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Nucleus substitution between *Petunia* species using gamma ray-induced androgenesis

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Summary. The ovaries of two different *Petunia* species: *Petunia hybrida* (hort) and *Petunia parodii* (Steere) were irradiated with τ-ray doses ranging from 50 to 1,000 Gy before pollination. Seed setting occurred after 4 days preculture on a non-sterile medium. Ovaries transformed into fruits were then cultivated aseptically with the following results: (1) τ-ray doses ranging from 200 to 1,000 Gy led to the development of two types of plants: haploids $2n = x = 7$ and overdiploids $2n > 2x = 14$. (2) The androgenetic origin of haploids was ascertained by using genetic markers. The origin of overdiploids is discussed. (3) Androgenetic haploids contained the chloroplasts of the irradiated female parent. No visible change of cp DNA patterns was observed after irradiation. (4) The four possible androgenetic events were successfully obtained between the two *Petunia* species: *hybrida* haploids with *hybrida* or *parodii* cytoplasm, and *parodii* haploids with *parodii* or *hybrida* cytoplasm.

Key words: *Petunia hybrida* – *Petunia parodii* – Androgenesis – Ovary irradiation – Chloroplast DNA

Introduction

The three main causes of spontaneous or induced development of haploid plants are abnormal embryo development, abnormal fertilization and disturbed gametogenesis, usually induced by in vitro culture. This latter method is currently used to obtain haploid plants in most species. Nevertheless, in vitro anther culture of *Petunia* produces mostly homozygous triploid plants (Raquin 1982). Unpollinated ovary culture only give results with *Petunia axillaris* (De Verna and Collins 1984). Haploids of *Petunia hybrida* were successfully obtained by in vitro culture of ovaries pollinated with irradiated pollen (Raquin 1985). A combination of pollen irradiation and in vitro haploid embryo rescue has also been recently performed for muskmelon (Sauton and Dumas de Vaulx 1987).

A major goal of plant breeding is to produce hybrid seeds. Haploids save the considerable time necessary to obtain the homozygous lines used as parents of these hybrids. In situ androgenesis (i.e. development of the haploid male nucleus in the cytoplasm of the embryo sac) may, furthermore, permit rapid nuclear cytoplasm exchanges, including the transfer of nuclei to cytoplasms carrying male sterility. Such results were obtained with spontaneous androgenetic haploids of maize (Goodsell 1961) and petunia (Singh and Cornu 1976).

Although pollen irradiation is probably the oldest and most widely used technique for inducing in situ parthenogenetic haploids, only a few attempts at embryo sac irradiation have been performed in order to promote in situ androgenesis, apart from the pioneer work of Gerassimova (1936) on *Crepis tectorum* and Ehrensberger (1948) on *Antirrhinum majus*. Following the preliminary success (Raquin 1986), we report here the induction of androgenesis by in vitro culture of *Petunia* ovaries irradiated before pollination. Reciprocal nuclei plastid transfers were carried out between *Petunia hybrida* hort and *Petunia parodii* Steere.

Materials and methods

Plant material

Three different genotypes were used in these experiments: two genotypes of *Petunia hybrida* and one inbred line of *Petunia parodii*. The *hybrida* RLk 22-1 line is homozygous for the recessive genes hf_1, *fl*, ht_1, ph_2, *po*, an_2 and an_4 (Fig. 1, A2). The location and function of these genes was previously described by Cornu and Maizonnier (1982). One recessive gene involved in

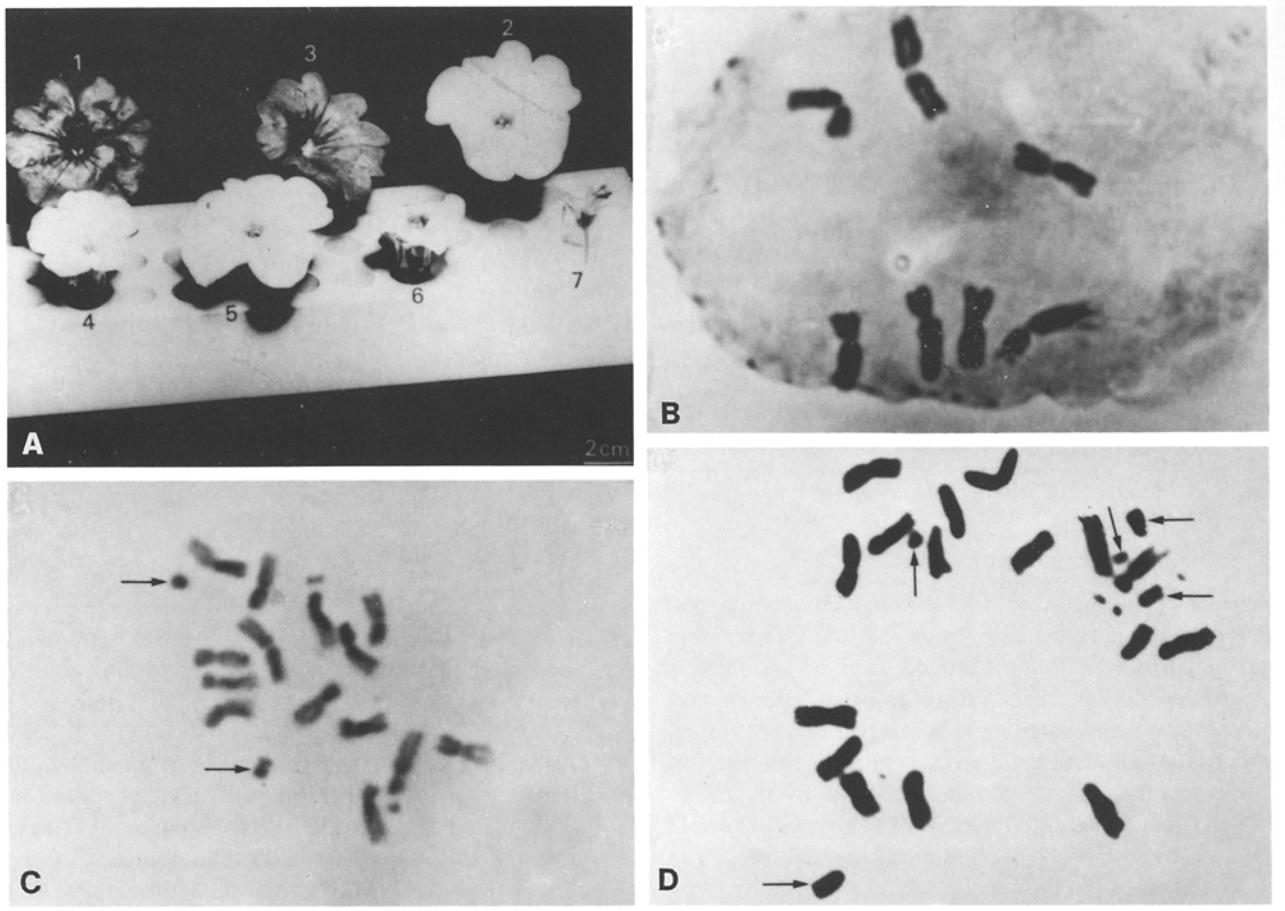

Fig. 1 A–D. Gamma-induced androgenesis. **A** *1* – parent multidominant $TG_5 \times T^+R67$; *2* – parent multirecessive RLk 22-1; *3* – sexual hybrid; *4* – progeny issued from irradiated $TG_5 \times T^+R67$ pollinated with RLk 22-1; *4* – androgenetic haploid; *5–7* – maternized overdiploids; *5* – no difference with RLk 22-1, normal fertility; *6* – only P_0 as dominant marker of the mother plant, sterility; *7* – Hf_1 and An_2 as dominant markers of the mother plant. Flowers generally aborted before opening. **B** – haploid chromosome number of A4. **C** – chromosome number of A_5 2n = 14 chromosomes + 2 fragments. **D** – chromosome number of A_7 2n = 16 chromosomes + 5 fragments. The *arrows* indicate the fragments

the biosynthesis of anthocyanins is carried by each of the seven chromosomes. The F_1 hybrid $TG_5 \times T^+R_{67}$ is homozygous for the dominant alleles Hf_1, Ht_1, An_2 and heterozygous for *Fl*, Ph_2, P_0 and An_4 (Fig. 1, A1). This hybrid possesses good seed setting ability after heavy irradiation of its ovaries. None of the multidominant lines tested up to now had this ability. These two *hybrida* genotypes possessed the same chloroplast DNA. The inbred line of *Petunia parodii* is homozygous dominant for Hf_{11} (a different allele dominant to hf_1), Ht_1 and PH_2.

Very poor seed setting occurred on RLk 22-1 ovaries with τ-ray doses of more than 200 Gy. Therefore, the hybrid RLk 22-1 × Pp was used as female parent on *hybrida* cytoplasm and the reciprocal hybrid Pp × RLk 22-1 as female parent on *parodii* cytoplasm for crosses F, G and H. The chloroplast DNA of *Petunia parodii* is different from cp DNA of *Petunia hybrida* (Kumar et al. 1982).

Irradiation and culture of ovaries

Flowers were emasculated 24 h before dehiscence of the anthers and harvested 2 days later. Whole flowers were irradiated by τ-rays of $^{60}C_0$. The dose rate decreased from 50 to 45 Gy × mn^{-1} during the experiment. Flowers were pollinated after irradiation and precultured for 4 days on medium A. Non-sterile culture may be carried out on this medium for 5 to 6 days at 26 °C without contamination. Ovaries transformed into fruits were then surface-sterilized and cultivated on medium B, as previously described (Raquin 1985). Cultures were incubated at 28 °C with 16 h/day illumination, $\approx 40\ \mu Exm^{-2} \times S^{-1}$.

Calli and embryos emerged from the ovules after 4–8 weeks of culture and were subcultured on medium C. Viable plants were planted in the greenhouse. Chromosome counts were made in the root tip after Feulgen staining.

The three media used for these experiments had the following components in common: half concentration of Murashige and Skoog inorganic salts, Fe EDTA 10^{-4} *M*; pH was adjusted to 5.8.

Medium A contained 0.12 *M* sucrose, 2.5×10^{-3} *M* phydroxybenzoic acid methyl ester (preservative) and $6\ g \times l^{-1}$ Bacto agar DIFCO. Medium A was not sterilized. $2\ ml \times l^{-1}$ of a 2% calcium hypochlorite solution was added after boiling.

Medium B contained Morel and Wetmoore vitamines, 5×10^{-3} *M* IAA, 0.17 *M* Maltose and $6\ g \times l^{-1}$ agarose (Sigma type 1A low EEO).

Table 1. In vitro culture of *Petunia* ovaries irradiated before pollination. Doses of γ-rays and results of intraspecific and interspecific crosses. Phy: *Petunia hybrida*, Pp: *Petunia parodii*

Cross	Type of cross	Dose of γ Ray (Gy)	Ovaries cultivated	Embryo produced	Viable plants			
					Total	n	2n	>2n
A	Phy × Phy	50–100	66	79	22	0	21	1
B	Phy × Phy	200	295	232	4	2	0	2
C	Phy × Phy	300	291	154	5	1	0	4
D	Phy × Phy	500	123	89	1	1	0	0
E	Phy × Phy	750	128	53	3	0	0	3
F	Phy × Phy	1,000	137	105	11	8	0	3
G	Phy × Pp	1,000	191	68	3	2	0	1
H	Pp × Phy	1,000	156	158	6	2	0	4
I	Pp × Pp	1,000	209	70	2	1	0	1

Crosses A–F irradiated ♀ = TGs × T + R67 (multidominant hybrid) ♂ = RLK 22-1
Cross G irradiated ♀ = RLk 22-1 × Pp ♂ = Pp
Cross H irradiated ♀ = Pp × RLk 22-1 ♂ = RLk 22-1
Cross I irradiated ♀ = Pp × RLk 22-1 ♂ = Pp

Medium C contained only 0.06 *M* sucrose, 4 g × l^{-1} agar and 2 g × l^{-1} gelrite. Media B and C were autoclave-sterilized (20 min at 118 °C).

Isolation of chloroplasts and chloroplast DNA:

A simple and rapid procedure was used to analyse cp DNA, starting with 1–3 g of young leaves. Material was homogenized at 4 °C in a Waring blender for 3 s twice at low speed in 10 ml/g fresh weight buffer A (50 m*M* TRIS-HCl, pH 8.0, 0.5 *M* mannitol, 3 m*M* EDTA, 0.1% BSA, 1 m*M* β-mercaptoethanol). The homogenate was filtered through a 35-μm nylon net without squeezing, and the filtrate was centrifuged at 100 g, 5 min (IEC rotor 210). The pellet was discarded and the supernatant was centrifuged at 1,200 g for 7 min (IEC rotor 210). The crude chloroplast pellet was resuspended in 5 ml of buffer A with a paint brush. Additional buffer A (10 ml/g fresh material) was added and the suspension was centrifuged again for 7 min at 1,200 g. The pellet was lysed with 2.5-ml buffer containing 50 m*M* TRIS-HCl, pH 8.0, 20 m*M* EDTA, 2% laurylsarcosinate and 1 mg/ml pronase (Boehringer Mannheim) for 1 h at 20 °C. The lysate was treated twice with a phenol-chloroform mixture and the nucleic acids precipitated from the aqueous phase overnight at −20 °C, after additions of 50 mg NaCl and 2.5 vol of cold ethanol. The nucleic acid pellet was recovered by centrifugation, dissolved in 300 μl of TSE (10 m*M* TRIS-HCl, pH 8.0, 50 m*M* NaCl, 1 m*M* EDTA) and precipitated with cold ethanol for 3 h at −20 °C. The last pellet was dissolved in 50–150 μl of TSE and the insoluble material was removed by centrifugation.

Chloroplast DNA analysis

One to these micrograms of cp DNA was digested in 30-μl reactions with sufficient enzyme to give complete digestion. The restriction enzyme preferentially used was BamHI (Boehringer Mannheim) because Kumar et al. (1982) used this enzyme to distinguish between *Petunia parodii* and *Petunia hybrida* cp DNAs. The restriction fragments were separated by electrophoresis in 0.7% agarose gels. The 1-kb ladder (Bethesda Research Laboratories) was used as a molecular weight standard.

Results

Irradiated ovaries gave rise to many calli and abnormal embryos, only a low proportion of which developed into viable plants. Results are reported in Table 1.

Ovaries irradiated with 50–100 Gy produced mostly normal or mutated hybrid diploid plants. Only one plant was counted as trisomic. No diploid hybrids were observed from ovaries treated with 200 Gy and higher. Mainly two types of plants were counted: haploids and overdiploids (plants with more than 2n = 2x = 14 chromosomes).

Haploids. Seventeen haploid plants were obtained (Fig. 1, A4 and 1B). All the 14 *hybrida* haploids expressed the seven recessive markers of the male parent and were, therefore, undoubtedly of androgenetic origin. The 3 *parodii* haploids had the *parodii* type of the pollinator and were, therefore, androgenetic.

No morphological differences were noted between *hybrida* haploids issuing from crosses with *hybrida* or *parodii* as female, and the same was true for *parodii* haploids issued from crosses with *parodii* or *hybrida* as female. The *parodii* or *hybrida* cytoplasm does not, therefore, affect the expression of the nuclear markers of these species.

Results of cp DNA analysis performed with BamH1 restriction enzyme are shown in Fig. 2. No difference was observed between the cp DNA pattern of the parental lines and those from androgenetic haploids obtained after irradiation with 200 Gy (3, 7) or 1,000 Gy (4, 5, 6, 8, 9, 10).

Overdiploids. Eighteen plants had a chromosome number greater than 2n = 2x = 14, from 2x plus 1 fragment to 2x

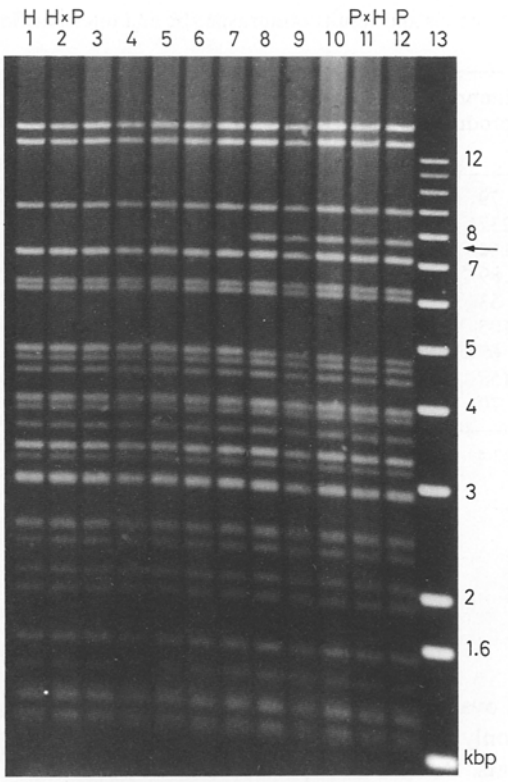

Fig. 2. BamHI restriction patterns of cpDNA from parental plants and androgenetics haploids. *Lanes 1 and 12:* parental lines, *Petunia hybrida* and *Petunia parodii*, respectively. *Lanes 2 and 11:* sexual hybrids, RLk 22-1 × Pp and Pp × RLk 22-1, respectively. *Lanes 3–5:* androgenetic haploids of *Petunia hybrida* with *hybrida* cytoplasm. *Lanes 6 and 7: Petunia parodii* haploids with *hybrida* cytoplasm. The plant of *lane 7* was obtained in a different experiment with 200 Gy irradiation of RLk 22-1 ovaries and possesses an additional chromosome fragment. *Lanes 8 and 9:* haploids of *Petunia hybrida* with *parodii* cytoplasm. *Lane 10:* a parodii androgenetic haploid with its own cytoplasm. *Lane 13:* 1-kb ladder molecular weight standard. The *arrow* indicates the specific additional fragment of *Petunia parodii* cp DNA

plus 7 chromosomes or fragments. The centromere was usually clearly visible on the fragments. These overdiploids were strongly "paternized" [very similar to the male parent, cf. Fig. 1 (plant A5)] but sometimes expressed one marker of the irradiated female (Hf_1, P_0, An_2). Only one of the plants derived from cross no. E (plant A7) expressed two dominant markers (Hf_1 and An_2).

Discussion

One hypothesis concerning the origin of the overdiploids is that there was spontaneous chromosome doubling of the male gamete. Similar findings were made after fusion of irradiated protoplasts of *Nicotiana sylvestris* with unirradiated protoplasts of *Nicotiana plumbaginifolia* (Framelaer et al. 1986). Regenerated plants showed 4x chromosomes of *plumbaginifolia* plus chromosomes and chromosome fragments of *sylvestris*. If true, the *Petunia* over-diploids obtained here have the homozygous diploid stock of the male parent and some chromosome fragments and (or) chromosomes of the female irradiated parent. Alternatively, heavy irradiation of the embryo sac may lead to more than one fertilization. The use of a mixture of pollen with different markers should make it possible to distinguish between these two hypotheses.

We showed that in situ androgenesis could be induced by ovary irradiation before pollination. From 200 Gy, no normal hybrids were obtained. Androgenetic embryos were rescued by in vitro culture of the ovaries. Androgenetic haploids contained chloroplasts from the irradiated female plant. Irradiation with 1,000 Gy did not modify the cp DNA patterns. Haploid nuclei of *Petunia hybrida* were transferred to *parodii* cytoplasm and vice versa. The four possible androgenetic events were successfully obtained between the two *Petunia* species: *hybrida* androgenetic haploids with *hybrida* or *parodii* cytoplasm and *parodii* androgenetic haploids with *parodii* or *hybrida* cytoplasm.

Similar experiments are now being carried out in order to transfer cytoplasmic male sterility. Gamma-induced androgenesis as well as spontaneous androgenesis screened by using genetic markers (Singh and Cornu 1976) or lethal mutants (Pelletier et al. 1987) complement the protoplast fusion as a means of rapidly creating isogenic homozygous lines with different types of cytoplasms. Gamma-induced androgenesis theoretically excludes the risk of mitochondrial recombination which is frequent with protoplast fusion (Belliard et al. 1979; Boeshore et al. 1983). Nevertheless, it is necessary to verify that high doses of τ-ray do not modify the mt DNA.

If the origin of overdiploid plants is confirmed, ovary irradiation may likewise provide a complementary method to "gamma fusion" for obtaining partial interspecific hybrids (Imamura et al. 1987; Gleba et al. 1988) and references therein.

DH seeds of RLk on *parodii* cytoplasm and *parodii* on hybrida cytoplasm are available.

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