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Production of somatic hybrids by electrofusion in *Solanum*

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Summary. Conditions are described for large scale electrofusion of mesophyll protoplasts of dihaploid *S. tuberosum* with those of diploid *S. brevidens.* Overall fusion frequencies of 20%-30% were achieved, and following fusion, large numbers of protoplast-derived calli were obtained. Putative somatic hybrid plants were selected from the regenerated shoots by examining their morphological characteristics. Twenty-one somatic hybrids were confirmed by isoenzyme analysis and six somatic hybrids were further confirmed by Southern hybridization. Tetraploid hybrids were obtained, but cytogenetic studies indicated that more of the regenerated hybrids were hexaploid than had previously been found following chemical fusion of the same partners. Some advantages of electrofusion over chemical fusion are discussed.

Key words: *Solanum tuberosum* – *Solanum brevidens* – electrofusion – somatic hybrids – cytology

Introduction

Potato (*Solanum tuberosum* L.) is amenable to a number of tissue culture techniques which offer new potential for improvement of the potato crop. One such technique is the production of somatic hybrids by protoplast fusion.

Recent advances in protoplast fusion technology have made this technique of more immediate value for potato breeding. In particular, the combination of dihaploid *S. tuberosum* breeding (by conventional methods) with somatic hybridisation offers new opportunities for introducing novel characters into potato (Fish et al. 1987; Austin et al. 1985a), and of synthesising superior, tetraploid potato cultivars (Ross 1986; Austin et al. 1985b).

Until recently, protoplasts were routinely fused together using chemical fusogens (Negrutiu et al. 1986; Menczel and Wolfe 1984; Kao and Michayluk 1974; Keller and Melchers 1973). In many cases, however, this method cannot be used because the target protoplasts cannot tolerate the fusogens (Chapel et al. 1984). When chemical fusogens have been used successfully, relatively low fusion frequencies have usually resulted, and this has led to the need to develop complementation selection or other schemes to recover the heterokaryons from a mixture of fused and unfused parental protoplasts (e.g. Hein and Schieder 1986; Gräfe and Müller 1983; Power et al. 1976). Unfortunately, it is often not possible or desirable to introduce a selectable marker into an agronomically useful genotype as this may result in other undesirable changes (de Vries et al. 1987).

If protoplast fusion frequencies could be increased substantially, then heterokaryon selection schemes might not be necessary. There has therefore been much interest in the technique of electrofusion, as it has been demonstrated that high fusion frequencies can be obtained (Tempelaar and Jones 1985a, b; Tempelaar et al. 1987; Bates 1985; Zimmermann and Scheurich 1981). Following developmental work on the technique of electrofusion (Tempelaar and Jones 1985a, b; Tempelaar et al. 1987) we have applied this approach to the production of potentially useful somatic hybrids of *Solanum* species.

In this paper we describe conditions for electrofusion, culture and production of somatic hybrids between an agronomically useful dihaploid *S. tuberosum* genotype (PDH 40 derived from cultivar Pentland Crown) and *S. brevidens,* a diploid wild species that is resistant to Potato Leaf Roll Virus (PLRV) (Jones 1979; Fish et al. 1987).

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Materials and methods

Plant material and protoplast isolation

Dihaploid *S. tuberosum* (PDH 40) and *S. brevidens* plants were grown and mesophyll protoplasts isolated as described by Fish et al. (1987). Purified protoplast preparations were resuspended in 8.5% mannitol, 1 mM $CaCl_2$, pH 5.6 at 1×10^6 ml^{-1} for electrofusion (Tempelaar et al. 1987).

Electrofusion

Electrofusion was carried out using a Zimmermann Cell Fusion System (GCA Corp, Chicago/IL, USA) connected to a multilamellar fusion chamber (Krüss, W. Germany) with parallel stainless steel electrodes 1 mm apart. All manipulations were done in a laminar flow hood and fusion was observed using an inverted microscope. The fusion chamber was sterilised in 70% ethanol before use.

The protoplasts were mixed at a ratio of 1:1, and 125–200 µl of the protoplast suspension (1×10^6 ml^{-1}) pipetted into the fusion chamber. The protoplasts were aligned into short chains by applying an alternating current collecting field of 100 Vcm^{-1}, frequency 1 MHz, and fusion was induced by one 10 µs direct current square pulse of 1,250–1,500 Vcm^{-1}. After applying the fusion pulse, the alternating current collecting field was re-applied, then smoothly ramped down to zero over the following 20 s.

After electrofusion, the protoplasts were carefully pipetted into a 5 cm petri dish containing 2.5 ml VkCLG medium to give a final culture density of $6-8 \times 10^4$ ml^{-1}. The culture dishes were examined with a microscope and the dishes with most fusion products were selected for further culture.

Protoplast culture, shoot regeneration and putative hybrid selection

Protoplast culture and shoot regeneration was as described by Fish et al. (1987) except that the split plate reservoir medium contained 1% w/v activated charcoal (Carlberg et al. 1983). One shoot was excised from every regenerating callus and transferred to medium MS 20 + 0.05 mg l^{-1} NAA to induce rooting (Fish and Karp 1986). Regenerated shoots that resembled either parent, or that had an abnormal phenotype were discarded. Putative hybrid shoots were selected by their morphological characteristics as described previously (Fish et al. 1987) and after replication in culture, single regenerants were potted in EFF compost (EFF products, Guildford) for further analysis.

Isoenzyme analysis

Putative hybrids were confirmed as somatic hybrids by examining the isoenzyme patterns for glutamate-oxaloacetate transaminase 2.6.1.1. (Got), 6 phosphogluconate dehydrogenase 1.1.1.44 (6Pgd) and glucosephosphate isomerase 5.3.1.9 (Pgi) as described by Fish et al. (1987).

DNA isolation

DNA was isolated from glasshouse grown parental and hybrid plants using the method of Dellaporta et al. (1983) as modified by R. Potter (Biochemistry Department, Rothamsted, unpublished results). Unless otherwise stated, all solutions are as listed in Maniatis et al. (1982).

Frozen leaf tissue (2 g) was ground to a fine powder in a cold pestle and mortar before adding 15 ml of extraction buffer (0.5 M NaCl, 0.1 M Tris.Cl pH 8.0, 50 mM EDTA pH 8.0, 10.5 µl β-mercaptoethanol), 1 ml 20% SDS and then the ground tissue was incubated at 65 °C for 10 min. 5 ml of 5 M potassium acetate was added and after a 30 min incubation on ice, the large debris was pelleted by centrifugation (Beckman J-21C, Ja20 rotor) at 25,000 g for 20 min. The supernatant was poured through Miracloth (Calbiochem) and the DNA precipitated by adding 10 ml isopropanol and incubating at −20 °C for 2 h. The DNA was pelleted by centrifugation at 20,000 g for 20 min and resuspended in 700 µl of 50 TE. Insoluble debris was removed by further centrifugation, and 0.7 g CsCl was then dissolved in the supernatant. The DNA was purified further by ultracentrifugation at 50,000 rpm (rotor Ti 80.4) on 50% CsCl gradients overnight. The DNA bands were then removed from the gradients, ethidium bromide extracted and after dialysis, precipitated overnight by 2–3 vol of cold ethanol at −20 °C. The precipitated DNA was pelleted by centrifugation, dried in a vacuum desiccator and taken up in 200 µl of TE.

DNA restriction and filter preparation

Approximately 7–10 µg DNA was digested with HindIII (BRL) according to the suppliers instructions. Equal amounts of the digested DNA were separated by electrophoresis on an 0.8% agarose gel and transferred to Biodyne A membranes by electroblotting (Biorad, Transblot). DNA was bound to the filters by baking at 80 °C for 2 h.

Probe preparation

The filters were probed with the HindIII-BamHI fragment of pGMOl, a cDNA clone of patatin (Mignery et al. 1984). Probes were routinely labelled to specific activities of $2-3 \times 10^9$ dpm by oligolabelling (Feinberg and Vogelstein 1983) with α P^{32} ATP (Amersham International).

Hybridisation to filters

The filters were prehybridised with prehybridisation solution for 4 h at 65 °C. The labelled probe (2.75×10^6 dpm/ml hybridisation solution) was mixed with sheared herring sperm DNA (100 µg/ml), denatured by boiling and added to the bags with the filters containing fresh prehybridisation solution. The filters were hybridised at 65 °C overnight with continuous shaking, and then washed twice with 2 × SSC and once with 0.5 × SSC at 65 °C. After drying and wrapping in cling film, the filters were exposed to Fuji X-ray film at −80 °C.

Chromosome analysis

Chromosome counts were made from rooted shoot cultures as described by Karp et al. (1982).

Results

Electrofusion

Using the electrofusion apparatus described (see "Materials and methods") it was relatively straightforward to fuse rapidly large numbers of protoplasts, and overall fusion frequencies of 20%–30% (defined as the no. of fused protoplasts ÷ total no. of protoplasts × 100) were obtained. Preliminary fusions using analytical electrodes (Tempelaar and Jones 1985 a, b) showed that PDH 40 and *S. brevidens* mesophyll protoplasts were sensitive to electric fields and were easily damaged. Bulk fusion was therefore induced by applying a relatively low voltage,

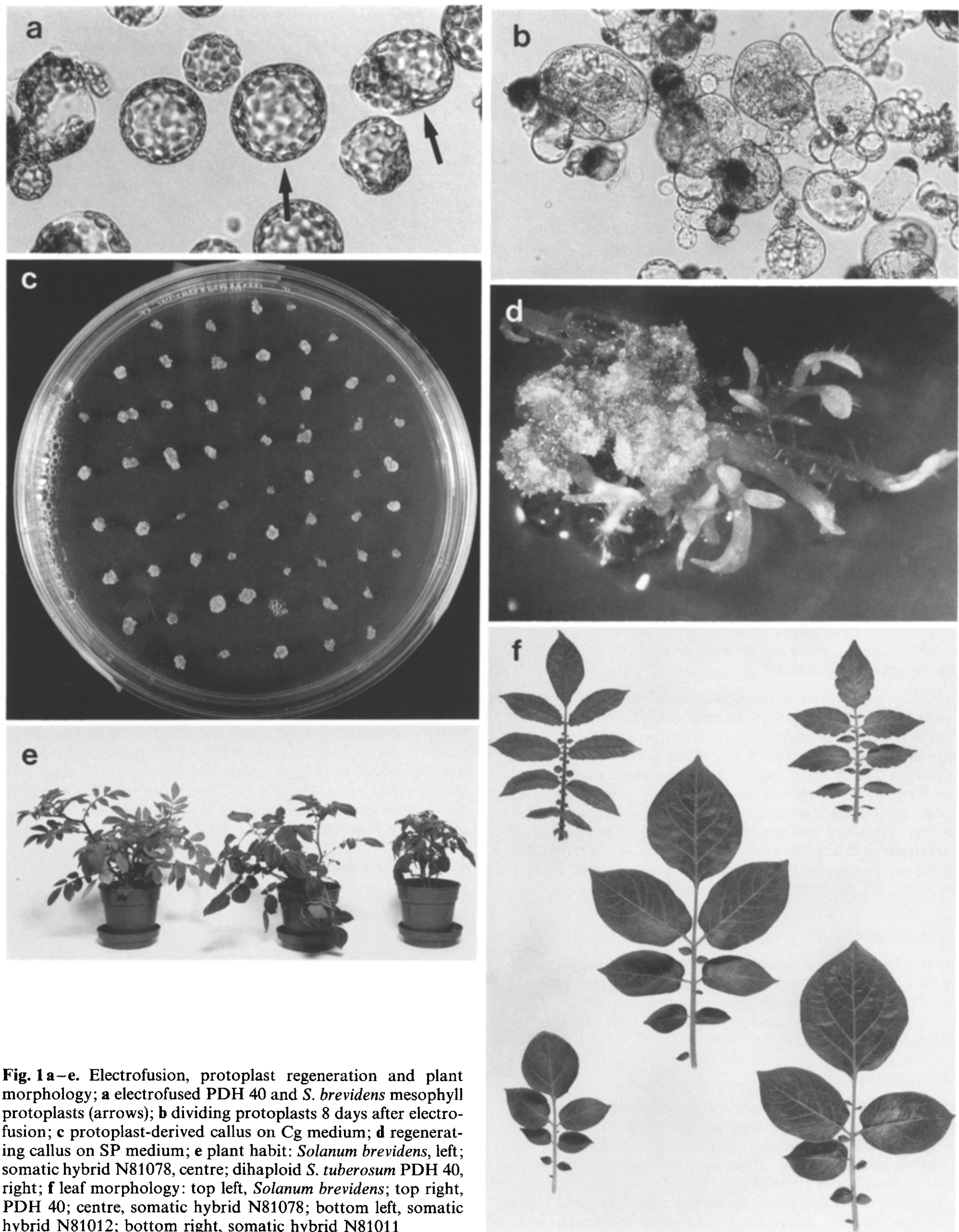

Fig. 1a–e. Electrofusion, protoplast regeneration and plant morphology; **a** electrofused PDH 40 and *S. brevidens* mesophyll protoplasts (arrows); **b** dividing protoplasts 8 days after electrofusion; **c** protoplast-derived callus on Cg medium; **d** regenerating callus on SP medium; **e** plant habit: *Solanum brevidens*, left; somatic hybrid N81078, centre; dihaploid *S. tuberosum* PDH 40, right; **f** leaf morphology: top left, *Solanum brevidens*; top right, PDH 40; centre, somatic hybrid N81078; bottom left, somatic hybrid N81012; bottom right, somatic hybrid N81011

short DC pulse (1×10 µs, 1,250–1,500 Vcm^{-1}). The inclusion of Ca^{2+} ions in the electrofusion medium was found both to improve protoplast stability and to increase the protoplast fusion frequency (Tempelaar et al. 1987).

Using the fusion conditions above, the majority of the fusion products were binary 1:1 fusions although multiple fusions also occurred (Fig. 1 a). After electrofusion, the fused protoplasts rapidly become round and there was little visual sign of protoplast damage as a result of the electrofusion process.

Protoplast culture and shoot regeneration

After electrofusion, the protoplasts were cultured in VkCLG medium and cell division began after 3–4 days. In some cases, however, sustained cell division was inhibited by the production of brown exudates that surrounded some protoplasts. The inclusion of 1% activated charcoal in the split plate reservoir medium reduced the inhibitory precipitates and promoted rapid protoplast division (Fig. 1 b).

Several thousand calli were recovered after transfer to solid Cg medium (Fig. 1 c) and the plating efficiencies (defined as the no. of calli on Cg medium ÷ total no. of protoplasts plated × 100) were similar to those obtained from chemical fusion experiments (≈0.5%, Fish et al. 1987). Regeneration was induced by transferring the calli from Cg to D medium, and shoot elongation was initiated by transfer to SP medium (Fig. 1 d). One shoot was excised from every regenerating callus (456 shoots in total), and rooting was induced by transfer to MS 20 + 0.05 mg l^{-1} NAA.

Morphological characteristics

Putative somatic hybrids were selected from the regenerated shoots after examining their morphological characteristics (Fish et al. 1987). The somatic hybrids typically grew extremely vigorously, had an erect habit (Fig. 1 e), variable anthocyanin pigmentation and elongated tubers. All the somatic hybrids produced leaves that were broader than those of either parent, with variable numbers of secondary leaflets (Fig. 1 f). Some somatic hybrids flowered, and produced characteristic large white flowers with purple streaks. The somatic hybrids obtained by electrofusion appeared similar to those somatic hybrids previously produced by chemical fusion (Fish et al. 1987).

Isoenzyme analysis

The hybrid nature of the putative somatic hybrids was confirmed by examining isoenzyme patterns for Got, 6Pgd and Pgi. The isoenzyme patterns of the somatic hybrids exhibited bands from both parents as well as

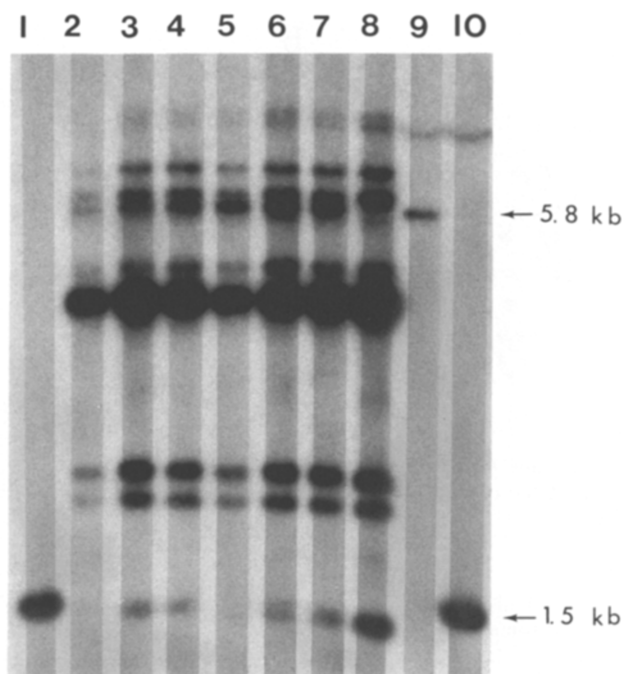

Fig. 2. Hybridisation patterns of patatin cDNA to parental and somatic hybrid, HindIII restricted DNA; lanes 1 and 10, pGMOI × 1 and × 4 reconstruction (11.5 kb marker); lanes 2–7 somatic hybrids (left to right) N84140, 81011, 81078, 81045, 84047, 81012; lane 8 pDH 40, lane 9 *S. brevidens*; note 5–8 kb band present in *S. brevidens*, absent in PDH 40, but present in all the somatic hybrids

additional new bands (data not shown), and were similar to those obtained for hybrids from chemical fusion (Fish et al. 1987). A total of 21 somatic hybrids were confirmed by isoenzyme analysis (See note added in proof).

Molecular analysis

When genomic DNA of the potato parent PDH 40 and the wild species *S. brevidens* was cut with the restriction enzyme HindIII, and probed with the HindIII-BamHI fragment from pGMOI, different hybridisation patterns were obtained (Fig. 2). The probe hybridised to a single, approximately 5.8 Kb fragment of DNA of *S. brevidens*, and to a number of different DNA fragments of PDH 40. Restricted genomic DNA from the somatic hybrids, similarly probed, gave characteristic hybridisation patterns that included all the bands from both parents. These results demonstrate that Southern hybridisation can be used to identify somatic hybrids unequivocally, and confirms the isoenzyme analysis.

Chromosome analysis

Chromosome counts from 15 of the 21 somatic hybrids are presented in Table 1. Eight of these 15 hybrid plants were tetraploid (Fig. 3 a) or aneuploid at the tetraploid

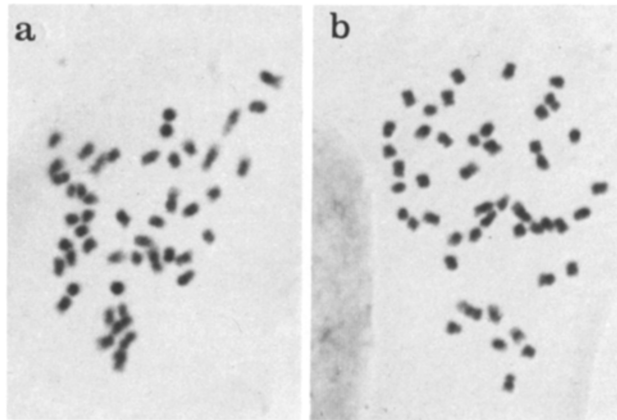

Fig. 3a and b. Chromosomes in root-tip cells of somatic hybrids; **a** tetraploid ($2n = 4x = 48$); **b** aneuploid at the tetraploid level ($2n = 47$)

Table 1. Chromosome counts from root tips of 15 electrofusion-derived somatic hybrid plants

Plant no.	Chromosome no.
N81011	47
N81012	46
N81013	89
N81025	45
N81031	47
N81036	75
N81045	78
N81068	69
N81078	48
N84015	71
N84079	74
N84042	48
N84111	71
N84118	47
N84140	48

level ($2n = 4x = 48 \pm$) (Fig. 3b). Six were aneuploid at the hexaploid level ($2n = 6x = 72 \pm$) and one hybrid was presumed aneuploid at the octaploid level ($2n = 8x = 96$), because of its high chromosome count ($2n = 89$). The tetraploid hybrid plants were completely stable, but in the somatic hybrids with high chromosome numbers some chromosome instability was observed.

Discussion

Sexual hybridization for the introduction of PLRV resistance from *S. brevidens* into *S. tuberosum* cannot at present be achieved directly by conventional means (Hermesen and Taylor 1979). The results presented in this paper illustrate that by application of electrofusion it is possible to regenerate many potentially useful somatic hybrid plants of *Solanum* spp by mass culture followed by later selection. Austin et al. (1985a) reported that PLRV resistance was expressed in similar somatic hybrids produced by chemical fusion. This is also the case for the electrofusion hybrids reported here. Full results from PLRV tests of somatic hybrids, and their characteristics in the field, will be presented in a subsequent paper (Gibson et al. 1988).

In the present work, a commercially available electrofusion apparatus and fusion chamber were used to fuse together large numbers of protoplasts extremely rapidly and under precisely controlled conditions. The conditions chosen were relatively mild (1 pulse of 10 µs, 1,250–1,500 Vcm^{-1}) in order to minimise damage to the protoplasts which are easily ruptured. Thus the overall fusion frequencies obtained (20%–30%) were not the highest attainable (Tempelaar and Jones 1985b), although similar frequencies have been reported in other bulk electrofusion systems (Watts et al. 1985; Bates et al. 1983; Kohn et al. 1983). Higher fusion frequencies have been achieved by using small scale analytical electrodes, but because of the small numbers of protoplasts involved, the recovery of somatic hybrid plants after electrofusion is more difficult (Tempelaar and Jones 1985a; Bates et al. 1983; de Vries et al. 1987; Zimmermann and Scheurich 1981). In general, application of longer fusion pulses at higher voltages results in higher fusion frequencies and more multiple fusions (Tempelaar and Jones 1985a). It should be noted that only half the total fusion products (10%–15%) will be heterofusions, and of these about half will be 1:1 fusions (i.e. 5%–7.5%) of the total protoplasts fused (Tempelaar and Jones 1985a, b).

Following electrofusion and culture, several thousand calli were recovered, from which 456 shoots were regenerated. Of these shoots, 21 were identified as somatic hybrids by examining their morphological characters and by isoenzyme analysis. Thus 4.6% of the regenerated shoots (approximately 1 in 20) were identified as somatic hybrids, compared with 2.6% (approximately 1 in 40) produced following chemical fusion of the same parental protoplasts (Fish et al. 1987). The total number of somatic hybrids produced after electrofusion was probably much higher than 21 as many hybrids with abnormal phenotypes would have been discarded. A more extensive isoenzyme analysis of all 456 regenerated shoots would undoubtedly result in more electrofusion somatic hybrids being identified (See note added in proof).

In this study, we have confirmed that DNA-DNA hybridisation analysis using a specific probe can be used to identify somatic hybrids. However, as Southern hybridisation analysis involves considerable time and effort (compared to isoenzyme analysis) we feel that it is best used either as a confirmatory procedure, or in situations where protoplast-derived regenerants show variable isoenzyme patterns (Fish et al. 1987). Species-specific re-

petitive DNA probes could also have been used (Saul and Potrykus 1984) to identify such hybrids. The latter approach is best employed for identifying inter-specific somatic hybrids from more distantly related species.

Chromosome analysis of 15 electrofusion somatic hybrids showed a larger number of hexaploid and octoploid regenerants when compared to similar hybrids produced by chemical fusion (Fish et al. 1987). At present, the reason for this is uncertain. One possible explanation is that more multifusion products were capable of surviving the milder conditions of electrofusion than from chemical fusion. This possibility is supported by the observation that protoplast damage was considerably less after electrofusion than chemical fusion.

From our experience of protoplast fusion of *Solanum* spp, it is clear that electrofusion has several technical advantages over chemical fusion. Electrofusion can be carried out extremely rapidly with few manipulations, and it is possible rapidly to optimise the fusion conditions for any batch of protoplasts used. In our hands (Tempelaar and Jones 1985a, b; Tempelaar et al. 1987), electrofusion gives much higher fusion frequencies for potato than is routinely possible by chemical fusion (Fish et al. 1987; Negrutiu et al. 1986). In addition, we are unable to use PEG fusion methods for these particular protoplasts because they caused lysis. However, the electrofusion frequencies obtained could almost certainly be increased further by using chemical facilitators (Tempelaar et al. 1987; de Vries et al. 1987; Ruzin and McCarthy 1986; Chapel et al. 1984). The ability to obtain high fusion frequencies eliminates a requirement for elaborate hetrokaryon selection schemes (e.g. using complementing selectable biochemical markers or a fluorescence activated cell sorter). In the present work, at least 5% of the regenerated shoots were somatic hybrids. This result is particularly relevant for fusion of crop plant protoplasts where selectable markers are either not available or difficult to introduce without altering the genotype of interest (de Vries et al. 1987). Thus the application of electrofusion techniques should increase the number of agronomically useful protoplast fusions that are possible and enable somatic hybrids to be selected at a later stage by hybrid vigour (Wenzel et al. 1979), morphology or isoenzyme analysis.

The somatic hybrids between dihaploid *S. tuberosum* and *S. brevidens* regenerated in this study are of considerable interest to potato breeders, and field characteristics of some of the plants have been assessed in a field trial (Fish et al. 1988). (The field trial has been approved by the UK Advisory Committee for Genetic Manipulation). The next step in the introgression of PLRV from *S. brevidens* into potato varieties is crossing hybrids with *S. tuberosum*. Many of the electrofusion hybrids flower, and are currently being used as female parents in crosses with male fertile UK cultivars of *S. tuberosum*.

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Note added in proof

We have identified a further 35 somatic hybrid plants from the regenerated shoots, thus the frequency of hybrids is 12.3%.