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Production of asymmetric hybrids between *Solanum tuberosum* and irradiated *S. brevidens*

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Summary. Asymmetric somatic hybrids were obtained by fusion of *Solanum tuberosum* (PDH40) protoplasts with 300- or 500-Gy irradiated protoplasts of *S. brevidens*. These radiation doses were sufficient to prevent the growth of the *S. brevidens* protoplasts. Putative hybrids were selected on the basis of phenotype from regenerated shoots and identified with a *S. brevidens*-specific probe. From these, 31 asymmetric hybrids were confirmed by morphological characteristics, isoenzyme patterns and RFLP analysis. The morphology of the asymmetric hybrids was intermediate between that of *S. tuberosum* and symmetric hybrids of both species (obtained without irradiation treatment). Chromosome counts from 17 asymmetric hybrids showed that the chromosome number of the hybrids ranged from 31 to 64. The asymmetric hybrids probably had one or two genome complements (i.e. either 24 or 48 chromosomes) from *S. tuberosum* and 7–22 chromosomes from *S. brevidens*. There was no clear correlation between the radiation dose and the degree of elimination of the *S. brevidens* genome.

Key words: Protoplast fusion – Gamma irradiation – Partial genome transfer – *Solanum tuberosum* – *Solanum brevidens*

Introduction

Sexual incompatibility barriers strongly restrict gene exchange between wild and cultivated species. Protoplast fusion has opened new possibilities for combining different plant genomes. Symmetric hybrids, i.e. the additive combination of the complete genomes of the fusion partners, often contain many unwanted traits of the wild species, and several backcrosses with the cultivated species are required to remove unwanted characters of the wild species (Ehlenfeldt and Helgeson 1987). It has been shown that X- or gamma-irradiation of one of the parental cells (the donor) can be used to produce asymmetric nuclear hybrids (Gupta et al. 1984; Dudits et al. 1987). The donor genome is fragmented and the asymmetric hybrids contain the complete genome of one of the parents (the recipient) and a small part of the donor genome. The advantages of this procedure is that hybrids arise with fewer unwanted donor traits, and that fewer backcrosses of the asymmetric hybrids are required to eliminate these. Asymmetric hybrids have now been obtained between a variety of species in both intra- and intergeneric fusion (Dudits et al. 1980, 1987; Gupta et al. 1984; Bates et al. 1987; Sidorov et al. 1987; Gleba et al. 1988; Wijbrandi et al. 1990a; Ratushnyak et al. 1991). The genetic makeup of these asymmetric hybrids is variable. In some cases much of the donor DNA is retained in the hybrids (Gleba et al. 1988; Famelaer et al. 1989; Wijbrandi et al. 1990a); in others, chromosome elimination appears to be more extensive (Dudits et al. 1980, 1987; Bates et al. 1987). The degree of elimination has in some cases been related to taxonomic relatedness of the genotypes combined (Famelaer et al. 1989; Yamashita et al. 1989; Wijbrandi et al. 1990b).

There are a number of different procedures for selecting somatic hybrids during culture. For example, fusion products have been selected by antibiotic resistance (Bates et al. 1987), resistance to chemicals (Dudits et al. 1987) and by metabolic mutants (Gleba et al. 1988) to favor the regeneration of hybrids. It is often

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not possible or desirable to introduce a selectable marker gene into an agronomically useful genotype because this may also introduce other undesirable changes (de Vries et al. 1987). When fusion products are selected on the basis of nuclear-encoded traits of both parents, the transfer of interesting traits may be limited by the positive selection of the chromosomes containing the selectable markers. When portoplast fusion frequencies are increased substantially, heterokaryon selection schemes are not so necessary. We have applied this approach to the production of somatic hybrids of *Solanum* species (Fish et al. 1987, 1988a). In addition, to aid in the post-fusion identification of hybridity we have used the previously identified species-specific sequences of *S. brevidens* to analyze the presence of donor DNA in putative asymmetric hybrids (Pehu et al. 1990).

The aim of this partial genome transfer program is to transfer virus resistance traits from the wild potato species *S. brevidens* to the cultivated species, *S. tuberosum*. *Solanum brevidens* is a wild, diploid non-tuber-bearing *Solanum* species, that has a wide resistance to viruses (Jones et al. 1990; Valkonen et al. 1992). It is not possible to make a direct sexual cross with *Solanum tuberosum* (Ramanna and Hermsen 1981). In the present article we report the recovery of asymmetric hybrids between *S. tuberosum* and gamma-irradiated protoplasts of *S. brevidens* after mass culture of fusion products and the subsequent identification of plantlets having *S. brevidens* DNA.

Materials and methods

Plant material

A dihaploid line (PDH 40) derived from *S. tuberosum* cv 'Pentland Crown' was used as recipient and the diploid, wild species *S. brevidens* Phil (CPC 2451) was used as the donor in donor-recipient fusion experiments. The plant material was maintained as in vitro shoots on MS20, Murashige and Skoog (1962) medium with 2% sucrose, with 0.05 mg/l BAP at 25 °C, a 16-h daylength and a light intensity of 100 $uEm^{-2}s^{-1}$.

Protoplast isolation, irradiation, fusion and culture

Mesophyll protoplasts were isolated from *S. tuberosum* PDH 40 and *S. brevidens* as described previously (Fish et al. 1988a). The protoplasts of *S. brevidens* were irradiated at 300 and 500 Gy by a [^{60}Co] source (120 Gy/min at the Gray Radiological Insititute, Watford, UK) in an electrofusion solution (9% mannitol + 1 m*M* $CaCl_2$) before the fusion. Putative hybrids that resulted from these fusions are designated 300H and 500H, respectively. Non-treated PDH40 and irradiated *S. brevidens* protoplasts were pelleted and resuspended in electrofusion solution to a density of 1×10^6 ml^{-1}. The protoplasts were mixed in 1:1 ratio, and 0.3 ml of the suspension was pipetted into the fusion chamber. Electrofusion, protoplast culture and shoot regeneration were carried out as described by Fish et al. (1988a), except that all of the shoots regenerated after culture were kept for analysis.

Morphological analysis

The criteria used for assessing plant morphology were chosen from those described in the NIAB guide for identifying potato varieties (NIAB 1975). The following characters were assessed: (1) general appearance, (2) habit and (3) anthocyanin pigmentation.

DNA isoltion

Total cellular DNA was isolated from in vitro-cultured materials using the method of Draper et al. (1988).

Dot-blot analysis

The DNA to be applied to the filter (0.1–1 μg per dot) was denatured by heating at 95 °C for 5 min. The sample was then chilled on ice and an equal volume of 20 × SSC was added. The sample was then applied to a DNA binding filter, Hybond-N (Amersham). The filter was denatured for 5 min in a 1.5 *M* NaCl + 0.5 *M* NaOH solution and neutralized for 1 min in a 1 *M* TRIS + 1.5 *M* NaCl, pH 7.5 solution. The filter was baked for 40 min at 80 °C. The *S. tuberosum*-specific probe pST10 and *S. brevidens*-specific probe pSB7 (Pehu et al. 1990) were labelled by the non-radioactive digoxigenin procedure according to the instructions of the supplier (Boehringer Mannheim, catalog no 1093657). Hybridization and immunological detection were also carried out following the procedures given by the supplier.

Chromosome analysis

Chromosomes were counted from rooted shoot cultures as described by Karp et al. (1982).

Results

With the culture procedures employed, calli could be obtained from protoplasts of *S. tuberosum* PDH40. These calli could regenerate shoots on shoot induction medium. The *S. brevidens* protoplasts that were irradiated with 300 or 500 Gy of gamma-rays failed to divide and did not form calli.

A total of nine fusion experiments were carried out. Each experiment included two fusions, one between PDH 40 and 300-Gy irradiated protoplasts of *S. brevidens* and the other between PDH 40 and 500-Gy irradiated protoplasts of *S. brevidens*. The plating efficiency ranged from 0.08% to 0.8%, the average being 0.3%. A total of 10,080 calli were transferred to regeneration medium. The regeneration efficiency from *S. tuberosum* protoplasts and fusion products of *S. tuberosum* with *S. brevidens* ranged from 0.3% to 2.4% between the different experiments. The rate of shoot regeneration and the fraction of calli that could form shoots decreased with increasing radiation dose. From the fusion between PDH 40 and 300-Gy irradiated protoplast of *S. brevidens*, 98 calli produced shoots. There were 72 calli with shoots from the fusion between PDH 40 and 500-Gy irradiated protoplasts of *S. brevidens*. Most of the regenerating calli produced several shoots. Plants excised from 49 fusion calli between PDH 40 and 300-Gy irradiated protoplasts of *S. brevidens* and 36 fusion calli between PDH 40

and 500-Gy irradiated protoplasts of *S. brevidens* were analyzed further.

The putative asymmetric hybrids were initially selected from regenerated shoots by examining leaf morphology, growth habit and anthocyanin pigmentation. The morphological characteristics of both parental genotypes and symmetric hybrids are distinct (Fish et al. 1987, 1988a, b). This enabled putative hybrids to be identified and selected from amongst the regenerated shoots. Thirty-one asymmetric hybrids from different calli were identified by dot-blot analysis of total cellular DNA. When the DNA was probed by *S. tuberosum*-specific probe pST10 (data not shown), all of the regenerants gave hybridization signals, whereas only the asymmetric hybrids produced hybridization signals when their DNA was probed by the *S. brevidens*-specific probes pSB7 (examples presented in Fig. 1).

The regenerative shoots were either from the non-irradiated PDH40 or resulted from fusion giving asymmetric hybrids between the two fusion parents. None of the regenerants was purely *S. brevidens* in origin. A total of 36% of the regenerants selected were

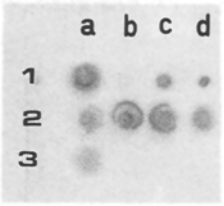

Fig. 1. Dot-blot analysis of the regenerants. Total DNA (100 ng) was from the fusion parents *S. brevidens* (*a1*) and PDH40 (*b1*), from asymmetric hybrids 10173, 40183, 10031, 10732, 50011, 10091 and 10251 (*c1*, *d1*, a2, b2, c2, d2, a3) and PDH40 protoplast-derived regenerants 40192, 40193 and 50023 (*b3*, *c3*, *d3*). The filter was probed with *S. brevidens*-specific prob pSB 7

Table 1. Morphological characters of asymmetric hybrids and parental plants in vitro and in the greenhouse

Genotype	General appearance[a]	Habit[b]	Anthocyanin on[c]		Roots[d]
			Stem	Leaf axil	
S. brevidens	V	B	Mot	Mot	G
PDH 40	V	B	A	A	G
10031	V	E	Mot	Mot	P
10091	Wk	Dom	Pr	Pr	N
10122	P	B	Mot	Mot	P
10173	P	E	Pr	Pr	P
10221	Wk	Prst	A	A	N
10251	P	B	A	A	P
10361	V	B	T	T	G
10423	V	B	P	P	G
10482	V	B	T	A	G
10531	V	E	Pr	A	G
10572	V	E	Pr	A	G
10611	P	Str	T	T	N
10712	Wk	B	T	T	P
10732	V	B	A	A	G
10751	P	B	T	T	P
20012	V	B	T	T	P
30012	V	B	T	T	G
30022	V	B	T	T	G
40021	P	B	Pr	Pr	P
40111	V	B	A	A	G
40162	P	B	Pr	Pr	P
40171	V	B	T	T	G
40183	V	B	T	T	G
50011	V	B	Pr	Pr	G
50061	P	B	Pr	Pr	P
50081	P	B	T	T	P
70021	V	B	T	T	G
70102	V	B	T	T	G
70120	P	B	Pr	Pr	P
80062	V	B	T	T	G
90063	V	B	T	T	G

[a] V, Vigorous; Wk, weak; P, poor
[b] B, Bushy; E, erect; Dom, domed; Prst, prostrate; Str, straggly
[c] Mot, mottle; A, absent; T, trace; Pr, present
[d] G, Good; N, no roots; P, poor

asymmetric hybrids. Of these hybrids, 45% grew rather poorly (poor rooting, small leaves and slow growth) (Table 1).

Four of the asymmetric hybrids were also confirmed by isoenzyme analysis of glutamateoxaloacetate transaminase (GOT) (data not shown). The GOT patterns of the asymmetric hybrids exhibited both parental bands and a new band of intermediate mobility similar to that found in somatic hybrids of *S. brevidens* and *S. tuberosum* PDH 40 (Fish et al. 1987).

The asymmetric hybrids differed in a range of phenotypic characters such as vigor, root-forming ability, leaf morphology and spontaneous tuber formation in vitro (Table 1). A total of 16% of the asymmetric hybrids produced tubers in vitro. No two hybrids appeared to be the same. In general, the asymmetric hybrids possessed phenotypic characters that were either similar to the non-irradiated parent PDH40, intermediate between the fusion parents or unique. For example, most of hybrids expressed anthocyanin pigmentation, but with less intensity than *S. brevidens*. PDH40 has no anthocyanin pigmentation. Other characters such as leaf shape and growth habit were quite distinct and typical only of the asymmetric hybrids (Figs. 2 and 3). The asymmetric hybrids had broader leaves when compared with PDH40 and *S. brevidens*, which both have narrow leaves. No

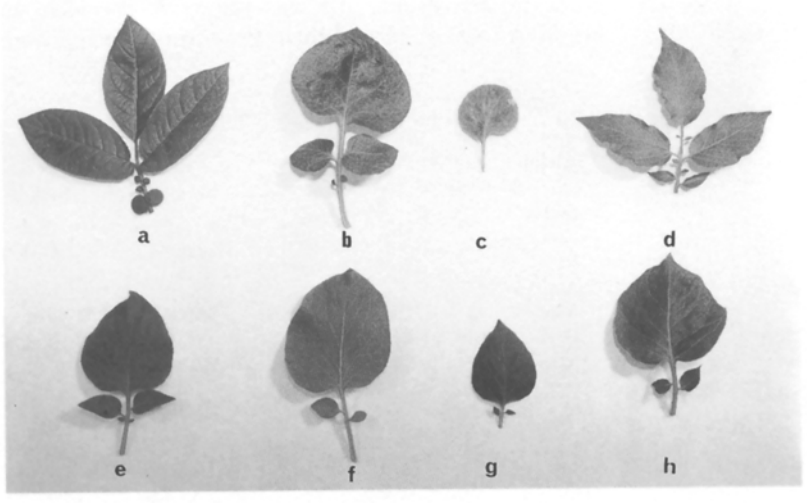

Fig. 2. Leaves of *S. brevidens* (*a*), PDH40 (+) *S. brevidens* asymmetric hybrids (*b*, *c* and *e–h*) and PDH40 (*d*)

Fig. 3a–d. Plant morphology. **a** PDH40, **b** *S. brevidens*, **c, d** PDH40 (+) *S. brevidens* asymmetric hybrids 10361 and 70102, respectively

Table 2. Chromosome counts of the asymmetric hybrids between *S. tuberosum* PDH40 and irradiated *S. brevidens*

Plant number	Irradiation of donor [dose (Gy)]	Chromosome number (± 2)[a]
10122	300	56
10171	300	62
10251	300	60
10361	300	40
10423	300	33
10482	300	42
10532	300	55
10572	300	52
10732	300	36
40111	500	34
40171	500	43
40183	500	31
50011	500	36
70021	500	46
70101	500	36
80062	300	61
90063	500	64

[a] Chromosome number is an average value from three independent counts

significant differences in general morphology and growth were found between 300H hybrids and 500H hybrids. Some of the asymmetric hybrids produced tubers in the greenhouse.

Chromosomal counts of 17 randomly selected asymmetric hybrids were made, and the numbers varied from 31 to 64 (Table 2). The counting of chromosomes was hampered by their small size and by poor growth of the roots.

Discussion

The experiments described above show that asymmetric somatic hybrid plants can be obtained from fusions between protoplasts of *S. tuberosum* and irradiated protoplasts of *S. brevidens*. The asymmetric hybrids were identified using a *S. brevidens*-specific probe. The morphology of these hybrids was intermediate between that of the symmetric somatic hybrids produced previously by Fish et al. (1987, 1988) and that of *S. tuberosum*. The asymmetric hybrid nature was confirmed by isoenzyme analysis and by RFLP analysis (Xu et al. in preparation). From the cytogenetic and morphological studies, we conclude that most tetraploid asymmetric hybrids probably had one diploid *S. tuberosum* genome (i.e. 24 chromosomes) and between 7 and 22 chromosomes from *S. brevidens*. For 5 asymmetric hybrids this was confirmed by RLFP analysis using linkage group-specific TG clones kindly supplied by S. Tanksley (Xu et al. in preparation). All of the 5 asymmetric hybrids (plants 10361, 10732, 40111, 50011 and 70021) analyzed with RLFP so far contain a complete set of *S. tuberosum* chromosomes and a partial set of the *S. brevidens* chromosomes. The asymmetric hybrids with chromosome numbers greater than 48 probably have two diploid *S. tuberosum* genomes (i.e. 48 chromosomes) and between 4 and 16 *S. brevidens* chromosomes, since the hybridization intensities of the dot-blots (probed by pST10) of these hybrids were stronger than those of other hybrids. Two of them (plants 10122, 10171) produced tubers in the greenhouse, which also suggests they have relatively more of the *S. tuberosum* genome than other hybrids. This was the case in the study of tuber production of hexaploid symmetric hybrids between *S. tuberosum* and *S. brevidens* (Fish et al. 1988b; Pehu et al. 1989).

There was no clear correlation between the radiation dose and the extent of elimination of the *S. brevidens* genome (Table 2). The irradiation of *S. brevidens* before fusion did not have the full desired effect of eliminating the donor genome to such an extent that only a small fraction was retained in the asymmetric hybrids. Generally, a relatively large fraction of the *S. brevidens* genome was retained, even in the 500H hybrids. Limited chromosome elimination, together with a lack of correlation between elimination and irradiation dose, have also been reported in other experiments on asymmetric hybridization (Gleba et al. 1988; Wijbrandi et al. 1990a).

In the work reported here it was not possible to select for asymmetric hybrids at an early stage because no selectable markers were available for PDH40 and *S. brevidens*. However, our work demonstrates that an efficient asymmetric hybridization program based on high fusion frequencies, partial selection (no *S. brevidens* only regenerants as a result of irradiation) and a post-fusion identification of hybrids by species-specific probes is feasible. Furthermore, it could be argued that the approach used here is more effective because the species-specific repetitive sequences (pSB7) used in the experiment is present in each of the *S. brevidens* chromosomes (Pehu and Lapitan, in preparation) and can thus give information on the transfer of each chromosome. When a selectable marker is used, selection is only for the presence of the chromosome/fragment in which it is present.

A higher percentage (36%) of hybrids amongst regenerants was obtained in our experiments than was obtained by Fish et al. (1988a) (13%) in comparable symmetric somatic fusion experiments for the same species combination and under the same culture conditions. This can be explained by the fact that 300- and 500-Gy irradiation can block the division of protoplasts from *S. brevidens* and thus result in the preferential selection of hybrids. In the initial selection of the asymmetric hybrids all shoots with abnormal phenotype were kept for further analysis, whereas such

shoots were discarded when the somatic hybrids reported by Fish et al. (1988a) were chosen.

Of the asymmetric hybrids identified 45% grew poorly, and all of these had poor root growth or no roots at all. The phenomenon may be a result of the unbalanced genome of the asymmetric hybrids.

The expression of virus resistance in the asymmetric hybrids between *S. tuberosum* and *S. brevidens* regenerated in this work is of interest. From preliminary virus tests on the asymmetric hybrids, 4 out of 52 plants from individual calli were resistant to potato virus Y. A study of the fertility of these hybrids is in progress.

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