Theor Appl Genet (1993) 87:75–80

THEORETICAL AND APPLIED GENETICS

Fertile somatic hybrids between *Petunia hybrida* and a wild species, *Petunia variabilis*

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Received: 24 September 1992 / Accepted: 1 March 1993

Abstract. Somatic hybrid plants were regenerated following electrofusion between leaf mesophyll protoplasts of *P. hybrida* (2n = 14) and a wild sexually incompatible species, *P. variabilis* (2n = 18). The selection of hybrids was based on the hybrid vigour, expressed both in the growth of the callus and at the shoot formation stage, resulting from the combination of parental genomes. Calli exhibiting vigorous growth were selected, and upon transfer to regeneration medium gave rise to shoots. Four regenerated plants from three calli had morphological characteristics intermediate between those of the parents. The hybrid nature of these plants was confirmed by chromosome counts as well as isozyme and DNA analyses. They had amphidiploid chromosome numbers (2n = 32) and were fertile. Following self-pollination and backcrossing with *P. variabilis*, large numbers of F_2 and BC_1 seedlings were obtained.

Key words: Somatic hybrid – *Petunia hybrida* – *Petunia variabilis* – Protoplast – Electrofusion

Introduction

Petunia (*Petunia hybrida*), possessing an extremely wide range of variation in floral color, pigmentation pattern and flower type, is one of the most popular ornamental flowers. Its extensive breeding, which originated in a cross between *P. axillaris* and *P. violacea*, has been carried out for more than one and a half centuries. There are many species in the genus *Petunia* and its related genera; however, they have been little utilized as genetic resources for petunia improvement, mainly due to the sexual incompatibility barrier.

Communicated by K. Tsunewaki
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Somatic hybridization via protoplast fusion is potentially useful in overcoming this barrier and so increasing the available genetic variation. In *Petunia*, somatic hybrids have been produced between sexually compatible species, *P. hybrida* and *P. parodii* (Power et al. 1978), between unilaterally cross-incompatible species, *P. inflata* and *P. parodii* (Power et al. 1979), and between sexually incompatible species, *P. parodii* and *P. parviflora* (Power et al. 1980). Cybrids of *P. hybrida* and *Nicotiana tabacum* have also been reported (Pental et al. 1986).

In the present study protoplast fusion was performed between *P. hybrida* (2n = 14) and a wild species, *P. variabilis* (2n = 18), in an attempt to transfer the latter's genetic traits to the former. This wild species has interesting characteristics, such as a branched, prostrate growth habit and a multifloral type. However, a sexual hybrid between the two has never been produced by conventional breeding. We describe here the regeneration and characteristics of somatic hybrids between *P. hybrida* and *P. variabilis* which were produced by electrofusion.

Materials and methods

Plant materials and protoplast isolation

Petunia (*P. hybrida*, inbred line W-1129) and a wild species, *P. variabilis*, grown in the greenhouse were used for protoplast isolation and fusion. Fully expanded leaves of both plants were sterilized in 1% sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. Then the sterilized leaves were cut into 1-mm-wide strips and incubated in an enzyme solution containing 1% Cellulase Onozuka RS, 0.5% Macerozyme R-10,9% mannitol and 10 mM $CaCl_2 \cdot 2H_2O$ (pH 5.6) for 1–2 h at 28 °C on a reciprocal shaker.

Protoplasts were separated from undigested tissue by filtration through a 53-μm nylon sieve and centrifuged at 80 g for 5 min. The supernatant was removed and the protoplast pellet was resuspended in 18% sucrose solution. Following centrifuga-

tion at 100 g for 10 min, viable protoplasts floating on the surface were collected and washed twice in 9% mannitol with 10 mM $CaCl_2 \cdot 2H_2O$ and finally adjusted to a cell density of 2×10^5/ml. Protoplast suspensions of *P. hybrida* and *P. variabilis* were mixed in a 1:1 ratio.

Protoplast fusion and culture

Protoplasts were fused by means of electrofusion using an SSH-1 Somatic Hybridizer (Shimadzu Corporation, Japan). A 1.5-ml aliquot of mixed protoplast suspension was introduced into the fusion chamber, aligned with an AC (alternating current) field of 2 MHz, 50 V/cm, and fused by applying a 30 μs DC (direct current) pulse of 1.25 kV/cm, twice. Following fusion treatment, the protoplast suspension was transferred to plastic Petri dishes (60 × 15 mm,) and an equal volume of two-fold-strength culture medium was added.

Fusion-treated protoplasts were cultured in MS medium (Murashige and Skoog 1962) supplemented with 2 mg/l naphthalene acetic acid (NAA), 1 mg/l benzylaminopurine (BAP), 1% sucrose and 9% mannitol at 25 °C in the dark. After 2 weeks, an equal volume of the fresh medium was added to the Petri dishes. When colonies were formed after 1 month of culture, they were transferred onto the regeneration medium (MS containing 0.1 mg/l NAA, 1 mg/l BAP, 3% mannitol, 3% sucrose and 0.2% Gelrite). Calli showing vigorous growth were transferred again to the regeneration medium without mannitol. Then shoots emerging from the calli were placed on the rooting medium (MS containing 0.05 mg/l NAA, 3% sucrose and 0.2% Gelrite). Finally rooted plantlets were transferred to sterilized soil and grown to flowering.

Chromosome counts

Chromosome numbers were determined after Feulgen staining. Root tips of regenerated plants were pretreated with 2 mM 8-hydroxyquinoline for 4 h at 20 °C, fixed in ethanol-acetic acid (3:1) for 12 h and hydrolyzed in 1 N HCl at 60 °C for 10 min before staining.

Isozyme analysis

Leaf tissues (100 mg) were homogenized in 50 mM HEPES buffer (pH 7.5) with 1 mM EDTA and 0.1% CHAPS. The homogenates were absorbed onto paper wicks and analyzed by ultrathin-layer isoelctric focusing. The gel was stained for peroxidase according to the procedure of Graham et al. (1964).

DNA analysis

Total DNAs were extracted from leaf tissues of regenerated plants and both parents as described by Rogers and Bendich (1985). After digestion with the indicated restriction endonucleases, DNA fragments were electrophoresed in 0.7% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham) by capillary transfer with 0.4 M sodium hydroxide. The membrane filter was neutralized and crosslinked with UV light. Hybridizing and detecting DNA followed the protocols of Boehringer Mannheim.

The clones from rice nuclear DNA containing the 17*s* ribosomal RNA gene (rDNA:Takaiwa et al. 1984), mitochondrial DNA (mtDNA) containing the ATPase α-subunit gene (*atpA*: Kadowaki et al. 1990) and 4.7 and 4.9 kb *Bgl*II fragments of chloroplast DNA (cpDNA) of *Brassica oleracea* (broccoli) were labeled by random primed incorporation of digoxigenin-labeled deoxuridine triphosphate according to the supplier's instructions (Boehringer Mannheim). These labeled DNA fragments were used as probes. The rDNA and mtDNA clones were kindly provided by Drs. F. Takaiwa and K. Kadowaki respectively.

Results and discussion

The fusion frequency observed in this experiment was about 5%. It was greatly affected by the strength of AC pulses. When the strength of the pulse exceeded the optimal condition (30 μs, 1.25 kV/cm), protoplasts were damaged and sometimes destroyed.

Fusion-treated protoplasts started cell division with a week and formed small colonies after 1 month of culture. These small colonies when transferred to regeneration medium solidified with 0.2% Gelrite grew to calli of 3–4 mm diameter. Calli showing vigorous growth on this medium were then picked up and transferred to regeneration medium without mannitol. Out of 76 calli transferred, five produced some shoots about 4 months after electrofusion treatment. Shoots were transferred to the rooting medium and roots formed within 2 weeks. In the primary experiments on the protoplast culture of both parents, the growth of the callus derived from the *P. hybrida* protoplast was better than that of *P. variabilis*, but shoots were regenerated only from *P. variabilis* under the same conditions.

Out of five regenerated plants, three had morphological characteristics intermediate between both parents (Fig. 1) while the others closely resembled *P. variabilis*. There were no variations in morphology among the three putative hybrids. When grown to the flowering stage these plants exhibited intermediate floral and leaf morphology, both in size and shape (Fig. 2). Their flower color, however, was magenta which was clearly different both from *P. hybrida* (white) and *P. variabilis* (light magenta). This color change was most likely caused by the predominant expression of genes determining magenta color and an increased thickness of the corolla. Another novel floral characteristic of the putative hybrids was that the outside lobes of the corolla were extremely bent (Fig. 1 A). There were no abnormalities, such as a sectored corolla or split blossom, of the type described in the somatic hybrid between *P. parodii* and *P. infrata* (Schnabelrauch et al. 1985).

Chromosome counts revealed that morphologically-putative hybrids had the expected amphidploid chromosome numbers of 32 (Fig. 3), which is equal to the sum of *P. hybrida* (2n = 14) and *P. variabilis* (2n = 18), while the other regenerated plants resembling *P. variabilis* had 2n = 18 chromosomes. Therefore, three out of five regenerated plants were assumed to be true somatic hybrids between *P. hybrida* and *P. variabilis*. These hybrids were produced by selection based on the hybrid vigour expressed both in callus growth and at the shoot formation

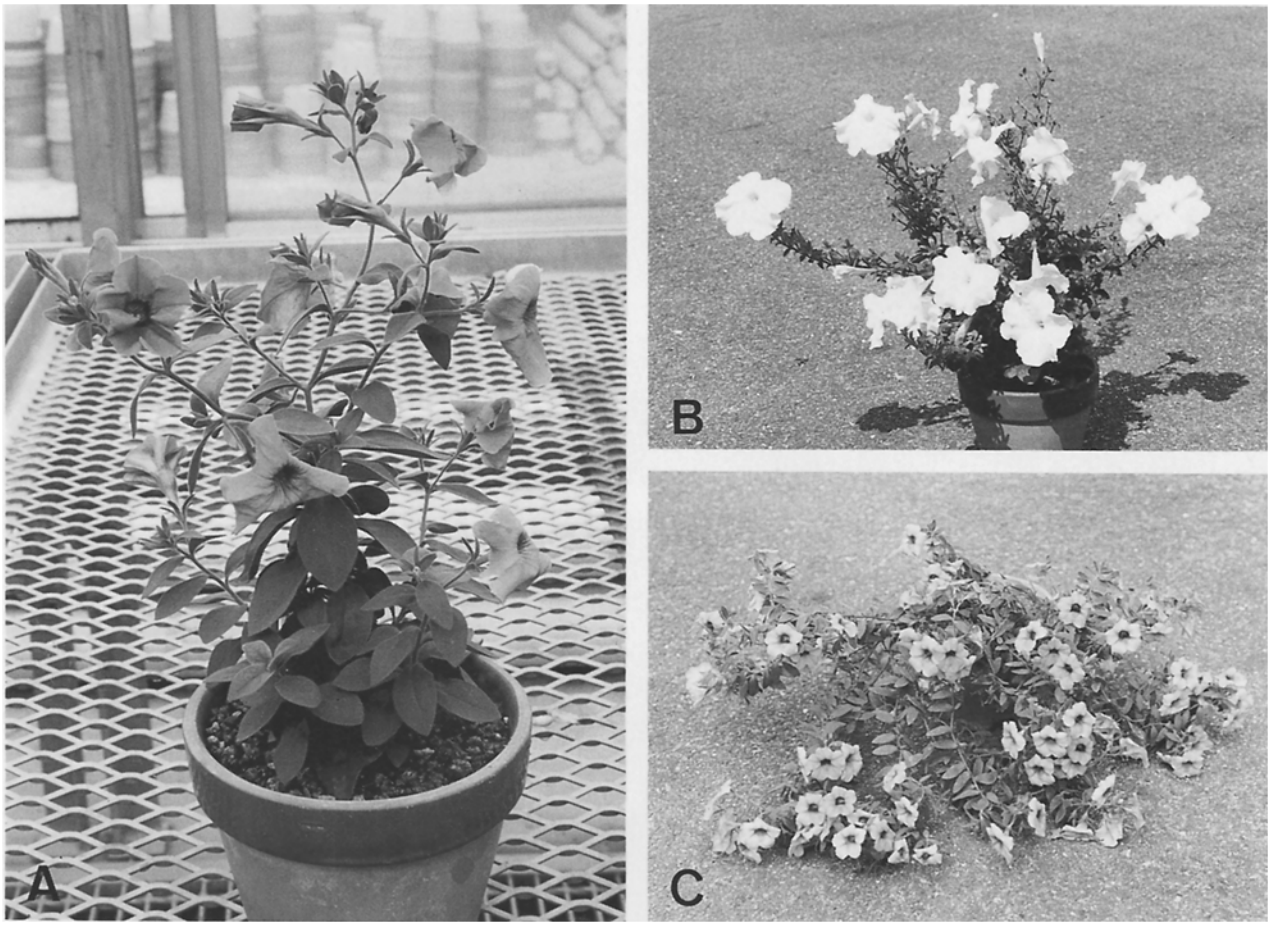

Fig. 1A–C. Flowering plants of the somatic hybrid (**A**), and its parents *P. hybrida* (**B**) and *P. variabilis* (**C**)

stage as described in *Datura* (Schieder 1978), *Brassica* (Taguchi and Kameya 1986) and an intergeneric hybrid between *Lycopersicon* and *Solanum* (Sakamoto and Taguchi 1991). This selction method is not completely reliable, but seems to be useful in cases where no other means of selection can be applied.

To confirm the hybridity of the regenerated plants, biochemical analyses of isozymes and DNA were carried out. Figure 4 shows the peroxidase isozyme patterns of the somatic hybrid and both parents. *P. hybrida* and *P. variabilis* each had three bands that were different from one another. The somatic hybrid had four bands consisting of two *P. hybrida*-specific bands and two *P. variabilis*-specific bands.

Figure 5A shows the results of nuclear rDNA analysis. The total DNAs were digested with *Eco*RI and hybridized to the labeled rDNA probe. In *P. hybrida*, three major fragments hybridized to the probe. *P. variabilis* had the same three fragments but with one additional faint fragment (5.8 kb). Four somatic hybrids were examined, two of which, SH-2a and SH-2b, were regenerated from same callus. They contained all of these fragments plus a novel fragment (7.0 kb). No clear difference was detected among the four somatic hybrids.

The total DNAs of both parents and the four hybrids were digested with *Eco*RI and hybridized to *atpA* (Fig. 5B). Somatic hybrids SH-2a and SH-2b had the same pattern, but they and two other somatic hybrids (SH-1, SH-3) showed different patterns from each other. The *P. hybrida*-specific fragment of 2.2 kb was not present in all hybrids and neither was the 2.3 kb *P. variabilis*-specific band. Rearrangements in mtDNA are known to be facilitated by protoplast fusion (Landgren and Glimelius 1990). Clark et al. (1986) have reported that petunia somatic hybrids derived from the same fusion product had variable mtDNA restriction patterns. Our results indicated that mtDNA rearrangements had occurred at the heteroplasmic state after protoplast fusion in the somatic hybrids between *P. hybrida* and *P. variabilis*.

For cpDNA analysis, we used two cpDNA (4.7 and 4.9 kb) probes and three restriction endonucleases

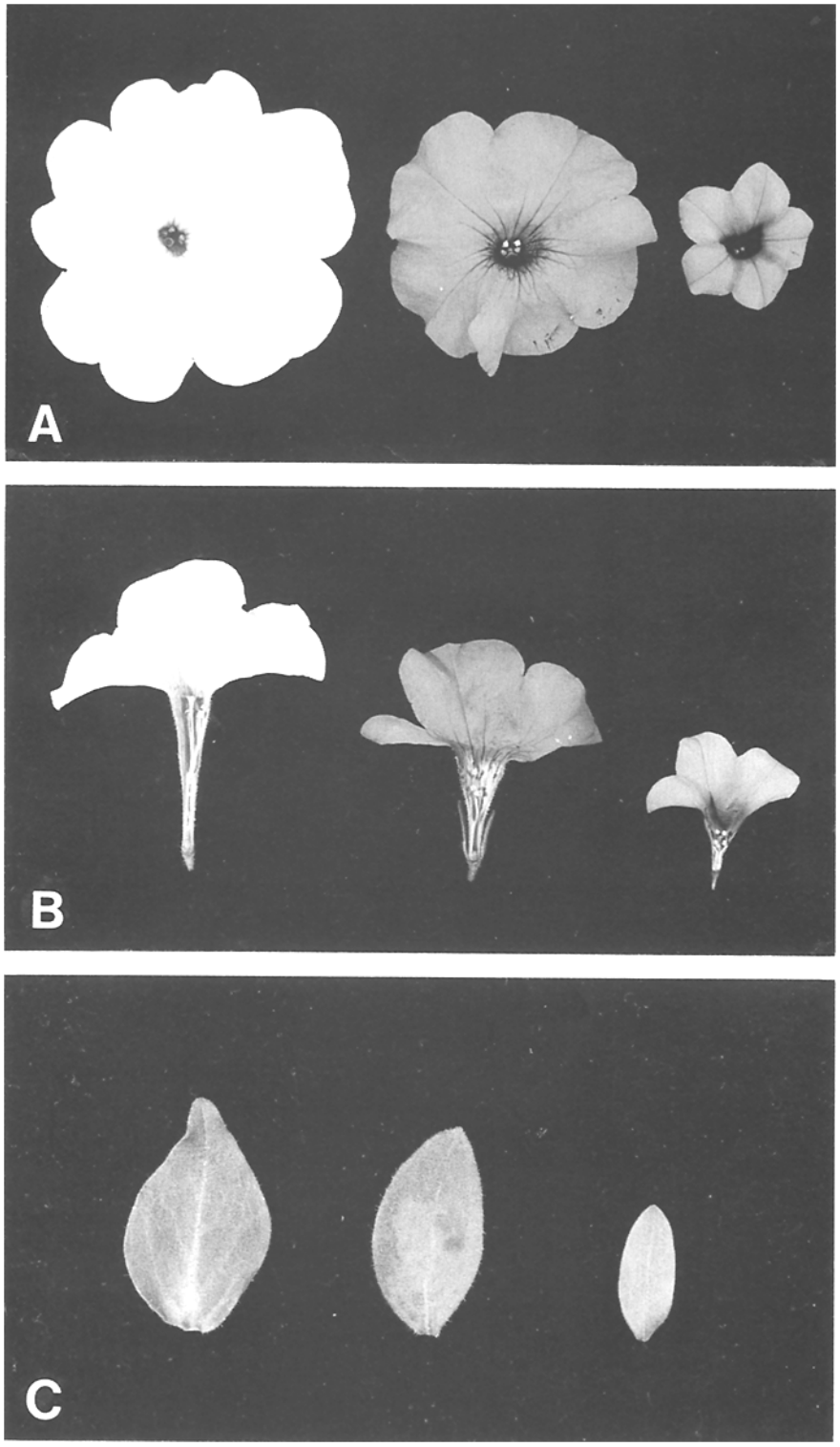

Fig. 2A–C. Floral (**A, B**) and leaf (**C**) morphology of *P. hybrida* (left), the somatic hybrid (center) and *P. variabilis* (right)

(*Bam*HI, *Eco*RI, *Hin*dIII). Both parents and all four hybrids could not obviously be distinguished by their restriction patterns, except that the *Hin*dIII digest of *P. hybrida* DNA produced a 7.0 kb faint fragment hybridizing to the 4.7 kb cpDNA probe (Fig. 5C). These results confirmed the conclusion of Kumar and Cocking (1982) that cpDNA is strongly conserved in the genus *Petunia*.

The somatic hybrids had about 34% pollen fertility as determined by the staining of freshly dehiscent pollen grains with 1% acetocarmine. The parents, *P. hybrida*

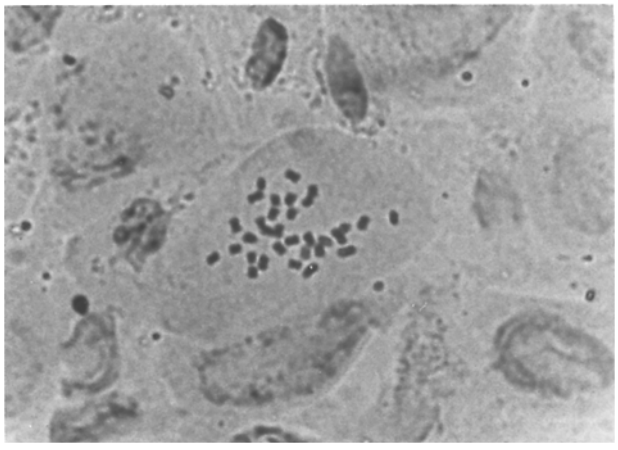

Fig. 3. Metaphase chromosomes (2n = 32) in a root-tip cell of a somatic hybrid between *P. hybrida* and *P. variabilis*

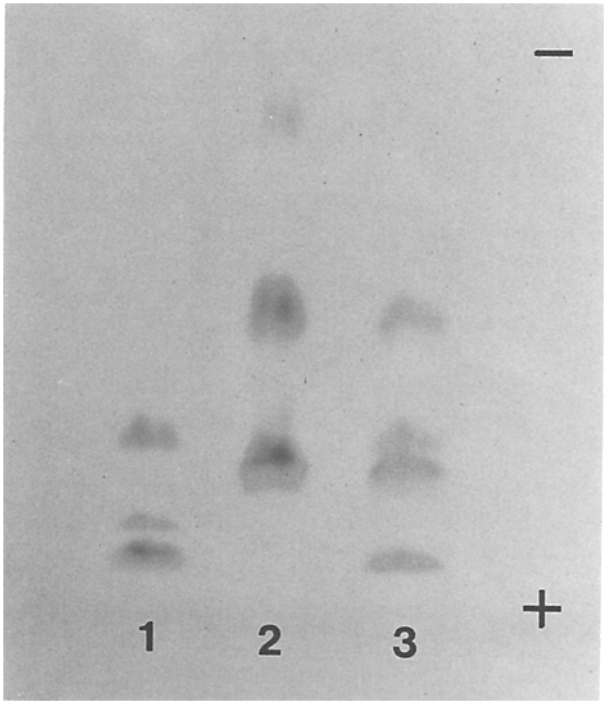

Fig. 4. Peroxidase isozyme patterns of *P. hybrida* (*lane 1*), *P. variabilis* (*2*) and the somatic hybrid between them (*3*)

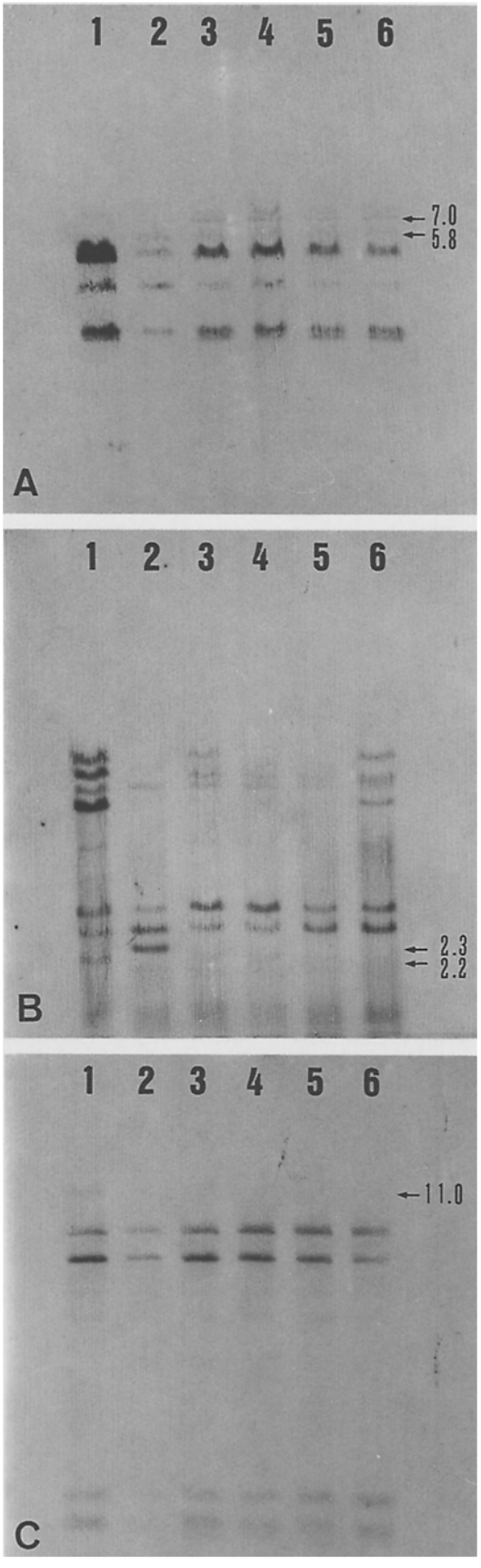

Fig. 5A–C. Southern-blot hybridization of *Eco*RI digests of total DNA to labeled rDNA fragments (**A**) and mtDNA fragments (**B**), and *Hin*dIII digests of total DNA to labeled cpDNA fragments (**C**). *Lane 1*, *P. hybrida; 2*, *P. variabilis*, 3–6, somatic hybrids SH-1, SH-2a, SH-2b and SH-3. Numerals indicate the size of hybrid bands in kbp

and *P. variabilis*, had pollen fertilities of 71% and 97% respectively. Although the pollen fetility of the somatic hybrid was low in contrast with those of the parents, large numbers of F_2 and BC_1 seedlings were obtained following self-pollination and backcrossing with *P. variabilis*. By contrast, no seeds obtained by backcrossing with *P. hybrida* germinated. F_2 plants were almost the same as somatic hybrids, and no morphological variation was found among them. Power et al. (1978) described that some variation was observed in the F_2 populations of somatic hybrids between *P. hybrida* and *P. parodii*. The discrepancy between their and our results seems to be caused by the different genetic backgrounds of the materials used for protoplast fusion. The morphological characteristics of BC_1 plants changed slightly toward those of *P. variabilis*.

Most somatic hybrids reported to-date between sexually incompatible species show extremely low fertility or complete sterility, creating a serious problem for plant breeders in utilizing these hybrids as breeding material. Fortunately, we were able to obtain fertile and reproductive somatic hybrids between *P. hybrida* and *P. variabilis*. These will be used for petunia improvement.

Acknowledgements. We thank Dr. T. Ando, University of Chiba, for his valuable comments on a petunia wild species and the members of Takii Plant Breeding and Experiment Station for their helpful suggestions and assistance.

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