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Efficient selection of somatic hybrids in *Nicotiana tabacum* L. using a combination of drug-resistance markers introduced by transformation

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Summary. Kanamycin resistance and chloramphenicol resistance were introduced separately to two different tobacco plants (*Nicotiana tabacum* L.) by *Agrobacterium*-mediated transformation. Leaf mesophyll protoplasts were prepared from the progeny of these transformants and were subjected to electrofusion. On the 10th day and the 20th day after the fusion treatment, respectively, kanamycin (100 mg/l) and chloramphenicol (30 mg/l) were added to the suspension of protoplasts. The parental protoplasts and an unfused mixture of these protoplasts failed to form colonies when this selection procedure was employed. However, three independent fusion experiments yielded more than 7000 double-resistant colonies with a frequency between 0.30% and 0.54%. All of the surviving colonies showed continuous growth in the presence of the two antibiotics. The majority of regenerated plants resembled, morphologically, a tetraploid tobacco plant, and their somatic chromosome numbers were $2n=96$. The leaf segments from these putative somatic hybrids formed calli and proliferated vigorously on a medium that contained both antibiotics. Southern hybridization permitted the detection of the DNA fragments that conferred kanamycin and chloramphenicol resistance on these somatic hybrid plants. The system described here can be considered to be a universal system for the selection of somatic hybrids and is applicable to various combinations of protoplasts in which preselected genetic markers are absent.

Key words: *Nicotiana tabacum* – Electrofusion – Selection of somatic hybrid – Chloramphenicol resistance – Kanamycin resistance

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Introduction

Protoplast fusion can result in both the formation of somatic hybrids and the transfer of cytoplasm between two species of plant. The major problem with this technique arises from the difficulty in selecting somatic hybrids originating from a population that consists of unfused protoplasts, homokaryons and heterokaryons. It is best if the selection of somatic hybrids can be made soon after the fusion, but such selection is not possible without appropriate markers. Although various systems for the selection of hybrids are available, most of them require certain unique cell lines as fusion partners and are not readily applicable to protoplast fusions that involve combinations of a wide range of plants. Selection of somatic hybrids can sometimes be simplified by the use of "universal hybridizers" (Lo Schiavo et al. 1983) which carry both a dominantly expressed trait, such as resistance, and a recessive trait, such as auxotrophy. This system has been successfully utilized to select an intergeneric somatic hybrid (Ye et al. 1987).

Recent advances in transformation in plants have facilitated the introduction of fragments of foreign DNA into a given species of plant. Resistance to antibiotics, such as kanamycin, is commonly used as a marker for selection of transformants (Fraley et al. 1986). Kanamycin resistance has been shown to be a dominant nuclear marker (Paszkowski et al. 1984; Hain et al. 1985), which can be introduced into the nuclear genomes of dicots as well as monocots (Potrykus et al. 1985; Lorz et al. 1985). The resistance to kanamycin was introduced to a nitrate reductase-deficient mutant of tobacco (*Nicotiana tabacum* L.), and the resulting plant was used as a "universal hybridizer" for fusions between tobacco and several other species of *Nicotiana* (Brunold et al. 1987). In this scheme, the selection of somatic hybrids was efficient,

and yet it was still dependent on the availability of a pre-selected genetic marker. It would be time-consuming and tedious to obtain such a nitrate reductase-deficient line of mutant cells in a given plant species.

The use of a second drug-resistance marker, introduced by transformation, would obviate such a tedious pre-selection process. In the present study, kanamycin and chloramphenicol resistance were separately introduced into two fusion partners by *Agrobacterium*-mediated transformation, and the combination of drug resistance was used as a marker for the selection of somatic hybrids. High-efficiency selection of somatic hybrids, achieved by this method, is described in this report.

Materials and methods

Plant materials. Nicotiana tabacum L. cv Wisconsin 38 was used as the starting material for transformation experiments.

Bacterial strains and plasmids. Agrobacterium tumefaciens LBA4404 (Hoekema et al. 1983) and the binary vector pGA472 (An et al. 1985) have been described previously. pTOK67 (Fig. 1) was obtained as follows: pGA515-47 (An 1986), which contains a chimeric chloramphenicol acetyltransferase gene, together with a kanamycin-resistance gene identical to the one in pGA472, was digested with HindIII and BamHI, treated with Klenow fragment of DNA polymerase I to give blunt ends and re-ligated with T4 DNA ligase, the kanamycin-resistance gene thus being deleted from pGA515-47. Procedures for handling of DNA and *Escherichia coli* (strain TB1) were those described by Maniatis et al. (1982). pGA472 and pTOK67 were introduced into LBA4404 by the freeze-thaw method described by An et al. (1988). *A. tumefaciens* was grown on AB media (Chilton et al. 1974) at 28 °C.

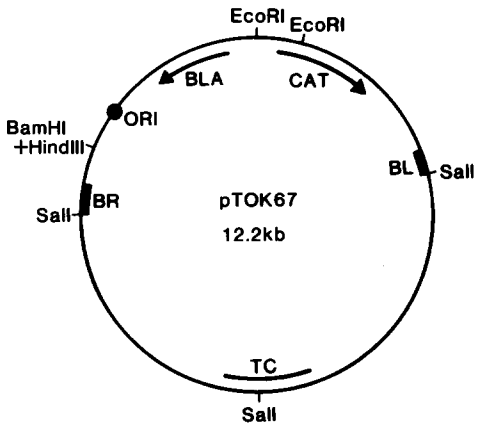

Fig. 1. *Structure of pTOK67.* pGA515-47 (An 1986) was digested with BamHI plus HindIII, treated with Klenow fragment of DNA polymerase I and re-ligated to delete the kanamycin-resistance gene. Abbreviations: CAT, chloramphenicol acetyltransferase; BLA, beta-lactamase; TC, tetracyline-resistance gene; BR, right border of T-DNA; BL, left border of T-DNA; ORI, origin of replication of ColE1

Transformation. Tobacco leaves were surface-sterilized, and 6-mm disks were prepared using a paper punch. The disks were incubated with approximately 10^8 cells of LBA4404 (pGA472) or LBA4404 (pTOK67) in 2 ml of liquid medium that consisted of Linsmaier and Skoog (1965) salts and 30 g/l sucrose (LS-R medium). After 48 h of co-cultivation, the bacteria were washed off and the disks were transferred to LS-S medium (LS-R medium plus 0.3 mg/l indole-3-acetic acid and 10 mg/l isopentenyl adenine) supplemented with 0.9% agar, 250 mg/l cefotaxim, and either 100 mg/l kanamycin or 30 mg/l chloramphenicol. Root induction of drug-resistant regenerates was achieved on LS-R agar (0.9%) medium. The plants were then transferred to soil and grown in a greenhouse. The medium used for germination of the seeds obtained from the transformants was the same as the medium used for induction of roots. Induction of calli from the explants of the seedlings was performed on an agar (0.9%) medium that consisted of Linsmaier and Skoog (1965) salts, 30 g/l sucrose, 3 mg/l indole-3-acetic acid, 3 mg/l naphthaleneacetic acid and 0.1 mg/l kinetin (LS-C medium). When appropriate, 100 mg/l kanamycin or 30 mg/l chloramphenicol were added to the media for germination of seeds and induction of calli.

Isolation of protoplasts. Leaf mesophyll protoplasts were isolated from fully expanded leaves of young plants. Leaves were surface-sterilized, cut into strips (1 mm in width), and incubated in a solution of 1.0% cellulase R-10 (Onozuka), 0.25% macerozyme R-10, 0.5% dextran sulfate (potassium salt) and 0.6 *M* mannitol, in the dark at 25 °C for 14 h. Protoplasts were filtered through one layer of Miracloth (Calbiochem), collected by centrifugation (200 rpm for 5 min) and resuspended in 0.6 *M* mannitol. This centrifugation-resuspension process was repeated three times.

Fusion of protoplasts and subsequent culture. Isolated protoplasts from two plants were mixed in equal numbers to give a final density of 5×10^4 protoplasts/ml. Fusion was accomplished by the use of an electrofusion system (Shimadzu model SSH-1, Kyoto, Japan). The fusion chamber (no. 04, Shimadzu) had two stainless electrodes, 4 mm apart, between which 1.8 ml of the suspension of protoplasts was introduced. About 5 min after the protoplasts were introduced into the chamber, the electrical force was applied. The electrical parameters for the fusion were as follows: AC field 1 MHz, 40 V; 400 V for the first DC pulse and 350 V for the second, each DC pulse being of 30-μsec duration, with an interval of 2 sec between the pulses. After the second pulse, no further electrical force was applied. About 20 min after the fusion, the protoplasts were gently collected with a pasteur pipette, rinsed once with 0.6 *M* mannitol, and resuspended in a liquid culture medium that consisted of Nagata and Takebe (1971) salts, 3 mg/l naphthaleneacetic acid, 1.0 mg/l 6-benzylaminopurine, 0.45 *M* mannitol and 17 g/l sucrose. The cultures were maintained in the dark at 25 °C for the first three days, illuminated with dim light (about 200 lux) for the next three days, and then transferred to bright-light conditions (about 2000 lux) at 25 °C. The cultures were diluted with an equal volume of fresh liquid medium every 10 days.

Selection of protoplasts for resistance to antibiotics. Ten days after the fusion treatment, when the majority of protoplasts had undergone one or two cell divisions, kanamycin sulfate was added to the culture medium at a concentration of 100 mg/l. After another ten days, chloramphenicol was added at a concentration of 30 mg/l. About 30 days after the fusion, cell colonies that had proliferated under this selection regime were transferred to LS-C agar medium supplemented with both antibiotics at the same concentrations as before.

Regeneration of plants. Colonies (about 10 mm in diameter), showing resistance to both antibiotics, were transferred to LS-S agar medium supplemented with 100 mg/l kanamycin and 30 mg/l chloramphenicol to induce shoots. Induction of roots was achieved by transferring the shoots to LS-R agar medium.

Isolation of DNA and Southern hybridization. DNA was isolated from fresh leaves frozen in liquid nitrogen and ground in a ceramic mortar. The ground leaves were suspended in an extraction buffer that consisted of 50 m*M* TRIS-HCl (pH 8.0), 10 m*M* EDTA, 1% sodium dodecyl sulfate, 0.75 *M* NaCl and 0.5% 2-mercaptoethanol. This suspension was extracted with a 1:1 mixture of $CHCl_3$ and phenol, pre-equilibrated with extraction buffer and then the aqueous phase was extracted with $CHCl_3$. DNA was precipitated by the addition of one-third volume of 10 *M* ammonium acetate and one volume of isopropanol. After centrifugation ($2000 \times g$) for 5 min, pelleted DNA was washed with 70% ethanol, and resuspended in TE buffer (10 m*M* TRIS-HCl (pH 7.5) and 1 m*M* EDTA). Further purification of DNA by ethidium bromide-cesium chloride equilibrium centrifugation and Southern hybridization was carried out according to the methods described by Maniatis et al. (1982). The probes used were the 2.1-kb BamHI-HindIII fragment from pGA472, which contains the coding sequence for the neomycin phosphotransferase gene, and the 0.8-kb HindIII fragment from pYEJ001 (obtained from PL Biochemicals), which contains the coding sequence for the chloramphenicol acetyltransferase gene.

Chromosome count. Root tips were collected from actively growing plants, pre-treated with 2 m*M* 8-hydroxyquinoline for 4 h at room temperature, and fixed in ethanol:acetic acid (3:1, v/v). After fixation, the root tips were washed three times for 2 min each with distilled water. The washed root tips were treated with a solution of 2% (w/v) cellulase R-10 and 0.5% pectolyase Y-23 in water for 30 min at 37 °C and then washed with distilled water twice. The root tips were placed on glass slides and gently dispersed with a fine needle. A small aliquot of fixative was then added and the preparations were air-dried. Before observation under the light microscope, the root tips were stained with aceto-orcein.

Results

The kanamycin-resistant fusion partner

Several kanamycin-resistant tobacco plants were obtained by the leaf-disk transformation method, using *A. tumefaciens* LBA4404 (pGA472). pGA472 carries a neomycin phosphotransferase (NPT) gene which can be expressed in plants. When the progeny of a particular transformant (plant no. 105) were grown on a kanamycin-containing medium, a typical, single-dominant, Mendelian segregation of the resistance to kanamycin was observed (Table 1). A kanamycin-resistant offspring of plant no. 105, which later turned out to be heterozygous with respect to the resistance gene, was used for the fusion.

The chloramphenicol-resistant fusion partner

The chloramphenicol acetyltransferase (CAT) gene is a powerful reporter gene in various organisms, but there are only a few published studies of the use of the CAT gene as a selection marker in plant transformation (De Block et al. 1984; An 1986). A chimeric CAT gene of pTOK67 (Fig. 1) was strongly expressed in and conferred chloramphenicol resistance on cells from *N. tabacum* (An 1986). We have used this gene as a selection marker. Leaf disks were co-cultivated with LBA4404 that carried pTOK67. Several chloramphenicol-resistant plants successfully regenerated from the leaf disks placed on a shoot-formation medium that contained chloramphenicol at 30 mg/l after the co-cultivation. Since rooting of these plants on a medium that contained chloramphenicol was not readily possible, they were allowed to form roots on a chloramphenicol-free medium, and then they were grown to maturity in soil.

Table 1. Inheritance of the kanamycin (Km) resistance (r) and the chloramphenicol (Cp) resistance introduction into tobacco

Generation	Phenotype	No. of plants	χ^2-value	*P*
S_1 of plant No. 105	Km^r	57		
	Km^s	23	0.598[a]	0.25–0.50
S_1 of plant No. 108	Cp^r	56		
	Cp^s	13	1.396[a]	0.10–0.25
F_1 of a cross No. 105 × 108	Cp^rKm^r	39		
	Cp^rKm^s	38		
	Cp^sKm^r	40		
	Cp^sKm^s	54	3.995[b]	0.25–0.50

[a] and [b] tested for 3:1 and 1:1:1:1 ratio, respectively

The seeds obtained from one of the regenerates (plant no. 108) were used for the genetic analysis of the resistance gene. First, the seeds were germinated on a phytohormone-free medium that contained chloramphenicol, as is usual in genetic analyses of kanamycin resistance, but there was little difference in growth between the resistant and sensitive seedlings; both grew equally well. Second, the cut leaves of the seedlings were placed on a callus-induction medium that contained chloramphenicol. In this case, only resistant explants formed calli (Fig. 2) and a 3:1 segregation ratio of the resistant and sensitive explants was observed (Table 1).

The sexual hybrids between the kanamycin-resistant transformant no. 105 and the chloramphenicol-resistant no. 108 were also subjected to genetic analysis, and a typical 1:1:1:1 segregation pattern of the two genetic factors was observed (Table 1). These data indicate that the chloramphenicol resistance of plant no. 108 was inherited as a single dominant trait, and the kanamycin- and chloramphenicol-resistance genes behaved as two independent Mendelian genes. A chloramphenicol-resistant offspring of plant no. 108, which later turned out to be heterozygous with respect to the resistance gene, was used as the other fusion partner.

Table 2. Formation of colonies on medium that contained both kanamycin and chloramphenicol

Protoplasts plated	Exp. I			Exp. II			Exp. III		
	Cell no.	Colony		Cell no.	Colony		Cell no.	Colony	
		No.	%		No.	%		No.	%
Km[a]	5×10^5	0	0	5×10^5	0	0	5×10^5	0	0
Cp[b]	5×10^5	0	0	5×10^5	0	0	5×10^5	0	0
Mixed	5×10^5	0	0	5×10^5	0	0	5×10^5	0	0
Fused	3×10^5	453	0.30[c]	1×10^6	1,811	0.36[c]	2×10^6	5,305	0.54[c]

[a] Protoplasts isolated from the kanamycin-resistant plant
[b] Protoplasts isolated from the chloramphenicol-resistant plant
[c] The denominator was one half of the cell number

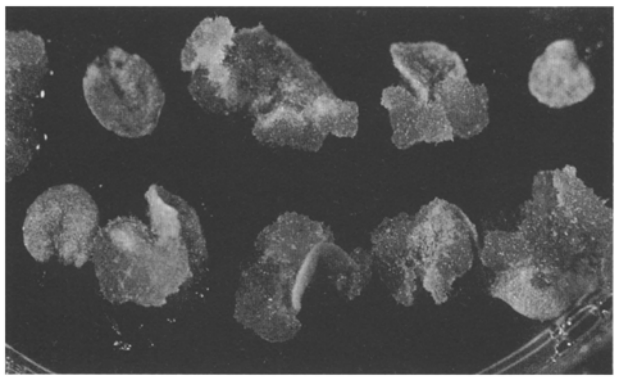

Fig. 2. *Assay of the chloramphenicol resistance of tobacco explants.* S_1 seeds of the chloramphenicol-resistant transformant were germinated under sterile conditions. Cotyledons were cut from the seedlings and placed on a callus-induction medium supplemented with 30 mg/l chloramphenicol. Calli appeared from the resistant explants between 2 and 3 weeks later

Fusion

Our electrofusion system, as described in Materials and methods, usually yields heterokaryons with a frequency ranging from 4% to 12% just after the fusion treatments, although the values tend to vary from experiment to experiment. The fusion between protoplasts isolated from the kanamycin- and chloramphenicol-resistant plants and the subsequent selection for resistance to the two antibiotics was conducted on three separate occasions. The results are summarized in Table 2. The cultures of kanamycin-resistant protoplasts, chloramphenicol-resistant protoplasts and the mixture of the two did not yield any colonies under our selection conditions. Colony formation was observed only after fusion of the mixture of the two different sets of protoplasts.

The number of surviving colonies was 453, 1811 and 5305 in the three separate fusion trials. The percentage of double-resistant colonies ranged from 0.30% to 0.54%, when one half of the total number of protoplasts subjected to fusion was used as the denominator in the calculation. The plating efficiencies of protoplasts were usually between 20% and 30%, when no selection or fusion treatment was applied. If these values are taken into consideration, the estimated frequency of formation of heterokaryon is between 1.00% and 2.70%.

All surviving colonies were subcultured on medium that contained both antibiotics for at least two cycles (for about 2 months). In no case was the loss of resistance detected. Thus, the resistance to the two antibiotics was stably expressed in these putative somatic hybrid cells.

Plant regeneration from putative somatic hybrid cells

The colonies showing double resistance were transferred to the shoot regeneration medium (LS-S) that contained both antibiotics. Numerous shoots were formed from these calli within 3 weeks. The shoots were cut and transferred to hormone-free medium (LS-R). From each colony, 1 regenerate was transplanted to soil. A total of 34 regenerates were evaluated further. Among them, 4 plants were morphologically abnormal and their growth was retarded. The rest of the plants showed morphology typical of a tetraploid tobacco and were self-fertile. The somatic chromosome number of the regenerates was examined (Table 3). The morphologically normal plants had 96 chromosomes (Fig. 3A), a number which corresponds to the sum of the chromosome numbers of the two fusion partners. The chromosome numbers of all of the morphologically abnormal plants deviated from this expected value of 96 (Fig. 3B, C, D).

Leaf segments (10 mm × 10 mm) were cut from each regenerate and cultured on callus-inducing medium (LS-C) that contained both antibiotics. All regenerates formed calli on this medium, but the kanamycin-resistant transformant, the chloramphenicol-resistant transformant and wild-type tobacco failed to form calli on this medium.

Southern hybridization

The DNA isolated from the somatic hybrids and from the parents of the fusion were analyzed by Southern

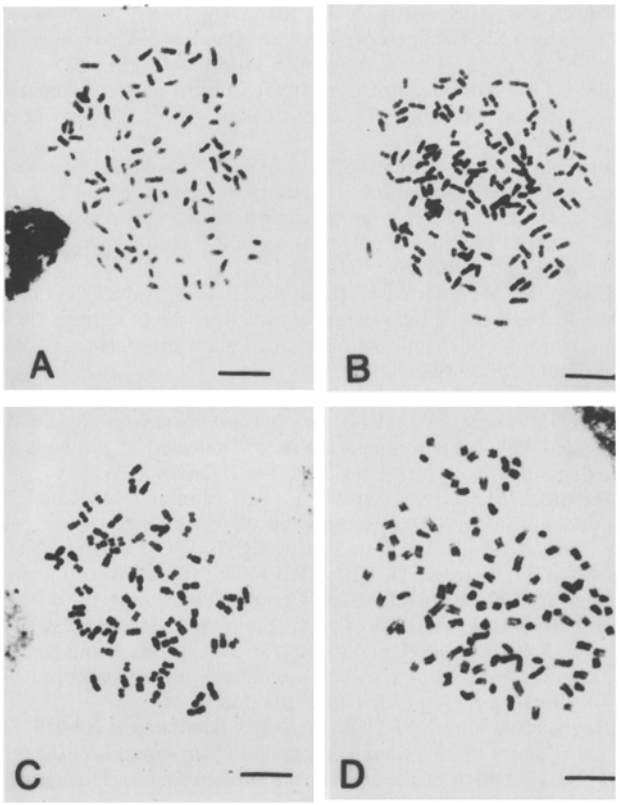

Fig. 3 A–D. *Chromosomes of the somatic hybrids.* **A** plant no. 14 ($2n=96$) representing the majority of the somatic hybrids; **B** plant no. 32 ($2n=$c. 144); **C** plant no. 31 ($2n=77$); **D** plant no. 34 ($2n=97$)

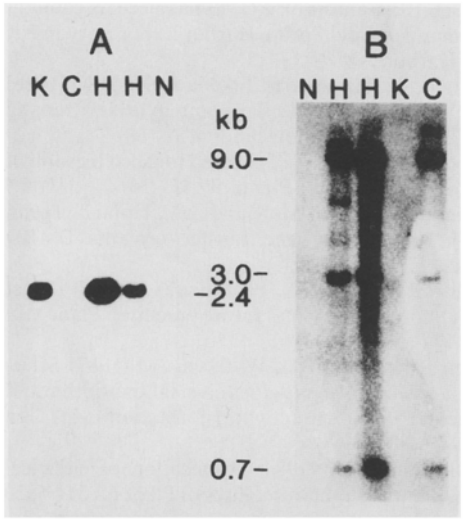

Fig. 4 A and B. *Southern hybridization.* DNAs were digested with **A** BamHI or **B** EcoRI, subjected to agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized to **A** the NPT probe or **B** the CAT probe. K, kanamycin-resistant parent; C, chloramphenicol-resistant parent; H, somatic hybrids; N, non-transformed tobacco

Table 3. Somatic chromosome number and morphological characteristics of the somatic hybrids

Plant	Chromosome no.[a]	Morphology	Fertility
11	96	normal	self-fertile
12	96	normal	self-fertile
13	96	normal	self-fertile
14	96	normal	self-fertile
15	96	normal	self-fertile
16	96	normal	self-fertile
31	77	abnormal	no flowers
32	c. 144	abnormal	self-fertile
33	c. 147	abnormal	sterile[b]
34	97	abnormal	sterile[c]

[a] Chromosome number was determined from at least five well-spread mitotic figures
[b] Few pollen grains (pollen fertility was around 5%)
[c] Almost no pollen grains

hybridization (Fig. 4). The NPT probe detected the 2.4-kb BamHI fragment in the kanamycin-resistant parent and the somatic hybrids, and this band was absent from the chloramphenicol-resistant parent. The CAT probe hybridized to three major EcoRI fragments (9.0, 3.0 and 0.7 kb) in the chloramphenicol-resistant parent and the somatic hybrids, but there was no fragment homologous to this probe in the kanamycin-resistant parent. These results clearly show that the somatic hybrids carried DNA fragments derived from each of the parents.

Discussion

The present study demonstrates that the kanamycin-resistance gene and the chloramphenicol-resistance gene together provide a convenient combination of markers for the efficient selection of somatic hybrids derived from fusion of protoplasts of plant cells. The kanamycin-resistance gene is the selection marker most frequently used in plant transformation experiments and has already been shown to be a powerful marker in protoplast fusion (Brunold et al. 1987). By contrast, the resistance to chloramphenicol conferred by the CAT gene has been utilized far less frequently and stable inheritance of resistance to chloramphenicol has not been reported. The present study demonstrates that the CAT gene can be used as a selection marker in plant transformation as well as in protoplast fusion, and that the chloramphenicol resistance introduced into tobacco was stably transmitted to the progeny in a Mendelian fashion.

Numerous colonies showing double resistance to kanamycin and chloramphenicol were obtained. Randomly chosen regenerates from these colonies exhibited

characteristics of a somatic hybrid: (1) resistance of leaf segments to both antibiotics; (2) morphology similar to a tetraploid tobacco plant, (3) chromosome number comparable to the sum of chromosome numbers of the two fusion partners; and (4) presence of DNA fragments that confer the resistance to each antibiotic. Other colonies which were not examined for these characteristics were probably also somatic hybrids.

The frequency of colonies of putative, somatic hybrid cells ranged from 0.30% to 0.54%, if the plating efficiency of control protoplasts is ignored. There are many reports of heterokaryon frequencies just after electrofusion (e.g. Bates and Hasenkampf 1985, Zachrisson and Bornman 1984), but few reports of an actual frequency of hybrid calli or hybrid regenerates. This value can be obtained only when both fusion partners carry appropriate genetic markers that act independently of one another, and when no mechanical selection force is applied. In the case of a combination of *N. glauca* and *N. langsdorffii*, where only the hybrid cells are capable of growing in a hormone-free medium, two reports have indicated their frequency of 0.2% (Morikawa et al. 1986) and 0.14% (Chapel et al. 1986) by electrofusion. Our results, in terms of frequency, appear to be comparable to or slightly better than these results.

In the present series of experiments, we isolated protoplasts from the S_1 progeny of transformants. Alternatively, the transformants themselves can be used as sources of protoplasts. In such a case, somatic hybrids between two plants can be obtained within a year: 3–4 months are required to obtain and grow transformants that are large enough for isolation of protoplasts, and about 6 months are required from the fusion to the evaluation of the regenerates. The present system is applicable to any combination of plants into which the genes for resistance to kanamycin, chloramphenicol, or other antibiotics, can be introduced by an appropriate transformation system.

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References

An G (1986) Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol 81:86–91

An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) New cloning vehicles for transformation of higher plants. EMBO J 4:277–284

An G, Evert PR, Mitra A, Ha SB (1988) Binary Vectors. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual A3. Kluwer Academic, Dordrecht, pp 1–19

Bates GW, Hasenkampf CA (1985) Culture of plant somatic hybrids following electrical fusion. Theor Appl Genet 70:227–233

Brunold C, Kruger-Lebus S, Saul MW, Wegmuller S, Potrykus I (1987) Combination of kanamycin resistance and nitrate reductase deficiency as selectable markers in one nuclear genome provides a universal somatic hybridizer in plants. Mol Gen Genet 208:469–473

Chapel M, Montane M-H, Ranty B, Teissie J, Alibert G (1986) Viable somatic hybrids are obtained by direct current electrofusion of chemically aggregated plant protoplasts. FEBS Lett 196:79–86

Chilton M-D, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681–1689

Fraley RT, Rogers SG, Horsh RB (1986) Genetic transformation in higher plants. CRC Crit Rev Plant Sci 4:1–46

Hain R, Stabel P, Czernilofsky AP, Steinbiss L, Herrera-Estrella L, Schell J (1985) Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. Mol Gen Genet 199:161–168

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Lo Schiavo F, Giovinazzo G, Terzi M (1983) 8-azaguanine resistant carrot cell mutants and their use as universal hybridizers. Mol Gen Genet 192:326–329

Lorz H, Baker B, Schell J (1985) Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199:178–182

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Morikawa H, Sugino K, Hayashi Y, Takeda J, Senda M, Hirai A, Yamada Y (1986) Interspecific plant hybridization by electrofusion in *Nicotiana*. Biotechnol 4:57–60

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

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722

Potrykus I, Shillito RD, Saul MW, Paszkowski J (1985) Direct gene transfer. State of the art and future potential. Plant Mol Biol Rep 3:117–128

Ye J, Hauptmann RM, Smith AG, Widholm JM (1987) Selection of a *Nicotiana plumbaginifolia* universal hybridizer and its use in intergeneric somatic hybrid formation. Mol Gen Genet 208:474–480

Zachrisson A, Bornman CH (1984) Application of electrical field fusion in plant tissue culture. Physiol Plant 61:314–320