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Culture and Selection of Somatic Hybrids Using an Auxotrophic Cell Line

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Summary. Protoplast fusions between *Nicotiana tabacum* and *N. paniculata* and between *N. tabacum* and *N. sylvestris* were obtained by polyethylene glycol and $Ca(NO_3)_2$ treatment. The protoplasts of one parent originated from cell suspensions, while the protoplasts of the other originated from leaf mesophyll. The heterokaryons were detectable by their intermediate phenotype, namely the green chloroplasts from mesophyll and the dense cytoplasm from suspension cells. They were isolated with micropipettes immediately after fusion using a micromanipulator and were transferred into a protoplast suspension of an auxotrophic cell line serving as a nursery. This mutant is not able to utilize nitrate and had to be supplemented with amino acids. The somatic hybrids were selected by a stepwise reduction of the supplements, which caused the death of the mutant cell colonies, while the autotrophic somatic hybrids continued to grow. The hybrid character of the selected colonies was confirmed by isoenzyme investigations.

Key words: Nurse culture – Protoplast fusion – Selection – Somatic hybrid

Introduction

A serious problem for the production of somatic hybrids is their selection, because not only the somatic hybrids, but also the unfused protoplasts will regenerate. Frequently somatic hybrids have been selected by complementation selection using protoplasts of auxotrophic, resistant and chlorophyll deficient mutants or of non-regenerating wild type lines (review Schieder and Vasil 1980). Because it is difficult to produce such mutants they are not available for every plant species. Moreover, mutagenic treatment may have an additional negative influence on interesting plant breeding material. Another possibility for the selection of somatic hybrids is the mechanical isolation of heterokaryons after fusion of the protoplasts and their individual culture in microdroplets (Kao 1977; Gleba and Hoffmann 1978) or their transfer into a protoplast suspension derived from chlorophyll deficient mutants (Menczel et al. 1978). The culture in microdroplets requires complex regeneration media which may differ among plant species, while cultivation in a nurse culture of albino protoplasts requires chlorophyll production by the somatic hybrids. The new selection method we describe is the culture of mechanically isolated heterokaryons in a protoplast suspension produced from an auxotrophic cell line. Protoplasts of the nitrate reductase deficient cell line cnx-68 of *Nicotiana tabacum* (Müller and Grafe 1978) were used as the nurse culture. The protoplast culture medium contained nitrate and was additionally supplemented with amino acids. The final selection of the somatic hybrids was carried out by reduction of the amino acid concentration, which caused the death of the auxotrophic cell colonies. In this paper we will demonstrate such a selection procedure with heterokaryons of *N. tabacum* (+) *N. paniculata* or *N. sylvestris*, respectively.

Material and Methods

Plant Material and Protoplast Preparations

Protoplasts of *N. paniculata* and of *N. sylvestris* were prepared from leaf mesophyll of aseptic shoot tip cultures. These cultures were established and maintained as described previously (Schieder 1975). The shoot tips were cultivated on agar medium B5 (Gamborg et al. 1968) without any plant hormones. For protoplast isolation leaf material was cut into small pieces with a razor blade and incubated in an enzyme solution made up with 1% cellulase R10 (Onozuka), 0.2% macerozyme R10 and 0.6 M mannitol at pH 5.5 for 5 h. Cell suspensions of the nitrate reductase deficient mutant cnx-68 of *N. tabacum* were cultivated on a shaker in the AA medium according to Müller and Grafe (1978) under continuous dim

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light at 150 rpm. They were transferred to fresh medium every 5 days. The protoplast preparation was carried out in the same enzyme solution as for the mesophyll material but for 16 h overnight.

After enzyme incubation the protoplasts of all species used were washed two times with 80% seawater (ca. 730 mOsm, North Sea, Sylt) and floated in 0.6 M sucrose (Krumbiegel and Schieder 1979). The protoplasts of *N. tabacum* floating in the supernatant and intended for the nurse culture were suspended in the protoplast regeneration medium V47 (Binding 1974), supplemented with the amino acids: arginine (174 mg/l), aspartic acid (266 mg/l), and glutamine (877 mg/l) giving a final protoplast density of ca. 10^4/ml. Two ml of this protoplast suspension were plated in plastic petri dishes of 60 mm diameter.

Fusion of Protoplasts

The protoplasts of *N. paniculata* or *N. sylvestris* and the mutant cell line cnx-68 of *N. tabacum* floating in sucrose and intended for fusion were diluted with 80% seawater and mixed. After centrifugation the pellets of the mixed protoplasts were transferred with a pipette between two droplets of the fusion mixture placed close together in a plastic petri plate. This lead to the fusion of the two droplets and the protoplast suspension. The fusion medium consisted of 0.1 M $Ca(NO_3)_2$, 25% polyethylene glycol (MW 6,000) and 0.45 M mannitol adjusted with KOH to pH 9. After 10 min incubation of the protoplasts with the fusion medium, 3 ml of 0.275 M $Ca(NO_3)_2$ at pH 6 was added. Five min later the whole mixture was removed by a pipette and transferred into a centrifuge tube. After centrifugation at 100 g the protoplast containing pellet was resuspended with V47 medium supplemented with the amino acids mentioned above and plated in plastic petri dishes.

Isolation of Heterokaryons and Cloning in Nurse Culture

Heterokaryons could be easily detected by their intermediate phenotype: green chloroplasts originating from mesophyll protoplasts and numerous cytoplasmic strands originating from suspension protoplasts. They were isolated immediately after fusion with micropipettes handled by a micromanipulator (Leitz, Germany). Twenty heterokaryons were picked up into one pipette and transferred into a petri dish containing protoplasts of the mutant cnx-68. In total 100 heterokaryons were transferred into one dish. After three weeks the developed cell colonies were diluted with 0.4% soft agar dissolved in V47 medium supplemented with or without amino acids. Three weeks later the cell colonies were transferred onto agar medium B5 (Gamborg et al. 1968) containing 0.5 mg/l each of 2,4-D (2,4-dichlorophenoxyacetic acid), BAP (6-benzylaminopurine) and NAA (α-naphtaleneacetic acid).

Selection and Identification of Somatic Hybrids

The somatic hybrids were selected by the reduction of the amino acids. This reduction lead to slower growth and to the death of the auxotrophic cell colonies, while the autotrophic somatic hybrids remained alive metabolizing the inorganic nitrogen. The selection was started either by the dilution with the soft agar, or after the transfer of the developed cell colonies onto agar medium B5 without amino acids. The presumed hybrids were further characterized by observing the electrophoretic mobility of esterase and malate dehydrogenase isoenzymes on polyacrylamide gels. For esterase isoenzyme investigations 10% gels, for malate dehydrogenases 10% gels with a 5% spacer were used. Separation was carried out at a constant voltage of 50 mV. The esterases were stained according to Brewbaker et al. (1968), and the malate dehydrogenases according to Scandalios (1969).

Results

By the described fusion method we could observe up to 3% heterokaryons. The protoplasts, particularly the heterokaryons, stuck neither together nor to the bottom of the petri dish; therefore, they could easily be isolated immediately after fusion (Fig. 1). At this time the heterokaryons could easily be identified because the cytoplasms of both partners were not yet mixed. Normally more than 100 heterokaryons could be isolated within one hour. The 100 heterokaryons transferred into one petri dish containing the mutant protoplasts were checked every day and could be identified by their green coloured chloroplasts for a long time. After 2 days 70% of the isolated heterokaryons were still alive. First divisions of the heterokaryons started after 3 days of culture. Almost 30% of the isolated heterokaryons showed divisions. After 12–14 days the green colour of the somatic hybrid colonies was so much reduced, that the heterokaryons could not longer be identified. At this time they had formed colonies of 8–16 cells. The selection of the hybrid colonies was carried out at two different times. The addition of soft agar without amino acids to the cultures led to a reduced amino acid concentration, which caused a reduced growth of only the mutant cell colonies (Fig. 2). The other alternative was the transfer of the developed colonies onto agar medium B5 which led to a stronger reduction of the amino acids and consequently to the death of the mutant cell colonies. No differences were observed between these two selection procedures. From 200 heterokaryons between *N. tabacum* cnx-68 and *N. paniculata* originally isolated, 29 (14.5%) formed colonies which were visible at the earliest after 4–5 weeks of culture by their larger size. After 3 months they were large enough to start the isoenzyme investigations. In the hybridization experiment between *N. tabacum* cnx-68 and *N. sylvestris* only 160 heterokaryons were isolated within 90 min and were transferred into a protoplast suspension derived from the mutant cell line cnx-68. From these 29 (18.1%) formed calluses.

The electrophoretic isoenzyme pattern of *N. tabacum* cnx-68 and *N. paniculata* differs for both the esterases and the malate dehydrogenases. All of the presumed somatic hybrids showed the same esterase pattern. The pattern was intermediate between that of both parental lines. The situation for the malate dehydrogenases was similar, but differences between the different somatic hybrid lines could be observed.

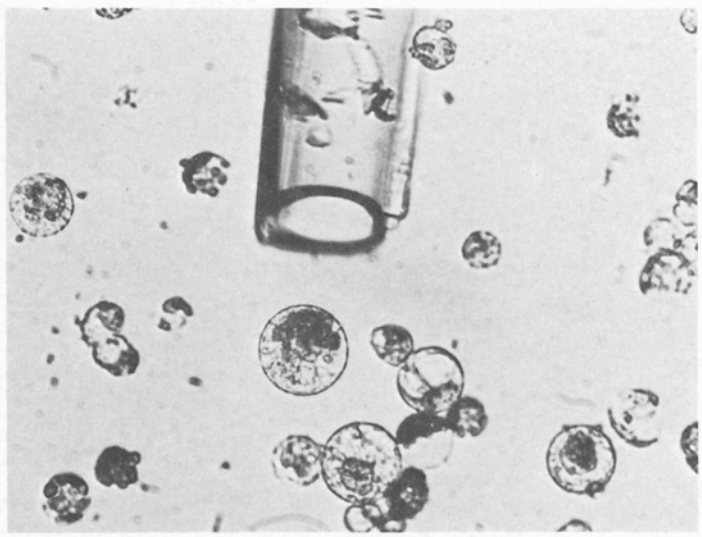

Fig. 1. Fused protoplast between *N. paniculata* and *N. tabacum* cnx-68 1 h after fusion; nearby an adjusted micropipette ×200

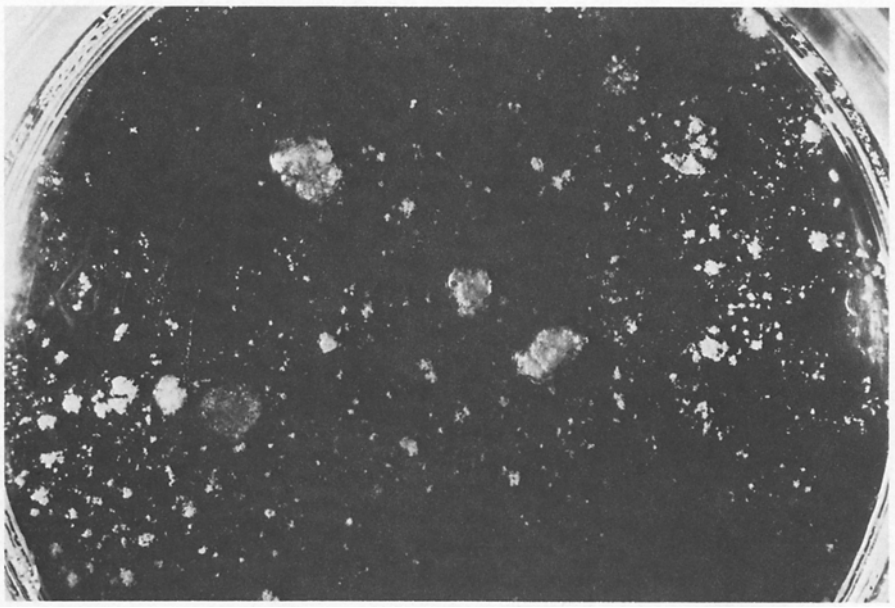

Fig. 2. Hybrid calluses between *N. paniculata* and *N. tabacum* cnx-68 selected by reduction of the amino acids, which caused a reduced growth of the mutant cell line ×3

Ten of the presumed somatic hybrids between *N. tabacum* cnx-68 and *N. sylvestris* were investigated. All ten showed an intermediate pattern in comparison to that of both parental lines.

The hybrid character of some identified hybrids between *N. tabacum* cnx-68 and *N. paniculata* was also confirmed by gas-chromatographic investigations according to Ninnemann and Jüttner (1981).

Discussion

The fusion method described here leads to an appropriate fusion rate and prevents protoplasts from continuing to clump for a longer time and that they stick to the bottom of the petri plate. Therefore the heterokaryons could be isolated earlier and easier than described in other communications (Gleba and Hoffmann 1978; Patnaik et al. 1982). The rate of hybrid callus formation of developing fusion bodies seems to be 100% as confirmed by the isoenzyme investigations. This confirms results reported by Kao (1977) in hybridization experiments between *Glycine max* and *Nicotiana glauca* but contradicts results reported by Menczel et al. (1978) in experiments to hybridize *N. knightiana* and *N. sylvestris*. In this case only 15% of the developed colonies could be determined as somatic hybrids.

The described hybridization method, carried out on model plant species of the genus *Nicotiana,* should also be transferable to other important crop plants such as potato. The somatic hybridization of selected dihaploid potato clones is one step of the theoretical potato breeding scheme proposed by Wenzel et al. in 1978. However, in potato clones with valuable characters normally no mutants for a complementation selection are available. Heterokaryons between *Solanum tuberosum* and *S. stenotomum* cultivated together with protoplasts of the nitrate reductase deficient mutant cnx-68 of *N. tabacum* showed some divisions (Hein, unpublished), but have not yet developed into cell colonies. This may be due to the culture medium or that the tobacco protoplasts can not serve for potato as a nurse culture

However, changes in the culture conditions or the use of mutants derived from other plant species or potato itself may overcome this problem. Besides, other auxotrophic cell lines from *Datura innoxia* (King et al. 1980), *Hyoscyamus muticus* (Gebhardt et al. 1981; Strauss et al. 1981), *Glycine max* (Roth and Lark 1982), *Nicotiana plumbaginifolia* (Marton et al. 1982), and *Petunia* "Mitchell" (Steffen, pers. commun.) already exist which also could be used for the nursing of potato heterokaryons. Such mutants may also be useful for the development of protoplasts or cells which have been genetically manipulated as for example in microinjection experiments.

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Literature

Binding, H. (1974): Regeneration von haploiden und diploiden Pflanzen aus Protoplasten von *Petunia hybrida.* Z. Pflanzenphysiol. **74,** 327–356

Brewbaker, J.L.; Upadhya, M.D.; Mäkinen, Y.; MacDonald, T. (1968): Isoenzyme polymorphism in flowering plants. III. Gel electrophoretic methods and applications. Physiol. Plant. **21,** 930–940

Gamborg, O.L.; Miller, R.A.; Ojima, K. (1968): Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158

Gebhardt, C.; Schnebli, V.; King, P.J. (1981): Isolation of biochemical mutants using haploid mesophyll protoplasts of *Hyoscyamus.* II. Auxotrophic and temperature-sensitive clones. Planta **153,** 81–89

Gleba, Y.Y.; Hoffmann, F. (1978): Hybrid cell lines *Arabidopsis thaliana* + *Brassica campestris:* no evidence for specific chromosome elimination. Mol. Gen. Genet. **165,** 257–264

Kao, K.N. (1977): Chromosomal behaviour in somatic hybrids of soybean – *Nicotiana glauca.* Mol. Gen. Genet. **150,** 225–230

King, J.; Horsch, R.B.; Savage, A.D. (1980): Partial characterization of two stable auxotrophic cell strains of *Datura innoxia* Mill. Planta **149,** 480–484

Krumbiegel, G.; Schieder, O. (1979): Selection of somatic hybrids after fusion of protoplasts from *Datura innoxia* Mill. and *Atropa belladonna* L. Planta **145,** 371–375

Marton, L.; Dung, T.M.; Menczel, R.R.; Maliga, P. (1982): Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia.* Mol. Gen. Genet. **182,** 301–304

Menczel, L.; Lázár, G.; Maliga, P. (1978): Isolation of somatic hybrids by cloning *Nicotiana* heterokaryons in nurse culture. Planta **143,** 29–32

Müller, A.; Grafe, R. (1978): Isolation and characterization of cell-lines of *Nicotiana tabacum* lacking nitrate reductase. Mol. Gen. Genet. **161,** 67–76

Ninnemann, H.; Jüttner, F. (1981): Volatile substances from tissue cultures of potato, tomato and their somatic fusion products – comparison of gas chromatographic patterns for identification of hybrids. Z. Pflanzenphysiol. **103,** 95–107

Patnaik, G.; Cocking, E.C.; Hamill, J.; Pental, D. (1982): A simple procedure for the manual isolation and identification of plant heterokaryons. Plant Sci. Lett. **24,** 105–110

Roth, E.J.; Lark, K.G. (1982): Isolation of an auxotrophic cell line of soybean (*Glycine max*) which requires asparagine or glutamine for growth. Plant Cell Rep. **1,** 157–160

Scandalios, J.G. (1969): Genetic control of multiple molecular forms of enzymes in plants. A review. Biochem. Genet. **3,** 37–74

Schieder, O. (1975): Regeneration von haploiden und diploiden *Datura innoxia* Mill. Mesophyll-Protoplasten zu Pflanzen. Z. Pflanzenphysiol. **76,** 462–466

Schieder, O.; Vasil, I.K. (1980): Fusion and somatic hybridization. In: Recent advances in plant cell and tissue culture (ed. Vasil, I.K.). Int. Rev. Cytol. (Suppl.) **11B,** 21–46. New York: Academic Press

Strauss, A.; Bucher, F.; King, P.J. (1981): Isolation of biochemical mutants using haploid mesophyll protoplasts: I A NO_3^- non-utilizing clone. Planta **153,** 75–80

Wenzel, G.; Schieder, O.; Przewoźny, T.; Sopory, S.K.; Melchers, G. (1979): Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. **55,** 40–55

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