*) HDg-73, (*8*) HDg-39, (*9*) HDg-57, (*10*) HDg-96, (*11*) HDg-88, (*12*) HDg-10. All of the somatic hybrid lanes show species-specific hybridizing bands from both parents. Size markers in kilobases are shown on the right

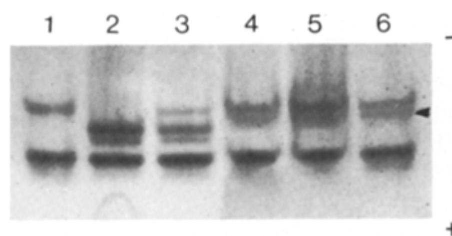

Fig. 3. Native polyacrylamide-gel electrophoresis (5% acrylamide) of leaf glutamate-oxaloacetate transaminases of *N. tabacum* (*dhfr*), *N. glutinosa* (*nptII*), and selected somatic hybrids. *Lanes* from left to right are: (*1*) *N. tabacum* (*dhfr*), (*2*) *N. glutinosa* (*nptII*), (*3*) a 1:1 mixture of leaf extracts of *N. tabacum* (*dhfr*) and *N. glutinosa* (*nptII*), (*4*) somatic hybrid HDg-1, (*5*) HDg-2, (*6*) HDg-3, (*7*) HDg-4 (*8*) HDg-5. A unique GOT isoenzyme band detected in all somatic hybrids examined is indicated by the *arrow* at the right of the figure. The direction of migration is towards the anode indicated at the lower right of the figure

Chloroplast segregation patterns

Using the cpDNA probe, species-specific Southern-hybridization patterns were obtained with total cellular DNA digested with *Xho*I or *Bgl*I. Analysis of genomic DNA of 41 *N. tabacum* (+) *N. glutinosa* somatic hybrids using pBal-9, summarized in Table 1, revealed that 16 of the 41 possessed chloroplasts derived from *N. tabacum* while 25 possessed chloroplasts from *N. glutinosa*. Using the chi-square test, these results are not significantly different from a random 1:1 segregation ($\chi^2 = 2.95, P < 0.05$). Thirty-two of the hybrids were screened using both types of DNA digests and results for both types of analysis were always in agreement. Hybridization results for parental species

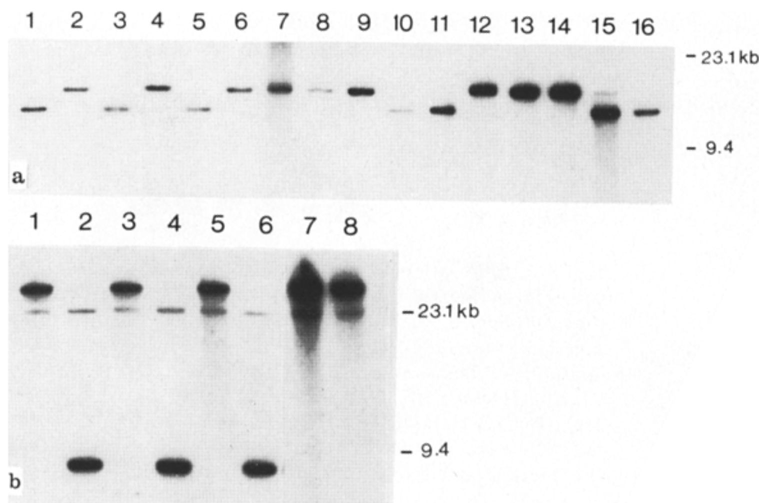

Fig. 4 a, b. Chloroplast inheritance: hybridization of the cpDNA probe (in pBal-9) to total cellular DNA of the parental species and somatic hybrids digested with either *Xho*1 or *Bgl*1 **a** Hybridization to *Xho*1-digested DNA. *Lanes* are (*1*) *N. tabacum* (*dhfr*), (*2*) *N. glutinosa* (*nptII*), (*3*) HDg-3, (*4*) HDg-5, (*5*) HDg-6, (*6*) HDg-9, (*7*) HDg-22, (*8*) HDg-24, (*9*) HDg-34, (*10, 11*) HDg-41, (*12*) HDg-46, (*13, 14*) HDg-48, (*15, 16*) HDg-53. **b** Hybridization to *Bgl*1-digested DNA. *Lanes* are (*1*) *N. tabacum* (*dhfr*), (*2*) *N. glutinosa* (*nptII*), (*3*) HDg-3, (*4*) HDg-5, (*5*) HDg-6, (*6*) HDg-9, (*7*) HDg-71, (*8*) HDg-102. Size markers in kilobases are shown on the right of each figure

and selected somatic hybrids are shown in Fig. 4a for *Xho*I digests and Fig. 4b for *Bgl*1 digests. The faint upper band visible for hybridization to *Xho*1 digests of HDg-53 in lane 15, Fig. 4a, but not for another digest of the same DNA sample in lane 16, Fig. 4a (and also other data not shown), is the result of a partial digest rather than to the presence of *N. glutinosa* cpDNA.

Analysis of mitochondrial genomes

Several heterologous mtDNA probes were tested in hybridization experiments with parental genomic DNA. Hybridization of the heterologous mtDNA probe (*cyt B*) to total cellular DNA digested with *Bam*H1, *Pvu*II, or *Pst*I produced species-specific patterns of hybridization. A population of somatic hybrids (40 individuals) was analysed and the results are also shown in Table 1. Patterns of hybridization for parental DNA and DNA of selected hybrids are illustrated in Fig. 5a, b for *Bam*H1 digests and Fig. 5c, d for *Pvu*II digests. Thirty-eight hybrids showed patterns of hybridization consistent with the presence of rearranged mtDNA. Results for the other two hybrids, HDg-41 (see Fig. 5b, lane 11) and HDg-84 (Fig. 5c, lane 13), were identical to *N. tabacum* in the case of both *Bam*H1 and *Pvu*II digests. HDg-41 was also analysed with *Pst*1-digested DNA and the pattern of hybridization was the same as for *N. tabacum* (data not shown). None of the somatic hybrids appeared to have mtDNA identical to *N. glutinosa*. The 38 somatic hybrids with rearranged mtDNA were further classified as one of three rearranged mtDNA types. Type R1 and type R2 plants shared the same pattern of *Bam*H1 hybridization, in which both parental-specific bands were detected (Fig. 5a, lanes 3–14; Fig. 5b lanes 3–10 and 12). R1-type plants all showed the same rearranged non-parental pattern of hybridization for *Pvu*II-digested DNA (example Fig. 5d, lanes 6, 7, 8, 10). Six somatic hybrids with R2-type mtDNA showed a different, unique, pattern of hybridization when *Pvu*II digests were probed (example Fig. 5c, lanes 3, 4, 5, 9). Three somatic hybrids, designated as type, R3 showed, a pattern identical to *N. tabacum* for *Bam*H1 digests but hybridizing bands corresponding to both parental types were observed for *Pvu*II digests (see Fig. 5c, lanes 6, 9). Ten additional hybrids showed the same pattern of *Bam*H1 hybridization as type R1 and type R2 hybrids, but since the *Pvu*II hybridization patterns were not tested they were not classified as rearranged mtDNA type R1, R2, or R3 but rather only as R.

Discussion

Seventy *N. tabacum* (+) *N. glutinosa* hybrids resistant to kanamycin and methotrexate were recovered from fusions of leaf mesophyll protoplasts of methotrexate-resistant *N. tabacum* and kanamycin-resistant *N. glutinosa*. Only fusion cultures produced double-resistant calli and these were recovered from fusions plated initially on control medium or those plated immediately after fusion on kanamycin- or methotrexate-containing medium. There was no apparent correlation between the order of exposure to the selective agent and the type of somatic hybrid obtained using the different treatments with regard to hybrid morphology, fertility, or the pattern of organelle inheritance.

Prior to this study, chloroplast inheritance among *N. tabacum* (+) *N. glutinosa* somatic hybrids had been examined in only a few plants, including a single sterile somatic hybrid described by Uchimiya (1982) and five

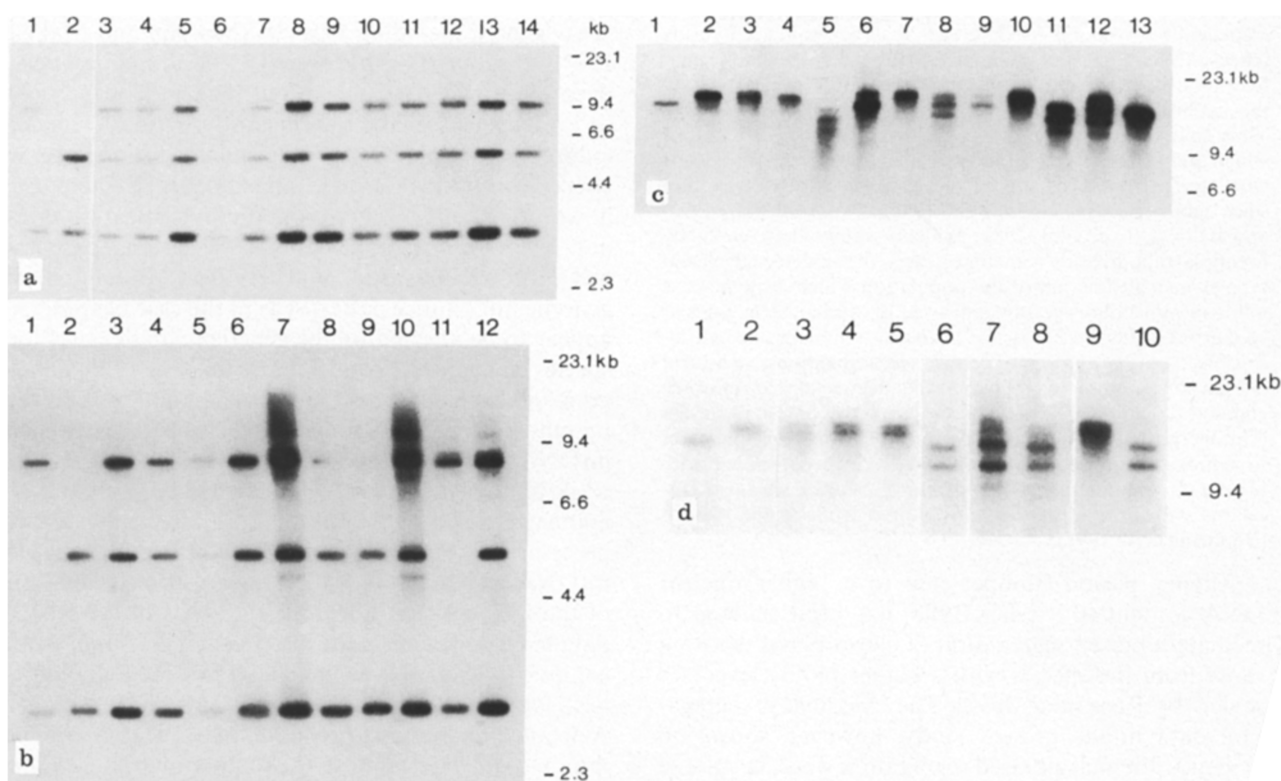

Fig. 5a–d. Mitochondrial genome analysis: total cellular DNA digested with either *Bam*H1 **(a, b)** or *Pvu*II **(c, d)** and hybridized with a heterologous *cytB* probe. **a** *Bam*H1-digested DNA. *Lanes* are *(1) N. tabacum (dhfr)*, *(2) N. glutinosa (nptII)*, *(3)* HDg-11, *(4)* HDg-6, *(5)* HDg-2, *(6)* HDG-37, *(7)* HDG-3, *(8)* HDG-34. *(9)* HDg-1, *(10)* HDg-4, *(11)* HDg-95, *(12)* HDg-94, *(13)* HDg-106, *(14)* HDg-7. **b** *Lanes* are *(1) N. tabacum (dhfr)*, *(2) N. glutinosa (nptII)*, *(3)* HDg-5, *(4)* Hdg-9, *(5)* HDg-24, *(6)* HDg-77, *(7)* HDg-104, *(8)* HDg-22, *(9)* HDg-97, *(10)* HDg-90, *(11)* HDg-41, (12) HDg-46. **c** *Pvu*II-digested DNA. *Lanes* are *(1) N. tabacum (dhfr)*, *(2) N. glutinosa (nptII)*, *(3)* HDg-87, *(4)* HDg-83, *(5)* HDg-53, *(6)* HDg-10, *(7)* HDg-88, *(8)* HDg-95, *(9)* HDg-39, *(10)* HDg-57, *(11)* HDg-48, *(12)* HDg-34, *(13)* HDg-84. **d** *Lanes* are *(1) N. tabacum (dhfr)*, *(2) N. glutinosa (nptII)*, *(3)* HDg-83, *(4)* HDg-88, *(5)* HDg-97, *(6)* HDg-7, *(7)* HDg-61, *(8)* HDg-71, *(9)* HDg-98, *(10)* HDg-106. Size markers in kilobases are shown on the right of each figure

somatic hybrids described by Horn et al. (1983), four of which derived from the same callus. In the present study chloroplast inheritance was examined in a population of 41 *N. tabacum* (+) *N. glutinosa* somatic hybrids. The results showed that more hybrids inherited *N. glutinosa* chloroplasts (G) than inherited *N. tabacum* chloroplasts (T) (25G:16T). However, the segregation ratio is not significantly different from a random 1:1 segregation pattern, according to the chi-square test. This is in contrast to the markedly non-random pattern of chloroplast segregation which we previously obtained for *N. tabacum* (*dhfr*) (+) *N. debneyi* (*nptII*) somatic hybrids, where 11/12 had only *N. debneyi* cpDNA (Sproule et al. 1991), and *N. tabacum* (*dhfr*) (+) *N. rustica* (*npt*II) somatic hybrids, where 20/21 had *N. rustica* cpDNA (Donaldson et al. 1993), using identical conditions for fusion and selection. In the present study, the more random chloroplast segregation most likely reflects the closer genetic relatedness of the parental species, i.e., *N. tabacum* and *N. glutinosa*, which are both members of the same *Nicotiana* subgenus, *Tabacum*, compared with the greater phylogenetic distance between the *Nicotiana* species in the previous somatic hybridizations between *N. tabacum* (subgenus: *Tabacum*) and either *N. debneyi* (subgenus: *Petunioides*) or *N. rustica* (subgenus: *Rustica*). In addition, we recently obtained a non-random pattern of inheritance among somatic hybrids between *N. tabacum* (*dhfr*) and the distantly-related species, *N. megalosiphon* (subgenus: *Petunioides*) using the same selection conditions (P. A. Donaldson, E. Bevis, R. Pandeya, and S. Gleddie, in preparation). In all these studies the potential influence of plastid input bias caused by the different donor tissues (see Rose et al. 1990) was eliminated since leaf mesophyll protoplasts were used for both parents.

Interestingly, biased inheritance of wild species chloroplasts in *N. tabacum* (+) *N. rustica* (Donaldson et al. 1993), and *N.*

tabacum (+) *N. nesophila* (Evans et al. 1982), somatic hybrids reflects the cytoplasmic composition of the respective sexual hybrids which can only be recovered using the wild species as the maternal parent (Evans et al. 1982; Douglas et al. 1983). Thus, limits to the range of nuclear-cytoplasmic combinations which are stable exist even for somatic hybrids. Somatic hybrids with rare plastid types should therefore be used with caution since both direct (Moll et al. 1990; Malone et al. 1992) and indirect (Iwai et al. 1981) evidence shows that seemingly homoplastidic hybrids sometimes carry the undetected plastid type as a small fraction of the population which may increase following selection pressure, meiosis, or a change in nuclear background after backcrossing. In *Brassica* interspecific hybridizations and related intergeneric hybridizations, a direct correlation is observed between the degree of chromosome elimination and the genetic distance of the parental species (Sundberg and Glimelius 1991). In *Lycopersion* increases in donor protoplast irradiation dose, which correlated with preferential elimination of donor chromosomes, was also correlated with preferential loss of donor plastids in the hybrids (Bonnema et al. 1992).

Higher plastid number due to a higher nuclear DNA content (Butterfass 1988) has been claimed to mediate a biased segregation of chloroplasts favoring those from the species with a higher ploidy level (see review by Rose et al. 1990). The chloroplast segregation data in the present study, however, shows no evidence for such a biased segregation since, in spite of the ploidy differences between the allotetraploid *N. tabacum* ($2n = 4x = 48$) parent and diploid *N. glutinosa* ($2n = 2x = 24$), segregation was not biased towards *N. tabacum* chloroplasts. Further evidence for a lack of this type of correlation between ploidy levels and biased segregation in *Nicotiana* interspecific hybridizations, was the biased segregation we observed previously for *N. tabacum* (+) *N. debneyi* and *N. tabacum* (+) *N. rustica* hybrids (Sproule et al. 1991; Donaldson et al. 1993) (obtained using the same protocols described in the present study) in spite of the similar ploidy levels of the parental species (Goodspeed 1954). Taken together these results imply that for interspecific hybridizations in *Nicotiana*, genetic distance may be more significant than parental ploidy-level differences in determining chloroplast inheritance. In *Brassica*, somatic hybridization of species with different ploidy levels in some cases showed a bias towards the inheritance of chloroplasts from the species with the higher ploidy level (Landgren and Glimelius 1990). Contradictory evidence which was obtained for *B. napus* (+) *B. juncea* hybridizations (i.e., biased segregation following fusion of parents with similar ploidy levels) was attributed to the small sample size of only six hybrids (Sundberg and Glimelius 1991).

The results of mtDNA analysis of the *N. tabacum* (+) *N. glutinosa* somatic hybrids showed that mitochondrial rearrangement was a common occurrence. Similarly, rearrangements in mtDNA following somatic hybridization, first reported in *Nicotiana* (Belliard et al. 1979; Nagy et al. 1983), are common in many genera including *Brassica* (Morgan and Maliga 1987), *Petunia* (Boeshore et al. 1983), and *Solanum* (Kemble et al. 1986). Our recent report of *N. tabacum* (+) *N. rustica* somatic hybrids also provides evidence for frequent mtDNA rearrangements in interspecific *Nicotiana* hybrids (Donaldson et al. 1993). There are, however, examples where somatic hybridization does not result in rearrangements between the parental mtDNAs (Kobayashi et al. 1991). Mitochondrial genome inheritance patterns, as in the case of cpDNA, appear to be affected by the genetic relatedness of the species being fused (Bonnet and Glimelius 1990; Landgren and Glimelius 1990; Bonnema et al. 1992). In the present study 38 of 40 somatic hybrids had rearranged mtDNA, two possessed mtDNA similar to tobacco, while none possessed mtDNA identical to parental *N. glutinosa* (based on RFLP data). These results do not necessarily indicate an overall bias towards tobacco mtDNA sequences since a detailed restriction analysis of the mtDNA was not done. Analysis of the RFLP data indicated some of the *N. tabacum* (+) *N. glutinosa* somatic hybrids shared similar, if not identical, types (e.g., type R1, R2, R3 etc.) of mtDNA rearrangements. A detailed restriction analysis of these mtDNAs will be required to determine if the mitochondrial genome rearrangements are indeed identical. Common types of mtDNA rearrangements for independent somatic hybrids are frequently observed following somatic hybridization (Kemble et al. 1986; Kothari et al. 1986; Bonnet and Glimelius 1990; Landgren and Glimelius 1990; Donaldson et al. 1993) and are considered as evidence for rearrangement hot-spots (Rothenberg and Hanson 1987).

The hybrids obtained were fertile, in contrast to other *N. tabacum* (+) *N. glutinosa* hybrids reported in the literature which were either sterile (Uchimiya 1982; Horn et al. 1983) or partially fertile (Nagao 1979) presumably due to the use of long-term suspension cultures or non-regenerating or mutant genotypes for fusion. A fertile amphidiploid hybrid recovered from a *N. tabacum* × *N. glutinosa* sexual hybrid (Clausen and Goodspeed 1925) was used in backcrosses to produce tobacco with enhanced viral resistance (Holmes 1938) but the N gene responsible for this trait has also been associated with significant undesirable traits (Chaplin et al. 1961; Nielson et al. 1985). Therefore, somatic hybrids from this study will be used in renewed attempts to exploit this wild species germplasm for tobacco improvement and for a study of the genetic determinants of disease resistance by the creation of isogenic lines in tobacco.

Acknowledgments. This research was jointly funded by Agriculture Canada and Imperial Tobacco Ltd. The authors would like to thank Dr. E. Galun for the gift of the cpDNA probe and Dr. L. Bonen for the gifts of heterologous mtDNA probes.

References

Aviv D, Bleichman S, Arzee-Gonen P, Galun E (1984) Intersectional cytoplasmic hybrids in *Nicotiana*. Identification of plastomes and chondriomes in *N. sylvestris* (+) *N. rustica* cybrids having *N. sylvestris* nuclear genomes. Theor Appl Genet 67:499–504

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

Boer, PH, McIntosh JE, Gray MW, Bonen L (1985) The wheat mitochondrial gene for apocytochrome b: absence of a prokaryotic ribosome binding site. Nucleic Acids Res 13:2281–2292

Boeshore ML, Hanson MR, Lifshitz I, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male-sterile and fertile plants. Mol Gen Genet 190:459–467

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*. Theor Appl Genet 84:435–442

Bonnett HT, Glimelius K (1990) Cybrids of *Nicotiana tabacum* and *Petunia hybrida* have an intergeneric mixture of chloroplasts from *P. hybrida* and mitochondria identical or similar to *N. tabacum*. Theor Appl Genet 79:550–555

Butterfass TH (1988) Nuclear control of plastid division. In: Boffey SA, Lloyd D (eds) Division and segregation of organelles. Society for experimental biology seminar series, vol 35. Cambridge, England, pp 21–38

Chaplin JF, Mann TJ, Apple JL (1961) Some effects of the *Nicotiana glutinosa* type of mosaic resistance on agronomic characters of flue-cured tobacco. Tobacco Sci 5:80–83

Charest PJ, Holbrook LA, Gabard J, Iyer VN, Miki BLA (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. Theor Appl Genet 75:438–445

Chen K, Wildman SG, Smith HH (1977) Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction 1 protein Proc Natl Acad Sci USA 74:5109–5112

Clausen RE, Goodspeed TH (1925) Interspecific hybridization in *Nicotiana*. II. A tetraploid *glutinosa-tabacum* hybrid, an experimental verfication of Winge's hypothesis. Genetics 10:278–284

Derks FHM, Hakkert JC, Verbeek WHJ, Colijn-Hooymans CM (1992) Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplasts interaction. Theor Appl Genet 84: 930–940

Dijak M, Sproule A, Keller W, Pandeya R, Gleddie S (1991) Transformation of *Nicotiana tabacum*, *N. debneyi*, and *N. rustica*: inheritance and protoplast expression of antibiotic resistance. Plant Cell Tiss Org Cult 25:189–197

Donaldson P, Sproule A, Bevis E, Pandeya R, Keller WA, Gleddie S (1993) Non-random chloroplast segregation in *Nicotiana tabacum* (+) *N. rustica* somatic hybrids selected by dual nuclear-encoded resistance. Theor Appl Genet 86: 465–473

Douglas GC, Wetter LR, Keller WA, Setterfield G (1981) Somatic hybridization between *Nicotiana rustica* and *N. tabacum*. IV. Analysis of nuclear and chloroplast genome expression in somatic hybrids. Can J Bot 59:1509–1513

Douglas GC, Wetter LR, Keller WA, Setterfield (1983) Production of sexual hybrids of *Nicotian rustica* × *N. tabacum* and *N. rustica* × *N. glutinosa* via in-vitro culture of fertilized ovules. Z Pflanzenzuchtg 90:116–129

Evans DA, Flick CE, Kut SA (1982) Comparison of *Nicotiana tabacum* and *Nicotiana nesophila* hybrids produced by ovule culture and protoplast fusion. Theor Appl Genet 62:193–198

Flick CE, Evans DA (1982) Evaluation of cytoplasmic segregation in somatic hybrids of *Nicotiana*: tentoxin sensitivity. J Hered 73:264–266

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal genes from wheat and barley. Nucleic Acid Res 7:1869–1885

Glimelius K, Chen K, Bonnett HT (1981) Somatic hybridization in *Nicotiana*: segregation of organellar traits among hybrid and cybrid plants. Planta 153:504–510

Goodspeed TH (1954) The genus *Nicotiana*: origins, relationships and evolution of its species in the light of their distribution, morphology and cytogenetics. Chronica Botanica Company, Waltham, Massachusettes, USA

Hamil JD, Pental D, Cocking EC (1985) Analysis of fertility in somatic hybrids of *Nicotiana rustica* and *N. tabacum* and progeny over two sexual generations. Theor Appl Genet 71:486–490

Holmes FO (1938) Inheritance of resistance to tobacco-mosaic disease in tobacco. Phytopathology 28:553–561

Horn ME, Kameya T, Brotherton JE, Widholm JM (1983) The use of amino-acid analog resistance and plant regeneration ability to select somatic hybrids between *Nicotiana tabacum* and *N. glutinosa*. Mol Gen Genet 192:235–240

Iwai S, Nakata K, Nagao T, Kawashima N, Matsuyama S (1981) Detection of the *Nicotiana rustica* chloroplast genome coding for the large subunit of Fraction 1 protein in a somatic hybrid in which only the *N. tabacum* chloroplast genome appeared to have been expressed. Planta 152:478–480

Kemble RJ, Barsby TL, Wong RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor Appl Genet 72:787–793

Kobayashi S, Ohgawara T, Fijiwara K, Oiyama I (1991) Analysis of cytoplasmic genomes in somatic hybrids between navel orange (*Citrus sinesis* Osb.) and 'Murcott' tangor. Theor Appl Genet 82:6–10

Kothari SL, Monte DC, Widholm JM (1986) Selection of *Daucus carota* somatic hybrids using drug resistance markers and characterization of their mitochondrial genomes. Theor Appl Genet 72:494–502

Kushnir SG, Babiychuk E, Bannikova M, Momot V, Komarnitsky I, Cherep N, Gleba Y (1991) Nucleo-cytoplasmic incompatibility in cybrid plants possessing an *Atropa* genome and a *Nicotiana* plastome. Mol Gen Genet 225:225–230

Landgren M, Glimelius K (1990) Analysis of chloroplast and mitochondrial segregation in three different combinations of somatic hybrids produced within *Brassicaceae*. Theor Appl Genet 80:776–784

Malone R, Horváth GV, Cśepló A, Búzás B, Dix PJ, Medgyesy P (1992) Impact of the stringency of cell selection on plastid segregation in protoplast fusion-derived *Nicotiana* regenerates. Theor Appl Genet 84:866–873

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and isolation of their somatic hybrids. Mol Gen Genetics 179:693–698

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6990–6964

Moll B, Polsby L, Maliga P (1990) Streptomycin and lincomycin resistances are selective plastid markers in cultured *Nicotiana* cells. Mol Gen Genet 221:245–250

Morgan A, Maliga P (1987) Rapid chloroplast segregation and recombination of mitochondrial DNA in *Brassica* cybrids. Mol Gen Genet 209:240–246

Nagao T (1979) Somatic hybridization by fusion of protoplasts. II. The combinations of *Nicotiana tabacum* and *N. glutinosa* and *N. tabacum* and *N. alata*. Jàpan Jour Crop Sci 48:385–392

Nagy F, Lázár G, Menczel L, Maliga P (1983) A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. Theor Appl Genet 66:203–207

Nielsen MT, PD Legg, CC Litton (1985) Effects of two introgressed disease resistance factors on agronomic characteristics and certain chemical components in burley tobacco. Crop Sci 25:698–701

Pehu E, Karp A, Moore K, Steele S, Dunckley R, Jones MGK (1989) Molecular, cytogenetic and morphological characterization of somatic hybrids of dihaploid *Solanum tuberosum* and diploid *S. brevidens*. Theor Appl Genet 78:696–704

Pelletier G, Primard C, Ferault M, Vedel F, Chetrit P, Renard M, Delourme R (1988) Use of protoplasts in plant breeding: cytoplasmic aspects. Plant Cell Tissue Organ Cult 12: 173–180

Perl A, Aviv D, Galun E (1991) Nuclear-organelle interaction in Solanum: interspecific cybridizations and their correlation with a plastome dendrogram. Mol Gen Genet 228:193–200

Rose RJ, Thomas MR, Fitter JT (1990) The transfer of cytoplasmic and nuclear genomes by somatic hybridisation. Aust J Plant Physiol 17:303–321

Rothenberg M, Hanson MR (1987) Recombination between parental mitochondrial DNA following protoplast fusion can occur in a region which normally does not undergo intragenomic recombination in parental plants. Curr Genet 12:235–240

Sproule A, Donaldson P, Dijak M, Bevis E, Pandeya R, Keller WA, Gleddie S (1991) Fertile somatic hybrids between transgenic *Nicotiana tabacum* and transgenic *N. debneyi* selected by dual-antibiotic resistance. Theor Appl Genet 82: 450–456

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within *Brassicaceae*. Theor Appl Genet 83:81–88

Sundberg E, Lagercrantz U, Glimelius K (1991) Effects of cell type used for fusion on chromosome elimination and chloroplast segregation in *Brassica oleracea* (+) *Brassica napus* hybrids. Plant Sci 78:89–98

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87–93

Thanh ND, Páy A, Smith MA, Medgyesy P, Márton L (1988) Intertribal chloroplast transfer by protoplast fusion between *Nicotiana tabacum* and *Salpiglossis sinuata*. Mol Gen Genet 213:186–190

Uchimiya H (1982) Somatic hybridization between male-sterile *Nicotiana tabacum* and *N. glutinosa* through protoplast fusion. Theor Appl Genet 61:69–72