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Combination of kanamycin resistance and nitrate reductase deficiency as selectable markers in one nuclear genome provides a universal somatic hybridizer in plants

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Summary. The combination in the nuclear genome of a dominant resistance marker (to select against unfused wild-type cells) and a recessive deficiency marker (to select against unfused mutant cells) in a cell line should provide a system for selecting fusion hybrids between the mutant line and any wild-type line. To test this idea, we fused protoplasts from a non-morphogenic cell line of *Nicotiana tabacum* which was kanamycin resistant (by transformation) and deficient in nitrate reductase (NR^-K^+) with protoplasts from *N. tabacum* cv. Petit Havana clone SR1, which provided resistance against streptomycin as an additional selectable marker ($NR^+K^-SR^+$). Putative hybrids were selected using a culture medium containing no available reduced nitrogen source and 50 mg/l kanamycin sulphate. After regeneration into plants, the hybrid character was demonstrated from: (i) the morphological variation of the regenerants; (ii) the chromosome number; (iii) the ability to grow on medium without a reduced nitrogen source and containing kanamycin sulphate at 50 mg/l; (iv) the presence of nitrate reductase activity; (v) the presence of the gene coding for neomycin phosphotransferase, which provides resistance to kanamycin sulphate; (vi) callus formation from leaves on medium containing 1 g/l streptomycin or 50 mg/l kanamycin sulphate; (vii) F_1 plants containing nitrate reductase and the gene for neomycin phosphotransferase. Fusions between the mutant cell line (NR^-K^+) and three wild-type tobacco species and subsequent cultivation on medium containing no available nitrogen source but 50 mg/l kanamycin sulphate resulted in callus formation with all combinations, while hybrid plants were only regenerated when *N. sylvestris* was the fusion partner.

Key words: Protoplast fusion – Nitrate reductase deficiency – Kanamycin – *Nicotiana tabacum* – *Nicotiana sylvestris*

Introduction

Despite numerous selection systems for somatic hybrids (Lazar 1983) it is evident that selection is still the bottleneck in the production of many desirable fusion hybrids (Harms 1983). The situation was greatly improved by the production of a nitrate reductase deficient (NR^-) and streptomycin resistant (SR^+) double mutant of *Nicotiana tabacum* (Hamill et al. 1983). Such a double mutant combining a recessive mutation in the nuclear genome (NR^-) and a dominant mutation in the chloroplast genome (SR^+) can be used as a universal hybridizer with any wild-type cells (Pental et al. 1986). Selection against unfused protoplasts of both types is possible by addition of streptomycin to a culture medium without a reduced nitrogen source.

The establishment of a comparable general selection system, but involving only the nuclear genomes, became feasible with the possibility of applying the method of direct gene transfer (Paszkowski et al. 1984; Hain et al. 1985) on NR^- mutants, using resistance against kanamycin sulphate (K^+) as a selectable marker. Kanamycin resistance has been shown to be a dominant nuclear marker (Paszkowski et al. 1984; Hain et al. 1985), which can easily be introduced into the genome of dicots as well as monocots by direct gene transfer (Potrykus et al. 1985; Lörz et al. 1985; Fromm et al. 1986). Protoplasts from the plant material thus produced can be used for hybrid formation with any wild-type protoplasts. Unfused transformed mutant protoplasts (NR^-K^+) can be eliminated by culture on a medium without an available reduced nitrogen source, whereas selection against unfused wild-type cells is provided by the addition of kanamycin sulphate to the culture medium.

In this paper we present evidence that somatic hybrids were selected with this procedure, using a non-morphogenic *N. tabacum* cell line resistant to kanamycin and lacking nitrate reductase (NR^-K^+) and *N. tabacum* which carried resistance to streptomycin as an additional selectable marker ($NR^+K^-SR^+$).

Materials and methods

Plant material. N. tabacum, cv. Petit Havana SR1, a streptomycin-resistant line (Maliga et al. 1973) was obtained from P. Maliga, Szeged, Hungary. The NR^- *N. tabacum* cell line nia-115 was kindly provided by A. Müller, Gatersleben, DDR. It is defective in the apoenzyme of nitrate reductase and therefore has an absolute requirement for a reduced nitrogen source (Mendel and Müller 1979; Mendel et al. 1981). Transformants resistant to kanamycin were produced from this line by the method of direct gene transfer (Shillito et al. 1985) using the plasmid p ABD 1 (Paszkowski et al. 1984). This plasmid contains the gene which codes for the aminoglycoside phosphotransferase II [APH(3')II] which confers resistance to aminoglycoside antibiotics (neomycin, kanamycin). One transformant was characterised by Southern blot analysis and used to produce a cell suspen-

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sion culture. This cell line (NR^-K^+) is here refered to as $niaK^+$. It was cultivated in liquid AA medium (Müller and Grafe 1978) on a rotary shaker at 26° C in the light. The cultures were subcultured once a week by 1:3 dilutions. Seeds of *N. sylvestris, N. glutinosa and N. repanda* were kindly provided by S. Reed, University of North Carolina, Raleigh, USA. Transformed *N. tabacum* cv. Petit Havana SR1 ($SR1K^+$, which is T_{2-1} of Paszkowski et al. 1984) was used as a control for Southern blot analysis. Shoot cultures of all tobacco species were established and maintained on T medium (Potrykus and Shillito 1986). This medium was supplemented with 50 mg/l kanamycin sulphate for the cultivation of $SR1K^+$.

Protoplast isolation. Leaf mesophyll protoplasts were isolated from sterile shoot cultures and purified as described by Potrykus and Shillito 1986. The washed protoplasts were suspended in TVL (0.1 M $CaCl_2$, 0.14 M sorbitol, 2 mM Tris, adjusted to pH 7 with HCl) at a density of 3×10^5/ml. Protoplasts from cell cultures of $niaK^+$ were isolated from cells harvested 3 to 4 days after subculture by incubation in a 3:1 mixture of 2% cellulase ONOZUKA R10, 0.5% Driselase, 1% Macerozyme R10, in 0.4 M mannitol and 0.16 M $CaCl_2$ containing 0.5% Mes and adjusted to pH 5.6 with KOH. Incubation was in plastic dishes (Corning, 9 cm) at 24° C for 3 h with constant shaking on a rotary shaker (Heidolph Taumler from Salvis AG, Reussbühl, Switzerland). Protoplasts were separated from undigested tissue by filtration through a 100 µm stainless steel sieve and subsequently washed three times and resuspended in TVL solution as for leaf mesophyll protoplasts.

Protoplast fusion. Protoplast suspensions from the different sources were mixed 1:1 to give a final population density of 1.5×10^5/ml of each fusion partner. The fusion treatment was according to Potrykus et al. (1984), but using polyethylene glycol (PEG) 6000 dissolved in TVL as fusion and TVL as washing solution. The culture medium was a 1:1 mixture of K3A and H medium (Potrykus and Shillito 1986). Cultivation was for 2 days in the dark at 26° C, followed by 7 days at 24° C in the light at 1000 lx. The protoplast-derived microcolonies were carefully washed once with 10 ml 0.16 M $CaCl_2$ in 0.5% Mes pH 5.6, adjusted with KOH, and followed by 10 ml K3 (Potrykus and Shillito 1986) medium. Material adhering to the bottom of the petri dish was loosened using a plastic pipette. After addition of 5 ml K3 medium the suspension was transferred to a 10 cm plastic petri dish (Corning) and mixed with 5 ml K3 containing 1.6% agarose (Sea Plaque, Marine Colloids, Rockland, Maine, USA) and 80 µg/ml kanamycin sulphate. After one further day the cells were subjected to selective conditions (50 mg/l kanamycin sulphate) according to Paszkowski et al. (1984), at 24° C in the dark. After 7 days 15 ml of the medium was replaced by 20 ml K3C medium (Potrykus and Shillito 1986). An identical replacement was done after 14 and 21 days, using K3E (Potrykus and Shillito 1986). After 4 weeks calli with a diameter of 2 to 3 mm were transferred to LS medium (Linsmaier and Skoog 1965) containing 0.05 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 2 mg/l NAA (naphthalene acetic acid), 0.1 mg/l 6BAP (6-benzyl aminopurine) and 50 mg/l kanamycin sulphate and subcultured every 4 weeks.

Plant regeneration. Plants were regenerated from calli under selective conditions by inducing shoots on LS medium containing 0.2 mg/l 6BAP and rooting these shoots on T medium (Potrykus and Shillito 1986), containing 50 mg/l kanamycin sulphate.

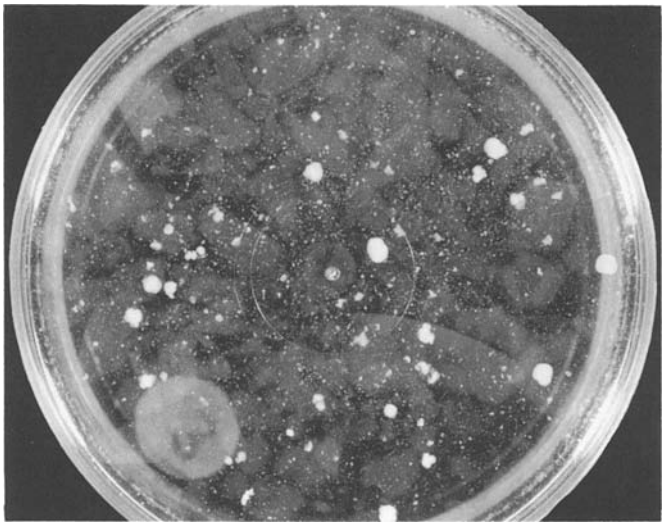

Fig. 1. Clones growing after polyethylene glycol (PEG) treatment of a mixture of SR1 and $niaK^+$ protoplasts under double selection conditions: lack of available nitrogen source and presence of lethal dose of kanamycin

Cytology. For the analysis of chromosomes, root tips were put into a saturated solution of paradichlorbenzene for 2 h. Subsequently they were fixed in acetic acid: ethanol, 1:3, v/v. The squashes were stained with lacto-propionic orcein.

Analytical methods. DNA isolation and Southern blot analysis were done according to Paszkowski and Saul (1986). Nitrate reductase activity was measured according to Neyra and Hageman (1975) using the extraction buffer of Egger (1986).

Results

Cultivation of PEG-treated mixtures of protoplasts from SR1 and $niaK^+$ in medium containing no available reduced nitrogen source but 50 mg/l kanamycin sulphate resulted in the formation of resistant calli growing in a background of small dead cell colonies (Fig. 1). Treatment of mixtures of 75000 of each type of protoplasts resulted in an average of 12 resistant colonies (4 independent experiments with 2 replicates in each). SR1 or $niaK^+$ protoplasts or mixtures of both types of protoplasts without PEG treatment did not grow under these conditions, nor did $niaK^+$ and SR1 protoplasts treated separately with PEG. Colonies which had grown to a diameter of 2 to 3 mm in the "bead culture" (Shillito et al. 1983) under selective conditions formed calli on LS medium containing 50 mg/l kanamycin sulphate. Transfer to shoot-inducing LS medium resulted in formation of shoots with normal and with distorted, thick leaves in 60% of these calli. These shoots formed roots on T medium containing 50 mg/l kanamycin sulphate. The plants thus formed were propagated as shoot cultures on T medium containing kanamycin sulphate. When transferred to soil (Fig. 2) these plants had a reduced growth rate compared with SR1 plants. Shoots from SR1 transferred to T medium

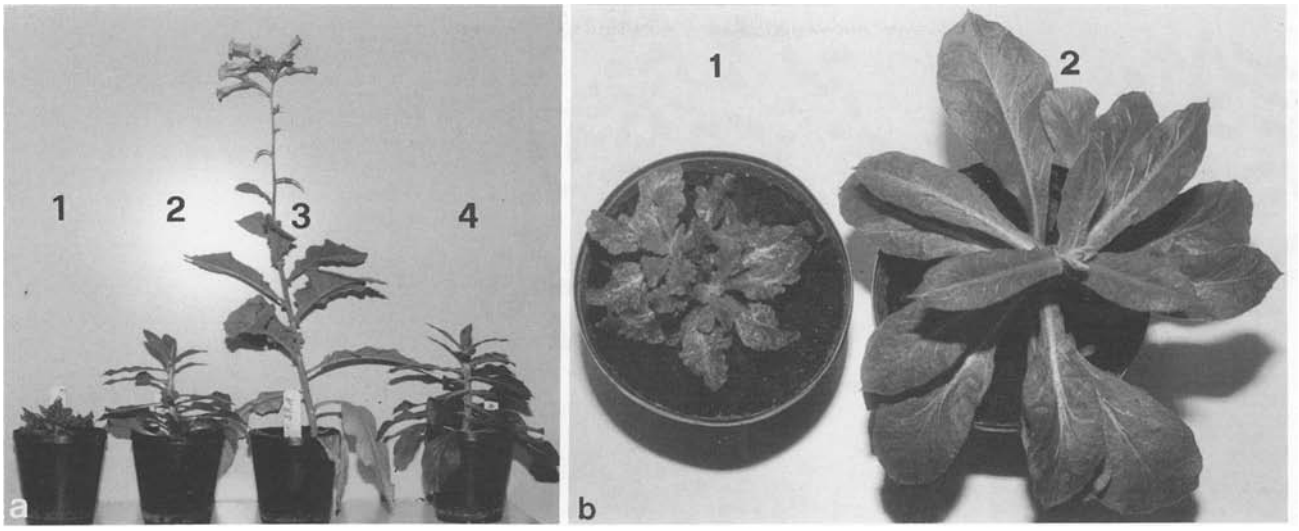

Fig. 2a, b. SR1 (3) and plants regenerated from fusion products between protoplasts from SR1 and niaK$^+$ (1, 2, 4). All plants were transferred at the same time from T medium (3) or T medium containing 50 mg/l kanamycin sulphate (1, 2, 4) to soil at a size of approximately 5 cm. Plant 1 with distorted leaves is defined as abnormal, plants 2 and 4 as normal. **a** Plants 1 to 4 viewed from the side; **b** plants 1 and 2 from above

Table 1. Nitrate reductase activity in leaves of *Nicotiana tabacum* SR1, resistant to kanamycin (SR1K$^+$) and of plants regenerated from protoplast fusion products between SR1 and an *N. tabacum* cell line resistant to kanamycin and lacking nitrate reductase activity (niaK$^+$). The enzyme activity was measured in extracts from fully developed leaves of plants grown in soil

Plant material	Nitrate reductase (nmol/mg protein per min)
SR1	9.3
SR1K$^+$	10.3
SR1 × niaK$^+$, normal	7.2
SR1 × niaK$^+$, abnormal	8.5

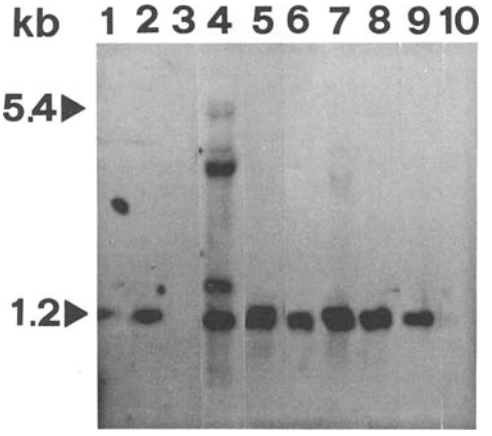

Fig. 3. Detection of the aminoglycoside phosphotransferase II (APH(3′)II) gene sequence in the DNA from leaves of plants regenerated from fusion products between SR1 and niaK$^+$. After restriction with *Eco*RV and electrophoresis of 5 µg DNA per slot DNA was transferred onto nitrocellulose filters and hybridized with the nick-translated *Hin*dIII fragment of p ABD I containing the gene for APH(3′)II. Lane 1, p ABD I, the plasmid used for transformation of the nia cell line, 1 copy; 2, 5 copies of p ABD I; 3, SR1; 4, SR1K$^+$; 5, shoot culture of abnormal-looking plant; 6, shoot culture of normal-looking plant; 7, 8, normal-looking plants grown on soil; 9, 10, F_1 plants grown from seeds of a selfed flower of a regenerant from a protoplast fusion between SR1 and niaK$^+$

containing kanamycin sulphate at 50 mg/l did not form roots and their leaves turned yellow and white. Taken together with the fact that the niaK$^+$ cell line did not grow under the selective conditions adopted and was non-morphogenic in the media applied, this demonstrates that information from both fusion partners must be present in the regenerated plants. It is therefore clear that the selection system described had led to the fusion hybrids expected. All results from the following analysis are also consistent with this interpretation. The leaves of the regenerated plants grown on soil contained nitrate reductase activity comparable with that measured in SR1 and SR1K$^+$ (Table 1).

Southern blot analysis demonstrated the presence of the hybrid APH(3′) II gene in regenerated plants and in shoot cultures and soil-grown plants derived from these (Fig. 3).

Leaf explants from the regenerated plants formed calli on B5 (Gamborg 1970) medium containing either 1 mg/ml streptomycin or 50 mg/l kanamycin sulphate, whereas *N. tabacum* leaf explants did not form calli under these conditions. Thus the presence of the cytoplasmic selectable marker, resistance to streptomycin, presents additional evidence for the hybrid character of the plants.

The chromosome number of *N. tabacum* is $2n=48$. In the root cells of plants regenerated from fusion products between SR1 and niaK$^+$ a chromosome number between 94 and 96 could be determined, indicating that the genomes of both parents were combined in these cells (Fig. 4).

All normal-looking plants and some of the ones with distorted leaves set flowers, but 99% of them (317 out of 320) were not fertile. The seeds had a very low germination frequency of less than 5% in soil.

Plants developing from seeds were more or less different from SR1. The variability of the progeny is demonstrated by the different shapes of the leaves taken from different offspring from one single selfed flower (Fig. 5). The leaves of these plants were examined for the presence of the APH(3′)II gene. This could be detected in the leaves of

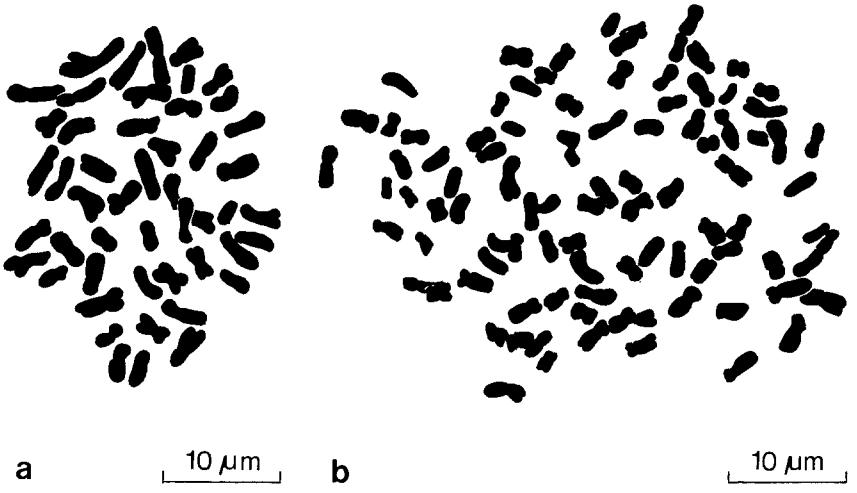

Fig. 4a, b. Metaphase chromosomes from root cells of SR1 (**a**; $2n=48$) and of plant regenerated from fusion products between SR1 and niaK$^+$ protoplasts (**b**; $2n=94$–96)

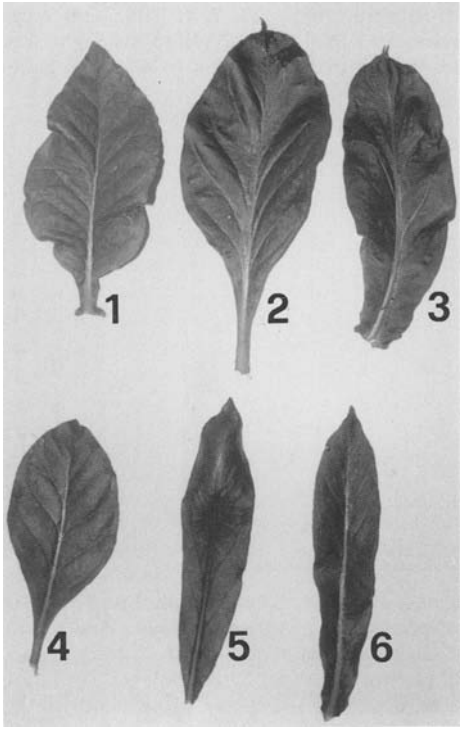

Fig. 5. Leaves from SR1 (1) and from five F_1 plants (2–6) grown from seeds of a selfed flower of a regenerant from a protoplast fusion between SR1 and niaK$^+$

all but one of these plants, indicating genomic rearrangement during meiosis (Fig. 3). Nitrate reductase activity at a level comparable to SR1 was, however, present in all of these plants (data not shown).

Subsequent experiments with different *Nicotiana* species demonstrated that the described selection method also functioned in interspecific combinations. Fusion of niaK$^+$ with protoplasts from *N. glutinosa, N. repanda* and *N. sylvestris* resulted in callus formation in medium without an available nitrogen source and containing 50 mg/l kanamycin sulphate. Only from the somatic hybrid with *N. sylvestris* was it possible, however, to grow complete plants resistant to kanamycin. These plants remained resistant to kanamycin in shoot cultures and grew after transfer to soil.

Discussion

The results presented in this paper show that the combination of a recessive (NR$^-$) and a dominant (K$^+$) nuclear marker in one genome provides an experimental system which can be applied to the selection of hybrids between protoplasts from this source and any wild-type protoplasts. The experimental system used contained two additional markers which were helpful in confirming the hybrid nature of the selected fusion clones: SR-1 was resistant to streptomycin and the cell line niaK$^+$ was non-morphogenic under the culture conditions used. This trait may be responsible, however, for the failure to produce hybrid plants from calli resulting from fusion with *N. glutinosa* and *N. repanda*. Hybrid plant formation between these two *Nicotiana* species and *N. tabacum* has been reported (Harms 1983). The method presented here should be a generally applicable selection system for somatic hybrids, because many NR$^-$ mutants are already available (Lazar 1983) or can conveniently be produced by selecting for chlorate resistance (Müller and Grafe 1978), and because transformation of these mutants for drug resistance is easy by the method of direct gene transfer (Paszkowski et al. 1984).

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