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J. O. Rasmussen · S. Waara · O. S. Rasmussen

# Regeneration and analysis of interspecific asymmetric potato – *Solanum* ssp hybrid plants selected by micromanipulation or fluorescence-activated cell sorting (FACS)

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**Abstract** Recipient protoplasts from three *Solanum tuberosum* genotypes, cv 'Folva' (2n = 4x = 48), cv 'Matilda' (4n) and '161:14' (2n), were electrofused with X-ray-irradiated donor protoplasts from two wild species *S. spegazzinii* (2n) or *S. microdontum* × *S. vernei* (2n). Prior to fusion, protoplasts were fluorescence-labelled with either fluorescein diacetate or scopoletin. Fusion products were identified by dual fluorescence and selected by micromanipulation or fluorescence-activated cell sorting (FACS). All putative hybrid plants were analysed by the random amplified polymorphic DNA (RAPD) technique. Our analysis demonstrates that each asymmetric hybrid plant has an individual and stable profile of donor-specific RAPD bands. The irradiation of donor protoplasts hampered the growth of selected heterofusion products in a dose-dependent way. Irradiation resulted in donor chromosome elimination, but not in a dose dependent way, in the tested interval. In asymmetric hybrids with the *S. spegazzinii* donor 33–68% of the donor-specific RAPD bands were missing, indicating a similar level of chromosome elimination. In asymmetric hybrid plants with the *S. microdontum* × *S. vernei* donor 74–95% of the donor RAPD bands were missing. Chromosome countings revealed that these hybrids had chromosome numbers equal to or below the chromosome numbers found in the tetraploid recipients. This is the first time that highly asymmetric hybrid plants between two tetraploid potato recipients and the donor *S. microdontum* × *S. vernei* have been obtained.

**Key words** Protoplasts · *Solanum tuberosum* · *Solanum* ssp. donor · RAPD · Chromosome elimination

## Introduction

Protoplast fusion is a powerful tool in potato breeding that is used to overcome sexual incompatibility barriers and to combine agronomically important traits (Pehu et al. 1989; Thach et al. 1993; Rasmussen et al. 1996). Interspecific protoplast fusions between *Solanum tuberosum* and wild *Solanum* species have been performed in order to enlarge the *S. tuberosum* gene pool with genes conferring resistance to pathogens (Pehu et al. 1989; Lentini et al. 1990; Mattheij et al. 1992) or abiotic stress (Cardi et al. 1993). However, fertility problems and an excess of undesired wild-type traits are commonly observed in interspecific somatic hybrids (Mattheij et al. 1992). These obstacles may be solved by use of a partial genome transfer technique such as asymmetric protoplast fusion.

It has been demonstrated that treating protoplasts (donor) with ionizing irradiation prior to fusion with non-irradiated protoplasts (recipient) results in a certain degree of donor chromosome elimination in the hybrids. Sjödin and Glimelius (1989) fused irradiated protoplasts from wild *Brassica* species with protoplasts from *Brassica napus* and obtained asymmetric hybrid plants conferring resistance traits from the wild species and with chromosome numbers not higher than that of the non-irradiated fusion partner. Both the elimination of a few donor chromosomes (McCabe et al. 1993) and the elimination of most of the donor chromosomes (Samoylov et al. 1996) in the asymmetric hybrids have been demonstrated . Xu et al. (1993) observed in asymmetric hybrids between *S. tuberosum* and *S. brevidens* that 2–17 of the 24 *S. brevidens* donor chromosomes were eliminated after irradiation with 300–500 Gy. It is likely that the degree of chromosome elimination

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J. O. Rasmussen (✉) · O. S. Rasmussen
Department of Molecular and Structural Biology, Aarhus University, C.F. Møllers Allé 130, DK-8000 Aarhus, Denmark

S. Waara
Department of Plant breeding, Swedish Agricultural University, Box 7003, S-750 07, Uppsala, Sweden

depends on the level of donor protoplast irradiation, since the number of dsDNA breaks in irradiated plant protoplasts increases with the irradiation dose (Schoenmakers et al. 1994a). However, additional factors other than irradiation doses also seem to be important for chromosome elimination, since clear correlations between irradiation doses and chromosome elimination have been demonstrated for some hybrid combinations (Melzer and O'Connel 1992; Schoenmakers et al. 1994b) and not for others (Dudits et al. 1987; Gleba et al. 1988; Xu et al. 1993). Samoylov et al. (1996) showed in asymmetric hybrids between *S. melongena* (recipient) and a *L. esculentum* × *L. pennellii* hybrid (donor) that most of the donor chromosomes were eliminated after a low irradiation dose. Asymmetric interspecific protoplast fusions have also resulted in the formation of potato cybrids after 100 Gy of irradiation (Perl et al. 1991).

Puite and Schaart (1993) and Schoenmakers et al. (1994a) observed a polyploidization of the recipient genome in the asymmetric hybrids. They suggested that polyploidization is necessary in order to buffer the negative effect of the additional genetic material from the donor.

Asymmetric hybrid plants from fusions between *S. tuberosum* genotypes (recipients) and wild *Solanum* species (donors) have all been reported to have a higher chromosome number than that found in the recipients. Donor chromosome elimination in asymmetric *Solanum* hybrids ranges from 0–65% (Fehér et al. 1992; Xu and Pehu 1993).

A major obstacle to the application of asymmetric protoplast fusion in potato breeding has been the lack of genotype-independent selection methods of putative hybrids. Selection strategies based on genetical transformed donor species with a selectable marker gene have contributed to our knowledge of the basic biological parameters involved in asymmetric protoplast fusion (Fehér et al. 1992; McCabe et al. 1993; Puite and Schaart 1993). Donor-specific morphological traits have also been used to select asymmetric hybrids after callus culture and shoot regeneration (Xu et al. 1993). Selection strategies that are independent of specific donor marker genes have been developed in symmetric *Solanum* (Waara et al. 1991a) and asymmetric *Brassica* (Sjödin and Glimelius 1989) protoplast fusion experiments. These selection strategies – micromanipulation and fluorescence activated cell sorting (FACS) – have never been used in experiments with asymmetric *Solanum* hybrids. Both methods are based on a selection of dual-stained heterofusion products shortly after fusion.

The identification and analysis of asymmetric hybrid plants have been performed by isozyme analysis (Sidorov et al. 1987) restriction fragment length polymorphic (RFLP) markers (Xu and Pehu 1993) and species-specific DNA probes (Xu et al. 1993; Samoylov and Sink 1996). The development of the random amplified polymorphism DNA (RAPD) method by Williams et al. (1990) has provided an easy, fast and powerful technique in the analysis of putative asymmetric hybrids. RAPD is a suitable technique for screening new hybrid combinations, and has been used to analyse *S. phureja* gene introgression into the *S. tuberosum* genome (Waugh et al. 1992) and to confirm the asymmetric hybrid nature of *S. melongena* (recipient) + *Lycopersicon esculentum* × *L. pennellii* (donor) hybrids (Samoylov et al. 1996). The number of donor-specific RAPD bands present in asymmetric hybrids may provide information on the extent of donor DNA introduced into the hybrid genome.

The aim of the study presented here was to produce asymmetric hybrids between three *S. tuberosum* recipient cultivars and two wild *Solanum* donor species with polygenically inherited resistance to the potato cyst nematode *Globodera pallida* and to evaluate micromanipulation and FACS, as selection methods, in the production of asymmetric *Solanum* hybrids. Different levels of irradiation were applied to the donor protoplasts in order to analyse the effects on hybrid cell growth and donor chromosome elimination. A dihaploid and two tetraploid recipient genotypes were used to analyse the influence of the recipient ploidy level on the growth capacity of the heterofusion products and on donor chromosome elimination and chromosome number in the asymmetric hybrids. RAPD analysis was used to identify the asymmetric hybrids and to estimate the extent of donor chromosome elimination in the hybrids.

## Materials and methods

### Plant material

Three *S. tuberosum* genotypes, cv 'Folva' ($2n = 4x = 48$ chromosomes), cv 'Matilda' ($2n = 4x = 48$ chromosomes) and cv '161 : 14' ($2n = 2x = 24$ chromosomes) (named 'Folva', 'Matilda' and '161 : 14', respectively), were used as recipients. Two wild potato species, *S. spegazzinii* Id. Nr. PI 320299 ($2n = 24$ chromosomes) (code V7) and *S. microdontum* × *S. vernei* ssp *balsii* ($2n = 24$ chromosomes) (code V8), were used as donors. All plants were grown in vitro under a regime of 16/8-h day/night at 20°/18°C with a light source of 80 $\mu E/m^2$ per second. Green plants were grown 4–5 weeks on MS media (Duchefa, Haarlem, The Netherlands) solidified with 0.3% gellan and supplemented with 1% sucrose for 'Matilda', 1% sucrose and 0.2 mg/l IBA for V7, 2% sucrose for '161 : 14' and 2% sucrose and 0.2 mg/l IBA for 'Folva' and V8.

SAN 9789 (Norfluorazon, Sandoz Agro, Basel, Switzerland)-bleached plants (Uhrig 1981) were grown 4–7 weeks on MS media solidified with 0.3% gellan and supplemented with 2% sucrose and 2.8 mg/l SAN 9789 for V8 and 3% sucrose and 4.2 mg/l SAN 9789 for V7.

### Protoplast isolation and culture

Prior to isolation of the protoplasts green V8 and 161 : 14 plants were preconditioned for 3 days at 8°C in the dark to improve protoplast stability.

Mesophyll protoplasts were isolated and washed according to Waara et al. (1991b) and resuspended in culture 8p-media as described by Glimelius et al. (1986) modified to a final concentration of 0.4 *M* glucose, 250 mg/l PEG 6000, 0.05% MES, 1 mg/l 2, 4-D and 0.25 mg/l BAP. After selection of putative heterofusion products by FACS or micromanipulation the protoplasts were cultured in 8p-media at 20°C, 16-h daylength with a light source of 10 $\mu E/m^2$ per second. After the first cell division the selected heterofusion products were diluted by 1 vol of 8p-media modified to 0.35 *M* glucose, 1 mg/l NAA and 0.25 mg/l BAP. Further dilutions were made depending on the growth rates of the cell populations. Microcalli were transferred to new media, depending on their individual size. Calli larger than 0.5 mm in diameter were transferred to 8p-media with 3.2% mannitol, 3.3% glucose, 1 mg/l NAA and 0.5 mg/l BAP solidified with 0.3% agarose and then transferred to a higher light intensity of 100 $\mu E/m^2$ per second after 2 days. When the calli reached a diameter of 1 mm they were transferred to cal-B media (Rasmussen and Rasmussen 1995). Calli larger than 3 mm were transferred to a modified R1-D regeneration media with 1% sucrose and 2.3% mannitol (R1-J) and cultured at 128 $\mu E/m^2$ per second at a 16-h daylength. Two individual shoots from each callus were rooted on MS media with 2% sucrose, 0.2% mg/l IBA and 0.8% Bacto agar.

### Fluorescence staining of protoplasts

Protoplasts from SAN 9789-bleached donor plants were fluorescein diacetat (FDA)-stained according to Waara et al. (1991a) and used for the micromanipulation selection experiments.

Green *S. tuberosum* protoplasts for FACS selection were scopoletin-stained in 2 ml CPW 16S and 1 ml W5 with 0.6 ml scopoletin (1 mg/ml scopoletin in 0.5 *M* mannitol, pH 9.0 with KOH) for 30 min and 32°C. Green donor protoplasts for FACS selection were stained with FDA prior to irradiation.

### Irradiation of donor protoplasts

Donor protoplasts were resuspended in 5 ml 8p-media after floating on CPW 16S and irradiated by X-rays generated by a Siemens Stabilipan 200 apparatus. The tube (TR 200f) was operated at 2 kV, 10 mA and the radiation was filtered through a 1-mm aluminium filter giving a dose rate of 7.5 Gy/min ($10.3 \times 10^{12}$ Bq). Protoplasts were irradiated for 20 or 40 min, giving a dose of 150 Gy or 300 Gy. After irradiation protoplasts were floated on CPW 16S and washed twice in W5.

### Protoplast fusion

Donor and recipient protoplasts were washed once in fusion buffer (0.5 *M* mannitol, 1 m*M* $CaCl_2$) and diluted to a density of $9 \times 10^5$ protoplasts/ml in fusion buffer. They were mixed 1 : 1 and transferred in 0.6-ml aliquots to the fusion chamber (BTX microslide 453, BTX, San Diego, Calif). The power was supplied by a BTX 200 Electro cell manipulator, giving an AC field of 100 V/cm and 1000 Hz for 35 s followed by a single DC pulse of 2 kV/cm for 70 µs. The cells were allowed to recover for 2 min and then transferred to 8p-media.

### Hybrid selection

#### *Micromanipulation*

Bleached, FDA-stained donor protoplasts were fused with green recipient protoplasts and resuspended in 2.5 ml 8p-media. Six to nine individual electrofused protoplast cultures were produced for each hybrid combination and irradiation dose. Fused protoplasts were cultured 48–80 h in 35-mm petri dishes (Falcon 1008). The protoplast cultures were diluted with 10 ml 8p-media modified to a final concentration of 0.35 *M* glucose, 1 mg/l NAA and 0.25 mg/l BAP and transferred to 60-mm petri dishes (Falcon 3004) prior to selection. A petri dish was transferred to an inverse Nikon microscope, which was equipped with a Nikon micromanipulator (Narashige, Japan) fit with a home-made glass capillary tube having a diameter of 100–120 µm and controlled by a manual pump (RI, Research instruments, England). Heterofusion products identified by their green FDA fluorescence and red autofluorescence from chloroplasts were picked by the glass capillary tube under UV light (Nikon, Super high pressure mercury lamp power supply, HB-10101HB) with filter B-2A (DM 510, Nikon Japan). Between 100 and 120 selected cells in 100–120 µl of 8p-media were transferred to a petri dish with a center well (Falcon 3037) with 2 ml $H_2O$ in the outer well to diminish evaporation of the culture media. The growth of the heterofusion products was observed daily under a microscope. When most of the intact heterofusion products had divided once, the culture was diluted by 1 vol of the modified 8p-media. After two to three cell divisions the culture was diluted by 200 µl of the modified 8p-media. After the development of small microcalli the whole culture was transferred to 2 ml fresh 8p-media in a 35-mm petri dish. Microcalli with a diameter larger than 0.5 mm were transferred, individually to solid media as described.

#### *FACS selection*

Irradiated donor protoplasts stained with FDA were fused with recipient protoplasts stained with scopoletin. After electrofusion treatment of a total of $5–7 \times 10^6$ protoplasts, the cells were diluted in 8p-media, supplemented with 400 mg/l claforan (Hoechst AG, Germany) and stored for 2 h in a 24-well multidish (Nunclon). The protoplasts were collected in FACS-sorting plastic tubes and diluted to a density of $5 \times 10^4$ protoplasts/ml. Heterofusion products were selected by fluorescence-activated cell sorting (FACS) using a FACSstarPlus flow cytometer, as described by Sundberg et al. (1991) with the following modifications. (1) To detect FDA emission we adjusted the laser to emit 200 mW at the 488-nm line. (2) Fluorescence emission was measured through a 530-nm bandpass filter. (3) Approximately 10 000 putative heterofusion products were selected and transferred into a total volume of 510 µl 8p-media. (4) Heterofusion products were cultured in a 48-well multidish (Nunclon).

### Analysis of putative hybrids

Regenerated putative hybrid plants were characterized by RAPD analysis, and verified asymmetric hybrid plants were analysed for chromosome number as described in Rasmussen and Rasmussen (1995).

### RAPD analysis

Decamer primers from Operon kit AT, AR, AQ and AS (Alameda, Calif.) were used in a RAPD primer screening analysis with DNA isolated from the donors, the recipients and a DNA mixture from both. Primers giving one or more distinct donor-specific RAPD bands were selected and used for the analysis of all regenerated plants from a certain fusion combination. All regenerated plants were analysed at least twice with the same primer.

## Results

Five asymmetric hybrid combinations, generated by electrofusion of recipient *S. tuberosum* protoplasts with X-ray-irradiated wild *Solanum* protoplast, were produced. Heterofusion products were selected by micromanipulation or FACS. Regenerated plants were assessed for amount of wild-species DNA using RAPD analysis. The frequency of regenerated hybrids is listed in Table 1. The number of donor-specific RAPD bands in the hybrids and their respective chromosome number are listed in Table 2. The size and the distribution of donor specific RAPD bands found in clones from one hybrid combination are listed in Table 3.

Irradiation of V7 and V8 protoplasts with doses of 150 Gy and 300 Gy resulted in total inhibition of microcallus formation in cultures of these cells (data not shown).

### Fusion combinations selected by micromanipulation

Three hybrid combinations were selected by micromanipulation, which all resulted in the regeneration of asymmetric hybrid plants (Table 1A).

### *S. tuberosum* cv 'Folva' + *S. spegazzinii* (V7)

In two fusion experiments V7 protoplasts were irradiated with 150 Gy or 300 Gy, and in a control experiment V7 protoplasts were not irradiated. The first microcalli in the non-irradiation experiment were transferred to solid media after 14 days of culture. When V7 protoplasts were irradiated, the growth of the heterofusion products was slow. On average the heterofusion products divided twice during a period of 14 days. The first microcalli could be transferred to solid media 20 or 25 days after electrofusion when donor protoplasts were irradiated with 150 or 300 Gy, respectively.

Of 573 heterofusion products selected from the non-irradiated experiment 243 (42.1%) could be transferred to solid media, and 192 (33.5%) continued growth and were transferred to regeneration media. Sixty-four randomly chosen calli in four petri dishes were used for regeneration. Seven calli regenerated shoots which were rooted. Only few heterofusion products with irradiated donor protoplasts started growth. In the experiment with 150 Gy, 774 heterofusion products were selected. In total 112 (14.5%) microcalli could be transferred to solid media, and 67 (8.6%) continued to grow and were transferred to regeneration media. Five calli regenerated one or more shoots during a regeneration period of 4–10 months.

In the 300 Gy experiment 1192 heterofusion products were selected. Calli from 113 of these (9.5%) could be transferred to solid media, and 55 (4.6%) continued to grow and were transferred to regeneration media. Four calli developed shoots which were rooted.

All of the regenerated plants were subcultured more than twice prior to RAPD analysis. Eighteen primers used for the 'Folva' + V7 combination generated 27 V7-specific RAPD bands (Table 2). Analysis of plants from the non-irradiation experiment revealed that all of the 14 subclones from 7 calli were hybrids and that they contained 81–100% of the total number of V7-specific RAPD bands. Only 1 plant (A) from each callus is listed in Table 2. Six plants (8A, 8B, 9A, 9B, 10A, 10B) from

**Table 1** Regeneration of putative asymmetric hybrids after selection by micromanipulation (A) or FACS (B). All rooted shoots were analysed by RAPD to identify asymmetric hybrids (*nd* not determined)

| Parents fused | Irradiation dose (Gy) | Number of selected hetero-fusions | Number of calli transferred to solid media | Number of calli transferred to shoot inducing media | Number of shoot-forming calli | Number of shoot-forming hybrid calli |
|---|---|---|---|---|---|---|
| **A: Selection by micromanipulation** | | | | | | |
| *S. tuberosum* cv Folva + | 0 | 573 | 243 (42.1%) | 192 (33.5%) | 7[a] | 7 |
| *S. spegazzinii* V7 | 150 | 774 | 112 (14.5%) | 67 (8.6%) | 5 | 3 |
| | 300 | 1192 | 113 (9.5%) | 55 (4.6%) | 4 | 1 |
| *S. tuberosum* cv 161 : 14 + | 0 | 600 | 212 (35.3%) | 181 (30.2%) | 3[a] | 3 |
| *S. spegazzinii* V7 | 150 | 534 | 18 (3.4%) | 9 (1.7%) | 3 | 2 |
| | 300 | 873 | 9 (1.0%) | 7 (0.8%) | 4 | 4 |
| *S. tuberosum* cv Matilda + *S. microdontum* × *S. vernei* V8 | 150 | 1110 | 332 (29.9%) | 52 (4.7%) | 4 | 1 |
| **B: Selection by FACS** | | | | | | |
| *S. tuberosum* cv Folva + *S. microdontum* × *S. vernei* V8 | 300 | 10 000 | 157 (1.6%) | 105 (1.1%) | 5 | 3 |
| *S. tuberosum* cv 161 : 14 + *S. microdontum* × *S. vernei* V8 | 300 | 10 000 | nd | 139 (1.4%) | 9 | 0 |

[a] From 64 cultured calli

**Table 2** RAPD analysis and chromosome number of asymmetric hybrids selected by micromanipulation or FACS. Two clones A and B from the same callus were analysed. Percentage in brackets

| Hybrid combination | Irradiation dose (Gy) | Hybrid code | Clone | Number of primers | Number of donor-specific RAPD bands | | Chromosome number |
|---|---|---|---|---|---|---|---|
| | | | | | In the donor | In the hybrid (%) | |
| *S. tuberosum* cv 'Folva' (2n = 48) + *S. spegazzinii* V7 (2n = 24) | 0 | 1 | A | 18 | 27 | 22 (81) | – |
| | | 2 | A | 17 | 25 | 24 (96) | 71 |
| | | 3 | A | 17 | 25 | 23 (92) | 73 |
| | | 4 | A | 18 | 27 | 27 (100) | – |
| | | 5 | A | 16 | 23 | 23 (100) | – |
| | | 6 | A | 17 | 25 | 25 (100) | 68 |
| | | 7 | A | 18 | 27 | 27 (100) | – |
| | 150 | 8 | A | 18 | 27 | 16 (59) | – |
| | | | B | 18 | 27 | 16 (59) | – |
| | | 9 | A | 18 | 27 | 13 (48) | – |
| | | | B | 18 | 27 | 12 (44) | – |
| | | 10 | A | 18 | 27 | 18 (67) | – |
| | | | B | 18 | 27 | 18 (67) | – |
| | 300 | 11 | A | 18 | 27 | 10 (37) | – |
| *S. tuberosum* cv 161 : 14 (2n = 24) + *S. spegazzinii* V7 (2n = 24) | 0 | 12 | A | 18 | 31 | 31 (100) | 72 |
| | | | B | 18 | 31 | 31 (100) | – |
| | | 13 | A | 18 | 31 | 24 (77) | – |
| | | | B | 18 | 31 | 25 (81) | – |
| | | 14 | A | 18 | 31 | 31 (100) | – |
| | | | B | 18 | 31 | 31 (100) | – |
| | 150 | 15 | A | 18 | 31 | 15 (48) | 54 |
| | | | B | 18 | 31 | 15 (48) | – |
| | | 16 | A | 18 | 31 | 14 (45) | 63 |
| | | | B | 18 | 31 | 11 (35) | – |
| | 300 | 17 | A | 18 | 31 | 12 (39) | – |
| | | | B | 18 | 31 | 13 (42) | – |
| | | 18 | A | 18 | 31 | 11 (35) | 67 |
| | | | B | 18 | 31 | 10 (32) | – |
| | | 19 | A | 18 | 31 | 11 (35) | – |
| | | | B | 18 | 31 | 11 (35) | 55 |
| | | 20 | A | 18 | 31 | 12 (39) | 51 |
| | | | B | 18 | 31 | 12 (39) | – |
| *S tuberosum* cv 'Matilda' (2n = 48) + *S. microdontum* × *S. vernei* V8 (2n = 24) | 150 | 21 | A | 16 | 27 | 7 (26) | 47 |
| | | | B | 16 | 27 | 7 (26) | 46 |
| *S. tuberosum* cv 'Folva' (2n = 48) + *S. microdontum* × *S. vernei* V8 (2n = 24) | 300 | 22 | A | 23 | 43 | 8 (19) | 45 |
| | | | B | 23 | 43 | 8 (19) | 44 |
| | | 23 | A | 23 | 43 | 10 (23) | 46 |
| | | | B | 23 | 43 | 9 (21) | 47/62 |
| | | 24 | A | 23 | 43 | 2 (5) | 48 |
| | | | B | 23 | 43 | 2 (5) | 48 |

3 calli in the 150 Gy experiment were true hybrids, revealing 44–67% donor-specific RAPD bands. The RAPD banding patterns seen in subclones A and B from the same callus were, with minor deviations, identical. One hybrid plant (11A) was regenerated from the 300 Gy experiment. RAPD analysis of 11A showed that 37% of the donor RAPD bands were present.

Regenerated plants that did not reveal any V7-specific RAPD bands in the first analysis, were tested with new primers. In total, they were tested for 52 V7-specific RAPD bands. Additional hybrids were not found (data not shown).

*S. tuberosum* cv '161 : 14' + *S. spegazzinii* (V7)

The dihaploid cv '161 : 14' was recipient. Similar to what we observed with the tetraploid 'Folva' recipient the growth of the heterofusion products was slow in experiments where donor protoplasts were irradiated.

Without donor irradiation 181 (30.2%) of the 600 heterofusion products selected developed into calli which could be transferred to regeneration media (Table 1). Sixty-four calli from four randomly chosen petri-dishes were cultivated for regeneration. Three calli regenerated shoots which were rooted. After the irradiation of the donor protoplasts with 150 Gy and

**Table 3** Donor-specific RAPD bands found in hybrid plants from the FACS-selected hybrid combination *S. tuberosum* cv 'Folva' + *S. microdontum* × *S. vernei* V8. The sizes of the donor-specific RAPD bands are given

| Hybrid | 22A | 22B | 23A | 23B | 24A | 24B |
|---|---|---|---|---|---|---|
| Primer | | | | | | |
| AT 03 | 340 | 340 | 340 | 340 | 340 | 340 |
| | 1460 | 1460 | 0 | 0 | 0 | 0 |
| AT 04 | 0 | 0 | 800 | 800 | 0 | 0 |
| AQ 02 | 0 | 0 | 500 | 500 | 0 | 0 |
| AQ 04 | 1590 | 1590 | 1080 | 0 | 0 | 0 |
| AQ 06 | 1170 | 1170 | 1170 | 1170 | 0 | 0 |
| | | | 550 | 550 | | |
| AQ 07 | 1460 | 1460 | | | | |
| | 610 | 610 | 610 | 610 | 0 | 0 |
| AQ 17 | 0 | 0 | 845 | 845 | 0 | 0 |
| AS 02 | 0 | 0 | 290 | 290 | 0 | 0 |
| AS 08 | 790 | 790 | 0 | 0 | 0 | 0 |
| AS 17 | 1210 | 1210 | 596 | 596 | 1210 | 1210 |

the selection of 534 heterofusion products, 9 (1.7%) calli could be transferred to regeneration media. Three calli regenerated shoots, which were rooted. When donor protoplasts were irradiated with 300 Gy, and 873 heterofusion products were selected we obtained 7 (0.8%) calli that could be transferred to regeneration media. Four calli regenerated shoots which were rooted.

Eighteen primers used in this hybrid combination generated 31 V7-specific RAPD bands (Table 2). All of the regenerated plants from the non-irradiation fusion experiment were hybrids. The subclones 12A, 12B, 14A and 14B generated all of the expected RAPD bands, while hybrid 13A had 77% and hybrid 13B had 81% of the total number of V7 RAPD bands. In the 150 Gy fusion experiment 4 asymmetric hybrid plants from 2 calli were regenerated. The subclones 15A and 15B from 1 callus showed the same RAPD banding pattern with 48% of the donor-specific RAPD bands. Another banding pattern was found in 16A and 16B. They showed the same banding profile for most of the primers, but 16B missed 3 bands compared to 16A. The percentage of donor-specific bands was 45% for 16A and 35% for 16B. With 300 Gy of irradiation all eight subclones from 4 calli were hybrids with 32–42% donor-specific RAPD bands.

Chromosome analysis revealed that the symmetric and asymmetric hybrids possessed a chromosome number higher than the sum of the parental plants.

### *S. tuberosum* cv 'Matilda' + *S. microdontum* × *S. vernei* (V8)

From the fusion combination between 'Matilda' and the 150-Gy-irradiated V8 1110 heterofusion products were selected (Table 1). Microcalli from 332 (29.9%) could be transferred to solid media, and 52 (4.7%) were transferred to regeneration media. After 2–6 months on regeneration media 4 calli regenerated shoots. Two rooted shoots from each callus were subcultured four times prior to RAPD analysis.

Sixteen primers generating 27 V8-specific RAPD bands were used in the analysis of all 8 clones. Two subclones 21A and 21B from the same callus were hybrids. They gave rise to 7 (26%) V8-specific RAPD bands. Clones 21A and 21B were analysed again after two subcultures, and no changes in the RAPD band patterns were observed. Chromosome analysis revealed that clone 21A had 47 chromosomes and clone 21B had 46 chromosomes.

## Fusion combinations selected by FACS

Two hybrid combinations were selected by FACS (Table 1B). One hybrid combination regenerated asymmetric hybrid plants.

### *S. tuberosum* cv 'Folva' + *S. microdontum* × *S. vernei* (V8)

From the fusion experiment between 'Folva' and V8, in which an irradiation dose of 300 Gy was used, 10 000 putative heterofusion products were selected by FACS. The quality of the FACS-selected protoplasts was high, with few damaged cells. We could transfer 157 (1.6%) microcalli to solid media (Table 1B), and 105 (1.1%) calli could be transferred to regeneration media. Five calli generated shoots. Rooted shoots were subcultivated three times prior to RAPD analysis.

Twenty-three primers generating 43 V8-specific RAPD bands were used in the analysis of this hybrid combination (Table 2). Six plants from 3 calli were asymmetric hybrids. Chromosome countings revealed that the asymmetric hybrids contained between 44 and 48 chromosomes (Table 2). Hybrid 23B was apparently a mixoploid. The chromosome number was different in subclones 22A and 22B, which were from the same callus.

Analysis of the distribution of donor-specific RAPD bands in these six hybrids (Table 3) revealed that a 340-bp band generated by the primer AT03, was found in all subclones. Other RAPD bands were found in subclones only from 1 callus, like the 790-bp band with primer AS08 in 22A and 22B and the 845-bp band with primer AQ17 in 23A and 23B. Hybrid 24A and 24B shared a 1210-bp band, generated by primer AS17, with the subclones 22A and 22B. Subclones from the same callus showed the same pattern of donor-specific RAPD bands, except for subclones 23A and 23B, which shared 9 identical donor-specific bands and, in addition 23A showed a 1080-bp band. Hybrid 22A was transferred to the greenhouse, where leaf material was

harvested for a further RAPD analysis. The same RAPD banding pattern as found in the in vitro-grown plants was observed (data not shown).

*S. tuberosum* cv '161 : 14' + *S. microdontum* × *S. vernei* V8

From the fusion experiment between the dihaploid 161 : 14 and V8 irradiated with 300 Gy 10 000 heterofusion products were selected by FACS. Of the selected cells 139 (1.4%) calli could be transferred to regeneration media, and 9 developed shoots. Two subclones from each callus were rooted and subcultured for more than 1 year prior to RAPD analysis. All subclones were analysed by 10 primers, giving a total of 17 V8-specific RAPD bands. None of the subclones had V8-specific RAPD bands.

## Discussion

Asymmetric hybrid plants derived from fusions between *S. tuberosum* and wild *Solanum* species were obtained in four different fusion combinations. Two procedures – micromanipulation or FACS – were used to select heterofusion products.

The growth rate of the selected heterofusion products from the irradiation experiments with V7 protoplasts was slower with increasing irradiation doses. The number of fusion products that were able to develop into microcalli and to continue growth was also reduced by increased doses of irradiation of V7 protoplasts (Table 1). The number of calli that could be transferred to regeneration media was reduced from 33.5% at 0 Gy to 8.6% at 150 Gy and 4.6% at 300 Gy when the tetraploid 'Folva' was recipient. With the dihaploid '161 : 14' as recipient, 30% of the cultured heterofusion products could be transferred to regeneration media at 0 Gy, while 150 Gy and 300 Gy reduced this fraction to 1.5% and 0.8%, respectively.

Several factors may work together and hamper the growth rate and microcallus formation. Ionizing irradiation of living cells results in the formation of free radicals, which are highly reactive and cytotoxic (Hall et al. 1992) and which may reduce the relative plating efficiency. Chromosome damage in the asymmetric heterofusion products as a result of irradiation may also inhibit or delay mitosis (Kastan et al. 1992). Schoenmakers et al. (1994a) observed that irradiation retarded cell division in tomato + irradiated potato hybrids. Xu et al. (1993) found in experiments with asymmetric *S. tuberosum* + *S. brevidens* hybrids that increased irradiation doses resulted in a decreased fraction of calli that could form shoots. The difference in relative plating efficiency observed in our study (Table 1) between the dihaploid and tetraploid recipients fused with irradiated V7 protoplasts could be due to a higher genomic buffer capacity in the tetraploid recipient to stand up to chromosomal rearrangements. The hampered growth of asymmetric heterofusion products exclude a selection strategy based on hybrid vigour, as reported in interspecific symmetric protoplast fusion experiments (Preizner et al. 1991).

Several methods have been used for analysing the extent of donor chromosome elimination in assymmetric hybrids. RFLP probes have been used in the verification of asymmetric hybrids (Fehér et al. 1992) and to estimate the level of donor DNA elimination (Xu and Pehu 1993). Dot blot analysis using species-specific repetetive DNA probes for calculating the amount of donor DNA in the hybrids has been reported by Imamura et al. (1987) and Puite and Schaart (1993). Analysis of asymmetric *Nicotiana* hybrids by dot blot and chromosome counts (Paistuch and Bates 1990; Kovtun et al. 1993) revealed that the dot blot value is correlated with the number of chromosomes in the hybrids.

We assume that RAPD will amplify DNA sequences distributed randomly over the donor genome and that RAPD analysis will provide a reliable basis for an estimate of the donor DNA content in the asymmetric hybrids. It is likely that the 23–43 donor-specific RAPD bands (Table 2) represent DNA sequences randomly distributed over the whole donor genome. We conclude that the number of RAPD bands found in the single asymmetric hybrid related to the total number of donor-specific RAPD bands found in the non-irradiated donor cells is a suitable and reliable estimate of the amount of donor DNA in the hybrid cells. RAPD analysis of asymmetric hybrids has recently been reported by Samoylov et al. (1996). Waugh et al. (1992) have also demonstrated the potential of RAPD markers for monitoring and identifying the presence of *S. phureja* gene fragments in a potato cultivar. In addition RAPD is a suitable method to analyse a great number of plants from different hybrid combinations compared to the labour-consuming RFLP and dot blot analysis.

In the micromanipulation experiments all of the regenerated shoots from the non-irradiated combinations were hybrids (Table 1). These results are in agreement with a previous report by Waara et al. (1991a) demonstrating that micromanipulation is an efficient and reliable method by which to select symmetric hybrids.

Fusions between 150-Gy- or 300-Gy-irradiated V7 protoplasts and either the tetraploid 'Folva' or the dihaploid '161 : 14' resulted in asymmetric hybrid plants.

RAPD analysis revealed that 32%–67% of the donor-specific RAPD bands were found in the hybrids. This indicate that approximately 33%–68% of the donor chromosomes were eliminated. However, a clear coherence between the irradiation dose and the percentage of donor-specific RAPD bands could not be demonstrated in this study.

Xu and Pehu (1993) analysed asymmetric hybrids between *S. tuberosum* and *S. brevidens* irradiated with 300–500 Gy and estimated the level of donor DNA elimination to range from 10–65%. Fehér et al. (1992) found for the same hybrid combination after 200 Gy irradiation that 0–32% of the donor DNA was eliminated in the asymmetric hybrid plants. Puite et al. (1993) could not regenerate shoots from this hybrid combination, but analysis of highly polyploid asymmetric calli revealed that 18–62% of the *S. brevidens* DNA was eliminated in experiments with 500 Gy irradiation. Our results indicated a similar level of V7 donor chromosome elimination after 150 Gy–300 Gy irradiation.

Chromosome analysis of asymmetric hybrids between '161:14' and V7 revealed that these hybrids contained more than 51 chromosomes. These hybrids might have originated from triple fusions, or the dihaploids might have doubled the chromosome number in order to buffer a putative effect from the fragmented donor genome.

From the hybrid combination 'Matilda' + V8 we could regenerate two asymmetric hybrid shoots with 26% V8 RAPD bands. The hybrids had 46 and 47 chromosomes, respectively.

The 'Folva' + V8 hybrid combination selected by FACS resulted in the regeneration of asymmetric hybrids with 5–23% donor-specific RAPD bands (Table 2). All six subclones had 44–48 chromosomes, although 23B apparently was a mixoploid. Donor DNA fragments introgressed into the recipient chromosomes or existing as minichromosomes have been demonstrated in asymmetric *Nicotiana* hybrids by use of genomic in situ hybridization (GISH) (Parokonny et al. 1992).

Donor RAPD band patterns found in subclones originating from the same callus were quite similar as those demonstrated in Table 3. The RAPD bands were distributed in a way that indicates that each regenerating callus had incorporated a unique number of V8 chromosome fragments. RAPD analysis of clone 22A, grown in the greenhouse, showed an unaltered RAPD profile after testing with the primers listed in Table 3. Identical banding patterns between subclones from the same callus, which were found in analysis repeated after each of three subcultivations, provided strong support for a stable integration of donor DNA in the hybrids. This observation is in contrast to the results of Fehér et al. (1992) who found that subclones from the same regenerated shoot could still lose donor DNA during subcultivation.

In two hybrid combinations with irradiated V8 donor protoplasts the donor chromosome elimination was estimated to be 74–95%. These results show a stronger chromosome elimination than that found in asymmetric 'Folva' + V7 hybrids after the same irradiation doses. Our results indicate that chromosome elimination was more dependent on the donor species than on the irradiation dose. Coherence between irradiation doses and chromosome elimination has been found in asymmetric tomato hybrids (Melzer and O'Connell 1992), while Xu et al. (1993) did not find a correlation between irradiation dose and chromosome elimination with asymmetric *Solanum* hybrids. Parameters like taxonomic relatedness between the fusion parents (Gleba et al. 1988) and different chromosome repair efficiencies (Schoenmakers et al. 1994a) may both contribute to the contradictory observations.

In our experiments all of the initial selected fusion products were cultured, and these represent a population of heterofusion products with fused nuclei, unfused nuclei and asymmetric hybrids with an efficient chromosome elimination. For all asymmetric hybrid combinations we did regenerate plants without donor-specific RAPD bands. These regenerants could be non-fused or homofused escaper cells or asymmetric hybrids with a negligible amount of donor DNA below the RAPD detection limit. Another explanation could be that they are cybrids originating from heterofusion products, where the nuclei did not fuse into a heterocaryon.

This is the first report on the regeneration of highly asymmetric potato + *Solanum* ssp hybrids with chromosome numbers nearly identical to those of the tetraploid recipients. In this respect the best recipients in these experiments were the tetraploid potato cultivars. Donor chromosome elimination was more related to the donor species than to an increased irradiation dose. Increased irradiation doses influenced negatively the ability of the heterofusion products to develop microcalli. The selection of heterofusion products by FACS and micromanipulation were efficient methods. The advantages of these methods are that they are not based on the use of specific morphological traits or the introduction of artificial genetic markers in the donor species. Both selection methods provide the possibility to produce asymmetric hybrid plants between a broad range of fusion combinations. The only constraint to the donor species is the ability to isolate protoplasts.

The asymmetric hybrids 21A/B, 22A/B and 24A/B are now being subjected to analysis for fertility, morphological changes and stability of donor DNA during sexual crossings. The perspective of the present work is to transfer the polygenically inherited *G. pallida* resistance traits from wild species to commercial potato cultivars. Resistance analysis of the hybrids are in progress.

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