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Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*

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Abstract

Protoplasts from diploid S. tuberosum and diploid S. phureja were electrofused followed by selection of the heterokaryons with a micromanipulator. Visual identification of the heterokaryons was facilitated by fluorescein diacetate staining of the protoplasts from one of the parents, which was grown on herbicide containing medium to induce bleaching of the chlorophyll. In total, 840 heterokaryons showing red (chlorophyll) and yellow-green (fluorescein) fluorescence were selected and cultured at relatively low densities using various feeder systems. Finally, 18 putative hybrid plantlets were obtained and grown to maturity. DNA histograms indicated that the plants were hexaploid, octoploid or mixoploid. With Giemsa C-band pattern analysis of the chromosomes the hybrid character and the combinations of the chromosome sets of all plants investigated could be established.

Introduction

Although commercial potato cultivars are in general tetraploids, breeding at this level is hampered by the high number of quality demands, the low chance of obtaining the desired characters after sexual crossing and the need for recurrent backcrossing. Breeding at the diploid level accelerates the selection process considerably; somatic fusion of two diploid lines may circumvent the problem of segregation and could lead to desired properties such as high yielding tetraploids (Wenzel 1980). Following this method incongruity barriers can be overcome and important traits (e.g. disease resistances) from wild species can be introduced.

Somatic hybridization of potato species has been reported for tetraploid S. tuberosum and diploid S. brevidens between which a crossability barrier exists (Barsby et al. 1984). Transfer of resistance to potato leaf roll virus from S. brevidens into S. tuberosum by somatic fusion has also been achieved (Austin et al. 1985a), while an intraspecific fusion of two S. tuberosum diploids has been realized (Austin et al. 1985b). Recently, the phenotypic variation of hybrid plants, obtained after fusion of protoplasts of a tetraploid S. tuberosum and a diploid S. brevidens has been described (Austin et al. 1986).

In view of a joint project with the Foundation for Plant Breeding (SVP) at Wageningen on the feasibility of somatic cell hybridization in potato breeding programmes (Roest and Puite 1986) we describe here the somatic hybridization of a diploid line of S. tuberosum and of S. phureja using electrofusion. Recently, some reports have pointed to the potential of this fusion method for Nicotiana (Bates and Hasenkampf 1985, Kohn et al. 1985, Puite et al. 1985).

Materials and Methods

Plant material

The diploid SVP1 line (SH 77-78-1994) of S. tuberosum and the diploid SVP5 line (PH 77-1445-2242) of S. phureja were used. Micropropagation of these lines, protoplast isolation, culture and regeneration followed with some modifications the procedure as described for the tetraploid cultivar Bintje (Bokelmann and Roest 1983).

SVP1 and SVP5 plantlets were grown on MS medium supplemented with 1% and 3% sucrose, respectively. Plantlets of SVP5 were bleached by adding the herbicide SAN 9789 (3 mg/l) to the medium (Uhrig 1981). From the in vitro cultured shoots 1 to 2 g of fresh shoot material (leaves and stems) was excised, cut into small fragments and preplasmolysed in 10 ml of 0.5 M mannitol during about 10 min. The enzyme treatment was carried out overnight in petri dishes on a rotary shaker (30 rotations per min) using 1% cellulase (Onozuka R-10) and 0.2% macerozyme (R-10) in V-KM culture medium (Bokelmann and Roest 1983) at half strength, supplemented with 0.04 M mannitol and 0.04 M glucose, resulting in an osmolality around 500 mOsm/kg. The protoplasts were washed in V-KM salts at half strength supplemented with 0.32 M NaCl at an osmolality of approximately 500 mOsm/kg, and floated on 0.43 M sucrose at about 500 mOsm/kg. The bleached protoplasts were stained with fluorescein diacetate (FDA) (8 µg/ml) during 30 min. Free FDA was removed by washing with W5 solution (Sidorov et al. 1981) and with 0.34 M mannitol.

Electrofusion, selection and culture of heterokaryons

One ml of a 1:1 mixture of the SVP1 and FDA stained SVP5 protoplasts (in total 3.10^4) in 0.34 M mannitol was pipetted into the multi-electrode fusion chamber.

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The design, electronic circuit and the AC and DC settings used are given in Puite et al. 1985. The heterokaryons showing both the red (chloroplast) and green-yellow (FDA) fluorescence from the SVP1 and SVP5 protoplasts, respectively, could easily be identified, even 2 days after electrofusion. Directly after fusion and removal of the electrode array 1 ml of an adapted culture medium (V-KM with 0.34 M glucose) was added to the fusion mixture. The protoplasts were thus cultured in liquid V-KM medium at half strength, in the absence of coconut milk and 2,4-D, and supplemented with 0.17 M mannitol and 0.17 M glucose, NAA at 0.1 mg/l and zeatin at 0.5 mg/l, casein hydrolysate at 375 mg/l and the antibioticum cefotaxime at about 250 µg/ml (osmolality around 400 mOsm/kg).

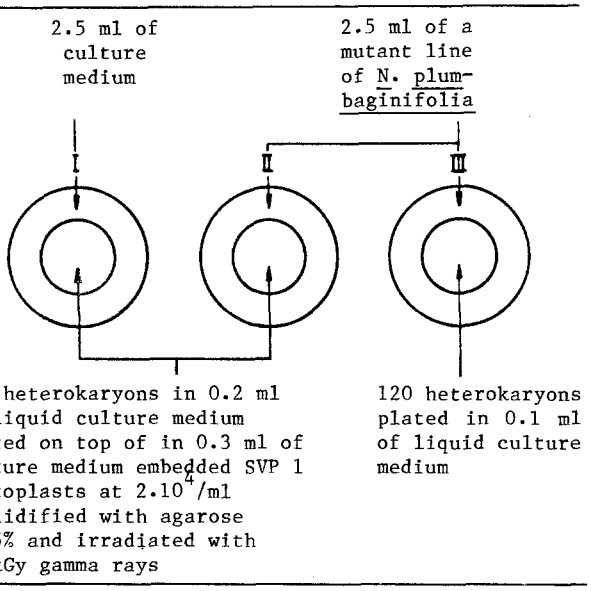

Fig. 1. Scheme of feeder systems used

One and two days after electrofusion a total of 840 heterokaryons were selected with a Leitz micromanipulator. Each time 120 heterokaryons were transferred into the center well of Falcon dishes (no. 3037) in 0.1 or 0.2 ml of liquid culture medium using various feeder systems (Fig. 1). Twelve days after electrofusion 0.2 ml of fresh liquid culture medium was added to the center well and 3 days later the developing cell aggregates were transferred with a micropipette to 1.5 ml of fresh culture medium and embedded in agarose (0.2%). Four weeks after electrofusion the cell colonies were picked up with tweezers and plated on top of a solid growth medium. Two weeks later the calli were transferred to shoot initiation and subsequently to shoot elongation medium. For plantlet production some shoots were excised from the calli and subcultured on a medium for root formation. Finally the rooted plants were transferred to soil and grown to maturity.

DNA histograms and chromosome analysis

DNA histograms were measured using a FACS IV Cell Sorter (Becton Dickinson, Sunnyvale USA) equipped with a Spectra Physics argon ion laser, model 164-05. Leaf material was treated according to Galbraith et al. (1983) with some modifications. The "chopping buffer" was the one used by Malmberg and Griesbach (1980) and the nuclei in the filtrate were stained with ethidium bromide (25 µg/ml). Excitation of the fluorochrome-DNA complex with the 488 nm line of the laser resulted in a fluorescence diagram representing the relative DNA content of the nuclei.

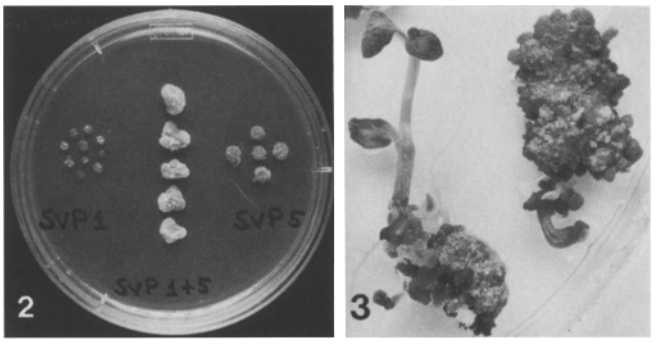

Fig. 2. Putative hybrid calli (SVP 1 + 5) and calli from the parental lines
Fig. 3. Putative hybrid plantlet

Giemsa C-band pattern analysis of chromosomes from shoot and root tip cells was carried out and compared with the parental karyotypes following Pijnacker and Ferwerda (1984). More consistent C-banding was obtained when the slides were immersed in 0.2 M HCl for 1 h at room temperature before the barium hydroxide step (Summer 1972).

Results

Electrofusion and culture of heterokaryons

The percentage of heterokaryon formation after electrofusion was 6 ± 1%. Collection of heterokaryons after two days was more efficient then after one day (plating efficiencies 6 and 3%, respectively) (Table 1). No substantial difference in plating efficiencies was observed between the various feeder systems used.

Plated heterokaryons showed the first cell divisions after 5 days and the developing cell aggregates were 0.5 to 1 and 1 to 2 mm in diameter, 2 and 4 weeks after electrofusion, respectively. From 840 selected and cultured heterokaryons 41 putative hybrid calli were obtained growing much more vigorously on solid growth medium than the calli obtained from protoplasts of the parental lines, which indicates hybrid vigour (Fig. 2). Ultimately 13 out of 41 calli, transferred to shoot initiation and shoot elongation medium, regenerated adventitious shoots (Fig. 3). Via root formation of excised shoots 18 plantlets derived from 7 calli were transferred to soil and grown to maturity.

Table 1. Plating efficiency of cultured heterokaryons using different feeder systems, 3 weeks after electrofusion

Feeder system	Selection of heterokaryons after 1 or 2 days	Number of plated heterokaryons	Number of microcalli	Plating efficiency (%)
I	1	120	2	1.7
	2	120	9	7.5) 4.6
II	1	120	4	3.3
	2	120	7	5.8) 4.6
III	1	120	6	5
	2	120	4	3.3) 5.4) 5.3
	2	120	9	7.5
I+II+III	1	360	12	3.3
	2	480	29	6.0
		840	41	4.9

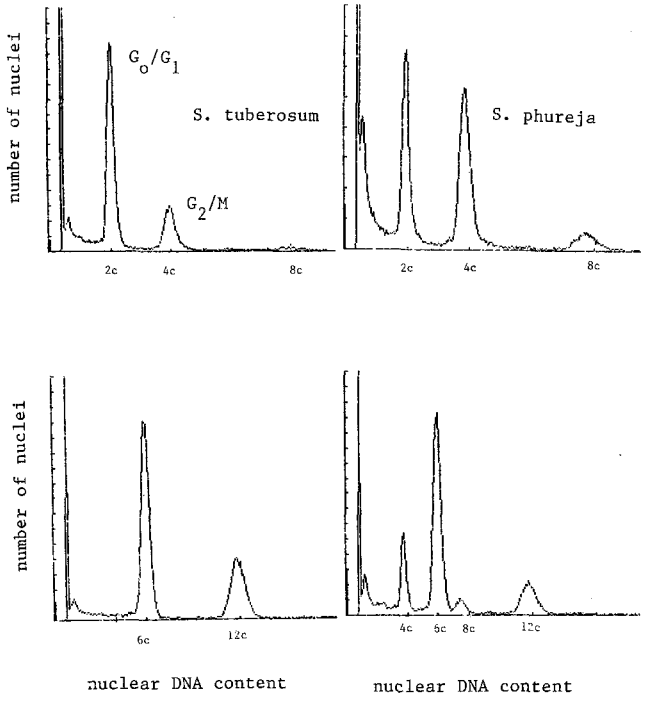

Fig. 4. DNA histograms of leaf material of in vitro cultured plantlets of SVP 1 and SVP 5 (top) and two putative hybrid plants (bottom)

Flow cytometric and cytological analysis

DNA histograms were measured from the in vitro grown parental lines and the putative hybrid mature plants (Fig. 4). Variation coefficients of the DNA peaks varied between 3.5 to 5.3 %. Based on the position of the peaks the ploidy level has been determined (Table 2).

The nuclear DNA content of the regenerants was either at the hexaploid or octoploid level and also indicated the presence of aneuploidy and mixoploidy. These results were confirmed more specifically by the chromosome counts (Table 2).

The karyotypes of the SVP1 and SVP5 plants differed in two distinct C-banding patterns. The C-band(s) of the satellite arm of the nucleolar chromosomes (chromosome 2) of SVP1 were much smaller than those of SVP5 and the band on the short arm of chromosome 4 of SVP1 was more distinct than that of SVP5 (Fig. 5a,b). It may be remarked that the satellites of SVP1 were polymorphic and that the karyotype of SVP5, being a S. phureja, was rather similar to the karyotype of S. tuberosum cv. Gineke described by Pijnacker and Ferwerda (1984) and that the reverse applied to SVP1. With the aid of these differences the combination of chromosome sets in the karyotypes of the regenerants could be established (Fig. 5c; Table 2). The eight regenerants investigated appeared to be hybrids.

Morphological characteristics

Morphological characterization of the hybrid plants revealed a robust and intermediate (with respect to the shape, hairyness and curliness of the leaves) phenotype of some plants, which proved to be hexaploids. The majority of the plants were grossly aberrant from the parental lines, many being small tiny plants and dwarfs (Fig. 6).

Discussion and conclusions

It is clear that electrofusion is a suitable technique in obtaining somatic hybrid potato plants. We have selected the heterokaryons after fusion with a micromanipulator at a rate of 100/h. Mass sorting of heterokaryons would be possible by means of a flow cytometer/cell sorter (Afonso et al. 1985). Flow sorting of plant protoplast and culture to plants have already been achieved (Verhoeven et al. 1985).

While potato protoplasts are usually cultured at a density of about 3.10^4/ml, these experiments showed that 120 heterokaryons could be succesfully cultured in 0.1 or 0.2 ml of culture medium. Regeneration at these low densities of 0.6 to 1.2 10^3/ml can be ascribed to the preculture during 1 or 2 days at an almost optimal density of 1.5 10^4/ml before selection of the heterokaryons and to the feeder systems used. Besides, hybrid vigour and the visual selection of the heterokaryons may play a role.

Most of the hybrids were hexaploids and octoploids and no tetraploid plants were obtained. Although fusion of three or four protoplasts instead of only two should not be excluded, the low fusion frequency of 6% would allow only a small fraction of these. Other explanations are that diploid G2 and tetraploid G1 cells, which are present in the leaves of both and one of the parents, respectively, as revealed by the DNA measurements (Fig. 4), are involved in the fusions and lead to higher ploidy

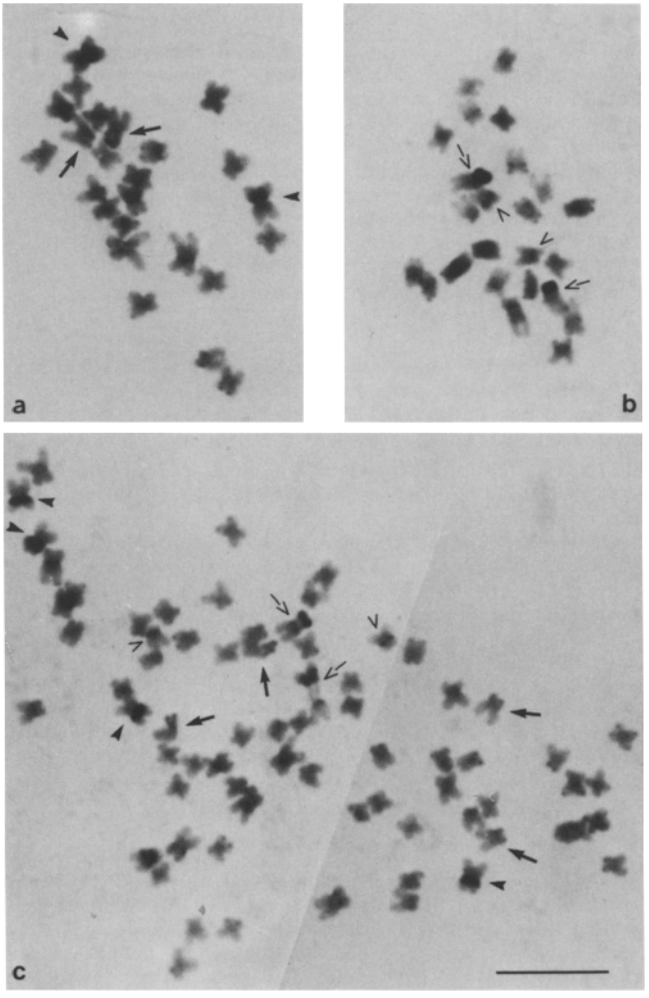

Fig. 5. Karyotypes of SVP 1 (a), SVP 5 (b) and one of the hexaploid regenerants (c), consisting of 2 x SVP1 and 1 x SVP5. Arrow: chromosome 2 (nucleolar chromosome). Arrow head: chromosome 4. Bar represents 10 µm.

Table 2 Ploidy level based on DNA histograms and chromosome analysis of plants regenerated from 7 putative hybrid calli after somatic hybridization of Solanum tuberosum and S. phureja (2x)

Callus	Ploidy level of plants with Fast	Moderate	Retarded growth	Chromosome analysis Genome	Number
1	6x[1], M[2]		6x[3], M	1) 4T + 2PH 2) 2T + 2PH 4T + 2PH 3) 4T + 2PH	72 45-47 72, some 71 67-69
2	6x[4], 6x[5], 6x[6]	6x, M	6x[7]	4) 2T + 4PH 5) 2T + 4PH 6) 2T + 4PH 7) 2T + 4PH	65-66 69, some 68 71 68-69
3	8x[8]	8x, 8x		8) 4T + 4PH	90-96
4		M	M		
5		M			
6			8x		
7			M/8x		

M = mixoploid T = S. tuberosum PH = S. phureja

levels or that doubling of chromosome sets takes place in the heterokaryons or later on during culture (Sree Ramulu et al. 1984). The example of mixoploidy (Table 2) points to either a doubling of one of the parental chromosome sets in a tetraploid hybrid cell or a loss of a diploid chromosome set in a hexaploid hybrid cell. The various combinations of chromosome sets indicate that both parents can contribute in an equal way to the higher ploidy levels. Chromosomal instability occurs during culture as indicated by loss of chromosomes. Wether particular chromosomes have disappeared is under investigation. The results further demonstrate that even slight differences in C-banding patterns are sufficient to identify a hybrid fusion.

The question whether somatic hybridization can really contribute to a potato breeding program cannot be answered yet and will require more extensive somatic hybridization experiments.

Fig. 6. Somatic hybrids (hexaploids) in the centre, parental plants SVP1 and SVP5 at the left and at the right, respectively

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