Mol Gen Genet (1984) 198:7–11

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Interspecific protoplast fusion to rescue a cytoplasmic lincomycin resistance mutation into fertile *Nicotiana plumbaginifolia* plants

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Summary. A lincomycin-resistant cell line, LR105, was isolated in a mutagenized (0.1 mM N-ethyl-N-nitrosourea) callus culture initiated from a haploid *Nicotiana sylvestris* plant. The regenerated plants had an abnormal morphology and did not set viable seeds.

Transfer of lincomycin resistance was attempted from the original *N. sylvestris* nuclear background into *Nicotiana plumbaginifolia* by protoplast fusion, since it was expected that resistance would be cytoplasmically coded. LR105 protoplasts were irradiated with a lethal dose (120 J kg^{-1}; ^{60}Co source), fused with sensitive *N. plumbaginifolia* protoplasts and the colonies grown from the fused population were screened for lincomycin resistance. Expression of resistance was expected only if the cytoplasm of the irradiated cells had mixed with nonirradiated cytoplasm, and was reactivated as a result of cell fusion (Menczel et al. 1982).

Plants were regenerated in 44 resistant clones. Plants in 41 clones had a *N. plumbaginifolia* nuclear genome. In three clones somatic hybrids were obtained. The resistant *N. plumbaginifolia* cybrid plants were fertile, unlike the original LR105 plants. Lincomycin resistance was inherited maternally in the eight clones in which crosses were made. In these clones the introduction of *N. sylvestris* chloroplasts into a *N. plumbaginifolia* nuclear background was confirmed by the *Sma*I restriction endonuclease pattern of the chloroplast DNA. The involvement of chloroplast DNA in determining lincomycin resistance is therefore implied.

Introduction

Selection in cultured plant cells resulted in the isolation of novel phenotypes, including cell lines resistant to herbicides and antibiotics, and overproducers of specific amino acids (reviewed in Bourgin 1983; Negrutiu et al. 1984; Maliga 1984a). Utilization of a mutation requires that it be available in fertile plants. In some of the mutant cell lines the regeneration ability has been lost, in others it was maintained but the plants were partially or fully sterile (reviewed in Maliga 1984a).

Different strategies have been applied to introduce the new mutations into fertile plants. Partial sterility could be corrected by crossing the regenerated plants with the original wild-type form (Cséplő and Maliga 1982; Keil and Chaleff 1983). However, back-crossing is not feasible if regeneration ability has been lost, or the plants are fully sterile. Mutant alleles coding for defective nitrate reductase apoenzyme and coenzyme have been rescued from nonmorphogenic mutant cells in fertile heterozygous plants by protoplast fusion (Márton et al. 1983). In this paper we describe the rescue of another mutation, lincomycin resistance. This trait was cytoplasmically coded, unlike the nitrate reductase deficiency mutations which were nuclear.

A new mutation, lincomycin resistance, was available in a *N. sylvestris* regenerate, LR105, which did not flower (this paper). Since the mutation was expected to be cytoplasmic (Cséplő and Maliga 1982, 1984), cell fusion was used to transfer the resistant cytoplasm into a normal nuclear background. To be able to prove that the resistance was acquired as a result of organelle transfer, *N. plumbaginifolia* was chosen as the recipient. The chloroplasts and mitochondria of the two species can be distinguished by their DNA restriction patterns.

Materials and methods

Callus culture and plant regeneration. N. sylvestris calli were induced from sterile leaves of haploid (n = 12) plants on RMO medium (Maliga et al. 1973). Shoots were induced from the LR105 calli on the same medium. The shoots were rooted and maintained as plants on RM salts (Murashige and Skoog 1962) containing 3% sucrose and 0.7% Bacto-Agar (RM medium). *N. plumbaginifolia* shoots were regenerated from protoplast-derived calli on RMOP medium (Menczel et al. 1982). The shoots were rooted, and the plants were maintained on RM medium. The cultures were incubated at 28° C; illuminated for 16 h at 1,000 lx. Cell culture procedures cited above, and in the section on protoplasts have been summarized in a technical paper (Maliga 1984b).

Mutagenic treatment of N. sylvestris calli. For mutagenic treatment callus pieces (about 20 mg) were placed into liquid RMO medium containing 0.1 mM N-ethyl-N-nitrosourea (NEU, filter sterilized) for 24 h. Incubation was carried out in the dark. After treatment the calli were washed in distilled water.

Protoplast isolation, fusion and culture. N. sylvestris LR105 and *N. plumbaginifolia* protoplasts were prepared from leaves of sterile plants (Menczel et al. 1981). LR105 proto-

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plasts were inactivated by irradiation (^{60}Co; 120 J kg^{-1}, dose rate 0.042 J kg^{-1} s^{-1}) as described in Menczel et al. (1982). Irradiated LR105 protoplasts were fused with *N. plumbaginifolia* protoplasts in a 1:2 ratio. Protoplast fusion was induced by polyethylene glycol (Menczel et al. 1981).

The protoplasts were cultured in K_3 medium (Kao et al. 1974) as modified by Nagy and Maliga (1976) containing 0.4 M glucose as an osmotic stabilizer. The cultures were diluted weekly, altogether 20-fold during a 1 month time period. The K_3 medium used for dilution contained decreasing concentrations (0.4–0.2 M) of glucose. After 1 month the colonies were plated into a selective lincomycin medium (1,000 mg l^{-1} lincomycin-HCl in RMOP medium containing 0.2 M glucose). Fresh protoplast cultures were incubated in dim light (100 lx, 16 h, 27° C). Selection for lincomycin resistance was carried out at 1,000 lx.

Testing of plants (leaf assay) and seedlings for antibiotic resistance. The phenotype of plants was determined by placing leaf sections onto a selective RMO or RMOP medium containing 1,000 mg l^{-1} lincomycin-HCl, 100 mg l^{-1} clindamycin phosphate and 1,000 mg l^{-1} streptomycin sulfate, respectively. Calli forming on the leaf section were green or white, indicating resistance or sensitivity, respectively.

For the seedling assay the seeds were surface-sterilized (Maliga et al. 1982) and germinated on a selective RM medium (RM salts, 3% sucrose, 100 mg l^{-1} lincomycin, 0.5% Bacto-Agar). Green and white seedlings were classified as being resistant and sensitive, respectively.

Cytology. The chromosomes in root tip cells were stained with acetocarmine after colchicine treatment (0.05%, 3 h).

Biochemical methods. Leaf esterase isoenzyme patterns were determined as previously described (Menczel et al. 1982). Chloroplast DNA was prepared according to Vedel et al. (1976). DNA was digested with *Sma*I restriction endonuclease, and the fragments were separated on 0.7% agarose gels (Nagy et al. 1981). The isolation and restriction of mitochondrial DNA by *Bam*HI were carried out as published by Nagy et al. (1983).

Results

Isolation of the lincomycin-resistant clone, LR105, in N. sylvestris

On RMO medium the *N. sylvestris* callus is pigmented green. Lincomycin (1,000 mg l^{-1}) and clindamycin (7-chloro-7-oxy-lincomycin, 100 mg l^{-1}) prevented formation of chlorophyll resulting in white calli (Cséplő and Maliga 1982). However, the antibiotics did not seriously inhibit callus growth. Four cell lines resistant to clindamycin bleaching were visually identified as green sectors in the mutagenized cultures (1013 calli screened). Resistance in one of them, LR105, was stable during two subsequent culture cycles on the selective medium. No resistant sectors were found in 2250 nonmutagenized calli.

Five plants regenerated from the LR105 cell line were tested for lincomycin and clindamycin resistance. All plants were resistant to both antibiotics by the leaf assay (data not shown). In subsequent tests only lincomycin was used. For this reason the line will be referred to as lincomycin resistant.

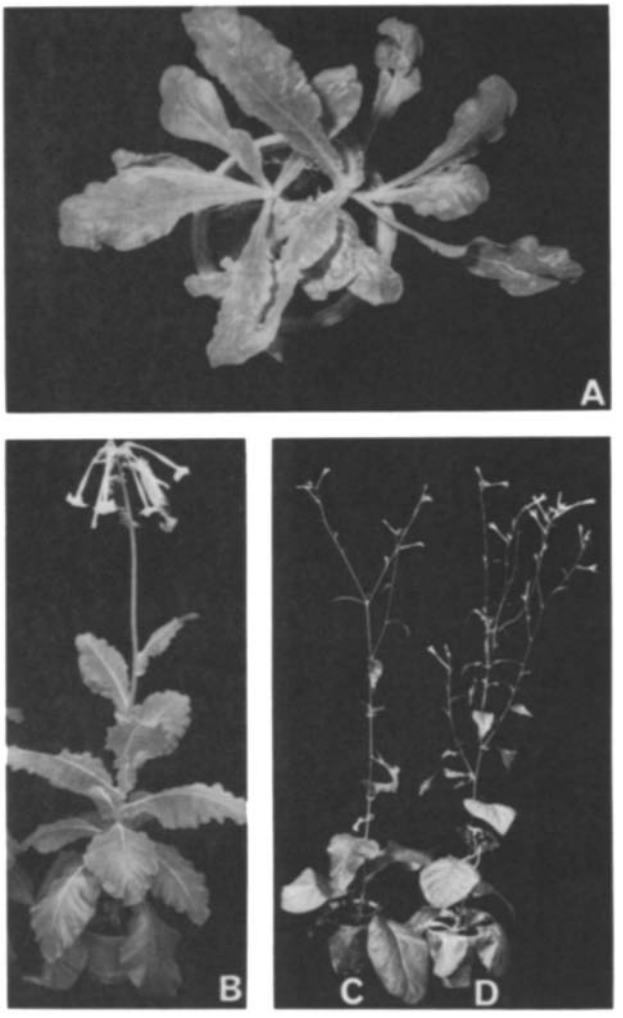

Fig. 1A–D. Morphology of the parental plants and of the tissue culture regenerates. **A** LR105; **B** wild-type *N. sylvestris*; **C** diploid *N. plumbaginifolia* cybrid; **D** wild-type *N. plumbaginifolia*

The LR105 plants grew slowly in the greenhouse, and had an abnormal morphology (Fig. 1A). The chromosome number in the root tip cells varied between 20 and 24. (The diploid chromosome number in *N. sylvestris* is $2n=24$). Due to the lack of flowering no back-cross could be made to normalize the plants or to determine the mode of inheritance of the lincomycin resistance factor.

Protoplast fusion to transfer the lincomycin resistance into N. plumbaginifolia

Irradiated, lincomycin-resistant *N. sylvestris* LR105 protoplasts were fused with *N. plumbaginifolia* (lincomycin sensitive) protoplasts. The irradiation dose used prevents colony formation. Mixing of the irradiated cytoplasm with nonirradiated cytoplasm was expected to help recovery of the irradiated portion, and allow expression of lincomycin resistance (Menczel et al. 1982). Accordingly, after fusion small colonies were grown from the fused protoplast population and were screened for lincomycin resistance (see Materials and methods). The resistant green calli were identified visually among the sensitive white ones. The frequency of the resistant calli was about 5×10^{-3} in the fused population. The lincomycin resistance of the green calli obtained was retested twice on the selective medium.

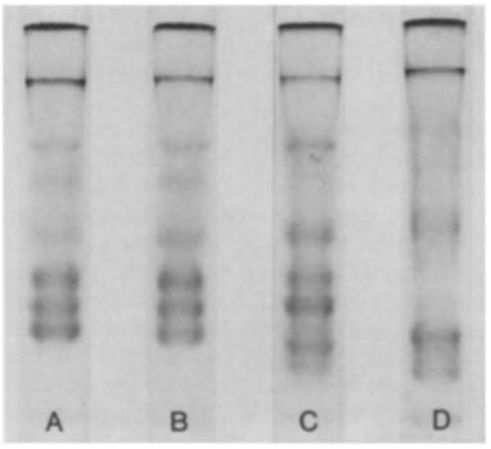

Fig. 2A–D. Leaf esterase isoenzyme patterns to identify parental-type plants and somatic hybrids. **A** Wild-type *N. plumbaginifolia;* **B** resistant *N. plumbaginifolia* regenerate; **C** somatic hybrid; **D** LR105 (*N. sylvestris*)

Characterization of plants regenerated from the resistant clones after protoplast fusion

Plants were regenerated in 90 out of the 109 resistant clones. The 19 clones in which no shoot formation could be induced were discarded.

Regenerated plants (three from each clone) were studied further in a random sample of 44 lincomycin-resistant cell lines. These plants were all resistant to lincomycin. They were cross-resistant to clindamycin (100 mg l^{-1}), but sensitive to streptomycin (1,000 mg l^{-1}; data on leaf assays not shown).

The regenerated plants were further characterized with respect to their nuclear genetic composition. The appearance of two types of resistant clones could be predicted: *N. plumbaginifolia* lines, which acquired the cytoplasmic lincomycin resistance by cell fusion, or were new spontaneous mutants (these could be distinguished by their chloroplast DNA, as discussed below) and somatic hybrids which were formed by the fusion of the irradiated *N. sylvestris* nucleus with a *N. plumbaginifolia* nucleus subsequent to protoplast fusion (for discussion of the fate of nuclei and organelles in heterokaryons see Medgyesy et al. 1980; Sidorov et al. 1981; Maliga et al. 1982). Classification was based on leaf esterase isoenzymes by which the parental species and the somatic hybrids could be distinguished (Fig. 2). Plants in 41 clones (93%) were identified as *N. plumbaginifolia.* In three clones (7%) somatic hybrids were obtained. The morphology of the plants was in agreement with the isoenzyme data.

Chromosome numbers have been determined in some of the *N. plumbaginifolia* type regenerates. The plants were diploid ($2n=20$) in most clones (14 out of 22), tetraploid in four clones, and aneuploid (31–38 chromosomes) in an additional four clones. Regeneration of polyploid and aneuploid plants is the consequence of tissue culture-induced chromosome instability, a phenomenon well documented in flowering plants (Bayliss 1980).

Inheritance of lincomycin resistance in the regenerated N. plumbaginifolia plants

Plants from eight diploid clones were grown to maturity (Fig. 1C), selfed and crossed with wild-type *N. plumbaginifolia* plants. The resistance or sensitivity of the progeny was determined by the seedling assay (see Materials and methods). Seedlings that were able to form green pigment were classified as resistant whereas white seedlings were scored as sensitive (Fig. 3). Lincomycin resistance was inherited maternally in each of the eight clones tested (Table 1).

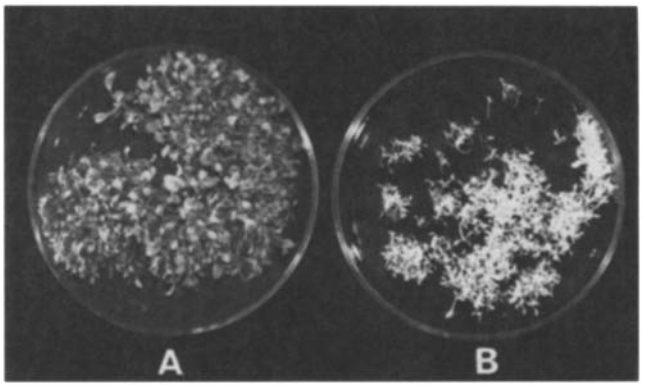

Fig. 3A, B. Seedling assay to test lincomycin resistance. **A** Resistant; **B** sensitive

Table 1. Maternal inheritance of lincomycin resistance in the *N. plumbaginifolia* regenerates[a]

Line[b]	Selfs	F_1[c]	RF_1[d]
Np/LR105/1	163/0	158/0	0/314
Np/LR105/17	12/0	147/0	0/72
Np/LR105/35	681/0	206/0	0/161
Np/LR105/37	72/0	388/0	0/63
NP/LR105/44	648/0	212/0	0/392
Np/LR105/47	759/0	829/0	0/430
Np/LR105/91	76/0	143/0	0/98
Np/LR105/94	389/0	277/0	0/209

[a] The number is given of green/white, that is resistant/sensitive seedlings germinated on lincomycin (100 mg l^{-1})-containing RM medium

[b] Line description: Np refers to the nucleus; in brackets the source of cytoplasm is indicated followed by the isolation number

[c] F_1 was obtained by pollinating the regenerates with wild-type pollen

[d] RF_1 is the reciprocal F_1, in which the resistant plants were used as pollinators

Chloroplast and mitochondrion types in the resistant N. plumbaginifolia cybrids

The chloroplast type (*N. plumbaginifolia* or *N. sylvestris*) was determined in plants representing ten independent clones, including the eight diploid clones listed in Table 1, and two tetraploid clones. Chloroplast identification was based on the species-specific restriction pattern of chloroplast DNAs (Fig. 4). The *N. plumbaginifolia* plants in each of the ten clones carried *N. sylvestris* type chloroplasts. None of the lines studied, therefore, was a new spontaneous mutant.

The mitochondrial DNA pattern of the ten clones mentioned above was analysed. The restriction pattern of all the ten clones proved to be of nonparental, recombinant type (data not shown).

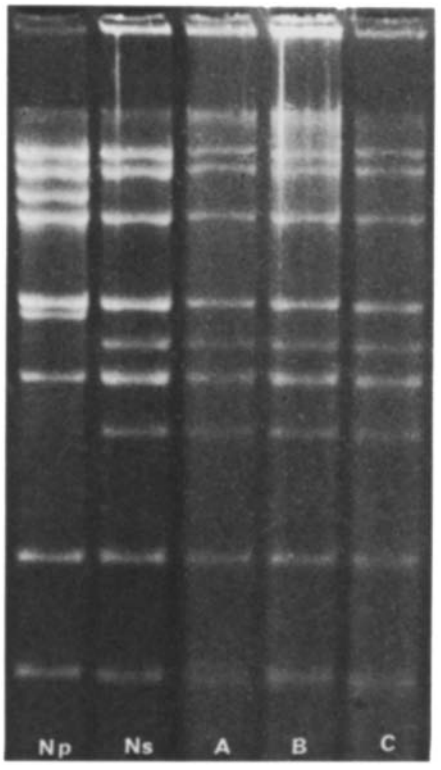

Fig. 4A–C. Identification of the chloroplasts in the regenerated plants by the *Sma*I restriction pattern of the chloroplast DNA. Np, *N. plumbaginifolia*; Ns, *N. sylvestris;* **A–C** three of the resistant *N. plumbaginifolia* regenerates

Discussion

There are several reports on tissue culture-induced morphological and karyotypic changes in plants, e.g., in *Nicotiana* (Melchers 1965; Sacristán and Melchers 1969; Maliga et al. 1979; Prat 1983). This would suggest that the abnormalities of the LR105 line could be a consequence of the use of tissue culture for selection. Multiple mutations induced by the mutagen NEU could also be the reason for the sterility and abnormalities (Hagemann 1982). NEU treatment was shown to be the reason for such changes in regenerated *N. plumbaginifolia* plants in a different experiment (Cséplő and Maliga 1984).

Lincomycin resistance has been transferred from the *N. sylvestris* LR105 line into a *N. plumbaginifolia* nuclear background by marker rescue from irradiated protoplasts. The lincomycin-resistant *N. plumbaginifolia* plants were fully fertile, and could not be distinguished morphologically from the original sensitive plants. Abnormalities of the LR105 line therefore were separable from lincomycin resistance.

Transfer of resistance could be achieved without an increase in the chromosome number in the recipient *N. plumbaginifolia* plants, many of which remained diploid after plant regeneration. In these plants, and in their selfed progeny lincomycin resistance was the only *N. sylvestris* trait expressed. The feasibility of transferring resistance without transferring chromosomes from the donor indicates that the resistance is cytoplasmically coded. This was further confirmed by showing that lincomycin resistance was maternally inherited after transfer (Table 1). Maternal inheritance is the rule of transmission of cytoplasmic genes in *Nicotiana* (Gillham 1978; Kirk and Tilney-Bassett 1978; Sears 1980).

Selection for lincomycin resistance resulted in the cotransfer of resistance and the LR105 (*N. sylvestris*) chloroplasts (Fig. 4), suggesting the involvement of chloroplast DNA in determining lincomycin resistance. Coding for resistance by the chloroplast DNA is in full agreement with the maternal inheritance of the trait. Mitochondria, however, are also maternally inherited. Donor and recipient mitochondria used in this experiment can be distinguished by their DNA (Nagy et al. 1983). The role of mitochondria in determining lincomycin resistance could not be clarified since in the cybrids only nonparental, recombinant mitochondria were found. Experiments aimed at locating cytoplasmic streptomycin resistance gave similar results. Cosegregation could be used to confirm the role of chloroplasts in conferring streptomycin resistance (Menczel et al. 1981, 1982, 1983; Maliga et al. 1982). Rearrangements in the mitochondrial DNA, however, made it impossible to draw any conclusions concerning the involvement of mitochondria (Nagy et al. 1981).

The frequency of cybrid formation depends on the effectiveness of the irradiation in preventing nuclear fusion in the heterokaryons. In the present experiment 93% of the resistant clones recovered were cybrid, having a new combination of the *N. plumbaginifolia* nucleus with the cytoplasmic factor from the irradiated *N. sylvestris*. The same irradiation dose (120 J kg^{-1}) gave about 57% cybrids in a different species combination (*N. tabacum* as donor, carrying streptomycin-resistant chloroplasts, was irradiated and fused with *N. plumbaginifolia* as recipient; Menczel et al. 1982). The difference in the frequency of nuclear segregation could be due to differences in the genome size and therefore in radiosensitivity (*N. tabacum* is an allotetraploid species whereas *N. sylvestris* is diploid). In the rest of the clones somatic hybrids were obtained as the result of nuclear fusion in the heterokaryons.

No segregation of lincomycin resistance was found in the seed progeny of the eight clones investigated. This indicates that lincomycin selection was effective, and no sensitive cytoplasmic determinant was present at the time of plant regeneration, from which time no further selection pressure had been applied. No segregation was found in the seed progeny either, after applying streptomycin selection pressure in chloroplast transfer experiments (Medgyesy et al. 1980; Menczel et al. 1982, 1983; Maliga et al. 1982). In the absence of selection pressure, however, chloroplast heterogenity was maintained through at least two seed generations (Fluhr et al. 1983). In the case of new lincomycin-resistant isolates chloroplast segregation took a longer time to complete. In that experiment, following a similar protocol, out of 17 clones segregation was found in the seed progeny of five (Cséplő and Maliga 1984). Differences in the speed of segregation may be explained by the different initial ratios of resistant and sensitive chloroplasts. A new mutation arises only in one of the chloroplasts present in a cell whereas in the case of transfer by cell fusion segregation starts from a roughly equal mixture of parental chloroplasts.

Acknowledgement. Part of this research was supported by OMFB grant No. 1982/F/2.3.1.

References

Bayliss MW (1980) Chromosomal variation in plant tissues in culture. In: Vasil IK (ed) Perspectives in plant cell and tissue culture. Int Rev Cytol, Suppl 11A. Academic Press, New York, pp 113–144

Bourgin JP (1983) Protoplasts and the isolation of plant mutants. In: Potrykus I, Harms CT, Hinnen A, Hütter R, King PJ, Shillito RD (eds) Protoplasts 1983. Lecture proceedings. Birkhäuser Verlag, Basel, pp 43–50

Cséplő A, Maliga P (1982) Lincomycin resistance, a new type of maternally inherited mutation in *Nicotiana plumbaginifolia*. Curr Genet 6:105–109

Cséplő A, Maliga P (1984) Large scale isolation of maternally inherited lincomycin resistance mutations, in diploid *Nicotiana plumbaginifolia* protoplast culture. Mol Gen Genet 196:407–412

Fluhr R, Aviv D, Edelman M, Galun E (1983) Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusions in *Nicotiana*. Theor Appl Genet 65:289–294

Gillham NW (1978) Organelle heredity. Raven Press, New York

Hagemann R (1982) Induction of plastom mutations by nitrosourea-compounds. In: Edelman M, Hallick RB, Chua NH (eds) Methods in chloroplast molecular biology. Elsevier/Biomedical Press, Amsterdam, pp 119–127

Kao KW, Constabel F, Michayluk MR, Gamborg OL (1974) Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 120:215–227

Keil RL, Chaleff RS (1983) Genetic characterization of hydroxyurea-resistant mutants obtained from cell cultures of *Nicotiana tabacum*. Mol Gen Genet 192:218–224

Kirk JTO, Tilney-Bassett REA (1978) The Plastids. Their chemistry, structure, growth and inheritance. Elsevier/North Holland, Amsterdam

Maliga P (1984a) Isolation and characterization of mutants in plant cell culture. Annu Rev Plant Physiol 35:519–542

Maliga P (1984b) Cell culture procedures for mutant selection and characterization in *Nicotiana plumbaginifolia*. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, New York, pp 552–562

Maliga P, Sz-Breznovits Á, Márton L (1973) Streptomycin resistant plants from callus culture of haploid tobacco. Nature New Biol 244:28–30

Maliga P, R-Kiss Z, Dix PJ, Lázár G (1979) A streptomycin-resistant line of *Nicotiana sylvestris* unable to flower. Mol Gen Genet 172:13–15

Maliga P, Lörtz H, Lázár G, Nagy F (1982) Cytoplast-protoplast fusion for interspecific chloroplast transfer in *Nicotiana*. Mol Gen Genet 185:211–215

Márton L, Biasini G, Sidorov V, Maliga P (1983) Nitrate reductase deficient plants in the progeny after selfing complemented somatic hybrids of *Nicotiana plumbaginifolia* mutants. In: Potrykus I, Harms CT, Hinnen A, Hütter R, King JP, Shillito RD (eds) Protoplasts 1983. Poster Proceedings. Birkhäuser Verlag, Basel, pp 100–101

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and isolation of their somatic hybrids. Mol Gen Genet 179:693–698

Melchers G (1965) Einige genetische Gesichtspunkte zu sogenannten Gewebekulturen. Ber Dtsche Bot Ges 98:21–29

Menczel L, Nagy F, Kiss Z, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor Appl Genet 59:191–195

Menczel L, Galiba G, Nagy F, Maliga P (1982) Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana*. Genetics 100:487–495

Menczel L, Nagy F, Lázár 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

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

Nagy JI, Maliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z Pflanzenphysiol 78:453–455

Nagy F, Török I, Maliga P (1981) Extensive rearrangements in the mitochondrial DNA in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*. Mol Gen Genet 183:437–439

Nagy F, Lázár G, Menczel L, Maliga P (1983) A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. Theor Appl Genet 66:203–207

Negrutiu J, Jacobs M, Caboche M (1984) Advances in somatic cell genetics of higher plants – the protoplast approach in basic studies on mutagenesis and isolation of biochemical mutants. Theor Appl Genet 67:289–304

Prat D (1983) Genetic variability induced in *Nicotiana sylvestris* by protplast culture. Theor Appl Genet 64:223–230

Sacristán MD, Melchers G (1969) The caryological analysis of plants regenerated from tumorous and other callus culture of tobacco. Mol Gen Genet 105:317–333

Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4:233–255

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplats. Planta 152:341–345

Vedel F, Quetier F, Bayen M (1976) Specific cleavage of chloroplast DNA from higher plants by EcoRI restriction endonuclease. Nature 263:440–442

Communicated by G. Melchers

Received August 9, 1984