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Asymmetric hybridization between *Nicotiana tabacum* and *N. repanda* by donor recipient protoplast fusion: transfer of TMV resistance

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Summary. Genetically asymmetric hybrids were recovered by fusion of *Nicotiana tabacum* protoplasts with irradiated protoplasts of kanamycin-resistant, nopaline-producing plants of *N. repanda*. Hybrid calli were selected by culture on media containing kanamycin and were regenerated. These plants were morphologically similar to *N. tabacum* but produced nopaline, indicating they retained genes from *N. repanda*. Esterase isozyme profiles also indicated that the plants are somatic hybrids, but are more similar to *N. tabacum* than *N. repanda*. Chromosome counts showed most of the hybrids had 55–62 chromosomes, which is consistent with extensive, although incomplete elimination of *N. repanda* chromosomes. The hybrids were largely male sterile, but about half of them set seed when crossed with *N. tabacum*. Chromosome numbers of the progeny and the pattern of inheritance of kanamycin resistance indicated the continued elimination of *N. repanda* genetic material in these backcrosses. The *N. repanda* parent used in these fusions gave a hypersensitive response to TMV, whereas the *N. tabacum* parent was TMV sensitive. When inoculated with TMV, plants from two hybrid clones gave a hypersensitive response. Plants from the other clones became systemically infected with the virus.

Key words: Somatic hybridization – Gene transfer – Chromosome elimination – Tobacco – TMV resistance

Introduction

Hybridization between *Nicotiana tabacum* and *N. repanda* is of interest because *N. repanda* is resistant to more diseases of cultivated tobacco than any other species in the genus (Burk and Heggestad 1966). Although Stavely et al. (1973) were able to recover hybrids between tetraploid tobacco and a *N. repanda* × *N. sylvestris* hybrid, direct sexual crosses between *N. tabacum* and *N. repanda* have never been successful (Reed and Collins 1978). Incompatibility reactions leading to embryo abortion and seedling lethality are part of the problem. Iwai et al. (1985) overcame these barriers and recovered a *N. tabacum* × *N. repanda* hybrid using a combination of ovule and callus culture, but the plant that regenerated was sterile. More recently, Shintaku et al. (1988) used pollen and egg cell irradiation plus ovule culture to recover *N. tabacum* × *N. repanda* hybrids. Six hybrids reached maturity and flowered, but no progeny were recovered.

Protoplast fusion offers an alternative avenue for hybridization between *N. tabacum* and *N. repanda*; however, this approach has also proven difficult. Evans et al. (1981) prepared somatic hybrids between *N. tabacum* and two other species in the section *Repandae* (*N. nesophila* and *N. stocktonii*) but were unsuccessful in regenerating *N. tabacum* (+) *N. repanda* hybrids. Nagao (1982) did recover *N. tabacum* (+) *N. repanda* hybrids, but in ten fusion experiments hybrid plants were regenerated from only three calli. These plants were morphologically intermediate between the two species and displayed *N. repanda*'s hypersensitive response to TMV, but produced no progeny when either selfed or backcrossed.

If the incompatibilities encountered in hybrids between *N. tabacum* and *N. repanda* are genetic, then partial elimination of *N. repanda* chromosomes could improve the vigor and fertility of the hybrids. Gupta et al. (1984) showed that genetically asymmetric hybrids can be formed by fusing protoplasts of a recipient species with irradiated protoplasts of a donor species and selecting for a nuclear trait of the donor. In the species combination they used (*Datura* and *Physalis*), irradiation re-

sulted in improved hybrid regeneration, presumably through the elimination of most of the donor (*Physalis*) chromosomes. This approach has now been used by a number of groups to produce inter- and intrageneric hybrids that are partially fertile (Bates et al. 1987, Dudits et al. 1987, Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989).

This study describes the recovery of asymmetric hybrids following fusion of *N. tabacum* protoplasts with irradiated protoplasts of *N. repanda*. Hybrids were recovered by selection for kanamycin resistance, which had been introduced into *N. repanda* by *Agrobacterium*-mediated transformation. Two of the hybrids displayed *N. repanda*'s hypersensitive response when inoculated with TMV. Although largely male-sterile, many of the hybrids produced viable seeds when backcrossed with *N. tabacum*. The inheritance of kanamycin resistance among the backcross progeny has been used to evaluate the transmission of *N. repanda* chromosomes in these crosses.

Materials and methods

Plant material

Seeds of *Nicotiana repanda* and *Nicotiana tabacum* var 'Xanthi', genotype *nn* were obtained from Dr. Verne Sisson, USDA Tobacco Research Laboratory, P.O. Box 1555, Oxford, NC, USA. *N. repanda* was transformed to kanamycin resistance by the leaf disc transformation procedure (Rogers et al. 1986) using *Agrobacterium tumefaciens* carrying pTiB6S3-SE with pMON200 cointegrated (the *Agrobacterium* strain was the generous gift of Dr. Stephen Rogers, Monsanto). For transformation, leaf discs of *N. repanda* were dipped briefly in a suspension of *Agrobacterium* and then cultured for 48 h on feeder plates prepared by layering *N. tabacum* suspension culture cells over solidified RMB medium [Murishige and Skoog salts and B5 vitamins supplemented with 3% sucrose, 1 mg/l benzyladenine, and 0.8% agar (Menczel et al. 1981]. After 48 h the leaf discs were transferred to fresh plates of RMB supplemented with 0.5 mg/ml carbenicillin plus 0.3 mg/ml kanamycin. Kanamycin-resistant calli that grew from the leaf discs were regenerated by successive transfers on RMB + 0.3 mg/ml kanamycin. Shoots were rooted on solidified P medium [Murashige and Skoog salts, vitamins, and 3% sucrose but with KNO_3, NH_4NO_3, and $MgSO_4$ reduced to one-fifth (Maliga 1982)] supplemented with 0.1 mg/ml kanamycin.

Protoplast isolation, irradiation, fusion, and culture

Mesophyll protoplasts were isolated from *N. tabacum* as described previously (Bates et al. 1987). Mesophyll protoplasts were isolated from leaves of kanamycin-resistant *N. repanda* plants by the same procedures but with the following modifications. Leaves from aseptically grown plants were cut into narrow (1 mm) strips and digested overnight (27 °C) with 0.2% Cellulysin plus 0.02% Pectolyase Y23 in CPW salts (Frearson et al. 1973) modified by reducing $CaCl_2$ to 1 m*M* and supplemented with 0.4 *M* mannitol. Protoplasts were purified by passage through a 70 μm nylon screen and flotation on 17% sucrose. Protoplasts were irradiated just prior to the sucrose flotation by exposing them to a ^{137}Cs source for 30 min (total gamma dose, 120 Gy).

The *N. tabacum* and *N. repanda* protoplasts were washed twice with 0.5 *M* mannitol, resuspended in 0.5 *M* mannitol + 0.5 m*M* $CaCl_2$ at a density of 4×10^5 protoplasts/ml, and mixed together to give a five-fold excess of *N. tabacum* protoplasts. Electrofusion was carried out as described previously (Bates et al. 1987). Following fusion, the 0.25-ml aliquots of the protoplasts were transferred to petri dishes and diluted with 0.33 ml of 0.5 *M* mannitol and 0.33 ml of K_3 medium (Nagy and Maliga 1976) containing 0.4 *M* glucose. The protoplasts were cultured in the light (20 $\mu E/m^2s$) at 27 °C.

Selection and regeneration

Beginning 5 days after fusion, the culture medium was diluted by successive additions of CM (callus medium: Murashige and Skoog salts, B5 vitamins, 1 mg/l benzyladenine, 1 mg/l NAA, and 3% sucrose) until at 14 days the cultures were three times their original volumes. Agarose-bead cultures (Shillito et al. 1983) were initiated by solidifying the protoplast cultures through the addition of an equal volume of CM containing 1.8% Sea Plaque agarose (FMC Corp) plus 200 mg/l kanamycin sulfate (Gibco). Slices of the agar were transferred to large petri plates and flooded with CM containing 100 mg/l kanamycin. After 4–6 weeks of selection, kanamycin-resistant calli were transferred to solid CM + 100 mg/l kanamycin for further growth. Shoot regeneration was induced by transferring the calli successively onto RMA (RMB supplemented with 0.1 mg/l NAA) + 100 mg/l kanamycin followed by RMB + 100 mg/l kanamycin. Plantlets were rooted on P medium + 100 mg/l kanamycin.

Esterase and nopaline analyses

Esterase isozymes were separated on polyacrylamide gels as described by Bates and Hasenkampf (1985). Nopaline assays were performed by paper electrophoresis and phenanthrenequinone staining of leaf extracts as described by Rogers et al. (1986).

Chromosome counts

Root tips were soaked in 1 mg/ml colchicine for 2 h and then fixed overnight in 3:1 ethanol:acetic acid (Carnoy's solution) and stored in 95% ethanol at −20 °C. Finally, the roots were softened by pectinase treatment followed by HCl and then squashed and stained with acetocarmine.

Crossing and seedling tests for kanamycin resistance

Hybrid plants were used as the female parent in backcrosses with wild type *N. tabacum* var 'Xanthi' (genotype *nn*, kanamycin-sensitive, nopaline-negative). The plants were emasculated just before the corolla opened, followed by manual pollination and bagging of the flowers. Inheritance of kanamycin resistance was determined by surface-sterilizing the seeds and sowing them on P medium containing 1% sucrose and 100 mg/l kanamycin. Germination was allowed to proceed in the light (50 $\mu E/m^2s$) at 27 °C. Resistant seedlings germinated and grew normally, whereas sensitive seedlings never produced true leaves or secondary roots and bleached after 3–4 weeks.

Tests for TMV resistance

Leaves of plants to be tested were rubbed with a cotton swab dipped in a slurry consisting of TMV particles diluted into 0.1 *M* phosphate buffer (pH 7) plus 10% w/v Carborundum powder. Presence or absence of local lesions was scored 4 days later.

Results

Recovery of hybrid clones

Four fusions were carried out between *N. tabacum* and irradiated *N. repanda* protoplasts. The following controls were included in each experiment: *N. tabacum* protoplasts cultured alone, irradiated *N. repanda* protoplasts cultured alone, and *N. tabacum* protoplasts mixed with irradiated *N. repanda* protoplasts and cultured without being fused. No kanamycin-resistant calli were recovered from any of these controls. A fourth control consisted of a mixture of *N. tabacum* and unirradiated *N. repanda* protoplasts that were fused, cultured, and selected for kanamycin resistance. A plating efficiency of 10% (fraction of *N. repanda* protoplasts in the initial sample recovered as calli after agarose-bead culture) was observed for this control. When fused mixtures of *N. tabacum* + irradiated *N. repanda* protoplasts were subjected to selection on kanamycin, the plating efficiency dropped to 1.5%, suggesting that about 15% of the *N. repanda* protoplasts were recovered as kanamycin-resistant hybrid clones.

Individual kanamycin-resistant calli recovered from fusions involving irradiated *N. repanda* protoplasts were transferred from the agarose-bead cultures, grown for several passages on CM, and then transferred to regeneration and finally to rooting media. Many of the calli would not regenerate or formed abnormal shoots that failed to root. However, rooted plants were established from 19 independent calli. Twelve of these clones regenerated vigorously and gave rise to numerous plants that survived transfer to soil.

Morphology of regenerated plants

All of the regenerants had the upright growth habit of *N. tabacum*; none of them grew as a basal rosette. Although similar in appearance to *N. tabacum*, the regenerants were shorter and displayed less apical dominance. Plants regenerated from the same clone were morphologically similar to each other, but considerable variation in stature, degree of branching, and leaf morphology existed between plants from different clones. The leaves ranged from ones similar to *N. tabacum* to elongate, strap-like leaves, irregularly-shaped leaves and thick succulent leaves (Fig. 1). Some plants had sectored leaves; plants from one clone were virescent.

All twelve of the vigorously regenerating clones gave rise to plants that flowered. Floral morphology was much closer to that of *N. tabacum* than *N. repanda*, although the corollas were shorter for some of the regenerants (Fig. 2). The flowers ranged from white to pink in color in plants from the different clones. All of the plants exhibited some degree of male sterility. Partial or complete feminization was encountered in plants from most of the clones, and in a few plants the stamens were missing entirely; others formed petalloid stamens (Fig. 3). Plants regenerated from two clones produced a small amount of pollen; acetocarmine staining indicated that 5% of this pollen was viable.

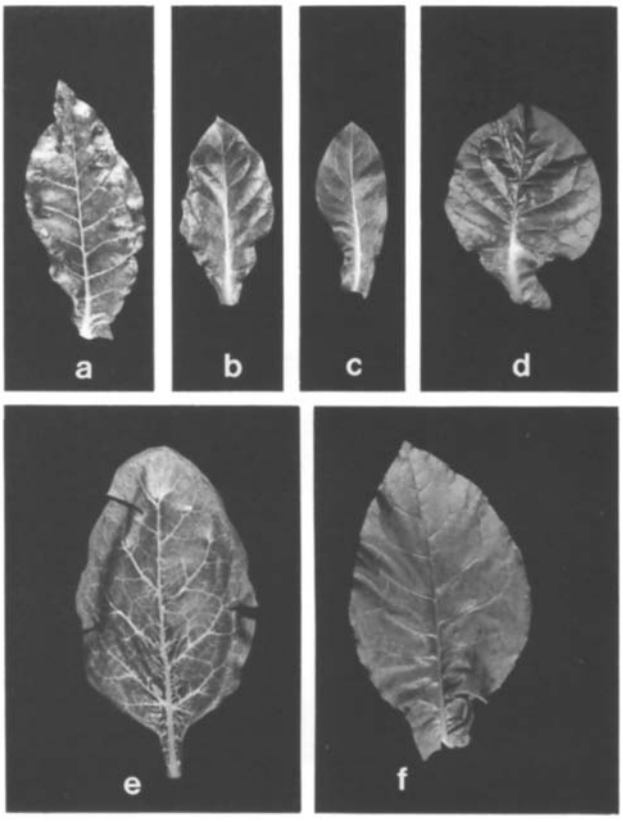

Fig. 1. Leaves of *N. tabacum* (+) *N. repanda* hybrids (**a–d**), *N. repanda* (**e**), and *N. tabacum* (**f**). The leaves were taken from lower portions of mature flowering plants and are shown at the same magnification

Biochemical analyses

The *N. repanda* fusion partner carried a functional nopaline synthase gene linked to the neomycin phosphotransferase gene, which encodes kanamycin resistance. The *N. tabacum* fusion partner was negative for nopaline. Thus, nopaline tests were used as a rapid screen for the presence of *N. repanda* genetic material in the hybrids. Nopaline was detected in leaf extracts from all of the hybrids (data not shown).

Esterase isozyme profiles were used to provide additional insights into the genetic constitution of the hybrids. *N. tabacum* leaf extracts, separated on polyacrylamide gels and stained for esterase activity, show one major band and two minor bands (Fig. 4); *N. repanda* has two major bands and two minor bands of esterase staining. Comparison of the two species indicates no esterase bands in common, although the *N. repanda* band showing the highest mobility overlaps with one of the minor

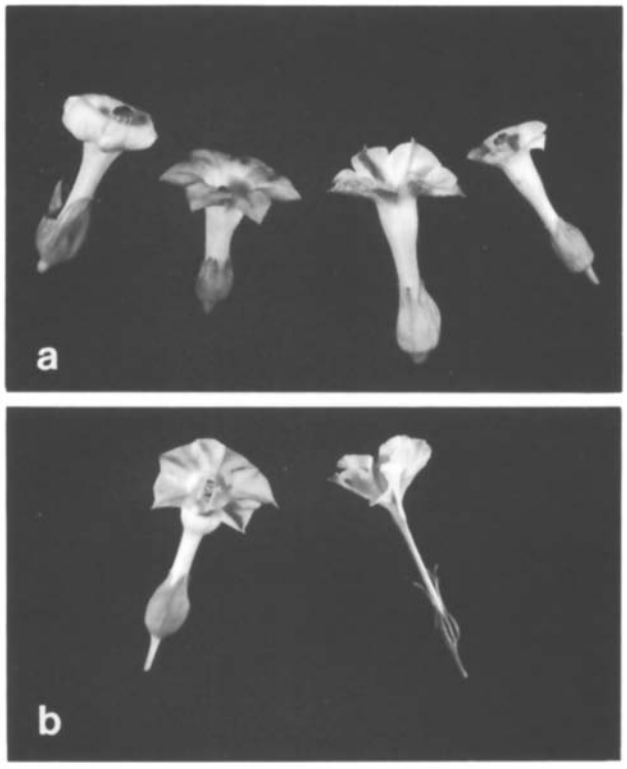

Fig. 2. Flowers of four hybrids (**a**) compared with *N. tabacum* (**b**, *left*) and *N. repanda* (**b**, *right*)

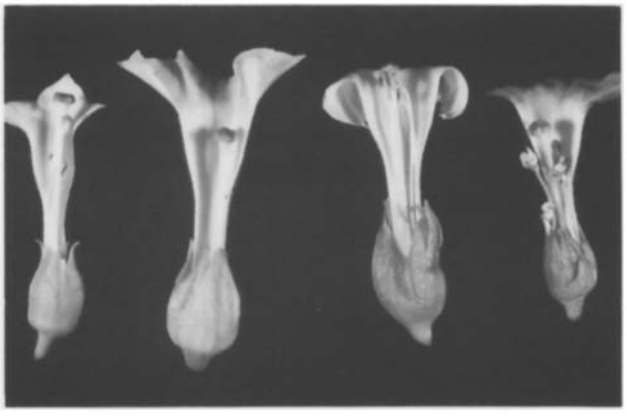

Fig. 3. Male sterility in *N. tabacum* (+) *N. repanda* hybrids. Left to right, stigmatoid anthers, no stamens, anthers with some pollen, and petalloid anthers

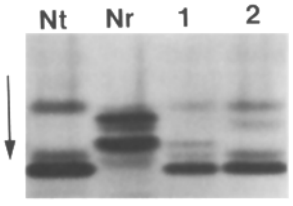

Fig. 4. Esterase Isozyme profiles for *N. tabacum* (*Nt*), *N. repanda* (*NR*) and two hybrids (*1*, *2*). The *arrow* indicates the direction of migration

Table 1. Chromosome numbers, fertility, and inheritance of kanamycin resistance among *N. tabacum* (+) *N. repanda* asymmetric hybrids. Fertility and inheritance were assessed by backcrossing the hybrids with *N. tabacum*

Clone	Plant	Chromosome Number	Viable Seeds/pod	Inheritance of Km^r Km^r:Km^s
1	989	56	33	10: 9
	992	53–58[a]	25	17: 8
2	978	58–60[a]	0	–
	1079	52, 56[a]	16	12: 3
3	1031	59	ND	ND
4	988	51–52	ND	ND
5	984	70, 90[a]	215	20:39
	1000	87	62	11:18
6	983	60	14	0:14
7	985	50–51	41	9:17
8	1089	80	ND	ND
9	1086	53, 60[a]	0	–
10	1076	69–75[a]	90	0:79
11	1023	62	0	–
12	995	62	0	–

ND, Not determined; Km^r, kanamycin resistant; Km^s, kanamycin sensitive

[a] Plants with mixoploid chromosome numbers

bands found in *N. tabacum*. Esterase isozyme profiles were examined for all twelve kanamycin-resistant clones that regenerated vigorous plants. Five clones regenerated plants that had all the *N. tabacum* esterase bands plus a band that comigrated with the high mobility major band of *N. repanda* (Fig. 4, lane 1). Plants from two clones had the *N. tabacum* bands, plus a band that comigrated with the slow, minor band of *N. repanda* (Fig. 4, lane 2). Plants from four clones had the three *N. tabacum* bands plus both additional bands described above (data not shown). One clone regenerated kanamycin-resistant, nopaline-positive plants that displayed only the three *N. tabacum* esterase bands (data not shown).

Chromosome numbers and inheritance of kanamycin resistance

Chromosome counts for the hybrids ranged from 51 to 90 (Table 1). Most of the clones regenerated plants having from 56 to 62 chromosomes; three clones yielded plants with more than 70 chromosomes. Six of the plants examined were clearly mixoploid, having variable chromosome numbers in the same root tip (i.e., 978, 1076) or in different root tips on the same plant (i.e., 992, 984, 1079, 1086). Two other plants (988 and 985) may also be mixoploid. In the three cases where chromsome numbers were determined for more than one plant regenerated from the same callus (clones 1, 2, and 5), the different plants had slightly different chromosome numbers.

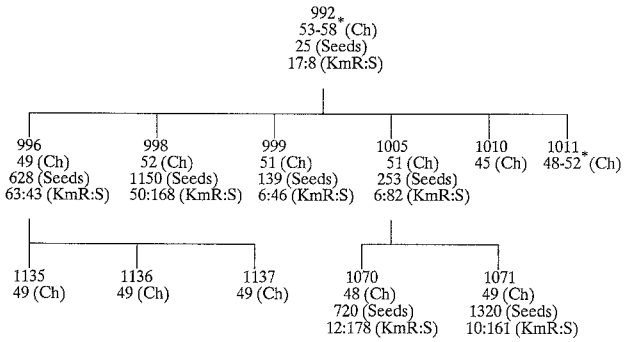

Fig. 5. Inheritance following backcrossing of hybrid 992 with *N. tabacum*. Progeny were scored for kanamycin resistance: sensitivity (KmR:S), and selected kanamycin resistant progeny were backcrossed again with *N. tabacum* as the pollen parent. *Ch* Chromosome number, *seeds* number of viable seeds per capsule, *asterisk* mixoploid plant

Plants from six clones formed viable seeds when backcrossed with *N. tabacum* as the pollen parent. Of the remainder, three clones (9, 11, and 12) yielded only sterile plants; plants regenerated from clones 3 and 4 were lost to fungal infections before seeds were collected, and plant 1089 (clone 8) has set pods but has not yet been analyzed for seed viability. Clone number 2 produced both sterile and fertile plants. In all cases the number of viable seeds produced was small compared with *N. tabacum*, which forms about 2000 seeds per capsule. Plants from clones 9 and 10, which formed a small amount of viable pollen, have been selfed and used a male parents in backcrosses with *N. tabacum*. So far no viable seeds have been recovered.

The inheritance of kanamycin resistance was determined by sowing surface-sterilized seeds on solidified P medium containing 100 mg/l kanamycin. Sensitive seedlings germinated but failed to produced true leaves or secondary roots, whereas resistant seedlings grew normally. The ratio of kanamycin resistant to sensitive (Km^r:Km^s) seedlings ranged from 4:1 to 1:2 for the different regenerants (Table 1). No kanamycin-resistant progeny were recovered from plants 983 and 1076. Plants regenerated from the same callus did not necessarily give the same ratio of Km^r:Km^s seedlings (compare, for example, plants 989 and 992 in Table 1); however, the number of seedlings analyzed was small.

The inheritance of kanamycin resistance has been followed for additional backcross generations for some of the hybrids. As an example, some of the backcross data obtained for plant 992 is shown in Fig. 5. This plant was mixoploid, having from 53 to 58 chromosomes and gave a 2:1 ratio of Km^r:Km^s seedlings. All of its BC_1, Km^r progeny had lower chromosome numbers, greatly improved seed sets, and were male sterile. Only one of the BC_1 plants examined was mixoploid; however, one plant had 45 chromosomes, which is three less than the 2N value for *N. tabacum*. The BC_1 plants all tested positive for nopaline and, with the exception of plant 1011, had only *N. tabacum* esterases. Similar to its female parent, plant 1011 had the *N. tabacum* esterase bands plus one *N. repanda* band.

Four of the Km^r, BC_1 plants were backcrossed with *N. tabacum*. The ratio of Km^r:Km^s progeny was lower than in the previous generation. The Km^r, BC_2 plants examined had either 48 or 49 chromosomes. Two of these BC_2 plants (1070, 1071) have reached maturity and been backcrossed. Although male-sterile, they show further improvement in seed sets and continued unstable inheritance of Km^r. The other three Km^r, BC_2 plants shown in Fig. 5 (plants 1135–1137) have not yet been backcrossed.

Analysis of the hybrids for TMV resistance

N. repanda gives a hypersensitive response when inoculated with TMV. The *N. tabacum* line used in these fusions lacks the *N* gene and is systemically infected when inoculated with TMV. All of the hybrids were tested for a hypersensitive response to TMV, only plants 1076 and 1086 gave a hypersensitive response. Plants that did not give a hypersensitive response eventually developed systemic TMV infections.

Discussion

Previous reports of hybridization between *N. tabacum* and *N. repanda*, whether by secual crosses plus ovule culture (Iwai et al. 1985; Shintaku et al. 1988) or by somatic cell fusion (Nagao 1982), have resulted in sterile plants. By contrast, the plants described in this report, although male-sterile, can be backcrossed when *N. tabacum* is used as the male parent.

Several lines of evidence indicate these plants are asymmetric hybrids containing a reduced amount of *N. repanda* genetic material. First, the plants morphology, especially their floral morphology, suggests a predominance of *N. tabacum* genetic material, while the fact that the plants are kanamycin resistant and positive for the presence of nopaline indicates they retain some *N. repanda* genes. Second, esterase isozyme profiles of the hybrids also suggest a predominance of *N. tabacum* genetic material: all the plants had a complete set of *N. tabacum* esterases; most also had one band (two bands in some hybrids) of staining that comigrates with an *N. repanda* esterase. Finally, *N. tabacum* and *N. repanda* both have 48 chromosomes, and all the hybrids had more than 48 but less than 96 chromosomes. Taken together, these observations suggest that the hybrids retain an incomplete set of *N. repanda* chromosomes.

The extent of chromosome elimination was quite varied. Clone 5 regenerated plants with chromosome numbers near 90 whereas clones 4 and 7 had only 51 to 52

chromosomes. The other clones gave plants with chromosome numbers in between these two extremes, but most of the hybrids had from 56 to 62 chromosomes. If these plants retain a full set of *N. tabacum* chromosomes, then they retain 15% to 30% of the *N. repanda* genome.

Not only did chromosome numbers differ between plants from different clones, but plants regenerated from the same clone had slightly different numbers of chromosomes. Moreover, a number of the plants had cells with different numbers of chromosomes when different root tips were examined. In some cases variations were observed within the same root tip. Together, these observations suggest genetic instability and ongoing chromosome elimination in the hybrids. Whether chromosome elimination is restricted to the callus phase or is also occurring in the regenerated plants is unclear. However, the large number of mixoploid plants recovered may provide circumstantial evidence that somatic chromosome instability in these lines does not end upon organogenesis.

Although seed sets were small, about half of the hybrids set viable seeds when backcrossed with *N. tabacum*. Inheritance of kanamycin resistance was used to follow the transmission of *N. repanda* genes in these crosses. Results for plants from four clones suggest a relatively stable inheritance of kanamycin resistance. However, the number of seedlings analyzed in these crosses was too small to permit any conclusion about the pattern of inheritance. Surprisingly, two of the hybrids yielded only kanamycin-sensitive progeny. Both of these hybrids tested positive for the presence of nopaline, but they have not been analyzed for the presence of a functional neomycin phosphotransferase gene.

Successive backcrosses of the kanamycin resistant, BC_1 progeny resulted in substantially improved seed sets and a concomitant reduction in chromosome number. Many of the lines of descent in these backcrosses also displayed a decrease in the proportion of kanamycin-resistant progeny. These observations indicate the continued loss of *N. repanda* chromosomes. Inheritance of kanamycin resistance was not unstable, however, in all the lines (see the results for plant 996 in Fig. 5), suggesting that it may eventually be possible to stabilize the inheritance of *N. repanda* traits in these hybrids.

Male sterility has previously been shown to result when the *N. repanda* cytoplasm is combined with an *N. tabacum* nucleus (Stavely et al. 1973). The mitochondrial DNA of the *N. tabacum* (+) *N. repanda* hybrids described here have not been examined; hower, Kumashiro et al. (1989) obtained male-sterile cybrid plants following donor-recipient fusions between *N. tabacum* and *N. repanda* and showed that sterility was due to the presence of a recombined mitochondrial genome. In the present report, two hybrid clones have regenerated plants with some viable pollen. If these plants can be used successfully as male parents in backcrosses with *N. tabacum*, then the male fertility of the hybrids may be improved. Efforts to secure this backcross are continuing.

The hypersensitivity of plants from two hybrid clones to inoculations with TMV suggests that asymmetric hybridization may provide a route for transferring disease-resistance genes from *N. repanda* to *N. tabacum*. However, the frequency of transfer of TMV resistance was low. Sjodin and Glimelius (1989) recently demonstrated a high frequency of transfer of resistance against *Phoma lingam* to *Brassica napus* by asymmetric somatic hybridization. Success was facilitated by direct selection of the hybrids for the disease-resistance trait. This approach should also be successful in *Nicotiana* if protocols for the direct selection of disease resistance genes can be designed.

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