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Fertile somatic hybrids between transgenic *Nicotiana tabacum* and transgenic *N. debneyi* selected by dual-antibiotic resistance

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Summary. A simple, yet effective selection system was used to produce fertile somatic hybrids between *Nicotiana tabacum* and *N. debneyi*. This approach utilized transgenic antibiotic-resistant *N. tabacum* and *N. debneyi* as donor plants for mesophyll protoplast fusions. Thirteen somatic hybrid plants were regenerated from calli capable of growth on medium containing both antibiotics. The majority of the hybrids displayed a range of leaf and floral morphologies and growth habits that were intermediate to those of the parental species, and had chromosome numbers varying from amphidiploid (2n = 96) to hypoaneuploid (2n = 60). Isoenzyme and RFLP analysis demonstrated the presence and expression of nuclear genes from both parents in all of the hybrids. Most plants are fully fertile. Thus, these plants differ from the male-sterile tobacco 'cybrids' and alloplasmic lines produced by transferring the *N. debneyi* cytoplasm to tobacco. A nonrandom pattern of cytoplasmic segregation in the fusion products occurred with a bias towards the presence of *N. debneyi* cp and mtDNA. Evidence for the presence of rearranged or recombinant cp and mtDNA in some of the hybrids was obtained. The somatic hybrids were successfully backcrossed to the *N. tabacum* parent and are now being tested for immunity to black root rot, a trait specific to *N. debneyi*, but not existent in the *N. tabacum* parental line.

Key words: *Nicotiana tabacum* – *N. debneyi* – Somatic hybridization – Transformation – Organelle inheritance

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Introduction

Somatic hybridization of a wide variety of higher plants is limited by the difficulty encountered in selecting heterokaryons following protoplast fusion. Different approaches have been utilized to solve this problem (see review by Evans 1983), but few have proven universally applicable. Cell sorting by fluorescent-activated cell sorters (FACS) is one of the more widely applicable methods of heterokaryon selection (Glimelius et al. 1986a), but its disadvantage is that cell sorters are expensive and their use requires specialized technical expertise. However, many plant species can now be readily transformed with dominant, nuclear-encoded, selectable marker genes such as neomycin phosphotransferase (*NPTII*), dihydrofolate reductase (*dhfr*), chloramphenicol acetyl transferase (*CAT*), hygromycin phosphotransferase (*HPT*), etc., which confer antibiotic resistance to their cells and tissues. This has led to novel, dual-antibiotic selection systems which have been described for the recovery of hybrids between two different *N. tabacum* plants (Komari et al. 1989), between different diploid potatoes (Masson et al. 1989), and for the recovery of somatic hybrids between *Medicago scutellata* and *M. intertexta* (Thomas et al. 1990).

Recently we have described the positive expression of methotrexate and kanamycin resistance in isolated leaf mesophyll protoplasts of *N. tabacum* cv Delgold and *N. debneyi*, respectively (Dijak et al. 1990). This report describes protoplast fusion experiments between these two genotypes and selection for the presence of nuclear heterokaryons using both selective agents. Cybrids between *N. tabacum* and *N. debneyi* have been studied extensively in the past. The *N. debneyi* cytoplasm alone, when transferred to tobacco following asymmetric hybridization, results in male-sterile tobacco (Kumashiro

and Kubo 1986; Asahi et al. 1988; Hakansson et al. 1988). This study, however, describes the selection for nuclear hybrids between these two species. A useful consequence of the transfer of *N. debneyi* nuclear material may be the concomitant transfer of black root rot resistance, an important disease of commercial tobacco, from the wild *N. debneyi* species into the economically significant Canadian tobacco cultivar Delgold.

Materials and methods

Plant materials

Antibiotic-resistant progeny of transgenic *Nicotiana tabacum* L. cv Delgold and *N. debneyi*, carrying either kanamycin resistance (*NPTII*) or methotrexate resistance (*dhfr*) genes, were used as protoplast donors. Introduction of the resistance genes and the expression of antibiotic resistance in mesophyll protoplasts of the transgenic plants has been described (Dijak et al. 1990). Selfed progeny (S_1) of kanamycin-resistant *N. debneyi* (N.d.K) and methotrexate-resistant *N. tabacum* cv Delgold (N.t.M) were germinated on agar-solidified B_5 medium (Gamborg et al. 1968) supplemented with either 150 mg l^{-1} kanamycin (Km) or 10 mg l^{-1} methotrexate (Mx). Resistant seedlings to be used as a source of protoplasts were selected after 4 weeks and transferred to sterile plastic containers (100 mm high, Magenta Corp.) containing 40–60 ml of agar-solidified B_5 medium without growth regulators or antibiotics.

Isolation and fusion of protoplasts

Mesophyll protoplasts were isolated from the preselected resistant plants grown in vitro as previously described (Dijak et al. 1990). For fusion, 10^6 protoplasts of each species were mixed in 16×125 mm glass tubes and centrifuged at $20 \times g$ for 5 min. Polyethylene glycol (PEG)-mediated fusion in glass tubes was performed according to Douglas et al. (1981 a), except that the fusion solution consisted of 25% (w/v) PEG MW 6,000 and 3% (w/v) $CaCl_2 \cdot 2H_2O$ (pH 6.9). The elution solution consisted of 2.5% (w/v) KCl and 0.2% (w/v) $CaCl_2 \cdot 2H_2O$ (pH 5.8). For postfusion culture, protoplasts were resuspended to a density of 1×10^5 ml^{-1} in NT medium (Nagata and Takebe 1971) containing 0.45 *M* glucose, 3 mg l^{-1} 1-naphthaleneacetic acid (NAA), and 1 mg l^{-1} benzylaminopurine (BA) at pH 5.8. The protoplast suspension (1 ml) was placed in 60×15 mm petri dishes over agarose underlayers containing 2 ml of medium (as above) solidified with 0.4% agarose (Seakem LE) and either 2–50 mg l^{-1} methotrexate, or 150–300 mg l^{-1} kanamycin. Protoplast cultures were incubated at 25 °C in darkness.

Selection of fusion products

After 7 days of culture, one-half of the liquid NT medium containing 0.45 *M* glucose was replaced with liquid NT medium containing 0.3 *M* glucose and either Km or Mx at the same concentration found in the agarose underlayer. At this time the cultures were moved to low light intensity (10 μE m^2s^{-1}) and a 16-h day length. The liquid suspension, containing all of the microcolonies which survived this initial selection, was transferred 7–10 days later by pouring onto a regeneration medium containing the antibiotic to which the microcolonies had not yet been exposed. The regeneration medium consisted of agar-solidified (0.8% w/v) MS medium (Murashige and Skoog 1962) containing 2% sucrose, 2 mg l^{-1} kinetin, and 0.1 mg l^{-1} NAA. The microcalli that subsequently survived and grew were then transferred, usually within 14–28 days, to fresh regeneration medium containing 150 mg l^{-1} kanamycin and 2 mg l^{-1} methotrexate. The calli were transferred monthly to fresh regeneration media containing both selection agents, until they formed shoots or died. Shoots that regenerated were transferred to rooting medium (growth regulator-free B_5 medium) containing 2 mg l^{-1} methotrexate and 150 mg l^{-1} kanamycin. Plants were transferred to peat pellets (Jiffy 7) after becoming well established in vitro. They were maintained in a mist bed with 80% rel. hum. at 25 °C day and 20 °C night temperatures under 16-h daylight (30 μE$m^{-2}s^{-1}$) supplied by fluorescent and incandescent lights.

Isoenzyme analysis

Leaf proteins were extracted in ice-cold 0.2 *M* TRIS-Cl (pH 8.5) with 30 m*M* DTT (Cleland's reagent), and were subjected to nondenaturing polyacrylamide gel electrophoresis (6.25% acrylamide) as described previously (Douglas et al. 1981 b). Gels were stained for peroxidase, glucosephosphate-isomerase, and glutamate-oxaloacetate transaminase isozymes.

Nucleic acid characterization

Total DNA was isolated from fresh green leaves using standard protocols. Genomic DNA (5–10 μg) was restricted with *Eco*RI or *Hind*III according to suppliers instructions. Digested DNA was electrophoretically separated on 0.8% agarose gels and transferred to nylon membranes (Nytran). For analysis of nuclear hybridity and patterns of inheritance of cytoplasmic organelles, the Southern blots were probed with pTA71, a wheat rDNA fragment (Gerlach and Bedbrook 1979), pBa1–9, a tobacco chloroplast DNA sequence (Aviv et al. 1984), and several mtDNA fragments including the wheat *COX*II (Bonen et al. 1984), *atp6* (Bonen and Bird 1988), and *cytB* genes (Boer et al. 1985). Probes were P^{32}-labelled according to Feinberg and Vogelstein (1983) and hybridized overnight at 65 °C in 1 *M* NaCl, 25 m*M* TRIS-Cl (pH 8), 1% sarkosyl, and 0.05 μg ml^{-1} of yeast tRNA. Final high stringency washes of the hybridized blots were performed at 65 °C in 0.1 × SSC, 1% SDS.

Pollen viability and somatic chromosome number

The percentage of pollen that stained with acetocarmine was determined following examination of pollen from at least six individual flowers per hybrid (1,000 pollen grains/flower). Root tips were excised from plants grown on a loose mixture of perlite and turface, incubated at 4 °C overnight in water, and fixed in 95% ethanol and acetic acid (3:1 v/v). Root tips were then hydrolyzed in 1 *N* HCL at 60 °C for 8–10 min and placed in Feulgen stain. Squash preparations were made in Carbol Fuchsin or acetocarmine before chromosome counting.

Results

Double-resistant putative hybrid calli were obtained from protoplast fusion experiments plated initially on kanamycin-containing medium and also from those in which fusions were plated initially on medium containing methotrexate. After transfer of microcalli from underlayers containing the first antibiotic to regeneration medium containing the second antibiotic, resistant calli were observed. A majority of these calli that survived exposure to the second selective agent continued to grow when transferred to regeneration medium containing both

Fig. 1 a–b. Somatic hybrid morphology. **a** *N. tabacum* cv Delgold (*left*), *N. tabacum* + *N. debneyi* somatic hybrid td-6 (*center*), *N. debneyi* (*right*). **b** *N. tabacum* cv Delgold (*left*), somatic hybrid td-5 (center), *N. debneyi* (*right*)

Table 1. Floral morphology, cytology, and fertility of *N. tabacum* + *N. debneyi* somatic hybrid plants

Hybrid plant	Chromosome no.	Corolla length[a]	Flower color[b]	Pollen viability (%)[c]
1	60	39	lp	1–25
1b	–	30	w	6–33
2	96	39	lp	90–94
3	60	40	lp	0–1
3b	–	29	w	11–28
4	96+	37	ip	not tested
5	96	40	lp	21–49
6	96+	26	tp	63–91
7	–	37	lp	1–46
8	–	39	lp	10–41
9	83–96+	40	lp	1–76
10	91–96	39	lp	1–53
11	–	27	lp	19–42
N. tabacum	48	64	tp	81
N. debneyi	48	14	w	88

[a] Average of lengths in mm of five flowers
[b] lp = light pink, w = white, ip = intermediate pink (pastel purple), tp = dark pink similar to tobacco flower color
[c] Range of % viability observed for six flowers (1,000 grains counted/flower)

methotrexate and kanamycin. All calli that continued to grow and that regenerated in the presence of both antibiotics developed into plants that were subsequently shown to be hybrids.

Assessment of the morphological and cytological characteristics of the somatic hybrids (outlined in Table 1) indicated a degree of variability among the individual plants. In height, growth habit, and leaf morphology the hybrid plants were predominantly intermediate to those of the parental lines. However, somatic hybrid td-6 (see Fig. 1 a), which had distinctly petiolated leaves and which produced multiple inflorescences from a rosette of leaves, resembled *N. debneyi* much more closely than did the other hybrids (e.g., see Fig. 1 b). As Table 1 shows, variability was also observed in flower size and also flower color, which included white (similar to *N. debneyi*), pale pink, pastel purple, and dark pink (similar to *N. tabacum*) (see Fig. 2 a; b). It should be noted that each of the somatic hybrid pairs td-1 and td-1 b and td-3 and td-3 b were derived, respectively, from single, double-resistant calli that produced shoots of two distinct types (both hybrid), a pink-flowering and a white-flowering type, and were thus designated as distinct hybrids and characterized individually. Leaves of many hybrid plants were intermediate in shape and size between those of *N. tabacum* and *N. debneyi*, however, occasional abnormalities such as fused leaves were observed (Fig. 2 c). Results of cytological examination of somatic chromosome numbers indicated considerable variability among the somatic hybrids, with some displaying the amphidiploid number of 96 ($2n = 48 + 48$) chromosomes (Fig. 3); however, other hybrids were aneuploid ($2n = 60–65$ or $84–86$) (Table 1).

All of the somatic hybrids displayed at least partial male and female fertility. Each produced dehiscent anthers, with varying amounts of viable pollen (Table 1) and, with the exception of the two white-flowering hybrids, td-1 b and td-3 b, all produced open-pollinated seed. Backcrosses (BCs) of the somatic hybrids with tobacco as the male parent resulted in seed set for eight of eight individual hybrid BCs performed.

Biochemical and molecular characterization of the plants provided evidence of the presence of nuclear genes

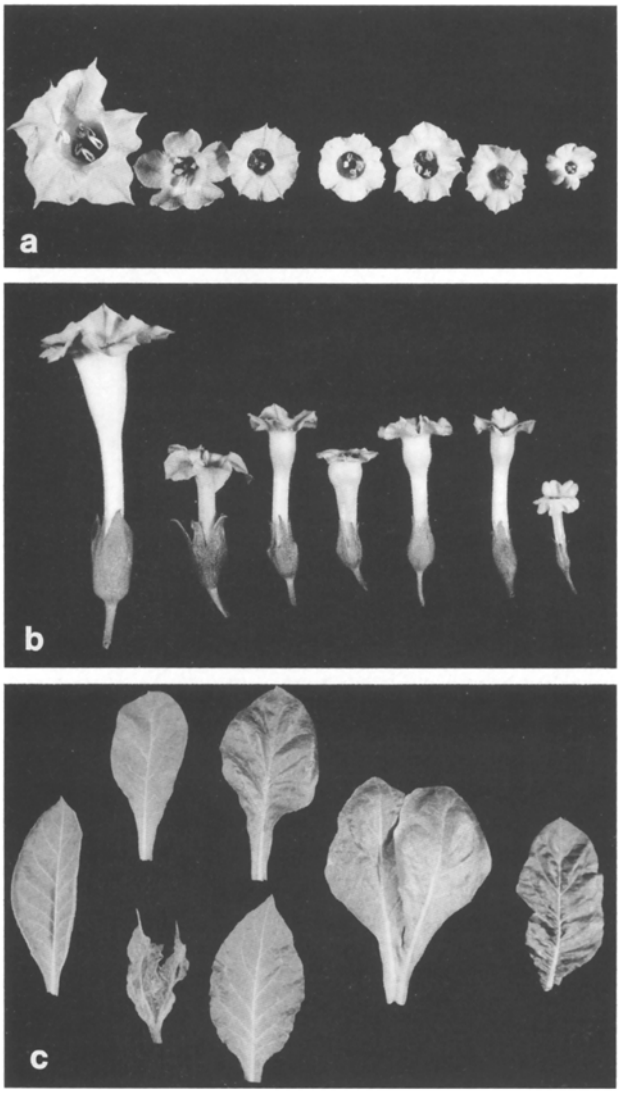

Fig. 2a–c. Floral and leaf morphology of somatic hybrids. **ab** Flowers of *N. tabacum* (*left*), five *N. tabacum*+*N. debneyi* somatic hybrids (*center*), and *N. debneyi* (*right*). **c** Leaves of *N. debneyi* (*right*), *N. tabacum* (left), and five somatic hybrid plants (*center*)

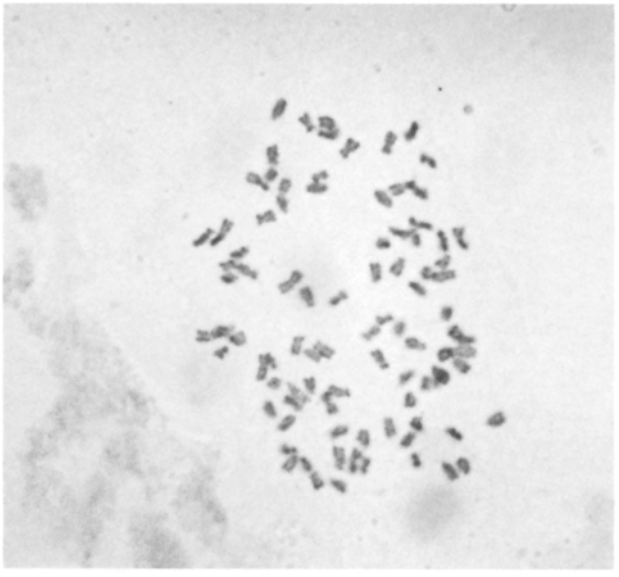

Fig. 3. Somatic chromosomes of hybrid td-2. Metaphase chromosomes of squashed root-tip cell carrying approximately 96 somatic chromosomes

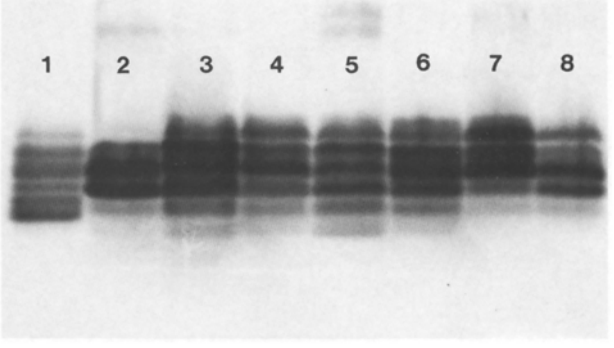

Fig. 4. Isoenzyme analysis of somatic hybrids. Leaf protein extracts of the somatic hybrids and of the parental species were electrophoresed in a 6.25% nondenaturing polyacrylamide gel and stained for peroxidase activity. *N. tabacum* (*lane 1*), *N. debneyi* (*lane 2*), *N. tabacum*+*N. debneyi* somatic hybrids (*lanes 3–8*)

from both parents. Isoenzyme banding patterns for peroxidase (shown in Fig. 4), glucosephosphate isomerase, and glutamate oxaloacetate transaminase (not shown) all showed a clear distinction between the parental forms of these enzymes. For each enzyme, the somatic hybrids possessed bands specific to both parents, and in some cases unique bands were detected indicating the expression of genes from both parents. Probing of genomic DNA with the heterologous wheat ribosomal DNA probe pTA71 was used to further confirm the nuclear hybridity of the plants. Bands representing rDNA sequences of both parental species occurred for each of the somatic hybrids (Fig. 5).

Chloroplast segregation in the somatic hybrids was tested by probing total digested DNA of the hybrids and of parental species with the *Nicotiana* chloroplast DNA probe pBa1–9. Using this probe, hybridization patterns identical to those for *N. debneyi* were obtained for all but one of the somatic hybrids tested (Fig. 6). A unique hybridization pattern was obtained with this plastid probe for somatic hybrid td-6. Total DNA of td-6 digested with *Hind*III and probed with pBa1–9 displays several unique bands not present in either parent and, in addition, displays most of the *N. debneyi*-specific bands (Fig. 6). pBa1–9 probing of *Eco*R1-digested DNA of td-6 shows unique hybridizing bands and possibly bands specific to

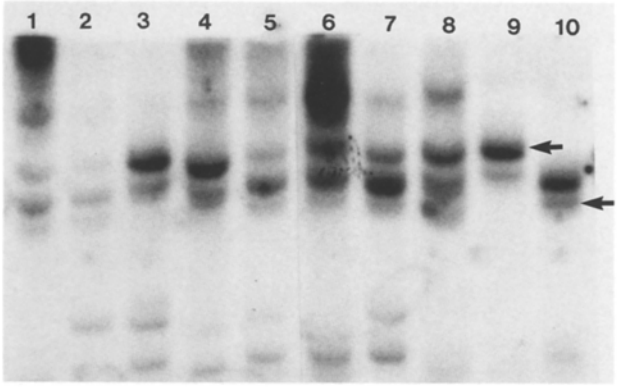

Fig. 5. Southern blot hybridization of *Eco*RI digests of total genomic DNA probed with pTA71. *Lanes 1–8* are *N. tabacum* + *N. debneyi* somatic hybrids. *Lane 9* – *N. tabacum*. *Lane 10* – *N. debneyi*. *Arrows* indicate species-specific parental bands

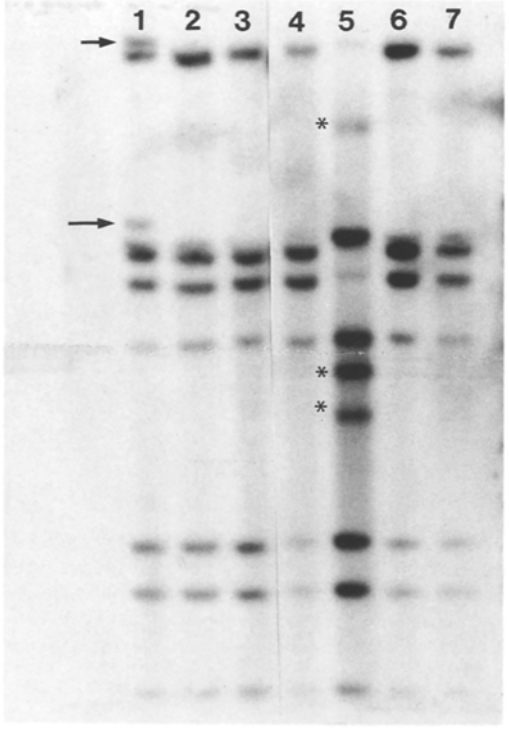

Fig. 6. Plastome analyses. Southern blot of *Hin*dIII digests of total genomic DNA probed with pBa1–9. *Lane 1* – *N. tabacum*, *lane 2* – *N. debneyi*, *lane 3* – somatic hybrid td 3, *lane 4-td-5*, *lane 5-td-6*, *lane 6-td-1b*, *lane 7* – td-3b. *Arrows* indicate *N. tabacum* chloroplast-specific bands. *Asterisks* represent novel bands found in hybrid td-6

N. debneyi, in addition to bands specific to *N. tabacum* (data not shown).

Total DNA of seven out of ten somatic hybrids probed with cloned mitochondrial-specific DNAs showed hybridization patterns identical to those for *N. debneyi* (Table 2). One hybrid, td-10, displayed unique hybridizing bands for *cytB* probing of *Eco*R1 digests,

Table 2. Cytoplasmic analysis of *N. tabacum* + *N. debneyi* somatic hybrid plants

Hybrid	cp DNA[a]		mt DNA[b]	
	*Hin*dIII	*Eco*R1	*atp6*	*cytB*
td-1	–	Nd	–	–
td-1b	Nd	Nd	Nd	Nd
td-2	–	Nd	Nd	–
td-3	Nd	Nd	Nd	Nd
td-3b	Nd	–	Nd	Nd
td-4	–	Nd	–	–
td-5	Nd	Nd	Nd	–
td-6	U	Nd + Nt	Nd	Nd
td-7	Nd	Nd	Nd	Nd
td-8	–	Nd	–	Nt
td-9	–	Nd	Nd	Nt
td-10	–	Nd	Nd	U

[a] Results of probing two separate genomic digests (*Hin*dIII and *Eco*R1) with pBA1-9

[b] Results of probing *Eco*R1 digests of genomic DNA with mtDNA fragments ATP6 or *cytB*. Nt = pattern of hybridization identical to *N. tabacum*. Nd = pattern of hybridization identical to *N. debneyi*, U = hybridization pattern is unlike either parent, Nd + Nt = hybridizing bands specific to *both N. tabacum* and *N. debneyi* are present but *no* unique bands observed

while the hybridization pattern for *atp6* probing of this plant is similar to *N. debneyi* for atp6 probing, however, the pattern resembled *N. tabacum* for *cytB* probing. The third plant which did not appear to contain unaltered *N. debneyi* mtDNA, td-8, was only probed with the *cytB* gene probe and resembled *N. tabacum* mtDNA.

Discussion

Selection of interspecific *N. tabacum* + *N. debneyi* plants was accomplished by using transgenic parental lines as the source of leaf mesophyll cells for protoplast isolation. Since each parental line expressed a different dominant nuclear marker gene conferring resistance to either kanamycin or methotrexate, heterokaryons were selected on media containing each selective agent alone, followed by selection on media containing both antibiotics. Thus, while the hybrids produced must carry at least a portion of the nuclear genetic material from both of the parental species, selection was not applied for the presence of other nuclear-encoded traits nor for any particular mitochondria or chloroplasts. The efficient selection of heterokaryons at an early stage following protoplast fusion resulted in tremendous time savings, since only hybrids reached the 'regeneration' stage of development. The major advantage of this approach to somatic hybridization is its applicability to any species or genotype that can be transformed with dominant selectable marker genes. Specifically, this permitted us to utilize the economically important tobacco cultivar Delgold and the disease-resistant wild species *N. debneyi* in this hybridization, and this

approach has also led recently to the selection of somatic hybrids between *N. tabacum* and *N. rustica* (P. Donaldson, A. Sproule, E. Bevis, R. Pandeya, W. A. Keller, and S. Gleddie, in preparation).

Somatic hybrid plants between *N. tabacum* and *N. debneyi* have not been previously described, although cybrid plants (male-sterile *N. tabacum* carrying the cytoplasm of *N. debneyi*) have been extensively studied (Kumashiro and Kubo 1986; Asahi et al. 1988; Hakansson et al. 1988). Transfer of the complete or partial cytoplasm of *N. debneyi* to tobacco by either asymmetric hybridization or alloplasmic substitution results in the production of male-sterile tobacco (Kumashiro and Kubo 1986; Sand and Christoff 1972). In these asymmetric hybrids, either of the parental chloroplast types may be present, but all possess rearranged mitochondrial genomes that are unlike either parent (Asahi et al. 1988). The mtDNA of those plants is thought to be responsible for the cytoplasmic male-sterile (CMS) phenotype (Belliard et al. 1979). The introduction of a single *N. debneyi* chromosome into the genome of those CMS plants can partially restore fertility (Hakansson et al. 1988), but the lack of such 'restorer' genes in the tobacco gene pool (Sand and Christoff 1972) has been demonstrated. In the present study the somatic hybrids between *N. tabacum* and *N. debneyi* are all partially or highly male fertile. Abberant anthers of the type reported for tobacco plants containing the *N. debneyi* cytoplasm (Belliard et al. 1979; Kumashiro and Kubo 1986; Hakansson et al. 1988) were not observed in our somatic hybrids. The results of probing genomic DNA of these somatic hybrids with mitochondrial gene probes revealed the presence of *N. debneyi* mtDNA in the somatic hybrids. Thus, it is likely that the hybrids also contain *N. debneyi* nuclear genes, which permit restoration of fertility, and the results of pTA71 probing and isoenzyme analysis further demonstrate that *N. debneyi* as well as *N. tabacum* nuclear genes are present and expressed in the hybrids.

Recombination between two different mtDNAs following protoplast fusion is a relatively frequent occurrence in *Nicotiana* (Belliard et al. 1979; Aviv and Galun 1987). Of five male-sterile tobacco plants obtained by fusion of *N. tabacum* protoplasts with *X*-irradiated *N. debneyi* protoplasts, examined by Asahi et al. (1988), all five possessed recombinant mtDNA. In the present study, when genomic DNA of somatic hybrid td-10 was probed with the mt *cytB* gene, unique hybridizing bands were detected, suggesting the presence of recombinant mtDNA or perhaps rearranged *N. debneyi* mtDNA. One other plant, td-9, may also contain recombinant mtDNA, although novel hybridizing fragments were not found, and only one of ten plants examined appears to contain *N. tabacum* mtDNA. Data for most of the other hybrids (7/10) suggest the presence of mtDNAs similar, if not identical, to the parental *N. debneyi* mtDNA.

Interestingly, the results of probing genomic DNA of the hybrids with cpDNA probes indicate that 11 of the 12 plants examined possess *N. debneyi* chloroplasts, while none appear to carry chloroplasts identical to those of *N. tabacum*. Chloroplasts are typically expected to sort out randomly following protoplast fusion, giving rise to plants containing one or the other of the parental types, but not both (Chen et al. 1977; Akada and Hirai 1986; Kumashiro and Kubo 1986). However, nonrandom patterns of chloroplast sorting-out have also been observed (Glimelius et al. 1981; Douglas et al. 1981 b; Bonnett and Glimelius 1983). Sometimes, but rarely, recombinant cpDNA (containing some but not all of the DNA from both of the parental cpDNAs) has been observed in products of protoplast fusions (Medgyesy et al. 1985; Thanh and Medgyesy 1989). Patterns of chloroplast inheritance in *N. tabacum* + *N. debneyi* cybrids (e.g., male-sterile tobacco) indicate random chloroplast segregation, with five of ten plants carrying chloroplasts identical with those of *N. tabacum* and the other five with chloroplasts identical to those of *N. debneyi* (Asahi et al. 1988). In contrast, none of the somatic hybrids in our study carry *N. tabacum* chloroplasts. The reason for this nonrandom pattern of cytoplasmic inheritance is not obvious, since the antibiotic resistance genes behaved as dominant nuclear loci in testcrosses of the donor plants (Dijak et al. 1990). Mesophyll protoplasts of each parental species were used in these fusions, which makes it improbable that differences in the state of differentiation of the plastids contributed to the biased inheritance of the *N. debneyi* cytoplasm.

Other factors that may lead to biased chloroplast segregation include differences in nuclear-cytoplasmic compatibilities (Glimelius et al. 1986 b) and, of course, selection protocols which favor the retention of one chloroplast type. In the case of *N. tabacum* + *N. debneyi*, the random sorting-out of chloroplasts in asymmetric hybrids carrying only tobacco nuclear genes (Asahi et al. 1988) suggests that there is no nuclear-cytoplasmic incompatibility between tobacco nuclei and *N. debneyi* chloroplasts. However, in the present study the non-random segregation could be due to incompatibility between the *N. debneyi* nuclear component of the hybrid nuclei and the *N. tabacum* chloroplasts. The probing data obtained for somatic hybrid td-6 suggest the presence of a recombinant cpDNA which, as mentioned, occurs only rarely. Nucleoplastic incompatibility was found to play a role in the selection of a recombinant 'potacco' plastid arising after the fusion of *N. tabacum* and *Solanum tuberosum* (Thanh and Medgyesy 1989). Thus, it is possible that both the biased inheritance of *N. debneyi* chloroplasts and the single instance of recombinant cpDNA observed here may both be due to incompatibilities between the tobacco plastids and the *N. debneyi* nuclear genes in the hybrids. Somatic hybrid td-6 possessing the

recombinant cpDNA was the only plant which came from fusions selected initially on kanamycin, while all of the other hybrids were from experiments in which methotrexate was used as the primary selective agent. Further studies are required to determine whether the precise selection protocols used in this study may have influenced the patterns of chloroplast inheritance. Detailed characterization of the recombinant cpDNA is in progress.

In conclusion, this study has resulted in the regeneration of fertile somatic hybrid plants between *N. tabacum* and *N. debneyi* using a simple, effective selection scheme. The level of fertility among these plants has allowed the production of selfed and backcross (BC_1) seed. Preliminary results of black root rot infection indicate that the somatic hybrids have inherited and express immunity to this pathogen (Brandle, personal communication). Further backcrosses and field/nursery evaluations with these lines are in progress to incorporate this germplasm in a tobacco improvement program.

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References

Akda S, Hirai A (1986) Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli. III. Random separation of two types of chloroplast genomes in hybrid callus. Jpn J Genet 61:437–445

Asahi T, Kumashiro I, Kubo T (1988) Constitution of mitochondrial and chloroplast genomes in male-sterile tobacco obtained by protoplast fusion of *Nicotiana tabacum* and *N. debneyi*. Plant Cell Physiol 29:43–49

Aviv D, Galun E (1987) Chondriome analysis in sexual progenies of *Nicotiana* cybrids. Theor Appl Gent 73:821–826

Aviv D, Bleichman S, Arzee-Gonen P, Galun E (1984) Intersectional cytoplasmic hybrids in *Nicotiana*. 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 PJH, 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

Bonen L, Bird S (1988) Sequence analysis of the wheat mitochondrial *atp6* gene reveals a fused upstream reading frame and markedly divergent N termini among plant ATP6 proteins. Gene 73:47–56

Bonen L, Boer PH, Gray MW (1984) The wheat cytochrome oxidase subunit II gene has an intron insert and three radical amino acid changes relative to maize. EMBO J 3:2531–2536

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

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

Dijak M, Sproule A, Keller WA, Pandeya RS, 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

Douglas GC, Keller WA, Setterfield G (1981a) Somatic hybridization between *N. rustica* and *N. tabacum*. II. Protoplast fusion and selection and regeneration of hybrid plants. Can J Bot 59:220–227

Douglas GC, Wetter LR, Nakamura C, Keller WA, Setterfield G (1981b) Somatic hybridization between *Nicotiana rustica* and *N. tabacum*. III. Biochemical, morphological, and cytological analysis of somatic hybrids. Can J Bot 59:228–237

Evans DA (1983) Agricultural applications of plant protoplast fusion. Biotechnology 1:253–261

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal genes from wheat and barley. Nuclic Acids 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

Glimelius K, Djupsjobacka M, Fellner-Feldegg H (1986a) Selection and enrichment of plant protoplast heterokaryons of Brassicaceae by flow sorting. Plant Sci 45:133–141

Glimelius D, Fahleson J, Sjodin G, Sundberg E, Djupsjobacka M, Fellner-Feldegg H, Bonnett HT (1986b) Somatic hybridization and cybridization as potential methods for widening of the gene pool of crops within Brassicaceae and Solanaceae. In: Horn W, Jensen CJ, Odenbach W, Schnieder O (eds) Genetic manipulation in plant breeding. de Gruyter, Berlin New York, pp 663–682

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

Komari T, Saito Y, Nakakido F, Kumashiro T (1989) Efficient selection of somatic hybrids in *Nicotiana tabacum* L. using a combination of drug resistance markers introduced by transformation. Theor Appl Genet 77:547–552

Kumashiro T, Kubo T (1986) Cytoplasm transfer of *Nicotiana debneyi* to *N. tabacum* by protoplast fusion. Jpn J Breed 36:39–48

Masson J, Lancelin D, Bellini C, Lecerf M, Guerche P, Pelletier G (1989) Selection of somatic hybrids between diploid clones of potato (*Solanum tuberosum* L.) transformed by direct gene transfer. Theor Appl Genet 78:153–159

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

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

Nagata T, Takebe I (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20

Sand SA, Christoff GT (1972) Cytoplasmic-chromosomal interactions and altered differentiation in tobacco. J Hered 64:24–30

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

Thomas MR, Johnson LB, White FF (1990) Selection of interspecific somatic hybrids of *Medicago* by using *Agrobacterium*-transformed tissues. Plant Sci 69:189–198