Theor Appl Genet (1996) 93: 809–815

R. Dirks · V. Sidorov · C. Tulmans

A new protoplast culture system in *Daucus carota* L. and its applications for mutant selection and transformation

Received: 10 December 1995 / Accepted: 23 February 1996

Abstract Petiole protoplasts from *in vitro*-grown carrot plants are a very good alternative to traditionally obtained protoplasts from suspension cultures. High plating and regeneration efficiencies were obtained in most of the breeding lines that were tested. The embedding of the protoplasts in alginate was crucial for initiating cell division and further development. Several streptomycin resistant and chlorophyll-deficient plant lines were selected for using the petiole protoplast system. Maternally inherited streptomycin resistance was demonstrated by sexual crosses. Protoplast fusion of several chlorophyll-deficient lines did not result in complementation, indicating the cytoplasmic nature of the mutations. Petiole protoplasts were used for direct transformation with plasmid DNA pNUN7 containing *NPTII* as a selectable marker. High transformation frequencies (up to 1%) were obtained after PEG treatment of the protoplasts. Kanamycin resistance was shown to be inherited as a single dominant nuclear trait.

Key words Protoplasts · Mutant · Streptomycin · Chloropyll deficiency · Transformation

Introduction

The culture and development of embryos from carrot protoplasts were first described by Kamya and Uchimya (1972). In their experiment, 50-day-old field-grown roots were used as a direct source of protoplasts. Since then, however protoplast culture and plant regeneration in carrot have only been reported from cell suspensions as donor material (see Grambow et al. 1972; Tanno-Suenaga et al. 1991; Kisaka and Kameya 1994).

The utilization of protoplasts from suspension cultures imposes some drawbacks with respect to genetic stability, especially when the regenerated material has to be incorporated in future breeding programmes. When cell cultures are kept for a long time in an undifferentiated stage, the capacity to regenerate plants will decrease over the course of time while the frequency of somaclonal variants will increase (Karp 1989). For breeding programmes it is therefore highly recommended to reduce the duration of the undifferentiated cell culture phase to a minimum. Cell suspensions have to be maintained by regular subcultures (usually once a week) or have to be reinitiated from callus cultures. The application of this method to many breeding lines is cumbersome and very laborious. The use of *in vitro* plants has the advantage that fully differentiated tissue is used and that the stock of the protoplast mother plants may not have to be maintained and multiplied no more often than every 6 weeks.

In this paper we describe a method for the culture of petiole protoplasts that is amenable to most breeding lines, and gives high protoplast yields (2×10^6 ppl/g tissue), high plating efficiencies (60–90%), and high regeneration frequencies (50–75%). The proposed culture system proved efficient for the production of cytoplasmic mutants and will be further used for cytoplasmic male-sterile transfer. Petiole protoplasts were also used for direct transformation with plasmid DNA carrying the NPTII gene. Progeny of the kanamycin-resistant transformants was obtained after selfing and was further analyzed by the polymerase chain reaction. With the petiole protoplast culture system, regeneration can be accomplished within 3 months after initiation of the culture.

Communicated by Y. Gleba

R. Dirks (✉) · C. Tulmans
Nunhems Zaden B. V., P.O. Box 4005, 6080 AA Haelen, The Netherlands

V. Sidorov[1]
Institute of Cell Biology and Genetic Engineering, Zabolotnogo street 148, Kiev, GSP-22 Ukraine

Present address:
[1] Monsanto, 700 Cherterfield Pkwy – North, St. Louis, MO 63178, USA

Materials and methods

Plant material

For the production of chlorophyll deficient variant lines, breeding line B4 001598 [Nantes type, cytoplasmic male-sterile (CMS)] was used. For the selection of transformants we used a Long Imperator type (line Nun 58). All other lines are listed in Table 2.

Seed sterilization

Seeds from various breeding lines were made aseptic by immersion in 70% ethanol for 1 min and by subsequent sterilization for 45 min in 4% commercial bleach After repeated washing with sterile water, the seeds were sown in petri dishes containing MS medium (Murashige and Skoog 1962) with 20 g/l sucrose and solidified with 9 g/l agar.

Plant propagation and rooting

Fully matured plants derived from seedlings or regenerants can be vegetatively propagated by transferring shoot apexes with 5–8 internodes onto MS medium containing 1 mg/l BAP (6-benzylaminopurine). After about 1 month, usually one to three newly formed shoots can be cut from the initial plant. The newly developed plants can then be rooted on MS medium supplemented with 20 g/l sucrose and 1 mg/l IBA (indole butyric acid).

Protoplast isolation and culture

Petioles from *in vitro* grown plants were cut into small pieces (2–3 mm^2) in MLP medium with the following composition: 1/2 MS macro-salts and FeEDTA, 2.2 g/l $CaCl_2.2H_20$ 74 g/l mannitol (pH 5.8 before autoclaving). The petiole tissue was then washed once more with MLP medium and subsequently digested in EKW enzyme solution [2% cellulase R10 Onozuka, 0.1% macerozyme, 0.1% $CaCl_2.2H_2O$, 0.06% MES buffer, 13% sucrose (pH 5.6, filter sterile)]. The digestion took place overnight in the dark at 27°C.

Protoplasts were separated from undigested tissues by passage through a 80-μm stainless steel sieve. The protoplast suspension was transferred to centrifuge tubes (10 ml) and overlaid with 1 ml W5 medium (Menczel et al. 1981) before centrifugation at 800 rpm for 7 min. Protoplasts were recovered from the interphase between W5 and EKW and were pelleted in W5 medium. After this step, the protoplasts were further treated and embedded essentially as described by Damm et al. (1989), then modified only by mixing 0.5 ml of the protoplast suspension with an equal amount of sodium alginate solution.

The alginate layers containing the protoplasts were incubated in 3 ml CPP medium (petri dishes: 6 cm diameter, Greiner TC quality) with the following composition: macro- and micro elements, organic acids and sugars according to Kao and Michayluk (1975), FeEDTA according to MS (Murashige and Skoog 1962), vitamins as in B5 (Gamborg et al. 1968), 74 g/l glucose, 250 mg/l casamino acids. Hormones were varied as described in Table 1. The CPP medium was adjusted to pH=5.6 and filter-sterilized. After 10 days of culture the medium was replaced by the same fresh culture medium

When the protoplast-derived colonies reached an average size of about 30 cells, the alginate layers were incubated in a solution containing 20 mM sodium citrate and 76 g/l mannitol (pH 5 8 before autoclaving). Following this treatment, all colonies were usually released within 2 h. Alginate residues and citrate solution were removed by centrifugation. The colonies were resuspended in CPPD medium, which has the same composition as the CPP medium as mentioned above, modified by replacing glucose with 30 g/l sucrose and 20 g/l mannitol. In general, colonies derived from one alginate layer were resuspended in 10 ml CPPD medium and from this suspension 1 ml was plated onto solidified medium. Hormones in CPPD medium were varied according to Table 3. Colonies were plated on the same medium solidified with 8 g/l agar (15 min autoclaving at 110°C). Greening was obtained in the light (2000 lux; 16-h day, 8-h night cycle, 27°C).

Plant regeneration

Green colonies were transferred to B5 medium containing 0.1 mg/l 2,4D and placed in the dark for 3–4 weeks. After this period they were transferred to the same medium without 2,4D and left in the dark for another 2 weeks. When embryogenesis occurred, the embryo containing calli were transferred into the light. Regenerated plants were rooted on IBA-containing medium as described above.

Mutagenesis and mutant selection

Protoplasts were mutagenized with freshly prepared *N*-nitroso-*N*-methylurea (NMU) dissolved in W5 medium at a concentration of 0.1–0.2 m*M*. After 1 h of exposure, the mutagen was removed by washing and centrifugation (twice). Mutagenized protoplasts were cultured as described above, except that for selection of the streptomycin-resistant mutants, 250 mg/l streptomycin was included in the CPP culture medium when the colonies reached a size of 10–15 cells and 1000 mg/l streptomycin was included in the CPPD medium.

Fusion-complementation analysis

Protoplasts of four chlorophyll deficient lines were fused to study complementation as described earlier (Sidorov and Maliga 1982). Petioles of in vitro-grown plant lines were used as a source of protoplasts. After fusion, protoplasts were imbedded in alginate and screened for greening on CPPD medium with 0.1 mg/l α-NAA and 0.2 mg/l zeatine.

Transformation and PCR analysis

The plasmid pNUN7 [a PBI 121 derivative (Clontech)] carrying the *NPTII* gene under the control of the CaMV 35S promoter was used for transformation. Direct gene transfer was performed with some modifications according to Dewulf and Negrutiu (1991). Aliquots of 2×10^6 protoplasts to which 20–40 µg of plasmid DNA was added were treated with PEG [40% PEG 4000, 0 4 *M* mannitol, 0.1 *M* Ca(NO3)2.4H_2O, pH 7.0] for 5 min. After gradual dilution and washing with W5 medium, the protoplasts were resuspended in 0.5 *M* mannitol solution and embedded into alginate as described before.

Selection pressure (100 mg/l of kanamycin) was applied after 3–4 days of culture. Plants from kanamycin-resistant calli were obtained on hormone-free medium lacking kanamycin and transferred to soil for further growth. The roots of putative transformants were collected, vernalized for 4 months at 4°C and planted again for seed production. The seeds produced were screened for kanamycin resistance on MS hormone free medium supplemented with 100 mg/l kanamycin. Genomic DNA was isolated from plantlets selected on kanamycin. Primers specific to the kanamycin resistance gene were used in a polymerase chain reaction (PCR) to detect the presence of the introduced gene. The PCR cycle was 1 min at 94°C. 2 min at 60°C and 2 min at 72°C for a total of 35 cycles. The amplification products were separated by agarose gel electrophoresis.

Ploidy determination

Ploidy level was determined by flow cytometry [Plant Cytometry Services, Schijndel (NL)].

Results

Protoplast culture and regeneration

In our first attempt we used petioles and leaf tissue as a source for direct protoplast culture. In both cases we obtained an average of 2×10^6 protoplasts per gram tissue. Freshly prepared petiole protoplasts look rather uniform and are shown in Fig. 1. The hormone variations that were tested in liquid CPP medium are described in Table 1. In practically all media, colonies were formed, but at a very low (< 0.1%) plating efficiency and highly unreproducible. When alginate was used as an embedding matrix, reproducible high plating efficiencies were obtained, especially with petiole tissue, and clear differences in the response to hormonal treatments were noted (Table 1). It was astonishing that even in medium without hormones or with either cytokinin or auxin, colony formation took place and microcolonies could be obtained up to a size of more than 10 cells. Because of the slow growth of these variants, only medium containing 0.1 mg/l 2,4D and 0.2 mg/l zeatine which gave the highest plating efficiency and very reproducible results, was used in subsequent experiments. In this medium, the first divisions occurred as early as by the second day of protoplast culture. Cell clumps of 10–15 cells per colony are usually obtained only after 15 to 20 days.

The CPP medium with 0.1 mg/l 2,4D and 0.2 mg/l was used as a standard medium in all further experiments.

The suitability of this medium was tested for a variety of breeding lines (Table 2). It is clear that genotypical differences do occur. In all the lines we have cultured so far, we have not been able to identify a genotype that did not respond to the standard culture medium. It is perhaps possible to set up a special culture medium for genotypes with a low response, thereby improving the plating efficiency as compared to what is obtained on the standard medium. It is always possible to select mutants of any line using the standard medium because of the high protoplast yields.

Protoplast-derived colonies were plated on CPPD medium supplemented with various compositions of hormones (Table 3). In general, about 20 000 colonies were plated on solidified medium with an average plating efficiency of 50%. Table 3 gives an estimation of the suitability of the plating media tested. It is difficult to estimate the possible number of colonies that have been formed. In both media with either 0.1 mg/l 2,4 D and 0.1 mg/l zeatine or 0.1 mg/l α-NAA and 0.1 mg/l zeatine, the plates were fully covered with colonies.

In the following experiments, only the combination with α-NAA was used because this also enables selection for greening ability when antimetabolites such as streptomycin are included in the culture medium.

Plant regeneration

In order to regenerate plants, the suitable classical approach to obtain embryogenesis was applied (see Materials and methods). For genotype 932156 an average regeneration frequency of 71% was obtained (average of three independent experiments with 80 calli per experiment); for genotype 803480 the average score was 35%. In all of the other genotypes tested we found regeneration frequencies between 10 and 90%. It was also observed that calli not regenerable in a first round of 2,4 D induction could, in several cases, be regenerated when returned to 2,4 D medium and then back to hormone-free medium.

Table 1 Results of various hormones on the plating efficiency of petiole protoplasts in CPP medium for breeding line 032057× 032058. Plating efficiency (PE) was determined 3 weeks after initiation of the culture

2.4D (mg/l)	Zeatine (mg/l)	PE (%)
0	0	4
0	0.1	16
0.1	0	11
0.1	0.1	43
0.1	0.2	51
0.2	0.1	51

Table 2 Estimated plating efficiencies of petiole protoplast cultures of various breeding lines of carrot

Genotype	Plating efficiency (%)
732243	30
903845	60
001600	5
001598	80
032204	10
91x92/5	70
003645	50

Table 3 Effect of hormonal treatment in CPPD medium. Genotype 932156 was used as the source of protoplasts; the plating efficiency was approximately 50%. About 20 000 protoplast derived colonies were plated per petri dish

2,4D	α-NAA	Zeatine	Observations
–	–	–	Few growing colonies; few embryos
0.01	–	–	Few colonies with root formation
0.01	–	0.1	Reasonable colony formation; root growth
0.05	–	–	Few colonies; yellow; root formation
0.1	–	0.1	Very good colony formation; yellow colour
	0.1	0.2	Very good colony formation; green

Selection for streptomycin resistance and genetic analysis of the regenerated line

Two experiments were undertaken for the selection of streptomycin-resistant lines (see Table 4).

Eight resistant variant colonies were identified by their greening ability in selective streptomycin concentrations. Of the four regenerants, one plant was tetraploid whereas the others were diploid. One of the diploid regenerants was further studied and designated as N6. Because of its back-

Table 4 Selection of streptomycin-resistant and chlorophyll-deficient lines in protoplast cultures. Protoplasts were exposed to NMU for 1 h

Number of plated protoplasts	NMU concentration (m*M*)	Number of (frequency) variant lines		Number of regenerants
		Streptomycin resistant	Chlorophyll deficient	
9×10^6	0.1	8 ($0.9\ 10^{-6}$)		4
22×10^6	0.2	2 ($0.1\ 10^{-6}$)		–
1×10^6	0.2		14 ($1.4\ 10^{-5}$)	8

[a] Approximately 80% of the mutagenized protoplasts were viable after 1 h (0.1–0.2 m*M* NMU)

ground (032058×03260), N6 is male-sterile (brown anther type). Therefore, it was backcrossed with a maintainer line designated as 35 W (B line or maintainer line). The progeny of this so-called backcross was tested for resistance to streptomycin (see Materials and methods). Of 100 seeds that were sown, 95 plants emerged and these were able to grow on MS medium supplemented with 500 and even 1000 mg/l streptomycin. Wild-type controls germinated but were not able to green and eventually died on streptomycin concentrations as low as 250 mg/l.

Full proof for maternal inheritance was obtained by backcrosses of the progeny of the previous cross with another maintainer (940406) line and a restorer line (940725), respectively. The progeny of both crosses was retested for resistance to streptomycin (Fig. 2). In both cases, resistance was confirmed, which indicates that the mutation is maternally inherited.

Isolation of chlorophyll-deficient lines and their analysis

A much higher mutation frequency was observed in an experiment for the selection of chlorophyll-deficient mutants (Table 4). Figure 3 shows the development of chlorophyll-deficient and wild-type plants from embryos.

Out of 14 selected chlorophyll-deficient lines 8 gave plants with normal morphology and were found to be diploid. Figures 4 and 5 show a wild-type and a chlorophyll-deficient line, respectively growing on hormone free MS medium. The nature of the chlorophyll deficiency of the 4 selected lines was studied by a fusion/complementation analysis that is based on the appearance of green (wild-type like) colonies if complementation takes place after protoplast fusion between two non-allelic chlorophyll-deficient lines. The protoplasts of the 4 lines were fused in pairwise combinations. Protoplast-derived cultures were obtained in all experiments; however no green nor variegated calli were identified in any of the fusion combinations.

Transformation and analysis of produced plants

Several successful experiments have been conducted with various genotypes. In all cases, from the 4×10^5 protoplasts plated after transformation into alginate, 2–5000 kanamycin-resistant calli could be identified. A piece of alginate with embedded protoplast derived colonies resistant to kanamycin is shown in Fig. 6 (upper). No growing colonies were observed from non-transformed protoplasts under the applied selection pressure (Fig. 6, left). Control plates without selection were completely filled with green calli (Fig. 6, right).

A few dozen of kanamycin-resistant callus lines were used for plant regeneration. Of these regenerants, eight randomly chosen plants were used for further analysis by PCR and were found to be true transformants. Four of these transformants (T0) were transferred to soil, vernalized and self-pollinated. T1 seeds were tested for kanamycin resistance.

Figure 7 shows the genetic segregation on medium containing kanamycin. In all cases, a 3:1 ratio of resistant to susceptible seedlings was observed, as was expected for a single nuclear dominant trait. The results of PCR analysis confirmed the presence of the *NPTII* gene in the resistant progeny (Fig. 8).

Discussion

Until now, carrot has been used as a model species for the induction of somatic embryogenesis, for protoplast culture and mutant selection, for intra-and interspecific hybridizations, as well as for transformation (see Dudits et al. 1976, 1977, Vergara et al. 1982, Droge et al. 1992). In all of the protoplast culture experiments that have been published so far, cell suspensions served as donor material. However, the plating efficiency of cell suspension-derived protoplast is highly variable, and the scores up to 20% that have been

Fig. 1 Freshly isolated petiole protoplasts

Fig. 2 Germination of progeny from respectively a resistant mutant (*left*) and a susceptible wild-type line (*right*) on MS medium supplemented with 1000 mg/l streptomycin

Fig. 3 Regeneration and identification of chlorophyll-deficient lines from protoplast culture

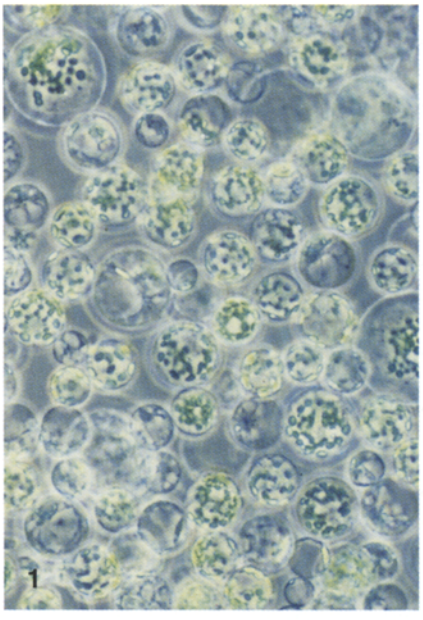
Fig. 4 Wild-type regenerant growing on MS medium

Fig. 5 Chlorophyll-deficient line isolated in protoplast culture

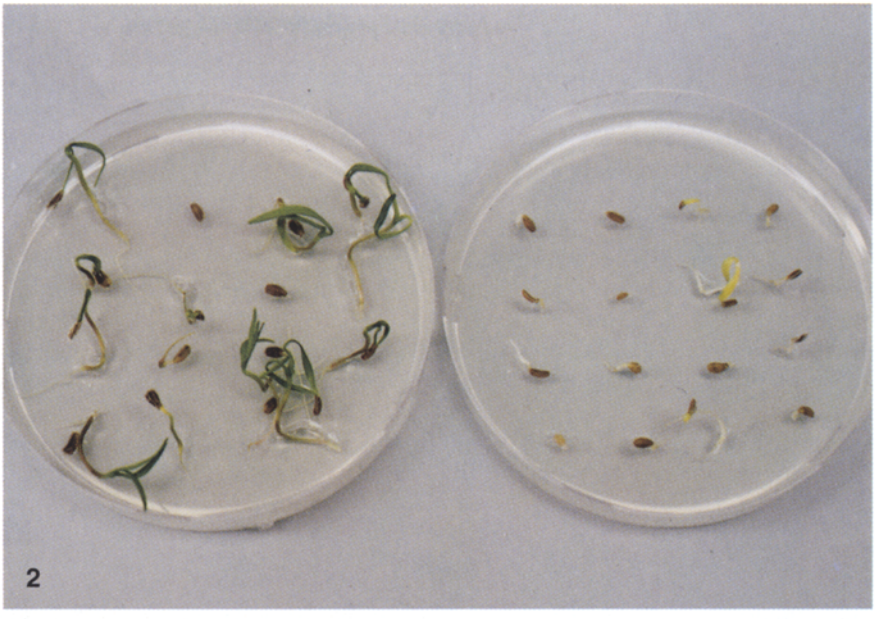
Fig. 6 Selection for kanamycin-resistant transformants in alginate. *Left* a slice of alginate with control cells (non-transformed and not growing). *Right* a control experiment without selection pressure: *upper* selection experiment, transformed protoplast-derived calli in the presence of 100 mg/l kanamycin

Fig. 7 Segregation for kanamycin resistance in the T1 generation of a transformant with plasmid pNUN 7

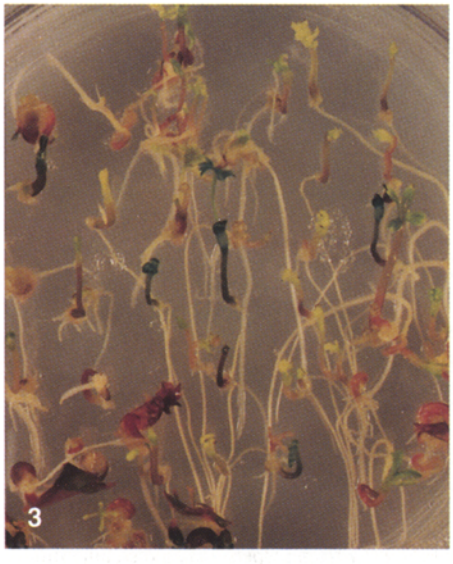
Fig. 8 Molecular analysis of transgenic progeny. *Lanes 1* and *6* λ molecular weight markers. *Lanes 2* and *5* presence of the *NPTII* gene in resistant progeny. *Lane 3* and *4* absence of PCR application in the susceptible seggregants



1



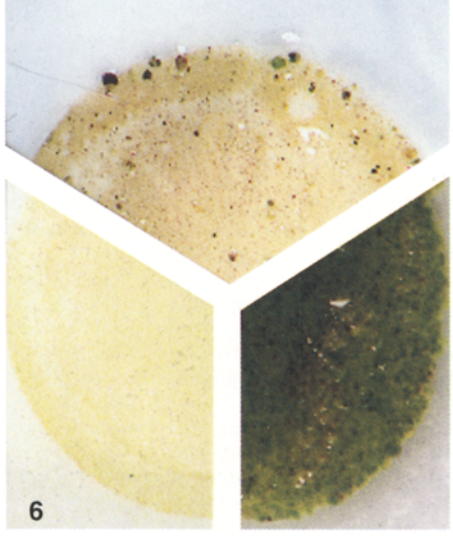
2



3

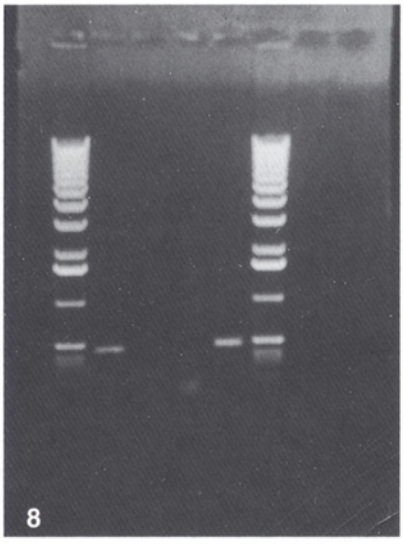
4

5



6

7



8

obtained (Dudits 1984) are really too low for a model species.

In order to avoid genetic instability such as chromosomal aberrations and mutations, it has been recommended that the undifferentiated phase of tissue culture experiment be reduced to a minimum (Karp 1989). Even prior to this, the loss of regeneration ability due to extended callus culture had been described Syono (1965). Smith and Street (1974) noted a decline in embryogenic potential and a selective advantage of non-totipotent cells during subculture.

In this paper we describe a general protocol for protoplast culture that is amenable to all genotypes, plant regeneration and mutant selection. The starting material are petioles from seedlings or vegetatively propagated plants. It is a simple and fast method that reduces the above mentioned problems. Because of these characteristics, the system approaches being a model species such as tobacco.

The high protoplast division rate and associated plating efficiency is solely due to the use of alginate as a matrix for embedding. It was surprising to note that, even in the absence of hormones or under conditions where only 2,4D or zeatine was administrated to the culture medium, cell divisions could take place and colonies could be formed. As far as we know, we are the first to report that normal cell divisions can take place from protoplast cultures incubated without the addition of growth regulators. It is not solely the act of embedding that triggers division – extensive experiments using the same media with agarose as embedding material completely failed (data not shown). The addition of $CaCl_2$ to the culture medium in concentrations present in the alginate layer or the addition of alginate layers without protoplasts to the liquid medium neither initiated nor stimulated divisions. It was only embedding in alginate that triggered cell division. We have also tried to induce cell division in the same liquid medium by adding dimethyl sulfoxide in concentrations that were successful in Hibiscus (Hahne and Hoffmann 1984), but again cell divisions did not take place. Recently, Giorgetti et al. (1995) reported the occurrence of somatic meiosis in embryogenic cell cultures in carrot. The protoplast culture system we describe could be of use to study whether or not somatic meiosis occurs during the first divisions. If this can be done, then the fate of single cell clones can be followed.

Although carrot has been used for mutant selection, and many variant resistant lines have been described, so far genetic transmission of the mutation has not been reported (Sidorov 1990).

In this paper we describe the selection of a streptomycin resistant mutant and the identification of chlorophyll-deficient variant lines. In both cases NMU was used as a mutagen. We found that protoplasts of petioles in carrot are extremely resistant to mutagenesis and not only to chemical but especially to UV, X or γ irradiation. Thus, preliminary results on "gamma-fusion" show that a large number of protoplasts can survive subsequent to undergoing irradiation with superlethal doses. The reason for this, the results, and the implications will be communicated in another paper.

Cytoplasmatic inheritance of streptomycin resistance was confirmed by genetic analysis. The nature of the chlorophyll deficiency was studied in fusion experiments. However, we never expected any of the chlorophyll-deficient mutations to be nuclear recessive since diploid protoplasts were used for mutagenesis. Complementation and formation of green (or variegated) calli could occur only when at least one line possessed a recessive nuclear-encoded pigment deficiency. Due to the very low frequency of chloroplast recombination (Medgyesy et al. 1985), complementation in fusion products can identify the cytoplasmic nature of chlorophyll-deficient mutations. The produced cytoplasmic mutants are unique material for manipulation of cytoplasmone-encoded traits and particularly interesting as a donor/recipient of CMS. The efficiency of the protoplast culture system was confirmed in experiments when petiole protoplasts were used for direct transformation with plasmid DNA. The production of seeds from transformant carrot lines and the inheritance of desired traits in the progeny show the suitability of the system for applied purposes.

Acknowledgements The authors are greatly indebted to Esther Schmitz for critically reading and correcting the manuscript.

References

Damm B, Schmidt R, Willmitzer L (1989) Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. Mol Gen Genet 217:6–12

Dewulf J, Negrutiu I (1991) Direct gene transfer into protoplasts: the chemical approach. In: Negrutiu I, Gharti-Chhetri G (eds) A laboratory guide for cellular and molecular plant biology. Birkhäuser, Basel Boston Berlin, pp 116–121

Droge W, Broer I, Puhler A (1992) Transgenic plants containing the phosphinotricin-*N*-acetyltransferase gene metabolize the herbicide L-phosphinotricine (glufosinate) differently from untransformed plants. Planta 187: 142–151

Dudits D (1984) Isolation and culture of protoplasts from carrot cell suspension cultures. In: Vasil (ed) Cell culture and somatic cell genetics of plants. Academic Press, Orlando London, pp 391–397

Dudits D, Kao KN, Constabel F, Gamborg OL (1976) Fusion of carrot and barley protoplasts and division of heterocaryocytes. Can J Genet Cytol 18:263–269

Dudits D, Hadlaczky G, Lévi E, Fejér, Haydu Zs, Lázár (1977) Somatic hybridisation of *Daucus carota* and *D. capillifolius* by protoplast fusion. Theor Appl Genet 51:127–132

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Giorgetti L, Vergara MR, Evangelista M, Lo Schiavo F, Terzi M, Nuti Ronchi V (1995) On the occurrence of somatic meiosis in embryogenic carrot cultures. Mol Gen Genet 246:657–662

Grambow HJ, Kao KN, Miller RA, Gamborg OL (1972) Cell division and plant development from protoplasts of carrot cell suspension cultures. Planta 103:348–355

Hahne G, Hoffmann F (1984) Dimethyl sulfoxide can initiate cell divisions of arrested callus protoplasts by promoting corical microtubule assembly. Proc Natl Acad Sci USA 81:5449–5453

Kameya T, Uchimiya H (1972) Embryoids derived from isolated protoplasts of carrot. Planta 103:356–360

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in luiquid media. Planta 126:105–110

Karp A (1989) Can genetic instability be controlled in plant tissue cultures? IAPTC Newl 58:2–11

Kisaka H, Kameya T (1994) Production of somatic hybrids between *Daucus carota* L. and *Nicotiana tabacum*. Theor Appl Genet 88:75–80

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82: 6960–6964

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 70:590–594

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

Sidorov VA (1990) Plant biotechnology. Cell selection (in Russian). Kiev, Nauk Duma

Sidorov VA, Maliga P (1982) Fusion complementation analysis of auxotrophic and chlorophyll-deficient lines isolated in haploid *Nicotiana plumbaginifolia* protoplast cultures. Mol Gen Genet 186:328–332

Smith SM, Street HE (1974) Decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota L.*) are serially subcultured. Ann Bot 38:223–241

Syono K (1965) Changes in organ forming capacity of carrot root calluses during subcultures. Plant Cell Physiol 6:403–419

Tanno-Suenaga L, Nagao E, Imamura J (1991) Transfer of the petaloid-type CMS in carrot by donor-recipient protoplast fusion. Jpn J Breed 41 25–33

Vergara MR, Biasini G, LoSchiavo F, Terzi M (1982) Isolation and characterization of carrot cell mutants resistant to α-amanitin. Z Pflanzenphysiol 107:313–319