Theor Appl Genet (1988) 76:237–243

A selection method for the synthesis of triploid hybrids by fusion of microspore protoplasts (n) with somatic cell protoplasts (2n)

D. Pental, A. Mukhopadhyay, A. Grover and A. K. Pradhan
Tata Energy Research Institute, 90 Jor Bagh, New Delhi-110 003, India

Received January 19, 1988; Accepted January 23, 1988
Communicated by G. Wenzel

Summary. Microspore protoplasts (n) isolated at the tetrad stage from plants of *Nicotiana tabacum* Km^+ ($2n=4x=48$) were fused with somatic cell protoplasts (2n) of WT *N. rustica* ($2n=4x=48$) to produce triploid plants. A total of 21.2×10^6 microspore protoplasts were fused with 11.2×10^6 somatic cell protoplasts using the high pH/Ca^{++} method. Microspore protoplasts did not divide and WT *N. rustica* protoplasts stopped dividing when the protoplast-derived colonies were transferred to a selection medium containing kanamycin. A total of 104 actively growing green colonies were recovered on the selection medium. Ninety-six of these colonies were tested for their hybrid nature by PAGE of peroxidases and were found to contain bands characteristic of both parents. Hybrid nature of the plants regenerated from some of the selected colonies was confirmed by IEF of leaf esterases, by NPT II activity assay and by hybridizing total DNAs restricted with *Eco*R I to a cloned 18s rDNA fragment. Root tip squashes of six of the hybrid plants revealed chromosome numbers ranging from 58–72. From chromosomal and biochemical analysis, it can be concluded that the procedure of fusing microspore protoplasts (n) of species A carrying a dominant selection marker with WT somatic cell protoplasts (2n) of species B can be a convenient selection method for the synthesis of triploid plants. The significance of triploids lies in their subsequent use for transferring alien chromosomes and genes of species A to species B.

Key words: Microspore protoplasts – Somatic protoplasts – Fusions – Triploids – *Nicotiana*

Introduction

Transfer of genetic information from wild to crop species is of considerable significance in crop improvement. This could be achieved through the production of alien addition and substitution lines by synthesizing triploids. O'Mara (1940) first proposed combining one set of chromosomes (n) of an alien parent A (genome designated as AA) with two full sets of chromosomes (2n) of a crop parent B (genome designated as BB) for synthesizing triploid hybrids (ABB). Savitsky (1975) produced triploid hybrids by crossing a tetraploid *Beta vulgaris* ($2n=4x=36$) with diploid *B. procumbens* ($2n=2x=18$). In the triploid hybrids, during meiosis, usually nine *B. vulgaris* bivalents and nine *B. procumbens* univalents were observed. *B. vulgaris* lines with chromosome additions of *B. procumbens* were recovered and were used for the transfer of nematode resistance from *B. procumbens* to *B. vulgaris* due to occasional trivalent associations in monosomic-addition plants. More recently, Rick et al. (1986) crossed *Lycopersicon esculentum* (LL) with *Solanum lycopersicoides* (SS) and doubled the chromosome number to produce an allotetraploid (LLSS). The allotetraploids were backcrossed to *L. esculentum* (LL) to produce allotriploids (LLS). In the allotriploids, during meiosis, LL chromosomes pair preferentially and S chromosomes segregate as univalents. These plants are being used for the production of alien addition and substitution lines that could be further used for introgression of important agronomic traits from *S. lycopersicoides* to *L. esculentum*.

Pental and Cocking (1985) proposed that triploid plants (ABB) could be produced by fusing protoplasts isolated from microspores at the tetrad stage (n) of species A with protoplasts isolated from the somatic cells (2n) of species B. Synthesis of triploids through gameto-

somatic fusions has an advantage over the conventional sexual crosses in bypassing pre- and post-zygotic sexual incompatibility barriers and also in avoiding extensive pollination and embryo rescue. Pirrie and Power (1986) synthesized triploids by fusing microspore protoplasts of *Nicotiana glutinosa* ($2n=2x=24$) with somatic cell protoplasts of *N. tabacum* ($2n=4x=48$). Microspore protoplasts were isolated at the tetrad stage and fused with leaf mesophyll protoplasts of nitrate reductase deficient *N. tabacum*. Only one colony was recovered in a selection medium that contained nitrate as the sole source of nitrogen. Plants regenerated from this colony were found to be triploid hybrids ($2n=5x=60$).

We report a selection method for producing a large number of gameto-somatic hybrids. The method involves fusing microspore protoplasts of parent A, carrying a dominant resistance marker (e.g. kanamycin resistance) in the nuclear genome, with WT somatic cell protoplasts of parent B. As microspore protoplasts do not divide in culture (Bhojwani and Cocking 1972), only triploid hybrids would be able to grow on a selection medium containing kanamycin. To test the efficiency of this selection method, *N. tabacum* Km^+ (Hain et al. 1985) was used for the isolation of microspore protoplasts. *N. tabacum* carries a kanamycin resistance conferring chimaeric gene, combining nopaline synthase promoter region with coding region of neomycin phosphotransferase gene (NPT II) of Tn5. Somatic cell protoplasts of WT *N. rustica* were used as the other fusion partner.

Materials and methods

Isolation of microspore protoplasts from Nicotiana tabacum

Nicotiana tabacum Km^+ (PT2) plants were grown in pots under natural conditions. Young buds, measuring around 7 mm, were surface sterilized with 0.1% mercuric chloride and washed thrice in autoclaved water. After surface sterilization, each bud was dissected and one of the anthers was crushed to ascertain the stage of meiosis. Only buds containing microspores at the tetrad stage (radial quartet cell stage), before any sporopollenin is laid down, were used for isolation of protoplasts. Anthers were separated from the filament tissue, cut into two halves and gently squeezed into an enzyme solution containing 2% Driselase (Kyowa Hakko Kogyo, Tokyo), CPW salts (Frearson et al. 1973) and 9% mannitol (CPW 9M), pH 5.8. Protoplasts were released after 15–30 min incubation in the enzyme solution at 25 °C on a shaker set at 50 rev/min. The protoplast suspension was sieved through a 45 μ stainless steel mesh to remove debris.

Isolation of cell suspension protoplasts of Nicotiana rustica

Nicotiana rustica V12 seeds sterilized with 95% ethanol for 3–5 min were germinated on MS medium (Murashige and Skoog 1962) solidified with 0.8% Agar (HiMedia, Bombay). Hypocotyl explants from 10 to 15-day-old seedlings were put on MS1 medium containing 2 mg/l NAA (naphthalene acetic acid) and 0.5 mg/l BAP (6-benzyl amino purine) for development of callus tissue that was transferred to liquid UM medium (Uchimiya and Murashige 1974) to raise cell suspension cultures. Cell suspensions were grown at 25 °C on a shaker set at 150 rev/min. For isolation of protoplasts, cells from 6-day-old suspension cultures were incubated in a solution containing 2% Cellulase R-10 (Yakult Honsha, Nishinomiya), 0.5% Macerozyme R-10 (Yakult Honsha) and 0.5% Driselase, in CPW 13M. Cells were incubated for 16–20 h at 20 °C on a shaker set at 50 rev/min. Enzyme solution with protoplasts was passed through a 45 μ steel sieve to remove macrodebris and centrifuged at $80 \times g$ for 5 min to remove the enzyme solution. The pellet was suspended in a solution of CPW salts with 21% sucrose and centrifuged at $100 \times g$ for 10 min. Viable, intact protoplasts were collected from the surface and suspended in CPW 9M.

Fusion and culture of protoplasts

Fusions were carried out between cell suspension and microspore protoplasts following the high pH/Ca^{++} method (Keller and Melchers 1973). A mixture of *N. tabacum* and *N. rustica* protoplasts was pelleted by centrifugation. After removing the supernatant, fusion-inducing solution was gently added to the protoplast pellet and tubes were incubated at 30 °C for 20 min. Following incubation, a CPW 13M solution containing an additional amount of $CaCl_2 \cdot 2H_2O$ (0.74%) was added to stabilize the protoplasts. After one wash in stabilizing solution, protoplasts were suspended in MS1 9M medium, counted and cultured in liquid medium at a density of 5×10^4/ml in sterile 3.5 cm diameter petridishes (Laxbro, Pune) and kept in dark for 4 days at 25 °C. Plating efficiency (considered as the no. of protoplasts that had undergone at least one division as a percentage of the number originally plated) was counted 5 days after culture of protoplasts.

After 12 days, dividing cell colonies were transferred to selection medium MS2 containing 0.1 mg/l NAA, 0.5 mg/l BAP, 100 µg/ml kanamycin sulfate. Cell colonies were cultured in liquid MS2 9M medium over solidified (0.8% Agar) MS2 4.5M medium. Contents of each 3.5 cm dish were transferred to a 9 cm diameter dish (Steriware, Faridabad). Subsequently, osmotic concentration of the medium was lowered by addition of fresh MS2 medium at 15 day intervals. Macroscopic green colonies growing on the selection medium were recovered from fusion dishes 45 days after initiation of the experiments. Throughput of colonies was calculated as the percentage of *N. rustica* somatic cell protoplasts that divided after fusogen treatment. Selected cell lines were grown on MS1 medium (0.8% agar) containing 50 µg/ml kanamycin sulfate. For regeneration of shoots, callus tissues were transferred to MS2 medium containing 50 µg/ml kanamycin sulfate or MS3 medium containing 2 mg/l IAA (Indole acetic acid) and 1 mg/l BAP. Regenerated shoots were maintained on MS medium with 50 µg/ml kanamycin sulfate and transferred to hydroponics to induce roots. For biochemical analysis, regenerated shoots were re-callused on MS1 medium.

Biochemical analysis of cell lines and regenerated plants

Selected callus lines, plants and callus tissues induced from selected plants were used for biochemical characterization. For analysis of peroxidases, callus tissues of the two parents and selected lines were used. Protein extracts were made with a buffer used for Fraction 1 protein analysis (Cammaerts and Jacob 1980). For 1 g callus tissue, 0.25 ml of extraction buffer was used. Extracts containing 70 μg protein (measured by the method of Lowry et al. 1951) for each tissue were loaded on a discontinuous polyacrylamide gel (vertical electrophoresis, Biorad Protean II) and run at a constant current of 2.5 mamp/channel at 4 °C. Gels were stained for peroxidase bands according to Lagrimini and Rothstein (1987).

For esterase analysis, extracts were made from shoots growing in vitro using the same buffer as was used for peroxidase analysis. For 1 g leaf tissue, 1 ml of extraction buffer was used. Isoelectric focusing of the soluble proteins was done using 3.5–10 pH range gel (cast in Ultramould LKB apparatus). Gels were run and stained as per Pental et al. (1984).

Assay for neomycin phosphotransferase (NPTII) was performed as described by Reiss et al. (1984) and modified by Schreier et al. (1985).

DNA from both parents and the selected lines was isolated from freeze-dried callus tissues by the method of Murray and Thompson (1980) and digested with various restriction endonucleases under conditions recommended by the suppliers (BRL, Bethesda). Electrophoresis was performed in the horizontal mode as described earlier (Pental et al. 1986). An 18s rDNA sequence cloned from wheat nuclear genome (Gerlach and Bedbrook 1979), carried on plasmid pTA71, was used as a probe. Nick translation of pTA71 and DNA/DNA hybridization was carried out as described by Evans et al. (1983).

Root-tips of plants grown in hydroponics were fixed in a solution containing 0.03% hydroxyquinoline and 0.05% colchicine for 5–6 h at 15 °C and then for 2 h at 4 °C. Roots were hydrolyzed in 1N HCl and squashed in 2% acetocarimine for visualizing chromosomes.

Results

Recovery of colonies on selection medium

Nicotiana tabacum Km^+ protoplasts isolated from microspores at the tetrad stage were densely cytoplasmic and uniform in size. Protoplasts isolated from the cell suspension of *N. rustica* were variable in size and had vacuoles. A total of 12 fusion experiments were performed, of which 6, namely 4, 7, 9, 10, 11 and 12, were carried through for the isolation of 'hybrid' cell colonies (Table 1). On an average 75% of somatic cell protoplasts survived fusogen treatment and 65% of these divided in MS1 9M medium by the 5th day. Actively growing green colonies were visible among pale yellow ones 20–30 days after transfer to the selection medium (MS2). A total of 104 green colonies were scored in the fusion dishes (Table 1). The frequency of 'hybrid' production thus turns out to be one per 5.25×10^4 dividing protoplasts of *N. rustica*. In control experiments a total of 3×10^6 *N. rustica* protoplasts were co-cultured with 2.8×10^6 microspore protoplasts without any fusogen treatment. No green colonies appeared in such cultures upon transfer to the selection medium. The green colonies maintained on MS1 medium for biochemical analysis were designated by the serial number of the experiment, followed by the number of the selection dish and lastly by the number of colonies selected in each dish. For example, in experiment 4, fusogen treated protoplasts were plated in five dishes designated as 4.1, 4.2 . . . 4.5. The colonies in each dish were referred to as 4.1A, 4.1B, 4.2A, etc. Whereas the colonies 4.1A, 4.1B could result from the breaking up of one hybrid colony during the first transfer from MS1 to MS2 medium, colonies 4.1A and 4.2A were certainly of independent origin as these came from two different culture dishes.

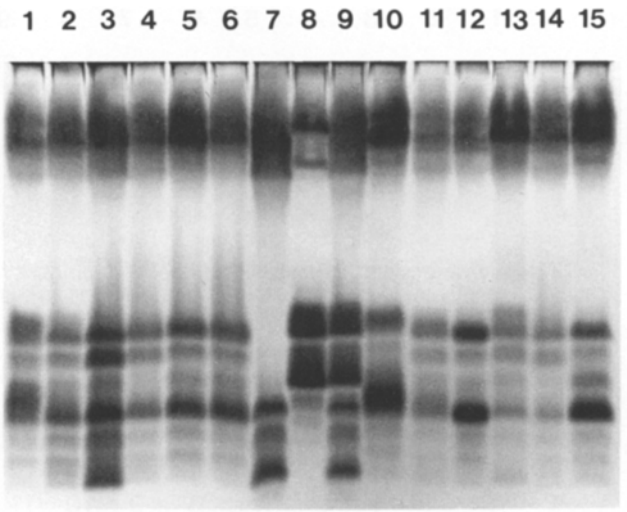

Fig. 1. Polyacrylamide gel electrophoresis of peroxidases extracted from callus tissues of *Nicotiana tabacum, N. rustica* and selected hybrid cell lines. *Lanes* from left to right are: (*1*) Cell line 10.4A, (*2*) 10.5A, (*3*) 10.6A, (*4*) 10.7A, (*5*) 10.8A, (*6*) 10.9A, (*7*) *N. tabacum*, (*8*) *N. rustica*, (*9*) 1:1 mixture of *N. tabacum* and *N. rustica* proteins, (*10*) 10.10A, (*11*) 11.1A, (*12*) 11.2A, (*13*) 11.3A, (*14*) 11.4A, (*15*) 11.5A

Table 1. Details of the no. of protoplasts fused and the no. of green colonies recovered on the selection medium in six different fusion experiments

Experiment no.	No. of *N. rustica* somatic cell protoplasts	No. of *N. tabacum* Km^+ microspore protoplasts	No. of Km^+ colonies
4	10^6	2×10^6	7
7	2×10^6	2.8×10^6	8
9	2.2×10^6	3.9×10^6	5
10	2×10^6	1.4×10^6	50
11	10^6	2.7×10^6	26
12	3×10^6	8.4×10^6	8

Analysis of selected cell lines

Ninety-six of the 104 selected cell lines grew well on MS1 medium and were analyzed for their peroxidase pattern. *N. tabacum* Km^+ and *N. rustica* showed distinct peroxidase patterns (Fig. 1). All 96 green colonies had peroxidase bands characteristic of both parents (Fig. 1).

DNAs isolated from callues tissues of *N. tabacum* and *N. rustica* were restricted with seven different restriction endonucleases, namely *Alu* I, *Cfo* I, *Eco*R I, *Hae* III, *Hind* III, *Sal* I and *Sma* I, and hybridized to nick translated 18s rDNA probe to generate species-specific patterns. In *Eco*R I digests distinct patterns were identified

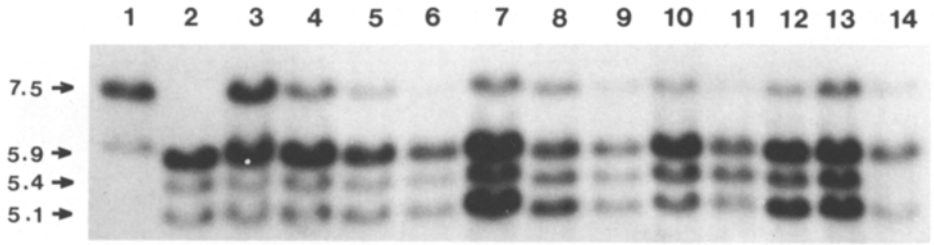

Fig. 2. Hybridization of ^{32}P-labelled 18s rDNA clone (pTA71) with DNAs isolated from callus tissues of *Nicotiana tabacum, N. rustica* and hybrid plants. DNAs were digested with *Eco*R I. Fragment length (kb) was calculated by running λ DNA digested with Hind III. *Lanes* from left to right are : (*1*) *N. tabacum,* (*2*) *N. rustica,* (*3*) 1.1 mixture of parental DNA, (*4*) 1:2 mix, (*5*) 1:4 mix, (*6*) hybrid 10.9C3, (*7*) 10.10B1, (*8*) 7.1A4, (*9*) 10.2C5, (*10*) 11.7A4, (*11*) 10.3A3, (*12*) 10.10B2, (*13*) 7.1A7, (*14*) 10.1F. Out of the four bands present in the physical mixtures of the parental DNAs (*lanes 3–5*), a 7.5 kb band is specific to *N. tabacum,* a 5.9 kb band is common to *N. tabacum* and *N. rustica* and the two other bands, 5.4 and 5.1kb, are unique to *N. rustica*

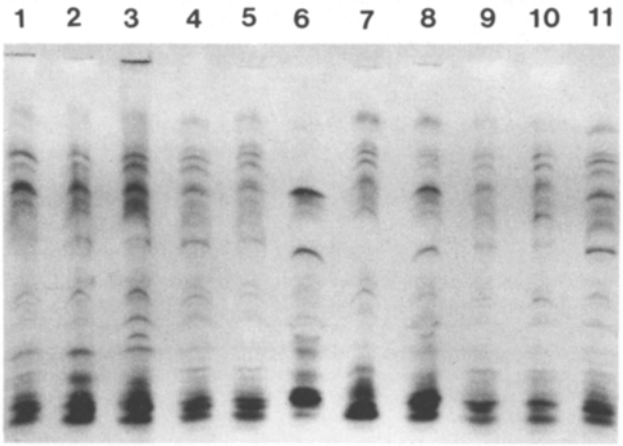

Fig. 3. Isoelectric focusing of leaf esterases of *Nicotiana tabacum, N. rustica* and hybrid plants. *Lanes* from left to right are: (*1*) hybrid 7.1A4, (*2*) 7.1A7, (*3*) 10.1F2, (*4*) 10.2C4, (*5*) *N. tabacum,* (*6*) *N. rustica,* (*7*) 1:1 mixture of *N. tabacum* and *N. rustica* proteins, (*8*) 10.3A3, (*9*) 10.6C5, (*10*) 10.9H5

for the two parental species (Fig. 2). DNAs extracted from 30 selected cell lines were hybridized with 18s rDNA probe. Band characteristic of both parents were observed in all the analysed lines, confirming their hybrid nature (Figure not shown).

Plant regeneration and characterization of regenerated plants

All 96 selected callus lines regenerated shoots on transfer to MS2 and MS3 medium. Shoot cultures were maintained on MS medium with 50 μg/ml of kanamycin sulfate. In cases where more than one plant was regenerated from a callus line, plants were numbered as 1,2,3 . . . following the number of the cell line. For example, plants 7.1A4 and 7.1A7 are two independent plants that were regenerated from cell line 7.1A. Leaf tissue of parental material and some of the selected shoot cultures growing in vitro were used for analysis of esterases by isoelectric focusing. The two parents showed distinctive esterase patterns (Fig. 3). Plants regenerated from the selected lines showed bands characteristic of both parents (Fig. 3). NPT II assay was done on leaf tissue of the two parents and five selected plants, namely 7.1A4, 10.1F2, 10.3A3, 10.6C3 and 10.9H1. *N. tabacum* Km^+ and the five selected plants showed NPT II specific band. No NPT II specific band was visible in *N. rustica.*

DNAs isolated from the callus tissues induced from the two parents and nine selected plants were digested with *Eco*R I and hybridized to 18s rDNA probe (Fig. 2). All the hybrids showed bands characteristic of both parents (Fig. 2). Preliminary studies of root tip squashes of a few of the hybrids, namely 7.3A7, 10.3A3, 10.6D6, 10.6C3, 10.1F1, 11.4B1, 10.1G1 and 10.6A4, revealed that these plants have chromosome numbers ranging from 58–72. Detailed analysis of mitotic chromosomes will be reported elsewhere.

Discussion

All the selected colonies and regenerated plants that were tested for isozyme and rDNA profiles showed hybrid characteristics. The selection procedure of fusing microspore protoplasts (n) of parent A carrying dominant selection marker with WT somatic protoplasts (2n) of parent B can be used for the recovery of gameto-somatic hybrids. In the present study, hybrid colonies were recovered in the selection medium at a frequency of 1 in 5.25×10^4 dividing protoplasts of *N. rustica.* This frequency is lower than that reported for the fusion of leaf mesophyll protoplasts of *N. tabacum* NR^-SR^+ with *N. rustica* cell suspension protoplasts (Pental et al. 1984) using a fusion method similar to the one used in this study. Protoplasts isolated from the microspore cells at the tetrad stage were found to be highly susceptible to fusogen treatment. After high pH/Ca^{++} treatment, very few microspore protoplasts survived. In preliminary in-

vestigations (data not reported in this paper) it was found that polyethyleneglycol treatment at various concentrations causes immediate lysis of microspore protoplasts. Perhaps electrofusion methods (Zimmermann and Scheurich 1981; Watts and King 1984; Bates 1985) could give a higher number of viable fusions and hybrids. Further work would be needed to improve fusion frequencies.

Although rDNA and isozyme profile studies of hybrid plants showed the presence of bands characteristic of both the parents, chromosomal analysis of some of the plants revealed numbers varying from 58–72. In an earlier study (Hamill et al. 1985), it has been shown that somatic hybrids of *N. rustica* ($2n=4x=48$) and *N. tabacum* ($2n=4x=48$) have chromosome numbers varying from 63–87. The loss of chromosomes seems to be a peculiar feature of *N. rustica* + *N. tabacum* hybrids. Contrary to this, fusion of *N. glutinosa* ($2n=2x=24$) microspore protoplasts with *N. tabacum* somatic cell protoplasts produced triploids with 72 chromosomes (Pirrie and Power 1986).

In many studies, irradiated (X or γ-irradiation) diploid protoplasts of parent A were fused with normal protoplasts of parent B to bring about limited gene transfer from parent A to parent B (Dudits et al. 1980; Gupta et al. 1982, 1984; Bates et al. 1987; Imamura et al. 1987; Muller-Gensert and Schieder 1987; Sidorov et al. 1987). While this method may be useful in cases where only organelle genome is to be transferred from parent A to parent B (Zelcer et al. 1978; Menczel et al. 1983; Aviv et al. 1984), its utility in transfer of nuclear genes from parent A to parent B is suspect. X or γ-irradiation would create lesions in the genome of the alien parent, leading to breakage of chromosomes and their premature elimination during the somatic phase. There could also be deleterious mutations at the loci to be transferred. In wide hybrids (genomic allopolyploids following the terminology of Stebbins 1950), the limiting factor to gene transfer would be the inability to retain alien chromosomes and lack of pairing of alien chromosomes of parent A with the homoeologous chromosomes of the crop parent B. In such a situation, weakening of the alien chromosomes with irradiations will even further lower the chances of their being transmitted through mitotic and meiotic divisions. A better strategy would be to irradiate the triploid (sesquidiploid) hybrids to bring about introgression in the generative phase of the hybrid plants.

Triploids could also be produced by fusing somatic cell protoplasts of species A and B. The resulting hybrid (AABB) could be sexually crossed with one of the parents, e.g. BB to produce sesquidiploid plants (ABB). However, to select hybrids of somatic cells, genetic markers will be needed in both the parents (Melchers and Labib 1974; Douglas et al. 1981) or a double mutant approach will have to be used (Pental et al. 1984; Brunold et al. 1987; Toriyama et al. 1987; Ye et al. 1987). Direct triploid synthesis is possible by fusing microspore protoplasts with somatic cell protoplasts. As microspore protoplasts do not divide, only one parent needs to have a marker gene for the selection of hybrids. Earlier, Pirrie and Power (1986) fused WT *N. glutinosa* microspore protoplasts with nitrate reductase deficient *N. tabacum* somatic cell protoplasts and recovered a hybrid colony on selection medium that had nitrate as the sole source of nitrogen. In comparison to the above method, we suggest the use of microspore protoplasts of species A transformed for a drug resistance marker for fusions with WT somatic cell protoplasts of species B. A large number of plant species belonging to dicotyledonae, e.g. *N. tabacum* (Herrera-Estrella et al. 1983; An et al. 1985), *Lycopersicon esculentum* (McCormick et al. 1986; Shahin et al. 1986; Chyi and Phillips 1987), *Medicago varia* (Deak et al. 1986), *Linum usitatissimum* (Basiran et al. 1987), *Gossypium hirsutum* (Umbeck et al. 1987), *Helianthus annuus* (Everett et al. 1987), *Brassica napus* (Fry et al. 1987; Pua et al. 1987), *Populus deltoides* (Fillati et al. 1987) have been transformed with chimaeric genes carrying drug-resistance markers using *Agrobacterium* mediated transformation (Horsch et al. 1985). Thus, it should be possible to introduce drug-resistance markers into wild species and use these for isolation of microspore protoplasts (n). A successful hybridization program using the selection procedure outlined in this communication would also require regeneration capability from callus to plant in a least one of the two parent species.

Acknowledgements. Technical assistance was provided by Mr. B. S. Yadav. We thank Dr. R. Hain and Dr. H. Lorz for *N. tabacum* (PT2) seed. The visit to Max-Planck-Institut, Köln was made possible by a Rockefeller Career Fellowship to D. Pental. Plasmid pTA 71 was supplied by Dr. R. A. Flavell.

Reference

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

Aviv D, Arzee-Gonen P, Bleichman S, Galun E (1984) Novel alloplasmic *Nicotiana* plants by "donor-recipient" protoplast fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genomes and either or both plastomes and chondriomes from alien species. Mol Gen Genet 196:244–253

Basiran N, Armitage P, Scott RJ, Draper J (1987) Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens:* regeneration of transformed shoots via a callus phase. Plant Cell Rep 6:396–399

Bates GW (1985) Electrical fusion for optimal formation of protoplast heterokaryons in *Nicotiana*. Planta 165:217–224

Bates GW, Hasenkampf CA, Contolini CL, Piastuch WC (1987) Asymmetric hybridization in *Nicotiana* by fusion of irradiated protoplasts. Theor Appl Genet 74:718–726

Bhojwani SS, Cocking EC (1972) Isolation of protoplasts from pollen tetrads. Nature 239:29–30

Brunold S, 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 208:469–474

Cammaerts D, Jacobs M (1980) A simple electrophoretic procedure for the determination of the polypeptide composition of the subunits of Fraction 1 protein. Anal Biochem 97:317–320

Chyi YS, Phillips GC (1987) High efficiency *Agrobacterium*-mediated transformation of *Lycopersicon* based on conditions favourable for regeneration. Plant Cell Rep 6:105–108

Deak M, Kiss GB, Koncz C, Dudits D (1986) Transformation of *Medicago* by *Agrobacterium* mediated gene transfer. Plant Cell Rep 5:97–100

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

Dudits D, Fejer O, Hadlaczky G, Koncz C, Lazar GB, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283–288

Evans IJ, James AM, Barnes SR (1983) Organization and evolution of repeated DNA sequences in closely related plant genomes. J Mol Biol 170:803–826

Everett NP, Robinson KEP, Mascarenhas D (1987) Genetic engineering of sunflower (*Helianthus annuus* L.). Biotechnology 5:1201–1204

Fillati JJ, Sellmer J, McCown BH, Haissig BE, Comai L (1987) *Agrobacterium* mediated transformation and regeneration of *Populus*. Mol Gen Genet 206:192–200

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130–137

Fry J, Barnason A, Horsch RB (1987) Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. Plant Cell Rep 6:321–325

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Gupta PP, Gupta M, Schieder O (1982) Correction of nitrate reductase defect in auxotrophic plant cells through protoplast mediated intergeneric gene transfer. Mol Gen Genet 188:378–383

Gupta PP, Schieder O, Gupta M (1984) Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. Mol Gen Genet 197:30–35

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

Hamill JD, Pental D, Cocking EC (1985) Analysis of fertility in somatic hybrids of *Nicotiana rustica* and *N. tabacum* and progeny over two sexual generations. Theor Appl Genet 71:486–490

Herrera-Estrella L, De Block M, Messens E, Hernalsteens JP, Von Montagu M, Schell J (1983) Chimaeric gene as dominant selectable marker in plant cells. EMBO J 2:987–995

Horsch RB, Fry JE, Hoffmann NL, Eichholtz NL, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Imamura J, Saul MW, Potrykus I (1987) X-ray irradiation promoted asymmetric somatic hybridization and molecular analysis of the products. Theor Appl Genet 74:445–450

Keller WA, Melchers G (1973) The effect of high pH and calcium on tobacco leaf protoplast fusion. Z Naturforsch 28:737–741

Lagrimini LM, Rothstein S (1987) Tissue specificity of tobacco peroxidae isozymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol 84:438–442

Lowry OH, Rosenbrough NJ, Farr AL, Randall AJ (1951) Protein measurements with the folin phenol reagent. J Biol Chem 193:265–275

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch RB, Fraley RT (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) unsing *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84

Melchers G, Labib G (1974) Somatic hybridization of plants by fusion of protoplasts. I. Selection of light resistant hybrids of "haploid" light sensitive varieties of tobacco. Mol Gen Genet 135:277–294

Menczel L, Nagy F, Lazar G, Maliga P (1983) Transfer of cytoplasmic male sterility by selection for streptomycin resistance after protoplast fusion in *Nicotiana*. Mol Gen Genet 189:365–369

Müller-Gensert E, Schieder O (1987) Interspecific T-DNA transfer through plant protoplast fusion. Mol Gen Genet 208:235–241

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

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

O'Mara JG (1940) Cytogenetic studies on triticale. I. A method for determining the effects of individual *Secale* chromosomes on *Triticum*. Genetics 25:401–408

Pental D, Cocking EC (1985) Some theoretical and practical possibilities of plant genetic manipulation using protoplasts. Hereditas (Suppl) 3:83–93

Pental D, Hamill JD, Cocking EC (1984) Somatic hybridization using a double mutant of *Nicotiana tabacum*. Heredity 53:79–83

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida*. Recovery of plants with *P. hybrida* nuclear genome and *N. tabacum* chloroplast genome. Mol Gen Genet 202:342–347

Pirrie A, Power JB (1986) The production of fertile, triploid somatic hybrid plants (*Nicotiana glutinosa* (n) + *N. tabacum* (2n)), via gametic: somatic protoplast fusion. Theor Appl Genet 72:48–52

Pua EC, Mehra-Palta A, Nagy F, Chua NH (1987) Transgenic plants of *Brassica napus*. Biotechnology 5:815–817

Reiss B, Sprengel R, Will H, Schaller H (1984) A new sensitive method for qualitative and quantitative analysis of neomycin phosphotransferase in crude cell extracts. Gene 30:217–223

Rick CM, DeVerna JW, Chetelat RT, Stevens MA (1986) Meiosis in sesquidiploid hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*. Proc Natl Acad Sci USA 83:3580–3583

Savitsky H (1975) Hybridization between *Beta vulgaris* and *B. procumbens* and transmission of nematode (*Heterodera schachtii*) resistance to sugarbeet. Can J Genet Cytol 17:197–209

Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J 4:25–32

Shahin EA, Sukhapinda K, Simpson RB, Spivey R (1986) Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes:* transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri plasmid T-DNA. Theor Appl Genet 72:770–777

Sidorov VA, Zubko MK, Kuchko AA, Komarnitsky IK, Gleba YY (1987) Somatic hybridization in potato: use of γ-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction. Theor Appl Genet 74:364–368

Stebbins GL Jr (1950) Variation and evolution in plants. Columbia University Press, New York

Toriyama K, Kameya T, Hinata K (1987) Selection of a universal hybridizer in *Sinapis turgida* Del. and regeneration of plantlets from somatic hybrids with *Brassica* species. Planta 170:308–313

Uchimiya H, Murashige T (1974) Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant Physiol 54:936–944

Umbeck P, Johnson G, Barton K, Swain W (1987) Genetically transformed cotton (*Gosypium hirsutum*) plants. Biotechnology 5:263–266

Watts JW, King JM (1984) A simple method for large-scale electrofusion and culture of plant protoplasts. BioScience Rep 4:335–342

Ye J, Hauptmann RM, Smith AG, Widholm JM (1987) Selection of a *Nicotiana plumbaginifolia* unisversal hybridizer and its use in intergeneric somatic hybrid formation. Mol Gen Genet 208:474–481

Zelcer A, Aviv D, Galun E (1978) Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male-sterile *N. tabacum*. Z Pflanzenphysiol 90:397–407

Zimmermann U, Scheurich P (1981) High frequency fusion of plant protoplasts by electric fields. Planta 151:26–32