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Asymmetric hybridization in *Nicotiana* by fusion of irradiated protoplasts

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Summary. Mesophyll protoplasts of a kanamycin-resistant, nopaline-positive *Nicotiana plumbaginifolia* seed line were inactivated by γ-irradiation and electrically fused with unirradiated mesophyll protoplasts of *N. tabacum*. Hybrids were selected on kanamycin and regenerated. Genetic material from *N. plumbaginifolia* was detected in these plants by the following criteria: (1) morphology, (2) esterase isozyme profiles, and (3) the presence of nopaline in leaf extracts. All of the plants regenerated were morphologically more similar to *N. tabacum* than to *N. plumbaginifolia,* and many were indistinguishable from *N. tabacum*. It was found that 37 plants displayed one or two esterases characteristic of *N. plumbaginifolia* in addition to a full set of esterases from *N. tabacum*. Based on their esterases, we have classified these plants as somatic hybrids. However, irradiation has clearly reduced the amount of *N. plumbaginifolia* genetic material that they retain; 24 plants were found that had only *N. tabacum* esterases but that produced nopaline and were kanamycin resistant. Genomic DNA from several of these plants was probed by Southern blotting for the presence of the authentic neomycin phosphotransferase gene (kanamycin-resistance gene) – all were found to contain the gene. These plants were classified as asymmetric hybrids. Finally, 25 plants were regenerated that were kanamycin sensitive, negative for nopaline, and contained only *N. tabacum* esterases. All of the regenerated plants, including this final category, were male sterile. As transferring the *N. plumbaginifolia* cytoplasm to an *N. tabacum* nuclear background results in an alloplasmic form of male sterility, all of the plants regenerated in this study appear to be cybrids irrespective of their nuclear constitution. Chromosome analysis of the asymmetric hybrids showed that most of them contained one more chromosome than is normal for *N. tabacum*. The somatic hybrids examined all had several additional chromosomes. Although male sterile, the asymmetric hybrids were female fertile to varying degrees and were successfully backcrossed with *N. tabacum*. Analysis of the resultant F_1 progeny indicated that the kanamycin-resistance gene from *N. plumbaginifolia* is partially unstable during meiosis, as would be expected for factors inherited on an unpaired chromosome.

Key words: Gene transfer – Asymmetric hybrids – Kanamycin resistance – Tobacco – Fertility

Introduction

Protoplast fusion facilitates the transfer of nuclear-encoded genes between plant species. In the last 10 years, progress in the development of protoplast fusion, culture, regeneration, and hybrid-selection systems has resulted in the formation of a large variety of interspecific somatic hybrids (see Evans et al. 1983; Gleba and Sytnik 1984 for reviews). However, if these hybrids are to be useful in breeding programs, they must have some fertility. With the exception of a few cases (see, for example, Schieder and Vasil 1980; Evans et al. 1981), interspecific somatic hybrids are sterile. An additional difficulty is their amphidiploid nature which requires an extensive program of backcrossing to introgress desirable genes into the target crop species.

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Abbreviations: Km^r: kanamycin resistant; Km^s: kanamycin sensitive; Nop^+: nopaline positive; Nop^-: nopaline negative

The formation of asymmetric somatic hybrids carrying the complete genome of the recipient species plus a few chromosomes or chromosome segments from a donor species may provide an approach to solving some of these problems. Several methods are available for forming asymmetric hybrids: egg transformation (Pandey 1975), the uptake of isolated chromosomes by protoplasts (Szabados et al. 1981), and the fusion of protoplast combinations in which most of the chromosomes of one species are spontaneously eliminated during development (Dudits et al. 1979). Chomosome elimination can also be induced in somatic hybrids by irradiation of one of the protoplast types prior to fusion. This method has been applied by several laboratories to the transfer of organelle-encoded traits between species (cybrid formation) (see Galun and Aviv 1983 for review). Irradiation has also been used to form asymmetric nuclear hybrids (Dudits et al. 1980; Gupta et al. 1982, 1984). For example, Gupta et al. (1984) documented the transfer of single chromosomes from *Physalis* to *Datura*. Hybrid identification was based on the restoration of chlorophyll synthesis in the chlorophyll-deficient *Datura* line.

The present study describes the formation of asymmetric somatic hybrids between two species of *Nicotiana*. Our approach was to fuse γ-irradiated mesophyll protoplasts of a kanamycin-resistant (Km^r), nopaline-positive (Nop^+) line of *N. plumbaginifolia* with wild-type protoplasts of *N. tabacum*. The *N. plumbaginifolia* plants had been stably transformed to kanamycin resistance by an *Agrobacterium* strain carrying a disarmed Ti plasmid (Horsch et al. 1984, 1985). After fusion, hybrid plants were selected on the basis of kanamycin resistance. Although irradiation induces the loss of *N. plumbaginifolia* chromosomes from the hybrids, selection on kanamycin allowed the recovery of plants retaining the chromosome (or chromosome fragment) encoding Km^r/Nop^+. These two genetic markers are dominant genes that can be identified both phenotypically and at the molecular level. Thus asymmetric hybrids were readily identified and the stability and heritability of the markers were assessed through a first sexual generation.

Materials and methods

Plant material

Seeds of *Nicotiana tabacum* var. Xanthi were kindly provided by Dr. Roy Chaleff (E. I. duPont de Nemours). Seeds of transformed *N. plumbaginifolia* were the generous gift of Dr. Robert Horsch (Monsanto). The *N. plumbaginifolia* seed line was transformed to kanamycin resistance by *Agrobacterium* carrying the cointegrating plasmid pMON200 (Horsch et al. 1985). In addition to the neomycin phosphotransferase gene (encoding kanamycin resistance), this plasmid contains an intact nopaline synthase gene. Both genes are expressed in the *N. plumbaginifolia* plants and have been stably inherited in a Mendelian fashion through several sexual generations both at Monsanto and in our laboratory.

Protoplast isolation and irradiation

Mesophyll protoplasts were isolated from *N. tabacum* as previously described (Bates and Hasenkampf 1985) except that the enzyme concentrations were reduced five-fold, and the digestion was carried out overnight. Mesophyll protoplasts were isolated from *N. plumbaginifolia* by the same procedures with the following modifications. The surface-sterilized leaves were cut into strips, and digestion was carried out in K3 medium (Nagy and Maliga 1976) containing 0.4 *M* sucrose, 0.5% Cellulysin, and 0.02% Pectolyase Y23. Live protoplasts were floated by centrifugation and then were washed with 0.5 *M* mannitol.

The *N. plumbaginifolia* protoplasts were γ-irradiated (^{137}Cs, 4 Gy/min) in the interval between the sucrose flotation step and the first mannitol wash. Radiation doses ranging from 50–100 Gy were used depending on the experiment. Preliminary experiments indicated that 20 Gy was sufficient to block completely the formation of macroscopic colonies by the *N. plumbaginifolia* line used in this study. However, at this dose, 5% of the protoplasts divided 2–3 times. None of the protoplasts divided more than once after receiving doses of 50 Gy or greater.

Protoplast electrofusion and culture

The *N. plumbaginifolia* (Km^r/Nop^+) and *N. tabacum* (Km^s/Nop^-) protoplasts were washed twice in 0.5 M mannitol and adjusted to a density of 5×10^5 cells/ml. In an attempt to maximize the yield of heterokaryons carrying the dominant selectable marker gene (Km^r), the two cell types were mixed together prior to fusion to yield a 3-fold excess of *N. tabacum* protoplasts. Protoplasts were fused by means of electric fields (electrofusion) using a Zimmermann Cell-Fusion Power Supply (GCA Corp.) and the "mass fusion chamber" described by Bates (1985). A 0.25 ml aliquot of the mixed protoplasts was introduced into the chamber, aligned with an AC field of 600 kHz, 100 V/cm, and fused by applying 2, 1,000 V/cm DC pulses. Compared with our earlier experiments on the electrofusion of suspension culture protoplasts (Bates 1985; Bates and Hasenkampf 1985), the mesophyll protoplasts proved more sensitive to electric-field-induced lysis. This result was especially true for the *N. plumbaginifolia* protoplasts. Lysis was adequately controlled if the AC field strength was turned down to 20 V/cm just prior to fusion and short (25 μs) DC pulses (1,000 V/cm) were applied to induce fusion. These conditions also reduce the fusion efficiency. However, counts of acetocarmine-stained nuclei showed 20%–25% binucleate and multinucleate cells after fusion versus an endogenous fusion percentage of 6.5% in untreated control cells.

Following fusion, the protoplasts were transferred to petri dishes. For culture, 0.33 ml each of 0.5 M mannitol and K3 medium (Nagy and Maliga 1976) containing 0.4 M glucose were added to the dishes. Protoplasts were cultured in the light (20 $\mu E/m^2 \cdot s$) at 27 °C.

Selection and regeneration

Beginning 5 days after fusion, the culture medium was diluted by successive additions of CM (callus medium: Murashigie and Skoog salts, 1 mg/l thiamine, 100 mg/l inositol, 1 mg/l benzyladenine, 1 mg/l NAA, and 3% sucrose) until at 14 days the cultures were 3 times their original volumes. Agarose-bead cultures (Shillito et al. 1983) were initiated by solidifying the pro-

toplast cultures through the addition of an equal volume of CM containing 1.8% Sea Plaque agarose plus 200 μg/ml kanamycin sulfate (Gibco). Slices of the agar were transferred to large petri plates and flooded with CM containing 100 μg/ml kanamycin. After 4–6 weeks of selection, kanamycin-resistant calli were transferred to solid CM + 100 μg/ml kanamycin for further growth. Shoot regeneration and rooting were achieved on the media described by Menczel et al. (1981) without the addition of 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 Otten and Schilperoort (1978).

Southern blotting

Genomic DNA was isolated from leaf material as described by Dellaporta et al. (1985) and digested with Eco RI and Bam HI. Fragments were separated by electrophoresis on 1% agarose gels and transferred to nitrocellulose by the Southern (1975) procedure. The blots were hybridized by standard procedures (Maniatis et al. 1982) with the 1.3 kb Eco RI/Bam HI fragment of the plasmid pMON200 as a probe (pMON200 was kindly provided by Dr. Robert Fraley, Monsanto Co.). This fragment contains the neomycin phosphotransferase coding region under the control of the nopaline synthase promoter and polyadenylation signals.

Chromosome counts

Root tips were soaked in 1 mg/ml colchicine for 3 h and then fixed overnight in 3 : 1 ethanol : acetic acid (Carnoy's solution). Finally, the roots were softened by pectinase treatment followed by HCl and then squashed and stained with acetocarmine. Flower buds were fixed with two changes of Carnoy's solution (24 h) and then squashed and stained with acetocarmine.

Crossing and seedling tests or kanamycin resistance

Hybrid plants were used as the female parent in backcrosses with wild type *N. tabacum* var. Xanthi (Km^s/Nop^-). 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 half-strength Murashigie and Skoog salts and vitamins containing 1% sucrose and 100 μg/ml kanamycin. Germination was allowed to proceed in the light (50 $\mu E/m^2 \cdot s$) 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.

Results

Recovery of hybrid clones

Interspecific fusions were carried out with mesophyll protoplasts from *N. plumbaginifolia* (Km^r/Nop^+) and *N. tabacum* (Km^s/Nop^-). The recovery of kanamycin-resistant calli following fusion of irradiated (50–100 Gy) *N. plumbaginifolia* protoplasts with unirradiated *N. tabacum* protoplasts ranged from 0.03 to 2 resistant calli per 1,000 *N. plumbaginifolia* protoplasts (average recovery was 0.7 resistant calli per 1,000). This variation in the efficiency with which resistant calli were recovered appeared to be due to day-to-day variations in protoplast quality.

Three different controls were used in each experiment: *N. tabacum* protoplasts cultured alone, irradiated *N. plumbaginifolia* protoplasts cultured alone, and *N. tabacum* protoplasts mixed with irradiated *N. plumbaginifolia* protoplasts and then cultured without being fused. No kanamycin-resistant calli were ever recovered from any of these controls.

Morphology of regenerated plants

In an initial experiment, the *N. plumbaginifolia* protoplasts were left unirradiated; in all subsequent experiments irradiation was applied. The morphology of plants regenerated from kanamycin-resistant calli differed distinctly depending on whether or not irradiation was applied before fusion.

A total of ten plants were regenerated from the experiment in which no irradiation was applied. Four were morphologically similar to *N. plumbaginifolia* and presumably resulted from the development of unfused protoplasts. The remaining six plants were intermediate between the two parental species, as would be expected for somatic hybrids. These plants had a partially rosette growth habit, larger and more clearly ovate leaves than *N. plumbaginifolia,* and slightly winged petioles (Fig. 1b) (*N. tabacum* var. Xanthi lacks petiole wings). The flowers of these plants were also intermediate in morphology (Fig. 2).

When irradiation (50–100 Gy) was applied prior to fusion, plants regenerated from kanamycin-resistant calli fell broadly into two morphological classes. Of 101 plants that grew to sufficient size to allow judgment of their morpholoy, 46 appeared similar or identical to *N. tabacum* (e.g., Fig. 1c). The remaining 55 looked more like *N. tabacum* than like *N. plumbaginifolia* but had abnormalities ranging from elongate, strap-like leaves to dwarfing and greatly thickened stems and leaves. Four plants produced spontaneous tumors on their stems.

Whether abnormal or not, most of these *N. tabacum*-like plants flowered successfully. Their flowers were similar to those of *N. tabacum* but showed variably shortened corollas and stamens and had protruding stigmas (Fig. 3). The anthers of all the plants were shrunken and shed little pollen.

Esterase isozyme analyses

Although the genetics of esterase isozymes in *Nicotiana* is not completely understood, esterase isozyme profiles nonetheless provide a convenient way of distinguishing

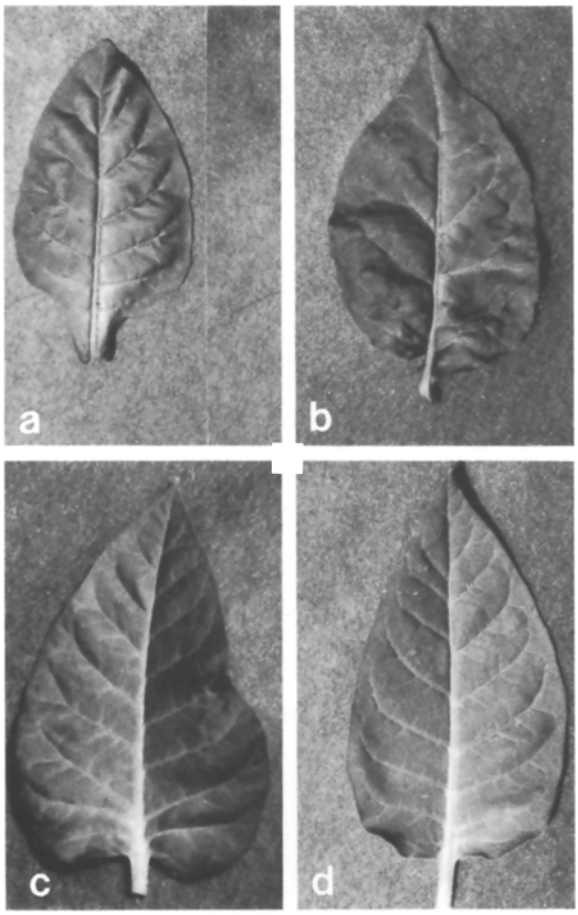

Fig. 1 a–d. Leaves of somatic and asymmetric hybrids. **a** *Nicotiana plumbaginifolia;* **b** plant 221, a somatic hybrid recovered from a fusion in which the *N. plumbaginifolia* protoplasts were not irradiated; **c** plant 203, an asymmetric hybrid recovered when irradiation was applied before fusion; **d** *N. tabacum*

Fig. 2. Flowers of *N. tabacum* (*left*), somatic hybrid 221 (*center*), and *N. plumbaginifolia* (*right*)

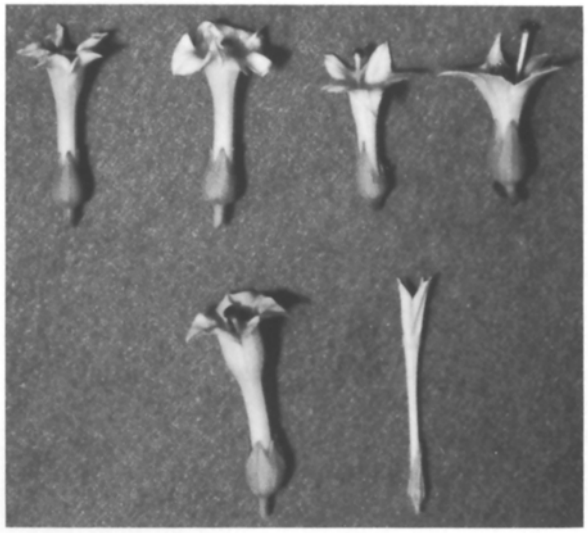

Fig. 3. Flowers of *N. tabacum* (*bottom left*), *N. plumbaginifolia* (*bottom right*), and four different asymmetric hybrids recovered from fusions involving irradiated protoplasts (*top*)

N. tabacum, N. plumbaginifolia, and their somatic hybrids (Menczel et al. 1982). We found that *N. plumbaginifolia* contains five bands not found in *N. tabacum,* whereas *N. tabacum* has four such species-specific bands. In both species, several other bands also differ qualitatively in their level of staining. Fig. 4 shows the esterase profiles of *N. tabacum, N. plumbaginifolia,* and two plants regenerated from kanamycin-resistant calli recovered after fusion. Plant 213 displayed all esterases of both species plus two unique bands. Such a profile is typical of those morphologically intermediate plants that were recovered when no γ-irradiation was applied before fusion and indicates that these plants are indeed somatic hybrids.

Esterase profiles were examined for 86 of the plants recovered from fusions involving irradiated protoplasts. Only two plants had balanced isozyme profiles similar to that of plant 213. However, 35 of the plants had a *N. tabacum* profile plus one or, in a few cases, two bands characteristic of *N. plumbaginifolia.* Thus, these plants also appear to be somatic hybrids although there has been some reduction in the amount of *N. plumbaginifolia* genetic material that they retain.

The remaining 49 plants had esterase profiles identical to that of *N. tabacum* (e.g., Fig. 4, plant 205). The recovery of plants such as these that have both the morphology and esterase profile of *N. tabacum* is important because these plants may be asymmetric hybrids – hybrids containing a very limited amount of genetic material from *N. plumbaginifolia.* Alternatively, these plants might have arisen from the loss of the kanamycin-resistance gene during plant regeneration or

by the escape of unfused *N. tabacum* protoplasts from selection. The following analyses indicate that some of these *N. tabacum*-like plants are kanamycin resistant, contain nopaline, and thus are indeed asymmetric hybrids.

Nopaline and Km^r assays

The *N. plumbaginifolia* plants used in these experiments carried an intact nopaline synthase gene linked to the kanamycin-resistance gene (Horsch et al. 1984, 1985). Thus the presence of nopaline in plants regenerated after fusion can be used as a rapid assay for the retention of genetic material from *N. plumbaginifolia.* A typical nopaline analysis is shown in Fig. 5.

As expected, most of the plants (27 of 37) displaying one or more esterases from *N. plumbaginifolia* were positive for nopaline (e.g., plant 221, Fig. 5). However, 24 of the plants having exclusively *N. tabacum* isozymes also contained nopaline (e.g., Fig. 5, plants 203, 205, and 207). Thus, these plants appear to be asymmetric hybrids. To be certain that they indeed contained the kanamycin-resistance gene, we performed two further tests. Leaf explants were placed on callus-inducing medium containing 100 μg/ml kanamycin. This leaf callus assay is a sensitive test for kanamycin resistance and is reported to be more reliable than the nopaline assay (Horsch et al. 1985), which can produce false positives. Eight nopaline-positive plants displaying *N. tabacum* morphology and esterases were tested; all were kanamycin resistant. Final proof was obtained by Southern blotting. Genomic DNA from four of these putative asymmetric hybrids (plants 203, 204, 205, and 207) was probed for the presence of the neomycin phosphotransferase gene. DNA from all three plants showed hybridization to the 1.3 kb Eco RI/Bam HI fragment of pMON200 that contains the authentic neomycin phosphotransferase gene (Fig. 6).

Twenty-five plants having *N. tabacum* esterase profiles tested negative for nopaline. Cultures of unfused mixtures of *N. tabacum* plus irradiated *N. plumbaginifolia* protoplasts never produced any kanamycin-resistant calli. Thus there appears to be no reason to believe that these nopaline-negative plants were regenerated from calli that escaped selection. A more likely explanation is that the clones giving rise to these plants lost the chromosome encoding Km^r/Nop$^+$ during plant regeneration. All of the plants, whether nopaline positive or negative, were male sterile and had a characteristic floral morphology (shortened corolla and stamens and protruding stigmas, see Fig. 3). This type of male sterility has been reported to result from the transfer of *N. plumbaginifolia* cytoplasm (mitochondrial genome) to a *N. tabacum* nuclear background (Menczel et al. 1986). The occurrence of this type of male sterility in

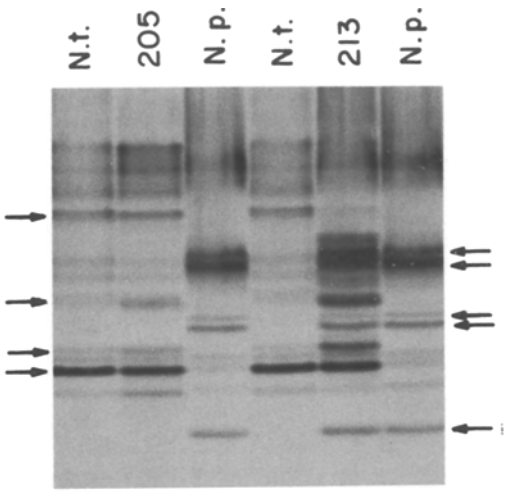

Fig. 4. Polyacrylamide gel electrophoresis of esterases from leaves. Nt: *N. tabacum;* Np: *N. plumbaginifolia;* 205: an asymmetric hybrid; 213: a somatic hybrid recovered when no radiation was applied. *Arrows* in the margins indicate the positions of esterase bands specific to each species

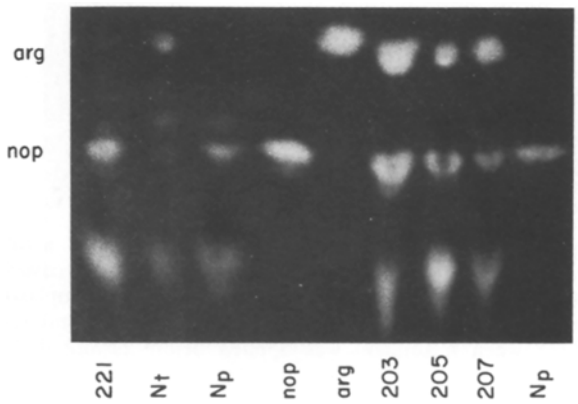

Fig. 5. Nopaline assay. Leaf extracts were separated by paper electrophoresis, stained with phenanthrenequinone, and viewed under UV light. Np: *N. plumbaginifolia;* Nt: *N. tabacum;* 203, 205, and 207: examples of asymmetric hybrids; 221: a somatic hybrid recovered when no radiation was applied; nop and arg refer to authentic nopaline and arginine standards, respectively

nopaline negative plants having exclusively *N. tabacum* esterases indicated that these plants did indeed develop from fusion products and should be classified as cybrids.

Fertility and inheritance of kanamycin-resistance

Selected somatic hybrids and asymmetric hybrids were self-pollinated and backcrossed to wild-type *N. tabacum.* Typical results are shown in Table 1. Plants 201–207 and 296 are asymmetric hybrids. Plants 234, 236, and 240 are somatic hybrids (recovered from

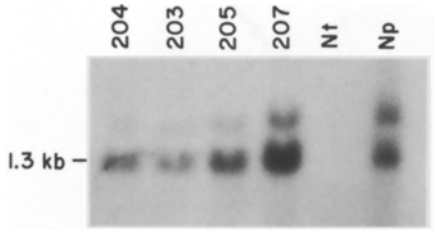

Fig. 6. Southern blot showing the presence of the neomycin phosphotransferase gene (kanamycin-resistance gene) in four asymmetric hybrids (plants 203, 204, 205, 207). Genomic DNAs were digested with Bam HI and Eco RI and probed with the Eco RI/Bam HI 1.3 kb fragment from pMON200 (Horsch et al. 1984, 1985) that corresponds to the neomycin phosphotransferase gene. Molecular weights were determined using λ Hind III fragments as standards (not shown). Signal intensities vary due to inadvertent overloading of some lanes. The second band of hybridization seen in overloaded lanes (207 and Np) at 1.6 kb is probably due to cross-hybridization of the probe to the promoter region of the nopaline synthase gene also present in these plants. Np: *N. plumbaginifolia* (Km^r/Nop^+); Nt: *N. tabacum* (km^s/Nop^-)

Table 1. Hybrid fertility and the inheritance of kanamycin resistance on backcrossing with wild-type *N. tabacum*. The numbers are averaged data for at least two capsules from each plant. Fertility was measured by determining the average number of seeds per capsule and comparing this figure with the number produced by *N. tabacum* (about 2,300 seeds/capsule) when emasculated and self-pollinated. Inheritance was assessed by germinating the seeds in the presence of 100 µg/ml kanamycin and scoring the proportions of seedlings that bleached (kanamycin sensitive) and that grew as normal green seedlings (kanamycin resistant). Chromosome numbers were determined using a combination of mitotic (root tip) and meiotic (anther) preparations. Plants 201–207 and 296 are asymmetric hybrids. Plants 234, 236, and 240 are somatic hybrids having a somewhat reduced complement of *N. plumbaginifolia* genes. Plant 221 is a somatic hybrid (recovered from a fusion not involving radiation treatment) displaying a complete set of both parental esterase isozymes. ND: not determined

Plant	Chromosome number	% Seed Set	% Germination	Resistant/sensitive seedlings	Ratio
201	49	53	92	9/105	1:11.7
203	49	59	93	19/256	1:13.5
204	49	12	92	5/68	1:13.6
205	47–50	54	95	23/371	1:16.1
206	50	65	89	11/95	1:8.6
207	48–49	59	94	4/123	1:30.8
296	ND	67	86	29/52	1:1.8
234	ND	9	56	5/15	1:3
236	ND	9	44	6/26	1:4.3
240	58–61	5	71	2/27	1:13.5
221	63–70	0[a]	–	–	–

[a] Plant 221 occasionally set capsules containing shrunken, inviable seeds

fusions involving irradiated *N. plumbaginifolia* protoplasts). Each of these somatic hybrids contained only a single esterase isozyme from *N. plumbaginifolia,* indicative of a reduced complement of *N. plumbaginifolia* genes. Plant 221 is a somatic hybrid, having a balanced isozyme profile, recovered from a fusion involving unirradiated protoplasts.

Due to their male sterility, self-fertilization failed in all of the plants tested, even when the contents of their anthers were daubed onto the stigma and the flowers were sprayed with 2,4-D to prevent premature fruit drop. However, backcrossing to *N. tabacum* was successful to some degree for all the plants except those somatic hybrids having balanced isozyme profiles (e.g. plant 221, Table 1).

The gene for kanamycin resistance is inherited in plants as a simple, dominant Mendelian trait (Horsch et al. 1984). If the hybrids contained one copy of the Km^r gene, a 1:1 segregation of resistant and sensitive progeny would be expected on backcrossing the hybrids with *N. tabacum.* As seen in Table 1, all of the hybrids (whether asymmetric or not) show an excess of kanamycin-sensitive progeny. The most promising ratio obtained (plant 296) is nonetheless significantly different than 1:1 ($P<0.05$). The fertility data (Table 1) are based on the number of seeds per capsule recovered when the hybrids were backcrossed to *N. tabacum,* compared with *N. tabacum* crossed with itself. The seed set varied considerably from plant to plant. As a group, the asymmetric hybrids (e.g., plants 201–207 and 296) had better seed sets than the reduced somatic hybrids (e.g., plants 234, 236, and 240), but some asymmetric hybrids (e.g., plant 204) had low seed sets. In some cases, seed set also varied substantially among capsules from the same plant. For example, plant 205 yielded seven capsules containing from 584 to 2,289 seeds per capsule (or 25%–98% fertility). This variation is probably due to occasionally inadequate pollination because capsules with full seed sets showed essentially the same segregation ratios as capsules with poor seed sets (data not shown).

Chromosome numbers

Chromosome numbers were determined utilizing a combination of mitotic and meiotic preparations. The results are shown in Table 1. Most of the asymmetric hybrids analyzed were found to have 49 chromosomes (in the case of meiotic preparations, 24 bivalents plus one univalent). This represents one more chromosome than is normal for *N. tabacum.* An example of a meiotic squash and a mitotic karyotype for one of these plants is shown in Fig. 7. Plant 206 has 24 bivalents plus two univalents (50 chromosomes). Two plants (205 and 207) appeared to be chimeras, having different numbers of

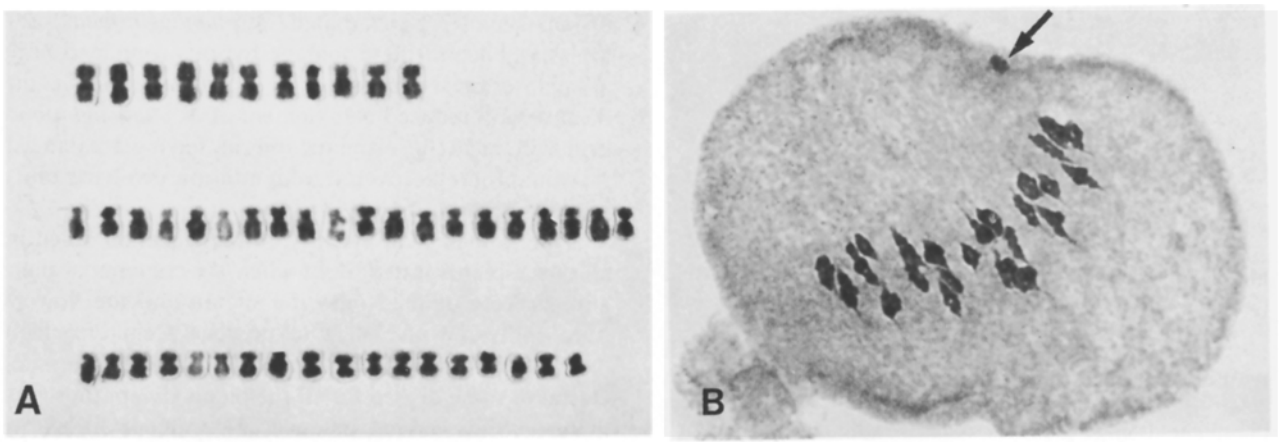

Fig. 7. **A** Karyotype of mitotic chromosomes from a root tip of the asymmetric hybrid, plant 204, which has 49 chromosomes. **B** Metaphase I of meiosis in an anther of plant 204, showing 24 pairs plus one unpaired chromosome (*arrow*)

chromosomes in different cells. Plant 296 was not analyzed.

Three somatic hybrids, recovered from fusions involving irradiated *N. plumbaginifolia* protoplasts, were analyzed. All of these plants had several additional chromosomes beyond the normal 48 expected for *N. tabacum*. One example, plant 240, (Table 1) appears to be a chimera having 58–61 chromosomes per cell. Plants 234 and 236 were not analyzed. The only somatic hybrid analyzed that resulted from fusions involving unirradiated protoplasts (plant 221, Table 1) has 63–70 chromosomes depending on the cell analyzed.

Discussion

The goal of this work was to use donor-recipient fusions to recover asymmetric nuclear hybrids. Selection relied on the presence of kanamycin resistance in the irradiated donor species. The results show the power of using this molecularly defined selectable marker gene for both the recovery and the subsequent identification of asymmetric hybrids. Hybrids were recovered with frequencies as high as 10^{-3}. Regardless of their morphology or isozyme content, the presence in the hybrids of DNA from *N. plumbaginifolia* (the donor) was readily detected by assaying for the presence of nopaline. The reliability of this assay was verified by a kanamycin-leaf-callus assay and by Southern blots showing the presence of the kanamycin-resistance gene.

Classification of the recovered plants

It was found that 24 plants (of a total of 86 analyzed) recovered from fusions involving irradiated protoplasts were indistinguishable from *N. tabacum* in terms of morphology and esterase isozymes, but were Km^r/Nop^+. These plants have been designated asymmetric hybrids. Other plants were recovered (37 of 86) that contained some esterases characteristic of *N. plumbaginifolia;* these plants probably retain somewhat more genetic information from the donor than do the asymmetric hybrids. Therefore, they have been designated somatic hybrids. However, in all cases where irradiation was applied, these somatic hybrids resembled *N. tabacum* more closely than they did *N. plumbaginifolia,* and, except for two plants, they did not contain a full complement of *N. plumbaginifolia* esterases. These observations indicate a reduction in the retention of genes from *N. plumbaginifolia* due to irradiation. A third category of plants (25 of 86) was also recovered. Most of these plants were identical to *N. tabacum* in terms of morphology, had *N. tabacum* esterases, and were Km^s/Nop^-. However, like the asymmetric and somatic hybrids, these plants displayed the male sterility that is characteristic of the transfer of cytoplasm from *N. plumbaginifolia* to *N. tabacum*. Thus, this final group of plants was classified as cybrids.

The categories of hybrids described here – asymmetric hybrids, somatic hybrids, and cybrids – are based solely on the presence or absence of esterase isozymes or Km^r/Nop^+ from *N. plumbaginifolia*. Undoubtedly, the assignment of individual plants to these categories could change if different isozymes had been examined. Although the morphology of the plants was generally correlated with the plants' isozyme content (most of the asymmetric hybrids and cybrids were quite similar in appearance to *N. tabacum* and most of the somatic hybrids displayed some morphological abnormalities), morphology was not an entirely reliable indicator because some of the asymmetric hybrids and cybrids also had morphological abnormalities. This situation may

reflect ploidy changes or somaclonal variation arising during tissue culture.

Male sterility

All of the plants recovered were male sterile, having characteristically shortened corollas and stamens and shrunken anthers that shed little or no pollen. Menczel et al. (1986) observed this type of male sterility in cybrids containing a *N. tabacum* nucleus and cytoplasm from *N. plumbaginifolia.* They were able to show that male sterility was not associated with the transfer of plastids; thus male sterility must be due to a nuclear/mitochondrial incompatibility. Nagy et al. (1983) found recombinant mitochondrial DNAs in each of 12 different *N. tabacum/N. plumbaginifolia* cybrid lines that they examined. Although we have not examined their mitochondrial DNAs, it seems likely that the plants recovered in our study also contain recombinant mitochondrial genomes and that incompatibility between these mitochondria and the *N. tabacum* nucleus results in male sterility. If this is the case then recombination between mitochondria in *N. plumbaginifolia* + *N. tabacum* somatic cell fusions must occur with very high frequency because all of the plants regenerated in this study, as well as those regenerated by Menczel et al. (1986), were male-sterile.

Separation of the genetic markers, Km^r/Nop^+, and esterases

Ten plants were recovered that had one or more esterases from *N. plumbaginifolia but were* Km^s/Nop^-. The recovery of these plants, together with the recovery of Km^r/Nop^+ plants both with and without *N. plumbaginifolia* esterases indicates that the Km^r/Nop^+ locus in the *N. plumbaginifolia* plants used in these experiments is not genetically linked to any species-specific esterases. The fact that esterases can be transferred independently of Km^r/Nop^+ also suggests that traits that are not directly linked to the selectable marker can nonetheless be transferred in donor-recipient fusions.

The recovery of Km^s/Nop^- plants was unexpected because the calli from which they were regenerated were selected for kanamycin resistance. Control cultures consisting of mixtures of irradiated *N. plumbaginifolia* and unirradiated *N. tabacum* protoplasts never yielded any kanamycin-resistant calli. Thus, the initial selection seems to have been efficient in forcing retention of the chromosome carrying Km^r/Nop^+. The recovery of Km^s/Nop^- plants probably reflects the elimination of the chromosome carrying Km^r/Nop^+ during mitosis in the cultured cells or in the regenerated plants. Of these two possibilities, elimination during callus growth appears the most likely, especially because kanamycin was removed when the calli were transferred to shoot-regeneration-medium, thus creating a period where chromosome sorting could occur in unorganized tissues in the absence of selection. All the regenerated plants were screened for nopaline when first transferred to soil, and many were retested as they matured. We have never observed any plants to revert from Km^r/Nop^+ to Km^s/Nop^- either wholly or in part. For example, leaves from 20 independent shoots of plant 203 were checked for nopaline after the plant had been in the greenhouse for 15 months – all the shoots were nopaline positive. These observations suggest that the Km^r chromosome is stable during mitosis in the regenerated plants but they do not constitute proof. Moreover, the known genetic instability of *N. tabacum* × *N. plumbaginifolia* sexual hybrids (Moav 1961) indicates that loss of the chromosome carrying Km^r/Nop^+ could occur in the regenerated plants as well as the calli.

Organization and inheritance of the N. plumbaginifolia genes in the asymmetric hybrids

Most of the asymmetric hybrids analyzed contained a single extra chromosome. The cytogenetic similarity of *N. plumbaginifolia* and *N. tabacum* (Goodspeed 1954) is such that we could not establish the origin of this extra chromosome unequivocally. However, in meiosis this extra chromosome appears unpaired, as a univalent, and is often off the methaphase plate. An unpaired chromosome would tend to be lost during meiosis, and so the strong skewing of inheritance of Km^r toward sensitive progeny suggests that this unpaired chromosome does carry the gene for Km^r.

Intergenomic translocations have been reported for animal somatic hybrids (Klobutcher and Ruddle 1981) and appear to have occurred in the *Daucus* + *Aegopodium* hybrids described by Dudits et al. (1979). However, we have no evidence that such translocations occurred in our experiments. We did not find any asymmetric hybrids that contained only 48 chromosomes, which would have provided circumstancial evidence for a translocation. Moreover, backcrossing a plant carrying an intergenomic translocation with *N. tabacum* would be expected to yield a stable 1 : 1 inheritance of Km^r. No plants giving a 1 : 1 inheritance were found. The only plant approaching this ratio (plant 296) died before it could be analyzed further; however, seeds and progeny from this plant have been retained for future analysis.

Two of the asymmetric hybrids (plants 205 and 207) appear to be chromosomal chimeras, having different numbers of chromosomes in different cells. Whether these plants are chimeric for Km^r is unknown. However, the presence of some cells in plant 205 having only 47 chromosomes means a *N. tabacum* chromosome has been lost in these cells. This observation suggests that, in addition to any possible inherent instability of the

Km^r chromosome in the hybrids, simply regenerating plants from tissue culture contributed to their chromosomal variability.

Previously, donor-recipient fusions have been used primarily for transferring organelles between species (Menczel et al. 1982; Galun and Aviv 1983; Menczel et al. 1986). Our results, like those of Dudits et al. (1980) and Gupta et al. (1984), show that single chromosomes can also be transferred by donor-recipient fusions as long as the donor contains a nuclear encoded marker gene. In contrast to the work of Dudits et al. (1980) and Gupta et al. (1984), we have followed the inheritance of this gene in a first sexual backcross. Due to the male sterility encountered in the hybrids, we have thus far been unable to stabilize the chromosome that has been transferred from *N. plumbaginifolia* to *N. tabacum*. However, if the problem of male sterility can be solved, the type of donor-recipient fusion described here might provide a one-step bridge for the interspecific transfer of nuclear-encoded traits.

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