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V. A. Sidorov · D. P. Yevtushenko · A. M. Shakhovsky
Y. Y. Gleba

Cybrid production based on mutagenic innactivation of protoplasts and rescuing of mutant plastids in fusion products: potato with a plastome from *S. bulbocastanum* and *S. pinnatisectum*

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Abstract A procedure for cybrid production, based on double treatment of donor protoplasts by physical and afterwards chemical mutagens at superlethal doses (γ-irradiation at a dose of 1000 Gy was applied for the inactivation of nuclei; 3–5 mM *N*-nitroso-*N*-methylurea was used for the efficient induction of plastome mutation) and the rescuing of mutant plastids after fusion with untreated recipient protoplasts, was developed. For identification of mutant donor-type plastids in fusion products a selection for streptomycin was performed. In two sets of experiments, in which *S. tuberosum* served as the recipient of foreign cytoplasm with the wild tuber-bearing species *S. bulbocastanum* and *S. pinnatisectum* as donors, a total of about 40 streptomycin-resistant colonies was isolated. Eight regenerants from the *S. tuberosum* + *S. bulbacastanum* fusion combination and four from *S. tuberosum* + *S. pinnatisectum* were further investigated using chromosome counting, analysis of esterase isoenzymes, restriction analysis of organelle DNA, and blot hybridization. All but one plant from both combinations were characterised as potato cybrids possessing exclusively foreign plastids and retaining a morphology typical of the recipient. Only in one line was rearranged mtDNA detected. The availability of potato cybrids facilitates the analysis of plastome-encoded breeding traits and the identification of the most valuable source of cytoplasm among the wild potato species. The described system for producing cybrids without genetic selectable markers in the parental material offers the possibility for the rescue of cytoplasmic mutations which are impossible to isolate by conventional approaches.

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V. A. Sidorov (✉)[1] · D. P. Yevtushenko · A. M. Shakhovsky · Y. Y. Gleba[1]
Institute of Cell Biology and Genetic Engineering Ukrainian Academy of Sciences, Zabolotnogo str., 148, 252022, Kiev, GSP-22, Ukraine

Present address:
[1] American Cyanamid Company, P.O.B. 400, Princeton, NJ 08543-0400, U.S.A.

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Introduction

The production of cybrids or alloplasmic-like plants by protoplast fusion in which the original cytoplasm (or part of it) is replaced by cytoplasm from another species has become of value both for fundamental research and more particularly for crop improvement. As reviewed by Medgyesy (1990), chloroplast and mitochondrial genomes can code such agriculturally important traits as pathotoxin- and herbicide-resistance, temperature tolerance, and male sterility; moreover, through nuclear-cytoplasmic heterosis they might have an important role in hybrid vigor. Functional interspecific cybrids have been obtained in *Nicotiana* (Zelcer et al. 1978; Aviv and Galun 1980; Sidorov et al. 1981), *Brassica* (Pelletier et al. 1983; Morgan and Maliga 1987), *Citrus* (Vardi et al. 1987, 1989), *Daucus* (Ichikawa et al. 1987; Tanno-Suenaga et al. 1988), and *Solanum* (Sidorov et al. 1989; Perl et al. 1990). Several intergeneric cybrids with a nucleus/cytoplasm of *Nicotiana*/*Petunia* (Glimelius and Bonnett 1986), *Niconiana*/*Atropa* (Kushnir et al. 1987), and *Nicotiana*/*Salpiglossis* (Thanh et al. 1988), which were also produced via protoplast fusion, indicate an apparently compatible nuclear-cytoplasmic composition even between remote species. Potato cybrids possessing the nuclear background of the cultivated potato and the cytoplasm of wild *Solanum* species can be directly used in breeding. The production of alloplasmic potato lines by sexual crosses, in order to use the chloroplast and mitochondrial genetic pool of wild potato species, is rather limited. At the same time, Hosaka and Hanneman (1988) showed that the domestic potato *S. tuberosum* ssp. *tuberosum* possesses a uniform chDNA (T type). Therefore cybridization is an important tool for broadening the cytoplasmic diversity within this crop.

In general, for efficient cybridization by the donor-recipient protoplast fusion procedure the presence of

selectable cytoplasmic markers (e.g., antibiotic resistance in the donor or chlorophyll-deficiency in the recipient) is usually required. A bank of chlorophyll-deficient cytoplasmic mutants retaining cultivar-specific heterozygosity was established earlier (Sidorov et al. 1990). However, the utilization of this, or other, type of mutant is quite often not suitable for breeding purposes and in any case is not always available.

In the present work an approach for direct cybrid production, based on double innactivation of donor protoplasts (by γ-rays for inactivation of the nucleus and by chemical mutagenesis for the induction of a high frequency of cytoplasmic mutations) and the rescuing of mutant plastids in the fusion products, is proposed. Following the fusion of double-treated protoplasts of wild potato species with non-treated *S. tuberosum* protoplasts the selection of putative cybrids on medium with streptomycin was performed. As a result, potato cybrids possessing chloroplasts of *S. bulbocastanum* and *S. pinnatisectum* were isolated.

Materials and methods

Plant material

Plants of *Solanum tuberosum* cv Zarevo were maintained as axenic shoot cultures on Murashige and Skoog's (1962) medium with 10–20 g/l of sucrose. Lines of the wild diploid tuber-bearing species *S. bulbocastanum* and *S. pinnatisectum* carrying various pathogen resistances were also subcultured in vitro as shoot cultures on the same medium.

Protoplast isolation, pretreatment, fusion and culture

Mesophyll protoplasts of cultivated potato and wild species were prepared by using 0.6% Cellulase, 0.6% Macerozyme and 0.2% Cellulisin in a solution of 0.5 M sucrose and 5 mM $CaCl_2$, pH 5.6. Before fusion the donor protoplasts (*S. bulbocastanum* or *S. pinnatisectum*) were γ-irradiated at doses of 1 000 Gy (100 krad) and afterwards treated with 3–5 mM NMU during 1–2 h. The fusion, protoplast culture, and shoot regeneration were carried out as described elsewhere (Sidorov et al. 1987). During culture the protoplast-derived colonies were exposed to selective media which contained at the first stage 0.5 mg/ml, and later 1 mg/ml, of streptomycin sulphate.

Isoenzyme analysis and chromosome counts

Were performed according to the protocols described by Sidorov et al. (1987).

Analysis of organelle DNA

The chDNA and mtDNA were prepared from isolated chloroplasts and mitochondria of aseptically-grown plants respectively (Sidorov et al. 1987). The chloroplasts and mitochondria were lysed in 1% Sarkosyl buffered with 50 mM Tris and 25 mM EDTA, pH 8.0, during 0.5 h at room temperature. DNA was digested with *Bam*HI or *Hin*dIII enzymes and separated by electrophoresis on 0.8% agarose slabs. For Southern-blot hybridization analysis total DNA was extracted from leaves, digested with endonucleases (Boehringer Manheim), electrophoresed on 0.8% agarose gels and blotted on to nylon filters. A radiolabelled 5.2-kb mitochondrial fragment of *Oenothera* containing 18*S*, 5*S* rDNA, 5′-ND5, and isolated from plasmid B6/2, was used as a probe (Brennicke et al. 1985).

Results

In two sets of experiments the recipient *S. tuberosum* mesophyll protoplasts were fused with double-treated (γ-rays plus NMU) mesophyll protoplasts of *S. bulbocastanum* and *S. pinnatisectum* at a ratio of 2:1. After screening for streptomycin resistance among protoplast-derived colonies the putative cybrid lines which became green were identified (Fig. 1A). A total of about 20 lines were isolated on selective medium in each fusion combination and only half of them gave rise to morphologically-normal plants. Eight regenerants from the *S. tuberosum* + *S. bulbocastanum* fusion combination and four from *S. tuberosum* + *S. pinnatisectum* were propagated for further analysis. After transferring plants to greenhouse conditions it was found that, to a great extent, all of them retained the phenotype of parental *S. tuberosum*. The appearance of potted plants of the wild

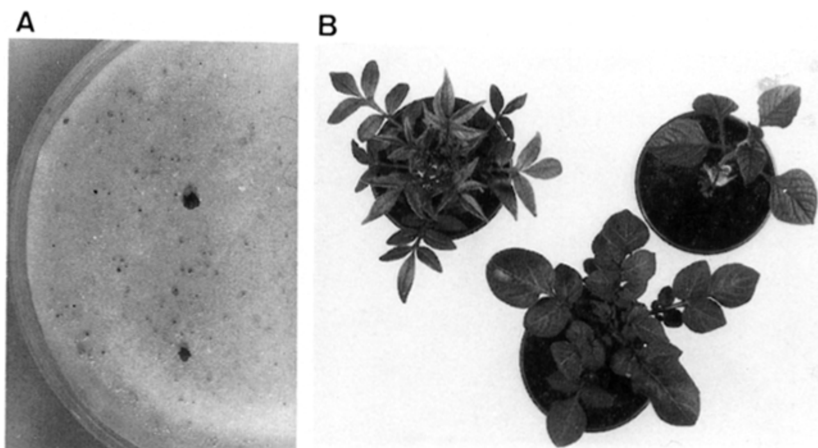

Fig. 1 A Protoplast-derived green calli on selective media with streptomycin. **B** The appearance of the two donor lines (*S. pinnatisectum*, top left and *S. bulbocastanum*, top right) and an example of a cybrid containing nuclear material of *S. tuberosum* and the cytoplasm of *S. pinnatisectum* (bottom center)

potato species and one of the *S. tuberosum/S. pinnatisectum* cybrid is shown in Fig. 1B.

The nuclear constitution of selected regenerants was studied by chromosome analysis and the analysis of multiple molecular forms of esterase. All lines revealed a chromosome number of 48 and an isoenzyme pattern similar to that of the original *S. tuberosum.*

The analysis of regenerated plants was mainly focussed on the characterization of organelle composition. Restriction analysis of chDNA indicated that all eight lines from the *S. tuberosum* + *S. bulbocastanum* fusion contained the plastome of *S. bulbocastanum* while four lines from *S. tuberosum* + *S. pinnatisectum* contained the plastome of *S. pinnatisectum.* The results of the analysis of several cybrid lines are presented in Fig. 2. No plastome recombination were found among the isolated cybrids. Chondriome composition was evaluated by restriction analysis of mtDNA (Fig. 3 A) and Southern-blot analysis of total DNA isolated from leaf material (Fig. 3 B, C). Surprisingly, 11 examined cybrids contained the chondriome of recipient plants and only in one line was any indication of an mtDNA rearrangement detected.

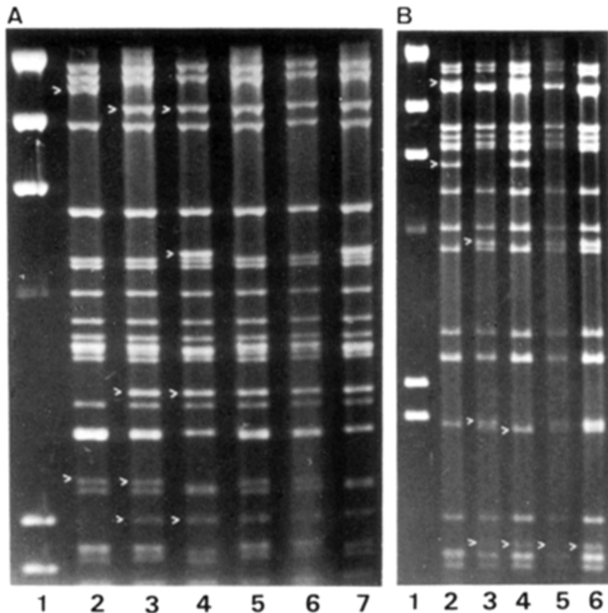

Fig. 2 Restriction pattern of chDNAs of putative cybrid lines and their parents after digestion with *Bam*HI (**A**) and *Hind*III (**B**). **A** *lane 1, λ/Hind*III; *2, S. tuberosum*; *3, S. pinnatisectum*; *4, S. bulbocastanum*; *5,* Sp1; *6,* Sp3; *7,* Sb5. **B** *lane 1, λ-Hind*III; *2, S. tuberosum*; *3, S. pinnatisectum*; *4, S. bulbocastanum*; *5,* Sp2; *6,* Sp3. *Arrows* indicate species-specific fragments

Discussion

One of the aims of this research was to develop a procedure for the production of cybrids without any genetic markers in the parental material. The prerequisite isolation of cytoplasmic mutants resistant to antibiotics, or other substances which can be beneficial for cybridization, is always time consuming. Chlorophyll-deficient plastome mutants used as a recipient of foreign cytoplasm might also be inappropriate for breeding purposes since deleterious mutations can arise during mutagenesis. The proposed procedure combines together two steps: the induction of chloroplast-coded

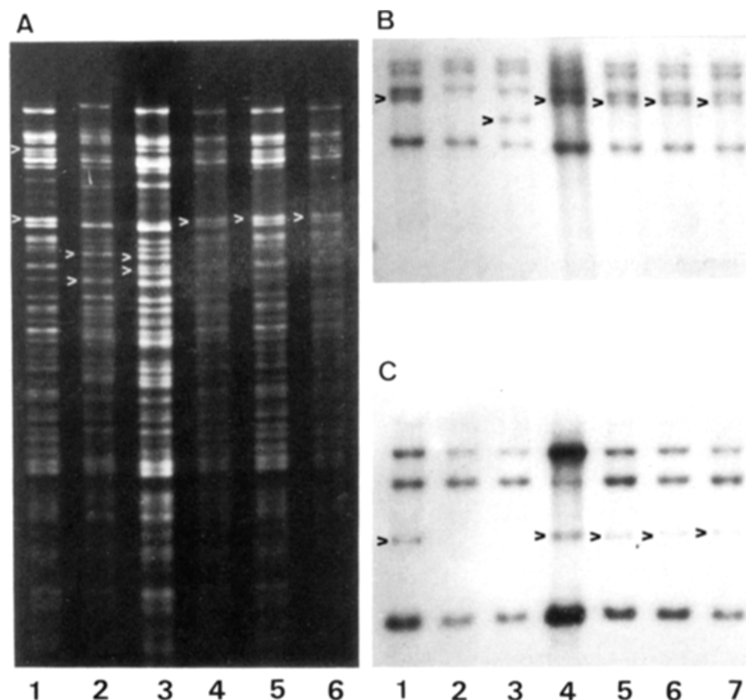

Fig. 3 Restriction analysis (**A**) and Southern-blot hybridization (**B, C**) of mtDNA of parental lines and isolated cybrids. **A** *lane 1, S. tuberosum*; *2, S. pinnatisectum*; *3, S. bulbocastanum*; *4, 5, 6,* cybrid lines Sp1, Sp5, Sb7. Digestion with *Hind*III. **B, C** *lane 1, S. tuberosum*; *2, S. bulbocastanum*; *3, S. pinnatisectum, 4, 5, 6,* cybrid lines Sp3, Sp5, Sb5, Sb1. The total DNA was digested with *Pst*I (**B**), *Sma*I (**C**) and probed with an *Oenothera* mtDNA *Bam*HI fragment

mutations in the donor material and the transfer of mutated plastids to the recipient by "gamma-fusion". Sorting out of organelles in the fusion products under selection pressure favourable for mutant plastids leads to the complete substitution of plastids by the mutant donor.

Due to the relatively low fusion frequency (from 15 to 30%) it is important to use an efficient plastome-targeted mutagen. Nitroso-urea-compounds are suitable for this purpose (Hagemann 1982). In order to increase the yield of cytoplasmic mutations superlethal doses of NMU were chosen. However we cannot exclude that another mutagen, or else γ-irradiation alone, might also be suitable for the efficient induction of cytoplasmic mutations. For complete inactivation of donor nuclear material γ-irradiation at doses of 1000 Gy was applied. As was reported earlier (Sidorov et al. 1987) at doses of 200 Gy the potato protoplasts were unable to divide but could still be used for the production of asymmetric nuclear hybrids.

We also focused on the use of one type of mutant resistant to streptomycin although other cytoplasmic selectable markers can be exploited. Furthermore, as expected, the application of superlethal doses of the mutagen provides a unique opportunity to induce novel types of cytoplasmic mutations which can be rescued in a different nuclear background.

The potential usefulness of the proposed procedure was demonstrated in fusion experiments with potato. Recently, the exploitation of protoplast fusion technology has led to the isolation of various interspecific nuclear hybrids of potato (Barsby et al. 1984; Helgenson et al. 1986; Puite et al. 1986; Fish et al. 1987; Sidorov et al. 1987; Seraff et al. 1991). However, their use for the production of new cultivars has been restricted since the introduction of only few valuable traits from wild to cultivated species is usually desirable. Therefore, numerous backcrosses or backfusions of such somatic hybrids with cultivated potato are required. "Gamma-fusion", as an alternative for the transfer of a limited amount of nuclear genetic material, gives unpredictable results if one requires the introgression of non-selectable traits. Our results indicate that immediate application can be achieved by cybridization which results in a reconstruction of the cytoplasmone while retaining the main cultivar-specific traits due to the presence of an unchanged nuclear background of the cultivated potato. Under the conditions used, two types of streptomycin-resistant cybrids, possessing the nucleus of *S. tuberosum*, cv Zarevo and the cytoplasm of *S. bulbocastanum* or *S. pinnatisectum*, were obtained. The nuclear constitution of all regenerants were classified by esterase isoenzymes, and chromosome number and morphology, as typical *S. tuberosum*. Analysis of organelle DNAs confirmed that all but one regenerant carried exclusively donor-type chloroplasts and recipient mitochondria. Only plants of one line contained *S. pinnatisectum* plastids and an altered mtDNA. The agronomic importance of the cybrids produced can be queried due to the suggestion that, while interspecific transfer of chloroplasts does not affect the functionality of cybrids, mitochondrial transfer has a deleterious effect on male fertility (Perl et al. 1991, Melchers et al. 1992). Although no differences in morphology between our cybrids, containing exclusively foreign plastids, and the original cultivated potato were found, further detailed study of these lines and their vegetative progeny is needed.

In the present experiments selection for antibiotic resistance did not result in the co-transfer of mitochondria; however, it has been reported (Menczel et al. 1983) that selection for streptomycin resistance of the donor gives 100% co-transfer of male sterility. Therefore, we assume that our technique for cybrid selection does not exclude the possibility of the simultaneous transfer of chloroplasts and mitochondria. Selected cybrids with recipient mitochondria can be used in future as an exclusive source of the *S. bulbocastanum* and *S. pinnatisectum* plastome.

From the results obtained in this report we conclude that the proposed double inactivation-fusion procedure is an efficient mechanism for the rescuing of cytoplasmic mutation and may be of general applicability in the production of cybrids. The availability of such potato cybrids makes possible the analysis of plastome-encoded breeding traits and the identification among wild potato species of the most valuable source of cytoplasm.

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