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Somatic Hybrids Between Unilateral Cross-Incompatible *Petunia* Species

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Summary. Somatic hybrid plants regenerated following the fusion of leaf mesophyll protoplasts of *Petunia parodii* with those isolated from a cell suspension of albino *P. inflata*. These two species exhibit a unilateral cross-incompatability with a pre-zygotic mode of reproductive isolation preventing hybridizations with *P. inflata* as the maternal parent. Selection of somatic hybrids relied on the fact that unfused or homokaryon protoplasts of *P. parodii* did not develop beyond the cell colony stage while those of the putative somatic hybrids and albino *P. inflata* parent produced callus. Green somatic hybrid calluses were readily identified against the white background of *P. inflata* following complementation to chlorophyll synthesis proficiency and continued growth in hybrid cells. Shoots, and ultimately flowering plants, were identified as somatic hybrids based on their floral morphology and colour, chromosome number and the fact that they segregated for parental characters. The frequency of somatic hybrid production was comparable to that previously established for two sexually compatible *Petunia* species.

Key words: *Petunia* – Unilateral cross-incompatibility – Protoplast fusion – Somatic hybridization

Introduction

In order to examine the potential of somatic hybridization in relation to species combinations which are difficult to cross sexually, we chose two species, *Petunia inflata* and *P. parodii*, where reciprocal crosses, using standard emasculation and pollination techniques, repeatedly fail. In this system, in spite of a high degree of chromosomal homology, minor genetic divergency leads to a pre-zygotic reproductive isolation which can only be overcome, at the sexual level, by bud pollination of *P. parodii* with *P. inflata* as the male parent. The interspecific hybrid with *P. inflata* as the maternal parent, cannot be produced (Sink et al. 1978).

In the genus *Petunia*, somatic hybrids of two species that are sexually compatible, *P. hybrida* and *P. parodii*, have been produced using two modes of selection. In the first selection scheme (Power et al. 1976a) a differential growth response to actinomycin D was linked with an inability of wild-type protoplasts of *P. parodii* to develop beyond the cell colony stage in certain media. Selection for somatic hybrids was possible in such media and in the presence of actinomycin D. In the second selection scheme (Cocking et al. 1977) somatic hybrid plants were recovered following the fusion of leaf protoplasts of wild-type *P. parodii* with those of albino *P. hybrida* cell suspension.

Somatic hybrids of *P. hybrida* and *P. parodii* were compared to their sexual counterparts with respect to flower colour segregation. Both types of hybrids segregated in a similar manner (Power et al. 1978).

We now describe the somatic hybridization of *P. parodii* W.C.S. with *P. inflata* Fries using the same selection principle (wild-type with albino) by substituting albino *P. hybrida* with albino *P. inflata*.

Materials and Methods

The albino *P. inflata* used in this study arose spontaneously during regeneration of *P. inflata* leaf protoplasts. The albino shoots were propagated axenically on M/S medium (Murashige and Skoog 1962) containing 0.009 mg/l IAA, 0.03 mg/l kinetin and 0.001 mg/l folic acid. Albino shoots were transferred to U/M medium (Uchimiya and Murashige 1974) solidified with 0.8 percent agar to

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induce callus which in turn was converted to a cell suspension in liquid U/M medium.

The cell suspension was maintained at 30° on a rotary shaker (80 cycles/min) and subcultured weekly, by transferring approximately 2 g fresh weight of cells to 100 mls of medium in 250 ml flasks. Protoplasts were produced from the cell suspensions 3-6 days after subculture. Protoplasts were released following on overnight incubation of cells, at 24° C and with shaking (20 cycles/min), in a filter-sterilized enzyme mixture consisting 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). The mixture was passed through a nylon sieve (64μ pore size) and protoplasts were then washed in a 13 percent (w/v) mannitol solution prior to suspension in culture medium. Protoplasts of *P. parodii* were isolated as previously described (Hayward and Power 1975).

Fusion of Protoplasts

Protoplasts of both species (at a density 2×10^5/ml) were suspended in 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 similar to that used for the somatic hybridization of *P. hybrida* and *P. parodii* (Power et al. 1976a) but with the following modifications. Protoplasts were dispensed in 3 ml volumes so that for each experiment there were 2 tubes containing albino *P. inflata* protoplasts, 2 tubes with *P. parodii* protoplasts, 8 tubes, each containing a mixture of equal volumes of each species, and one viability control for each species. All tubes, except the viability controls, were centrifuged (100 g, 10 min) and the supernatant was replaced by 5 ml of the high pH and calcium fusion solution (Keller and Melchers 1973) containing 9 percent (w/v) mannitol, instead of PEG as used previously. In the first of two experiments fusion was carried out at 25°C for 15 min while in the second the temperature was raised to 30°C.

After fusion, protoplasts were washed twice in 13 percent (w/v) mannitol containing inorganic salts (Frearson et al. 1973) and 0.74 percent (w/v) $CaCl_2.2H_2O$. Protoplasts were finally suspended in the M/S medium, 6 ml per tube. At this stage the post-fusion mixture control was prepared (Power et al. 1976a) and the protoplasts were plated, liquid on agar, with a final density of 5×10^4/ml. Dishes were stored at 27°C and provided with a continuous illumination of 1000 lux by daylight fluorescent tubes. After 14 and 28 days respectively, 1 ml aliquots of M/S medium containing 6 percent (w/v) and 3 percent (w/v) mannitol were added to each dish. Thereafter dishes were prevented from drying out by the addition of M/S medium lacking mannitol.

Results

It was expected that complementation in the somatic hybrid would be revealed in the form of actively growing green calluses after 6-8 weeks culture. Unfused or homokaryon cells of the albino *P. inflata* would produce only colourless calluses while based on previous experience (Power et al. 1976a; Cocking et al. 1977), *P. parodii* protoplasts/cells would not grow beyond the colony stage in the M/S medium used. Both species were known to possess the ability to regenerate shoots from callus (Power et al. 1976b).

Green calluses were visible after 30-45 days only in the dishes containing the fusion-treated mixture of protoplasts. These calluses were transferred to the surface of the M/S medium containing 3 percent (w/v) mannitol and 0.5 percent (w/v) agar. At this stage a total of 29 and 518 calluses for the two experiments were identified, respectively. Once the calluses had reached a diameter of 0.5 cm (33-75 days after fusion) those of the first experiment and a random sample of 50 from the second were transferred to either M/S medium with 1.0 mg/l zeatin as the sole growth regulator or to M/S medium with 2.0 mg/l IAA and 1.0 mg/l 6-BAP for shoot initiation. Shoots were rooted in M/S medium lacking growth regulators and the plants flowered in the greenhouse.

As shown in Figure 1, flowers and leaves of the somatic hybrids (*P. inflata* + *P. parodii*) were similar in appearance to the tetraploid, sexually produced F_1 (*P. parodii* × *P. inflata*).

In the first experiment, 8 of the 29 selected calluses produced shoots, and in all cases they were confirmed as somatic hybrids. In the second experiment, 10 calluses gave rise to mature plants of which 7 were somatic hybrids and 3 were *P. parodii* types. No plants resembling *P. inflata* were isolated in either experiment. Chromosome counts of somatic hybrids confirmed the expected tetraploid (4n = 28) number with some plants deviating from this value, as described for the *P. hybrida* + *P. parodii* somatic hybrids. Most of the somatic hybrids were self-

Fig. 1. Flowers and leaves of (left to right) tetraploid *P. parodii* (white), tetraploid F_1 hybrid (*P. parodii* × *P. inflata*) (light magenta), a somatic hybrid (*P. inflata* + *P. parodii*) (light magenta) and tetraploid *P. inflata* (magenta). The magenta flower colour corresponds to cyclamen purple (Royal Horticultural Society Colour Chart, 74A) (half size). The tetraploid parents (4n = 28) were produced following a 90 min treatment of seedlings with 0.3 percent (w/v) aqueous colchicine solution. The F_1 hybrid could only be obtained by bud pollination of *P. parodii* (4n) with *P. inflata* (4n) as the male parent, whilst the reciprocal cross proved impossible at both the 2n and 4n levels

fertile and, upon selfing, segregated for the parental flower colours (Fig. 1) and other plant and flower characters described previously (Sink et al. 1978). This segregation further confirmed the hybrid nature of the regenerated plants. These new somatic hybrids also intercrossed with both parents at the tetraploid level, although to achieve the test-cross to *P. parodii* (4n) bud pollination was necessary.

The emergence, in the second experiment, of *P. parodii* types may simply be the result of a breakdown of the selection due to cross feeding of cells. However, it is possible that in this system, with unilateral sexual incompatibility, loss of the *P. inflata* genome resulted in the production of parental types with hybrid cytoplasms.

Discussion

As a result of the relative ease (1 hybrid per 8.3×10^4 protoplasts) with which somatic hybrids were recovered, it is likely that somatic hybridization will be very effective for crossing *Petunia* species exhibiting this mode of sexual incompatibility.

More recent data (unpublished) also suggests that somatic hybrids between *P. inflata* and *P. parodii* can be readily produced following the fusion of protoplasts of a γ-radiation induced albino mutant of *P. parodii* with those of albino *P. inflata.*

It is not clear at this stage whether somatic hybridization can overcome all barriers to sexual reproduction in higher plants. Somatic hybrids have been produced between *Datura* species (Schieder 1978) exhibiting an embryo/endosperm breakdown. This incompatibility was also overcome by embryo culture.

It may be helpful when considering somatic hybridization, to determine the cause of sexual incompatibility, since in the cases examined to date and where somatic hybridization attempts have repeatedly failed (*Nicotiana* and *Petunia*) a strong zygotic incompatibility was detected (Zenkteler and Melchers 1978; Sink and Power 1978). In two other examples of somatic incompatibility *Datura/Nicotiana* and *Datura/Petunia* (Schieder 1977) these incompatibilities are probably post-zygotic. The technique of single heterokaryon culture, as used for *Arabidopsis/Brassica* (Gleba and Hoffman 1978), may identify those species combinations exhibiting post-zygotic incompatibility which could be overcome through somatic hybridization.

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