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Somatic Hybridization of Sexually Incompatible Petunias: *Petunia parodii, Petunia parviflora*

J.B. Power, S.F. Berry, J.V. Chapman and E.C. Cocking
Plant Genetic Manipulation Group, University of Nottingham, University Park, Nottingham (Great Britain)

Summary. Somatic hybrid plants were regenerated following the fusion of leaf mesophyll protoplasts of *P. parodii* with those isolated from a nuclear-albino mutant of *P. parviflora*. Attempts at sexual hybridization of these two species repeatedly failed thus confirming their previously established cross-incompatibility. Selection of somatic hybrid plants was possible since protoplasts of *P. parodii* would not develop beyond the cell colony stage, whilst those of the somatic hybrid and albino *P. parviflora* produced calluses. Green somatic hybrid calluses were visible against a background of albino cells/calluses, and upon transfer to regeneration media gave rise to shoots. Shoots and the resultant flowering plants were confirmed as somatic hybrids based on their growth habit, floral pigmentation and morphology, leaf hair structure, chromosome number and Fraction 1 protein profiles. The relevance of such hybrid material for the development of new, and extensively modified cultivars, is discussed.

Key words: *Petunia parodii* – *Petunia parviflora* – Sexual Incompatibility – Somatic hybridisation – Horticulture

Introduction

Somatic hybrids in the genus *Petunia* have been produced between sexually compatible species, *Petunia hybrida* and *P. parodii* (Power et al. 1976; Cocking et al. 1977) and unilaterally cross-incompatible species *P. inflata* and *P. parodii* (Power et al. 1979) following the fusion of leaf protoplasts of *P. parodii* with those of the appropriate cytoplasmic albino mutant partner. In the latter case prezygotic reproductive isolation was overcome through protoplast fusion. Current attempts at somatic hybridization are focussed on species combinations that are difficult or impossible to cross using conventional breeding techniques. Furthermore, the production of novel hybrids should ideally be of some value to the breeder in order that a particular species, or group of species can be improved. In the genus *Petunia*, extensive breeding programmes have produced ornamental *Petunia* (usually cultivars of *P. hybrida*), that possess an extremely wide range of floral pigmentations, patterns and flower types. However, the major deficiency in this group is the non-availability of a source whereby gross morphological changes (growth habit) can be introduced.

In an attempt to generate morphological change, and at the same time, to demonstrate an ability to produce somatic hybrids between sexually incompatible species, *P. parviflora* was chosen as one parental species. This species (x=9), reproductively isolated from all the regular Petunias (x=7), possesses a highly branched, prostrate growth habit, the introduction of which, would broaden the range of characteristics available to the plant breeder. We describe the somatic hybridization of *P. parodii* with *P. parviflora* following the fusion of wild-type leaf protoplasts (*P. parodii*) with those isolated from cells of a nuclear albino mutant (*P. parviflora*).

Materials and Methods

Production of Albino P. parviflora

Freshly harvested seeds of *P. parviflora* were irradiated using a Co^{60} source within an exposure range of 10-20 Krads. Seed populations exhibiting a minimum of 50% kill over the control (99% germination) were pricked out into vacapots and the plants grown to maturity in the greenhouse. Upon selfing individual plants, one heterozygous plant (subjected to an original seed irradiation of 13.8 Krads) was identified which segregated to give green: albino plants, in the ratio 3:1, following the germination of seed on sterilised moistened filter paper. Albino plantlets were transferred aseptically to petri dishes containing M/S Murashige and Skoog (1962) medium with kinetin (0.03 mg/l), folic acid (0.001 mg/l) and IAA (0.009 mg/l) solidified with 0.8 percent agar (pH 5.8) (Binding 1974). When seedlings were approximately 1.0 cm in

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length they were transferred to powder round jars (Beatson Clark and Co. Ltd. Rotherhan, England) containing the same medium, and maintained therein as axenic shoot cultures. Leaves of plants propagated in this way were transferred to the medium of Uchimiya and Murashige (1974) (UM) to produce callus. This callus was used as a source of albino *P. parviflora* protoplasts.

Protoplast Isolation

Protoplasts of *P. parodii* were isolated as previously described (Hayward and Power 1975). Callus (2-5 g fresh weight and 4 weeks after subculture) of *P. parviflora* was incubated in 20 ml of an enzyme mixture of 2 percent (w/v) Rhozyme, 4 percent (w/v Meicelase, 0.3 percent (w/v) Macerozyme in 13 percent (w/v) mannitol solution containing inorganic salts (pH 5.6) (Frearson et al. 1973). Following an overnight incubation at 24°C, with shaking (20 cycles/min), the mixture was passed through a nylon sieve (64 μ pore size) and the filtrate collected in a petri dish. Protoplasts were washed twice in 9 percent (w/v) mannitol containing inorganic salts.

Protoplast Fusion

Protoplasts of both species were suspended at a density of 2×10^5/ml in liquid M/S medium containing 2.0 mg/l NAA, 0.5 mg/l 6-BAP and 9 percent (w/v) mannitol at pH 5.8. The experimental design was as for the production of the somatic hybrid (*P. inflata* + *P. parodii*) (Power et al. 1979). Protoplast fusion was induced using the high pH/calcium method of Keller and Melchers (1973). The fusogen used here consisted of glycine-NaOH buffer, pH 10.4, to which was added 9 percent (w/v) mannitol and 0.74 percent (w/v) $CaCl_2.2H_2O$. All fusion treatments were for 15 minutes and together with subsequent washings, were carried out at 30°C. As in previous experiments (Power et al. 1979) viability and post-fusion mixture controls were prepared alongside the fusion-treated protoplasts. All protoplasts were plated liquid on agar, at a final density of 5×10^4/ml in 50 mm Nunc dishes (Gibco). Dishes were sealed and maintained at 27°C with a continuous illumination of 900 lux provided by daylight fluorescent tubes.

Results

Eight experiments were performed, as described above, and in only two experiments were putative somatic hybrid colonies observed. Following fusion, complementation should result in the production of green, actively growing calluses, whilst in the controls and post-fusion mixture controls (the two parents fused separately then mixed) (Power et al. 1976) no green calluses would be expected since wild-type *P. parodii* protoplasts do not grow beyond the small colony stage in the medium employed and albino *P. parviflora* protoplasts only give rise to colourless colonies and callus.

A total of 35 green colonies were identified 30 days after fusion and only in the fusion-treated plates. These small calluses were transferred to the surface of the M/S medium with 6 percent (w/v) mannitol solidified with 0.8 percent (w/v) agar then to the same medium but with 3 percent (w/v) mannitol. During this process 24 of the original green calluses reverted to a near-colourless appearance and became very slow growing and exhibited no morphogenic potential.

The remaining green calluses were transferred to M/S medium with IAA (2.0 mg/l), 6-BAP (0.5 mg/l) and no mannitol. From previous studies it was known that leaf protoplast-derived callus of *P. parodii* (Hayward and Power 1975) and wild-type *P. parviflora* (Sink and Power 1977) would regenerate on this medium. Three calluses, of separate origin, produced shoots approximately 18 weeks after fusion, whereupon the shoots were rooted in M/S medium without phytohormones and plants ultimately transferred to the greenhouse for flowering.

The floral and leaf morphology of the two parents and the somatic hybrid (*P. parodii* + *P. parviflora*) is shown in Figure 1. The somatic hybrid possessed pigmented flowers

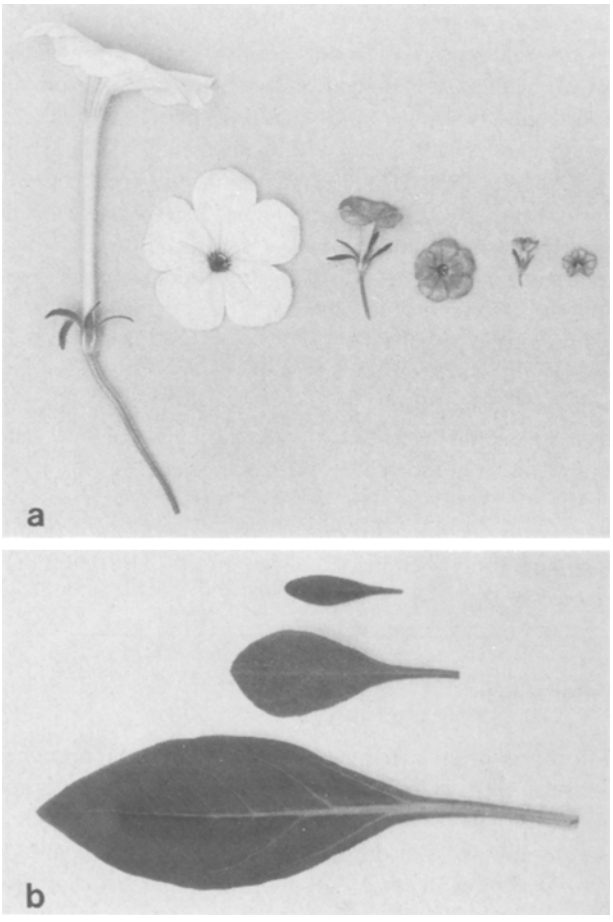

Fig. 1a and b. Flowers of (left to right) *P. parodii*, somatic hybrid (*P. parodii* + *P. parviflora*) and *P. parviflora*. The magenta flower colour of *P. parviflora* corresponds to redpurple group 68B and the somatic hybrid to red-purple group 68C of the Royal Horticultural Society Colour Chart; b Leaves of (top to bottom), *P. parviflora*, somatic hybrid and *P. parodii* (flowers, leaves half size)

and as shown in Figure 1, appeared intermediate for numerous characteristics including: – intensity of pigmentation, flower size, corolla shape and size, leaf shape and size, length of petiole and pedicel, and also trichome length was observed to fall between that of *P. parodii* (long, multicellular) and *P. parviflora* (short, multicellular). Both parental species are readily distinguished on the basis of the above mentioned characteristics (Figs. 1, 2).

As shown in Figure 2, the most recognisable and, from a breeding point of view, the most relevant feature was the branched, prostrate growth habit of the somatic hybrid which was clearly inherited from *P. parviflora*. In this respect at maturity a single plant of *P. parviflora* can attain a spread of 2-3 metres. In Figure 3 the growth habit can again be seen to be very similar to that of the *P. parviflora* parent whilst retaining the more prominent floral arrangement of *P. parodii*.

The somatic hybrid was further characterised with respect to its Fraction 1 protein profile. The small subunit (coded by the nucleus) can be distinguished for the two parents and as shown in Figure 4 the somatic hybrid possesses a nuclear-coded Fraction 1 protein profile consistent with the presence of both functional parental genomes (Kumar and Cocking 1980).

The somatic chromosome numbers of the regenerated plants were consistent for a given callus but of the three regenerating calluses only one approached the expected amphidiploid number of 4n=32. Over 50 plants were recovered from this callus and to date all plants examined possessed 31 chromosomes, as determined by root squashes, and the previously described characteristics. The remaining two regenerating calluses gave rise to very slow growing plants with 40 and 36 chromosomes respectively. Only the plants with 40 chromosomes had recognisable hybridity as regards their floral morphology. The plants with 36 chromosomes resembled *P. parviflora*.

The recovery rate of flowering somatic hybrids in this system was found to be 1 for every 2×10^7 protoplasts subjected to a fusion treatment.

The somatic hybrids had a pollen fertility count of 36% as judged by the staining of freshly dehisced pollen grains in 1 percent acetocarmine. The parents, *P. parodii* and *P. parviflora* had pollen fertility counts of 99 and 98 percent respectively.

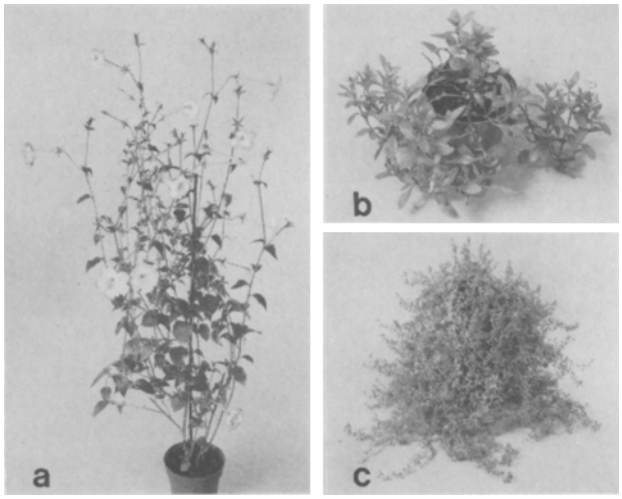

Fig. 2a-c. Flowering plant of a *P. parodii*, b somatic hybrid; c *P. parviflora*

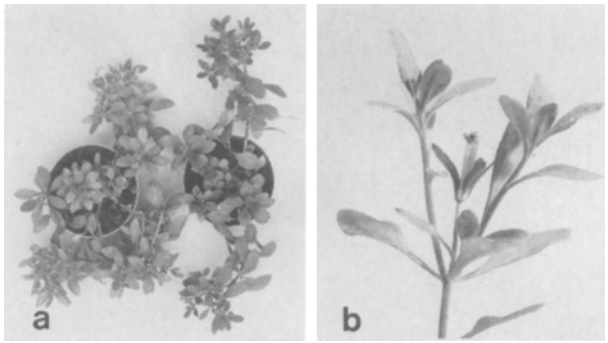

Fig. 3a and b. Somatic hybrids showing a prostrate growth habit, inherited from *P. parviflora* and b branched form and floral arrangement.

Discussion

In this sexually incompatible system the frequency with which somatic hybrids were produced was approximately 250 times lower than that for sexually compatible or unidirectionally cross-incompatible *Petunia* species combinations (Power et al. 1979). In this respect the choice of fusion agent reflected the fact that in the other *Petunia* somatic hybrid systems the throughput of hybrids was greater using the high pH method as compared with the other fusogens PEG and $NaNO_3$ (Berry and Power 1978). This was surprising since both PEG and high pH fusion methods induce approximately the same degree of protoplast fusion. The lowered recovery rate of somatic hybrids of *P. parodii* and *P. parviflora* may not be unexpected since the barriers to sexual hybridization of *P. parodii* with *P. parviflora* and indeed *P. parviflora* with any of the x=7 group (e.g. *P. hybrida, P. violacea, P. inflata* and *P. axillaris*), are two-fold and can only be explained on the basis of both a prezygotic incompatibility and a probable

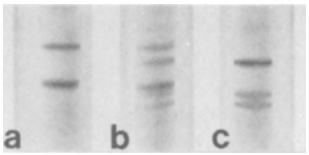

Fig. 4a-c. Small subunit (nuclear coded) Fraction 1 protein profile of a *P. parodii*; b somatic hybrid; c *P. parviflora*

embryo or embryo-endosperm disruption (Sink and Power 1978).

Somatic hybrids have been produced between species that are very difficult or impossible to hybridise conventionally e.g. *Lycopersicon esculentum* and *Solanum tuberosum*, (Melchers et al. 1978) *Datura innoxia* and *Atropa belladonna* (Krumbiegel and Schieder 1979); *Arabidopsis thaliana* and *Brassica campestris* (Gleba and Hoffmann 1979). However the full exploitation of somatic hybridization will follow from the availability of fertile, seed producing somatic hybrids.

The availability at present of somatic hybrid plants possessing one chromosome less than the true amphidiploid number is probably a reflection of the very low recovery rate so far achieved. In the other somatic hybrids between sexually compatible *Petunia* species which were produced using very similar mutants and experimental handling procedures, the true amphidiploid was the norm, suggesting that for this sexually incompatible system the amphidiploid (4n=32) can probably be produced given a higher throughput of somatic hybrids.

An attempt is now being made to incorporate the *P. parodii* + *P. parviflora* somatic hybrids in a conventional breeding programme with the aim of introducing new cultivars of *Petunia* possessing the novel growth habit of *P. parviflora* whilst retaining the comprehensive range of floral forms found in the other *Petunia*.

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Dr. J.B. Power
Department of Botany
School of Biological Sciences
The University of Nottingham
University Park
Nottingham NG7 2RD (England)