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Somatic hybridization in potato: use of γ-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction*

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Summary. Leaf mesophyll protoplasts of *Solanum pinnatisectum* (2n = 24) γ-irradiated at doses of 200 Gy and consequently unable to divide were fused with untreated protoplasts of genomic chlorophyll deficient mutant IvP 841-1 (2n = 24) containing the germplasms of *S. tuberosum* and *S. phureja*. Two types of plants differing in their pigmentation characteristics were selected. The regenerants of one group were identified as true somatic hybrids by using isozyme analyses of esterase and aspartate aminotransferase. The anthocyanin marker of *S. pinnatisectum* was phenotypically expressed in these regenerants and could be used as an additional selection trait for hybrid screening in this species combination. The regenerants of the second group were corrected for the gene controlling chlorophyll deficiency but contained species-specific isozymes of the potato cultivar only. Restriction analysis of chloroplast DNA revealed chloroplasts of the *S. pinnatisectum* type in all but one of the plants tested. The fusion experiments involving γ-irradiated protoplasts show that this approach in potato reconstruction has the advantage of producing a wide range of genetically novel plants.

Key words: interspecific somatic hybridization – γ-irradiation – *Solanum*

Introduction

Plant genome engineering by protoplast fusion may be a useful approach in the development of some crops by using the protoplast culture technique. Potatoes are one of the few crops already employed in this type of experimentation (Melchers et al. 1978; Butenko and Kuchko 1980; Binding et al. 1982; Austin et al. 1986). Most often, the aim in hybridization attempts is to introduce into potatoes a few valuable traits present in wild *Solanum* species such as pest resistance, adaptation potential, resistance to physiological stresses, etc. It was demonstrated earlier that γ-irradiation of one of the parental cells used for hybridization can be exploited for transfer/reconstitution of cytoplasmon (Zelcer et al. 1978; Aviv and Galun 1980; Sidorov et al. 1981; Menczel et al. 1982) as well as in producing asymmetric nuclear hybrids (e.g. Dudits et al. 1980; Famelaer et al. 1986).

We used γ-irradiated cells from a wild tuber-forming *Solanum pinnatisectum* species associated with some desired traits in order to transfer a limited amount of genetic material. The other parent was the genome chlorophyll deficient diploid line combining the germplasm of two taxonomic groups (Gp. *Tuberosum* and Gp. *Phureja*). *Tuberosum-Phureja* hybrids give high yields (Sanford and Hanneman 1982) and so is of potential interest for breeders. It can probably be improved by introducing disease resistance traits from *S. pinnatisectum*.

Materials and methods

Plant materials and culture conditions

The chlorophyll deficient diploid line IvP 841-1 was isolated in the Institute of Plant Breeding, Wageningen, The Netherlands and was kindly supplied by Dr. H. Pennings. The origin of this line as follows: (PM 13 × SuH 7310) × IvP *S. phureja* (PM, *Solanum phureja* Mares; SuH, haploid *S. tuberosum* var. Suchtelen). IvP 841-1 is the result of parthenogenesis after pollination of hybrids resulting from the first cross with IvP *S.*

* Dedicated to Prof. H. F. Linskens on his 65th birthday

phureja. Our earlier fusion experiments (Sidorov et al. 1986) demonstrated that the IvP 841-1 mutant is a genome recessive one. Axenic albino shoot cultures of IvP 841-1 were maintained on Murashige and Skoog's (1962) medium containing Morel's vitamines (Morel and Wetmore 1951) and 30 g/l sucrose. *Solanum pinnatisectum* (line 1–6) is a diploid, tuber-bearing wild species known for its high resistance to *Phytophtora* and potato viruses A and Y. Plants 1–6 were subcultured aseptically on Murashige and Skoog's medium with 10 g/l sucrose.

Protoplast isolation, fusion and culture

Mesophyll protoplasts of IvP 841-1 and 1–6 were prepared by using 0.8% Cellulisin and 0.3% Macerase in a solution of 0.5 M sucrose and 5 mM $CaCl_2$, pH 5.6. Before fusion, *S. pinnatisectum* protoplasts were γ-irradiated at doses of 200 Gy (Co^{60} source, dose rate 0.1 Gy/s). The fusions were carried out as described by Menczel et al. (1982). Protoplasts were cultured for 3 weeks (including two dilutions with fresh culture medium) in a liquid medium W-S-S (Sidorov et al. 1984) or S-W medium, which is similar to W-S-S but has another salt composition, namely, half the concentrations of the C medium used by Shepard and Totten (1977). The colonies were then plated onto a solid "greening" medium C and then individually transferred to regeneration medium according to the protocol of Shepard and Totten (1977). The shoots were rooted on Murashige and Skoog's (1962) basal medium with 10 g/l sucrose.

Isozyme analysis

Extracts for esterase and aspartate aminotransferase determinations were prepared from leaves of aseptically grown plants. The extraction buffer (6 ml/g leaves) contained the following: 0.05 M Tris-HCl, pH 8.0; 2 mM $MgCl_2$, 1 mM EDTA; 3 mM cystein; 15 mM 2-mercaptoethanol and 20% glycerol. For both types of isoenzymes, the extracts were subjected to electrophoresis on 6% polyacrilamide gels buffered with 0.05 M Tris-HCl, pH 7.5 and overlayered with 4% spacer gel containing 0.01 M Tris-H_3PO_4 buffer, pH 5.5, as described by Maurer (1971). The reservoir buffer contained 1 g Tris and 5.5 g 5,5′-diethylbarbituric acid per l, pH 7.0. A constant current of 5 mA was employed. For detection of esterase activity the gels were stained in 0.1 M phosphate buffer, pH 6.0, containing 400 mg Fast Blue RR salt and 200 mg of naphtylacetate per l (Brewbaker et al. 1968). Aspartate aminotransferase detection employed a pH 7.5, 0.2 M phosphate buffer (82 ml) containing 1 mg pyridoxal-5′-phosphate, 100 mg bovine serum albumin, 400 mg L-aspartic acid and 64 mg a-ketoglutarate to which 136 mg Fast Violet B salt dissolved in 18 ml of water was added (Wetter and Dyck 1983).

Analysis of chloroplast DNA

The chloroplast DNA (Ch DNA) was prepared from purified chloroplasts isolated from aseptically grown plants according to the method of Kolodner and Tewari (1975). The chloroplasts were lysed in 2% Sarkosyl and the DNA was then fractionated by CsCl density gradient centrifugation. Ch DNA was digested with Bam H I enzyme and separated by electrophoresis on 0.8% agarose slabs.

Chromosome analysis

Chromosome counts were made on root tips of regenerated plants. The material was treated with 0.05% colchicine (1 h, 10 °C–12 °C), fixed overnight in Carnoy's acetic acid-ethanol solution (1 : 3) and stained with 1% orcein in 45% acetic acid.

Results

Establishment of plants after protoplast fusion

Following fusion of protoplasts of the potato parent IvP 841-1 with 200 Gy irradiated protoplasts of *S. pinnatisectum* (this dose completely blocks cell division), 380 individual photosynthesizing cell colonies were isolated. A mixture of untreated protoplasts of the two parental types showed no growth. Following transfer of established clones to regeneration medium, the formation of two types of calli was observed. One had a bright green colour (241 clones), whereas the others (139 clones) were purple. Most of the colonies gave rise to apparently morphologically normal plants. Again, two distinct phenotypical classes were found. The plants of the first class had purple or reddish coloured stems and leaves, the other was green. A list of plant lines (protoclones) established to date is presented in Table 1. Surprisingly, the dark green calli gave rise to purple coloured shoots and vice versa. The green shoots developed exclusively from purple colonies. The plants obtained in both cases also showed a great deal of variation for characters such as growth vigour, root forming ability, leaf morphology, spontaneous tuber formation in vitro, etc. Cell clones regenerating abnormal flat leaf-like structures were also found, however shoots with normal morphology were later obtained from these. One protoclone (18r) with deep purple coloration after several subcultures gave rise to green shoots. Unusual behavior was exhibited by clone 3r. The plants derived from it showed intensive callusing on the underside of the leaves. Normal looking plants were frequently obtained from initially abnormal shoots, after propagation by stem nodes.

Chromosome analysis

Parental chromosomes were similar in size and morphology, therefore the study was limited to chromosome counts in regenerated plants for most of the lines (see Table 1). It was found that chromosome numbers in lines of different plant groups varied. Seven had mixoploid root cells but the main ploidy level was different. Thus, in some lines (18r, 32r) the most frequent chromosome number was 48 and in others (23 g) it was 84 or 72 (5r, 7g, 9g). The metaphase plates of two lines with chromosome numbers above 48 are shown in Fig. 1. The majority of cell lines had stable tetraploid levels, which is typical for most potato cultivars.

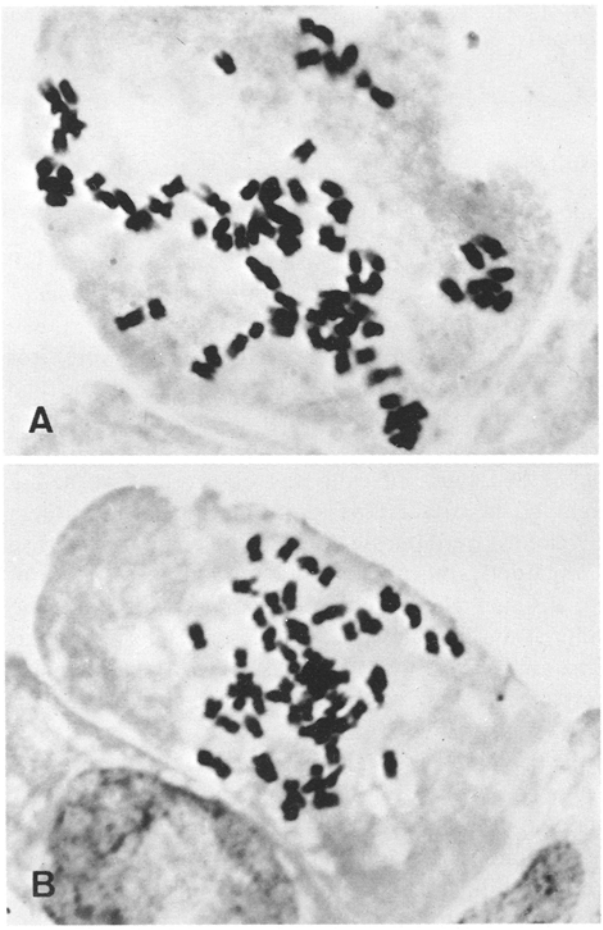

Fig. 1. Chromosome plates from root tips of plants 23 g (**a**) and 5r (**b**)

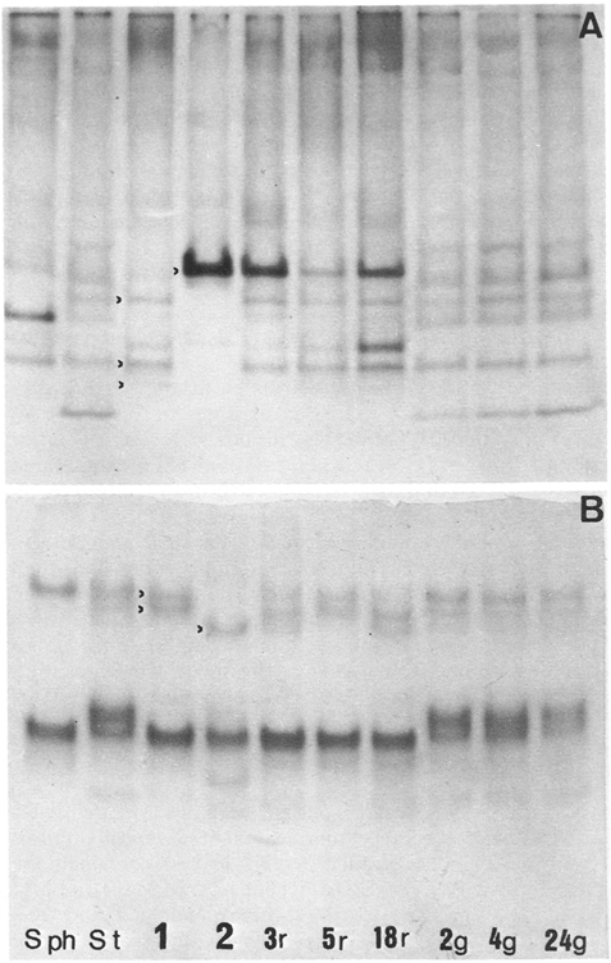

Fig. 2. Esterase (**a**) and aspartate aminotransferase (**b**) isoinzyme patterns: S ph: *S. phureja;* S t: *S. tuberosum* cv. Zarevo; 1: IvP 841-1; 2: *S. pinnatisectum;* 3r; 5r; 18r; 2g; 4g and 24g are clones derived from fusions. *Arrows* indicate the parent-specific bands

Isozyme analyses

The nuclear constitution of regenerated plants was determined from the analyses of the multiple molecular forms of esterase and of aspartate aminotransferase. Classification of plants as either somatic hybrids or parental types was possible since their isozyme patterns were characteristically different in the parents chosen (Fig. 2). The parental lines (including *S. phureja,* the germplasm of which is present in one of the parents, and *S. tuberosum* cv. Zarevo) were used as controls for determining the isozyme spectrum. The results of the analysis of six clones representing both phenotypic groups of hybrids are shown in Fig. 2 a, b. The bands specific for the parents used in fusion are present in the three clones with purple pigmentation (3r, 5r and 18r). These data confirm the hybrid nature of pigmented plants. On the other hand, esterase bands specific for *S. pinnatisectum* are absent in the patterns of clones with green colouring and the esterase isozymes of most of these lines were similar to those of the cultivated

Table 1. Phenotype and chromosome numbers of plants regenerated from fused protoplasts

Line of established plants	Chromosome no.
a) Green	
1g, 4g, 7g, 12g, 15g, 21g, 24g, 27g, 29g,	48
2g	60[a]
7g, 9g	72[a]
23g	72, 84[a], 87
6g, 17g, 28g, 33g	not studied
b) With purple pigmentation	
3r, 13r, 25r, 30r	48
5r	64, 72[a], 96
18r, 32r	36, 48[a], 56

[a] Most often observed chromosome number

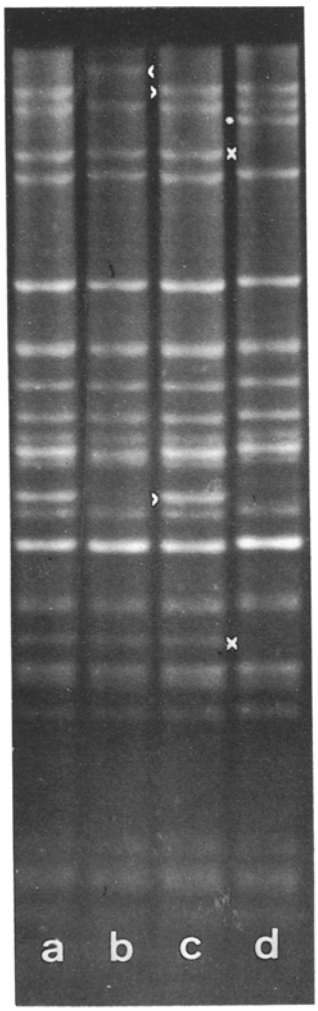

Fig. 3. Bam H I restriction pattern of Ch DNAs of plants produced by protoplast fusion and their parents: **a** 3r, **b** IvP 841-1, **c** *S. pinnatisectum*, **d** 15g. *Arrows* indicate species-specific fragments of corresponding parents. Note the presence of a novel fragment (asterisk) and absence of two non-species-specific fragments (x)

potato. The analyses of aspartate aminotransferase isozymes gave similar results (see Fig. 2 b).

Analysis of chloroplast DNA

Ch DNA was isolated from six plant lines derived from fused protoplasts. Our earlier results indicate that IvP 841-1 line possesses *S. phureja* Ch DNA. After digestion with the Bam H I restriction endonuclease, the fragments of Ch DNAs of all but one line analysed were identical to those of *S. pinnatisectum*. The Ch DNA restriction pattern of one such digest (line 3r) is presented in Fig. 3 a. One line (15g) showed a unique non-parental restriction pattern in which a novel fragment was present and two bands typical for both parents were absent (Fig. 3 d). In addition, only one of two species-specific Ch DNA bands of *S. pinnatisectum* was present in this plant. Experiments are in progress to further characterize the unusual type of Ch DNA.

Discussion

In this study, γ-irradiated protoplasts of *S. pinnatisectum* were used as a means of producing a wide range of somatic hybrids with variant genetic backgrounds. The selection procedure was based on restoration of photosynthetic ability in hybrid cells after fusion of the nuclear chlorophyll deficient mutant with γ-irradiated protoplasts of the wild type. Two callus lines differing in their pigmentation were obtained after hybridization and these gave rise to plants of diverse phenotypes which could be divided into two groups. The first had anthocyanin pigmentation and included seven lines, which judging from biochemical and cytogenetic data, were truly nuclear, probably symmetric, hybrids. Our data indicate that anthocyanin synthesis is expressed in true nuclear hybrids and is therefore a dominant trait. Although the degree of the expression varied at different stages of culture, the marker for purple stem and leaf is clearly manifested in hybrids and can be regarded as additional evidence for the hybrid nature of regenerants. This facilitates the screening of somatic hybrids in combinations with *S. pinnatisectum* as one of parents.

Aneuploidy and polyploidy among the hybrids may be the result of multiple protoplast fusion, of elimination of part of the genetic material by irradiation or of species-specific incompatibility. Obviously in some lines, the stabilization of genetic material is not complete at plant regeneration. In our experiments, abnormal shoots and organised structures similar to those observed in intergeneric hybrids of *Atropa* + *Nicotiana* (Gleba et al. 1983) were observed. After several passages, the stabilisation and normalisation of hybrid genomes occured and as a result phenotypically normal plants are formed. However, even at the whole plant stage the cells of different ploidy are obviously present, as indicated by chromosome counts in root cells of some hybrids. The deviation from normal development of tuber formation in vitro and the development of callus masses on the undersides of leaves in clone 3r is probably connected with imbalances in the hormonal levels of the clones in question.

The origin of the second group of plants, with morphology typical for the potato cultivar, cannot be completely explained. Biochemical data does not prove that these plants are derived from fusion products. At the same time, asymmetric hybrid production as a result of fusion of normal and irradiated protoplasts is the most likely explanation. If so, the plants must contain a small part of the genome of the irradiated cell responsible for chlorophyll synthesis, which was not detected by isoenzyme analyses. It was shown earlier

that even lower doses of irradiation than employed in our work can result in asymmetric hybrid formation (Sidorov et al. 1981; Gupta et al. 1984). On the other hand, this group of plants may result from reverse mutations or arise from wild type cells because of the chimerization of initial material. The wide range of genotypic variability noted in the material employed could be anticipated as somaclonal variations are well documented for potato. The last assumption, however, disagrees with data from the control experiment in which no photosynthesizing plants were found.

S. pinnatisectum Ch DNA was present in most lines shown to be hybrids. However, a rearranged Ch DNA was detected in one line. Rearrangement of plastid DNA has been previously observed in only one somatic hybrid of the *Nicotiana tabacum* + *N. plumbaginifolia* and was shown to be the result of recombination of parental DNA (Medgyesy et al. 1985). Our data indicate that Ch DNA rearrangements in somatic hybrids can be detected in the absence of selective pressure in favour of recombination, even though they occur quite rarely compared to alterations in the mitochondrial DNA (Belliard et al. 1979; Komarnitsky et al. 1986).

The results obtained in the present study demonstrates that the method of potato reconstruction via fusion of normal somatic and γ-irradiated protoplasts can be used for the selection of plants with new gene combinations. This approach has permitted us to produce genetically different symmetric and asymmetric hybrids which can facilitate the further development of new varieties of this species.

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Note added in proof. At present somatic hybrid plants have flowered and after pollination with potato cultivar yielded seeds.