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Southern and fluorescent in situ hybridization detect three RAPD-generated PCR products useful as introgression markers in *Petunia*

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Abstract Fluorescent in situ hybridization (FISH) was used to reveal the intrachromosomal organization of 11 RAPD markers localized on the genetic map of *Petunia hybrida*. The cloned RAPD markers were analyzed by means of Southern hybridization to determine their level of sequence repetition and their specificity in different *Petunia* species with 2n = 14 and 18 chromosomes. The same probes were then used in FISH experiments. Most of the RAPD clones studied showed high sequence repetition and no species specificity. Moreover, FISH analysis showed that these probes could belong to multilocus families as evidenced by the multiple FISH signals dispersed throughout the genome and present on every chromosome. Only 3 RAPD clones revealed species specificity at the chromosome level. Clones OPJ18-250 and V20-350 were only detected by FISH in the white-flowered species and clone OPV08-600 only in species with colored flowers. They were localized at one two or three pairs of fluorescent sites. The localization of OPJ18-250 at a unique site on chromosome VI give us the opportunity to compare genetic and physical distances.

Key words *Petunia* · RAPD markers · In situ hybridization · Physical mapping

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Introduction

Random amplified polymorphic DNA (RAPD) represents a powerful tool for establishing genetic maps. This type of genetic marker has been used by plant geneticists and breeders for cultivar identification, genetic diversity evaluation and marker-based selection (Hu and Quiros 1991; Hilu 1994). RAPD-based genetic maps have been created in several species such as conifers (Sederoff and Neale 1991; Tulsieram et al. 1992; Nelson et al. 1993; Binelli and Bucci 1994; Plomion et al. 1995), alfalfa (McCoy et al. 1991; Yu and Pauls 1993), *Arabidopsis thaliana* (Reiter et al. 1992) and rapeseed (Foisset et al. 1996). In our laboratory, an RAPD-based genetic map of *Petunia hybrida* was produced (Peltier et al. 1994) which consisted of 35 RAPD loci covering 296.2 cM with an average spacing of 8.2 cM. Twenty five of these RAPD loci were localized onto six identified chromosomes, while 10 loci remained not assigned. The RAPD method allows the identification of a large number of polymorphic DNA markers distributed throughout the genome, including both coding and non-coding regions (Williams et al. 1990). The majority of RAPD markers are amplified from highly repetitive chromosomal regions (Plomion et al. 1995), a significant proportion of which shows species specificity and some ability to detect introgression and alien chromatin/DNA fragments in intergeneric hybrids and their advanced generations (Bommineni et al. 1997). This characteristic of RAPD markers opens up new ways for the physical identification of chromosomes and alien chromatin and could thus help to elucidate genome relationships inside a particular species complex. The most accurate method for identifying the chromosomal location of a DNA segment is by in situ hybridization. The use of non-isotopic systems, particularly the fluorescent in situ hybridization method (FISH), is a powerful tool for the physical mapping of highly repeated DNA

sequences and also of low and unique sequence DNA (Rayburn and Gill 1985; Ambros et al. 1986; Clark et al. 1989; Schwarzacher and Heslop-Harrison 1991; Wang et al. 1995; Ten Hoopen et al. 1996).

In this investigation we applied a FISH-based procedure to study the intrachromosomal localization of 11 RAPD markers distributed on six of the seven genetic linkage groups of *Petunia hybrida*. These markers were also used for Southern analysis in order to detect their level of sequence repetition and their sequence homology within different *P. hybrida* lines and wild genotypes. *Petunia hybrida* is believed to be an interspecific hybrid derived from crosses between two groups of colored- and white-flowered 2n = 14 wild species. The group of colored-flowers species was represented by the wild species *P. integrifolia* and *P. inflata*, while that of the white-flowered species was represented by *P. axillaris* and *P. parodii*. The two wild species *P. linearis* and *P. parviflora* (both with 2n = 18) were assumed to be more distantly related to *P. hybrida* (Wisjman 1990).

Our aim was to determine whether RAPD could be converted into chromosomal markers useful to follow introgressions from the parental wild species to *P. hybrida*. It was also a good opportunity to compare how the physical locations of the chosen RAPD probes correspond to their previously established genetic ones. The distances established on genetic maps can differ from those observed on physical ones (Gustafson et al. 1990; Wang et al. 1991; Gustafson and Dillé 1992). This study provided useful data on the genome organization and the chromosomal location of RAPD markers among *Petunia* genotypes.

Materials and methods

Plant material

The *Petunia* species used were (Table 1):

- the most probable progenitors of *P. hybrida*: *P. axillaris*, *P. parodii*, *P. inflata* and *P. integrifolia* (with 2n = 14 chromosomes).
- two more distant *Petunia* species: *P. linearis*, and *P. parviflora* (with 2n = 18 chromosomes).
- an F_1 hybrid between *P. axillaris* and *P. inflata*.
- cultivars of *P. hybrida*: ST40, on which the RAPD-based genetic map was established, TB1-3, TLH7, and TLV1.

Chromosome preparation for in situ hybridization

Synchronization of mitosis

Root tips about 2 cm long were synchronized by the method of Pan et al. (1993) with minor modifications. The roots were placed into Hoagland solution (Gamborg et al. 1975) supplemented with 1.25 m*M* hydroxyurea (HU) for 18 h. After this period, they were rinsed three times with the spent medium without HU and transferred to fresh medium without HU. After 8 h, excised root tips were transferred to 0.05% colchicine for 90 min to accumulate metaphases. All treatments were performed at 27°C in the dark. After rinsing with distilled water, the root tips were fixed in 3 : 1 (v/v) 100% ethanol: acetic acid and stored in a freezer (−20°C) until used.

Preparation of metaphase spreads

The fixative was washed away with distilled water, and the digestion of meristems was carried out using an enzyme mixture (0.25% pectolyase Y-23, 3% cellulase onozuka R-10, 0.4 *M* sorbitol, pH 5.5) at 27°C. After 90 min of maceration, the root tips were transferred to fresh cold 45% acetic acid for 15 min. Single root tips were removed with a 20-µl pipette and placed onto clean slides before gentle squashing in one drop of 45% acetic acid. The slides were frozen in liquid nitrogen, the coverslips subsequently removed, then the preparations were dehydrated for 10 min each in a graded series of 70%, 95% and absolute ethanol, at room temperature. The air-dried slides were stored in sealed containers until use (up to 1 month).

RAPD analysis

DNA was prepared from young growing leaves according to Bernatzky and Tanksley (1986) except that the celsium chloride purification step was omitted. DNAs from five *Petunia* species (*P. linearis*, *P. integrifolia*, *P. inflata*, *P. axillaris* and *P. parodii*) and four *P. hybrida* lines (ST40, TB1-3, TLV1 and TLH7) were amplified using seven decamer primers. The amplification procedure followed exactly that of Peltier et al. (1994). Briefly, the 25-µl reaction mixture

Table 1 List of the main traits and origins of the plant material

Species	Code	2n	Corolla color	Origin[a]
P. parviflora		18	Purple	USA Michigan
P. linearis	S11	18	Violet pink	IGA S11
P. integrifolia	S13	14	Violet pink	Dijon S13
P. inflata	D1	14	Purple	Dijon, D1
P. axillaris	M1	14	White	USA Cornell
P. parodii		14	White	USA Michigan
P. hybrida	Lines	14		
	ST40		Clear pink	R40 (IGA)
	TB1-3		Purple	Velours rouges
	TLH7		Violet-white	Rose du ciel × *P. axillaris*
	TLV1		Clear violet	T1 × g
F_1 hybrid	code	14		
	D1 × M1		Clear violet	*P. inflata* × *P. axillaris*

[a] USA Michigan, Sink KC (East Lansing, Mich., USA); USA Cornell, Hanson M (Cornell University); IGA, Institute of Genetics (Amsterdam, The Netherlands); Dijon, SGAP, INRA (Dijon, France)

contained 40 ng of template DNA, 67 m*M* TRIS-HCl pH 8.8, 3 m*M* $MgCl_2$, 17 m*M* ammonium sulphate, 500 µ*M* of each dNTP, 30 ng of a single 10-mer oligonucleotide primer (Operon Technologies) and 1.5 units of *Taq* polymerase (BRL). Each reaction mixture was overlaid with a drop of paraffin oil to prevent evaporation. Samples for amplification were subjected to 30 repeats of the following thermal cycles: 1 min at 92°C, 1 min at 38°C and 1.5 min at 74°C on a Biometra® tribloc thermocycler. Fragments generated by amplification were separated according to size on 2.2% agarose gels run in 1 × TAE (40 m*M* TRIS acetate pH 7.8, 1 m*M* EDTA), for 4 h at 5 V/cm, stained with ethidium bromide and visualized by trans-illumination with UV light (312 nm). The reproducibility of each amplification was controlled by comparing the amplification patterns with results obtained by Peltier et al. (1994).

Cloning and labeling of RAPD markers

The chosen RAPD markers, amplified on *P. hybrida* line ST40 and used to construct the *P. hybrida* genetic map, were excised from low-melting-point agarose gels. They were purified by gene clean (Bio 101, Inc.) and cloned into pT7 Blue T-Vector (Novagen) following the manufacturer's instructions. For the restriction fragment length polymorphism (RFLP) analysis, the cloned RAPD markers were amplified from recombinant plasmids using the T7 and LV12 primers (Novagen) and labeled with [^{32}P]-dCTP ($1{,}11.10^{14}$ Bq/m.mole) using the random primed DNA labeling kit (Boehringer). For the in situ hybridization analysis, the same RAPD markers were labeled with digoxigenin-11-dUTP by polymerase chain reaction (PCR) amplification from the corresponding recombinant plasmids using the 10-mer oligonucleotide primer that generated each corresponding RAPD marker.

Southern analysis

Five micrograms of DNA per individual was digested with 20 U *Eco*RI restriction enzyme according to the manufacturer's instructions (Boehringer). DNA fragments were separated on 0.8% agarose gel in TAE buffer (Sambrook et al. 1989) and Southern-blotted on Biohylon Z + membranes according the manufacturer's instructions (Bioprobe). [^{32}P]-labeled RAPD markers were hybridized to the membranes in hybridization buffer (6 × SSC, 5 × Denhardt's, 0.1% SDS) at 65°C. After overnight hybridization, the filters were washed for 2 × 15 min at 65°C in 2 × SSC plus 0.1% SDS and then 2 × 15 min at 65°C in 0.2 × SSC plus 0.1% SDS. Autoradiograms were obtained after a 12- to 96-h exposure at −70°C.

In situ hybridization

The in situ hybridization reaction was modified from Benabdelmouna and Darmency (1997). The hybridization mixture was prepared to a final concentration of 10 ng/µl digoxigenin-labeled RAPD marker, 2 × SSC, 600 ng/µl of autoclaved herring sperm DNA, 10% dextran sulfate, 0.1% SDS, 1 m*M* EDTA and 50% deionized formamide. Slides were incubated with 100 µl of RNase A (100 µg/ml 2 × SSC) for 60 min, at 37°C, under a plastic coverslip in a humid chamber. After 3 × 5 min washes in 2 × SSC and 5 min in protease K buffer (20 m*M* TRIS-HCl, pH 7.5, 2 m*M* $CaCl_2$) at 37°C, slides were incubated with 100 µl of protease K solution (1 µg/ml) for 10 min at 37°C and 5 min in protease K stop-buffer (20 m*M* TRIS-HCl, pH 7.5, 2 m*M* $CaCl_2$, 50 m*M* $MgCl_2$). Chromosome preparations were then fixed in ethanol/acetic acid (3/1, v/v for 10 min at room temperature), rinsed 3 × 5 min in 2 × SSC, dehydrated by submerging for 3 min in 70%, 95% and 100% (v/v) ethanol and air-dried. The hybridization mixture was denatured at 70°C for 10 min and immediately quenched in ice for 5 min, then 20 µl was applied to each slide. A plastic coverslip was applied on the preparation and sealed with a rubber solution. The preparation and the mixture were denatured in a humid chamber at 80°C for 10 min and incubated overnight at 37°C. After hybridization, the rubber solution was pealed off, and the coverslips were removed by dipping the slides into 2 × SSC at 37°C. The slides were then washed twice in 50% formamide, 2 × SSC for 10 min each at 42°C, twice in 2 × SSC for 10 min each at 37°C, twice in 0.1 × SSC for 10 min each at 37°C, and in 2 × SSC for 10 min at room temperature. The sites of probe hybridization were detected by a high-sensibility detection system using a three-step immunodetection procedure. The slides were first washed in the detection buffer (PBS, 0.2% Tween 20) for 5 min at room temperature and then blocked with 200 µl 5% BSA (Bovine Serum Albumin Fraction V, Sigma) in detection buffer for 45 min at 37°C. The blocking solution was drained off and the slides incubated with 0.5 µg/ml monoclonal anti-Dig raised in mouse (Boehringer Mannheim) for 45 min, at 37°C, in a moist chamber. After 3 × 5 min washes at room temperature, the slides were incubated with 2 µg/ml Dig-conjugated anti-mouse antibody raised in sheep (Boehringer Mannheim) for 45 min at 37°C. The final washes were performed with 4 × SSC for 2 × 3 min at room temperature in the dark. Chromosomes were counter-stained with 5 µg/ml propidium iodide in 2 × SSC for 10 min at room temperature followed by washing in 2 × SSC. After the slides had been drained off, they were mounted in an antifade solution (90% glycerol in PBS, 1 mg/ml *p*-phenylene diamine), covered by a 24 × 34 mm coverslip and stored at 4°C for 1 day to stabilize the fluorescence. For visualization, slides were analyzed using a Leitz epifluorescent microscope with appropriate filter (I3: excitation 488 nm, emission 520 nm). Photographs were taken on Fujicolor 400 color slide film and on Fujicolor 400 print film. We mainly used a confocal microscope with appropriate filters. The results presented here were obtained from ten metaphases. Chromosome identification was according to the standard *Petunia hybrida* karyogram (Maizonnier 1971).

Results

PCR amplification, reproducibility and specificity of the RAPD markers

The patterns of RAPD amplification obtained in the *P. hybrida* lines and the wild species studied corresponded to those obtained by *Peltier* et al. (1994). Eleven of the mapped fragments were selected, those that belonged to the linkage map of *P. hybrida* ST40 line, including six linkage groups assigned to the corresponding chromosomes and one undetermined linkage group, as follows: V07-550 and J18-700 on chromosome I, V20-350 on chromosome II, J14-850 on chromosome III, V08-600 and J18-550 on chromosome V, J18-250 and U03-1100 on chromosome VI, H17-1100 and U03-550 on chromosome VII, and V07-600 on the non attributed linkage group. We examined the presence of the ST40-mapped RAPDs, in three *P. hybrida* lines and in the wild parental species according to both size and homology. The homology was confirmed by membrane hybridization using the mapped amplified fragments in line ST40 as a probe. Seven of the chosen RAPD markers were specifically amplified in *P. integrifolia* and/or *P. inflata*, representatives of the colored-flower group; while 2 markers, OPV20-350 and OPJ18-550, were specifically amplified in *P. axillaris* and *P. parodii*, which are representative of the

white-flowered group. The amplification origin of the remaining 2 markers, OPH17-1100, and OPV07-550 was not species-specific.

Genomic organization of the RAPD clones studied by Southern analysis

The selected RAPD markers were cloned and used as probes for Southern transfer of hydrolyzed genomic DNA in order to determine their level of sequence repetition. The *Eco*RI-digested DNAs from five wild species (*P. linearis*, *P. integrifolia*, *P. axillaris* and *P. inflata*), four *P. hybrida* lines (ST40, TB1-3, TLV1 and TLH7) and one F_1 hybrid (*P. inflata* × *P. axillaris*) were hybridized with the different clones. As shown in Fig. 1, three types of hybridization profiles were obtained: one to three major bands, multiple band patterns and smeared patterns.

The OPJ18-250 probe revealed a unique 4-kb *Eco*RI fragment observed as a strong signal in *P. axillaris* and *P. hybrida* line ST40 and a very faint signal in *P. integrifolia* and *P. hybrida* TB1-3. In *P. inflata* and the *P. hybrida* lines TLH7 and TLV1, a smeared pattern was observed in the high-molecular-weight region. However, no signal was detected in *P. linearis* (Fig. 1a).

The OPV08-600 probe revealed a multiple-band hybridization pattern in all the *Eco*RI-digested DNAs. In *P. axillaris* and *P. hybrida* TLH7, both with white flowers, several bands were absent that were present in the coloured-flower species (Fig. 1b).

The OPV20-350 probe revealed two to three strong bands only in *P. axillaris*. It produced a smeared hybridization pattern on *P. integrifolia*, *P. inflata* and the four *P. hybrida* lines, while no hybridization pattern was revealed on *P. linearis* (Fig. 1c).

Out of the 11 RAPD fragments used, 7 (namely OPJ14-850, OPU03-1100, OPV07-600, OPJ18-700, OPV07-550 and OPU03-550) produced a smeared and/or a multiple-band pattern on the different *Petunia* species tested (Fig. 1d, e). The last RAPD marker used, OPH17-1100, did not produce any hybridization pattern (not shown).

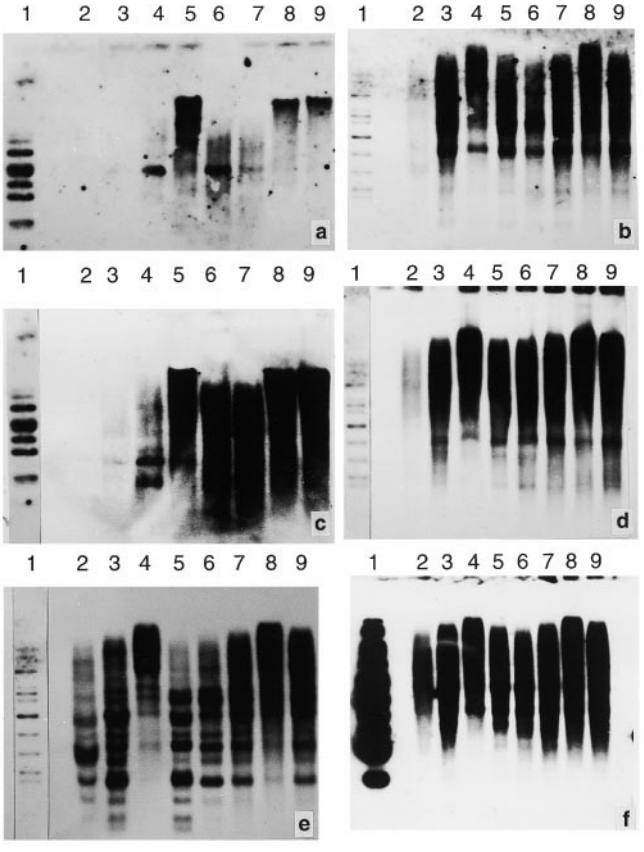

Fig. 1a–f Southern transfers of *Eco*RI-digested DNAs from different *Petunia* wild species and lines of *P. hybrida* probed with the following RAPD clones. **a** OPJ18-250, **b** OPV08-600, **c** OPV20-350, **d** OPJ14-850, **e** OPU03-1100, **f** OPV07-600. *Lane 1* 1-kb DNA ladder (BRL), *lane 2 P. linearis*, *lane 3 P. integrifolia*, *lane 4 P. axillaris*, *lane 5 P. inflata*, *lane 6 P. hybrida* ST40, *lane 7 P. hybrida* TB1-3, *lane 8 P. hybrida* TLH7, *lane 9 P. hybrida* TLV1

Intrachromosomal organization of the RAPD clones studied by FISH analysis

The intrachromosomal localization of the chosen RAPD markers was determined by fluorescent in situ hybridization (FISH) using the same probes as in Southern analysis. The probes were digoxigenin-labeled and hybridized with chromosome preparations from the different *Petunia* wild species and *P. hybrida* lines. Figure 2 shows the FISH patterns obtained.

In *P. axillaris*, *P. parodii* and *P. hybrida* ST40, the OPJ18-250 probe produced a specific 4-kb band in Southern analysis and revealed two FISH signals in the same species. The two fluorescent sites were localized on a chromosome pair with a submedian centromere, presumably corresponding to chromosome VI (Fig. 2a). No visible signals were obtained in *P. integrifolia* and *P. inflata*. As expected, when hybridized to the F_1 hybrid (*P. inflata* × *P. axillaris*) metaphase chromosomes, the same probe revealed only one signal carried by one chromosome of the same submedian centromeric chromosome pair (VI) (Fig. 2b).

The OPV08-600 probe, which produced a polymorphic banded pattern between the colored- and the white-flowered *Petunia* species in Southern analysis, also revealed species-specific FISH signals. Four pericentromeric hybridization signals on two chromosome pairs (III and V) in *P. integrifolia*, *P. inflata* and the *P. hybrida* lines ST40, TB1-3 and TLV1 were revealed (Fig. 2c), while no signals were produced on *P. axillaris* and the *P. hybrida* line TLH7.

The OPV20-350 probe, which showed three intensive bands in *P. axillaris* by Southern analysis, revealed six FISH signals only on the *P. axillaris* and *P. parodii*

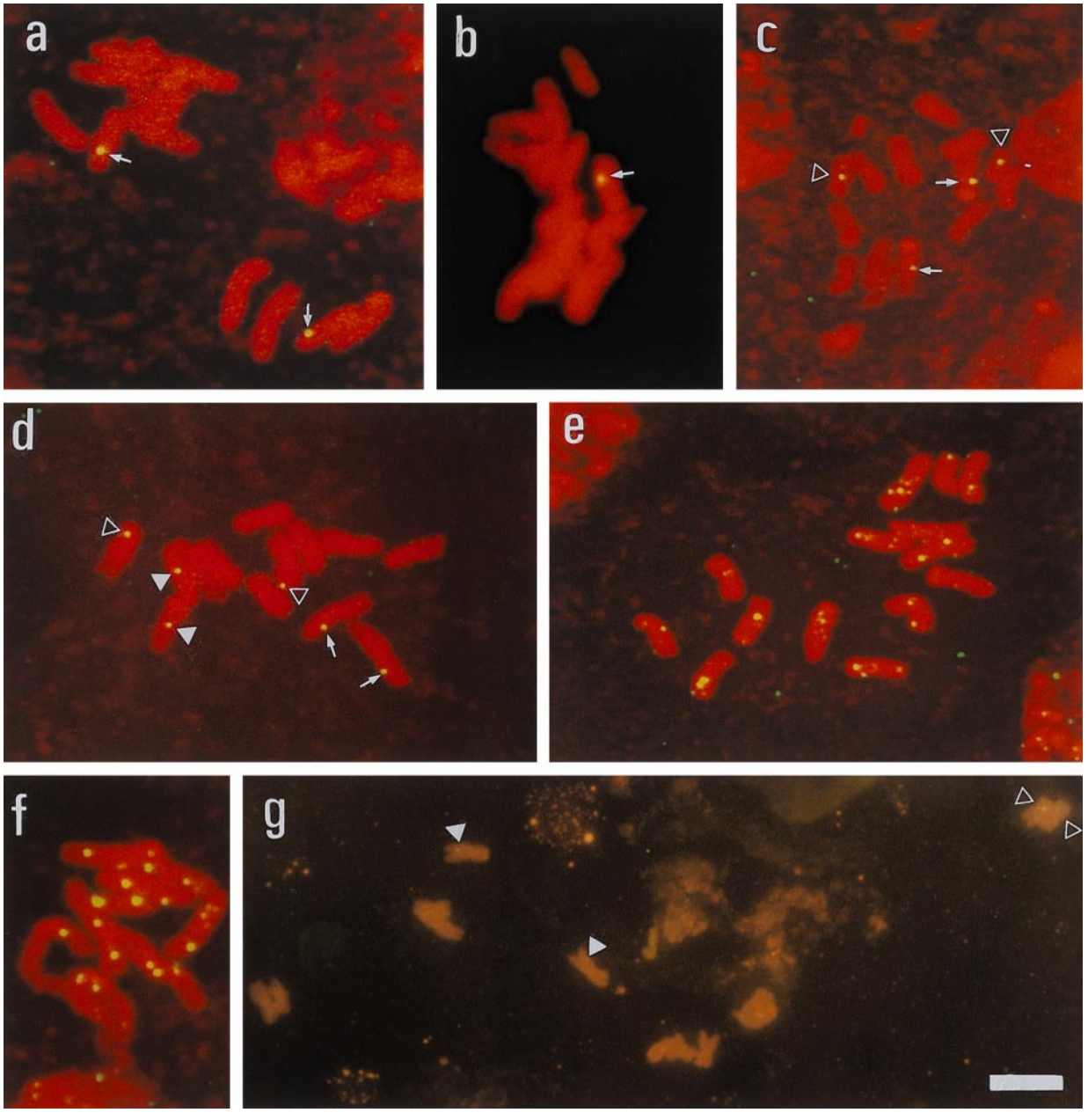

Fig. 2a–g Fluorescent in situ hybridization to somatic metaphases of different 2n = 14 *Petunia* genotypes. The chromosomes were counter stained with the red fluorochrome propidium iodide, and the yellow spots correspond to the hybridization sites of the different digoxigenin-labeled RAPD clones. These are indicated, for the different chromosomes, as *pairs of arrows*, *solid* and *open arrowheads*. Bar: 5 μm in **c**, **d**, **e**, **g**, and 4.5 μm in **a**, **b**, **f**. **a** *P. axillaris* metaphase probed with OPJ18-250, *solid arrows* indicate a pair of signals in the middle of the long arm of chromosome pair VI. **b** *P. inflata* × *P. axillaris* metaphase chromosome probed with OPJ18-250, *solid arrow* indicates a single signal in the middle of the long arm of chromosome VI. **c** *P. integrifolia* metaphase probed with OPV08-600; *solid arrowheads* indicate a pair of pericentromeric signals on the chromosome pair III, while *open arrowheads* indicate a pair of pericentromeric signals on the chromosome pair V. **d** *P. axillaris* metaphase probed with OPV20-350; *solid arrows* indicate a pair of signals located on the greatest metacentric chromosome pair I, *open arrowheads* indicate a pair of signals in a subterminal position carried by a chromosome pair with a median to submedian centromere (IV/VII) and *solid arrowheads* indicate a pair of signals in a subcentromeric position on the long arm belonging to the NOR-associated chromosome pair II. **e** and **f** *P. integrifolia* metaphases probed with OPV07-600 and OPJ14-850, respectively. **g** *P. hybrida* ST40 metaphase probed with OPV20-350; *open arrowheads* indicate a pair of signals in a subterminal position carried by a chromosome pair with a median to submedian centromere (IV/VII) and *solid arrowheads* indicate a pair of signals in a subcentromeric position on the long arm belonging to the NOR-associated chromosome pair II

chromosomes. These six sites were located on the greatest metacentric chromosome pair (I), in a subterminal position carried by a chromosome pair with a median to submedian centromere (IV/VII) and in a subcentromeric position on the long arm belonging to a NOR-associated chromosome pair (II) (Fig. 2d), respectively. This probe, when hybridized to *P. hybrida* line ST40 karyotypes, showed only four signals detected on two different chromosome pairs, identified as chromosomes II and IV/VII (Fig. 2g). Their localization was identical to that found in the parental species *P. axillaris* and *P. parodii* for the corresponding chromosomes.

Two clones (OPJ14-850 and OPV07-600) produced multiple hybridization signals on each of the 14 chromosomes of the different *Petunia* species and *P. hybrida* lines (Fig. 2e, f, respectively). Five other clones (OPJ18-550, OPJ18-700, OPU03-550, OPU03-1100 and OPV07-550) also revealed FISH patterns with multiple spots. Interestingly, these 7 RAPD clones all produced a smeared and/or multiple-band pattern in the Southern analysis. The last RAPD probe used (OPH17-1100), as in the Southern hybridization analysis, produced no detectable in situ hybridization signals (not shown).

Discussion

In this study, fluorescent in situ hybridization elucidated the intrachromosomal organization of 11 RAPD markers genetically mapped in *P. hybrida* ST40 line (Peltier et al. 1994). The RAPD patterns obtained were highly reproducible, although numerous studies have reported the altering effects of different amplification parameters (MacPherson et al. 1993; Jones et al. 1997). The amplification of the RAPD markers studied was revealed to be species-specific. However, as evidenced by Southern analysis, when the RAPD clones were used as probes, the most amplified sequences shared a high level of homology to all of the *Petunia* species studied. This data enabled us to consider that the amplification polymorphism observed between the different species was mainly due to polymorphism at the primer sites as the amplified sequences were present in all the studied genotypes. Our results demonstrate that the modification of the RAPD profiles results more from a mutation in the primer sites than from a deletion in the amplified region.

In Southern analysis, the 2 RAPD clones OPJ18-250 and OPV20-350 revealed differences in the organization and the level of the sequence repetition between species. They produced strong bands (1–3) in *P. axillaris* but a smeared pattern in the other *Petunia* species. They were not detected in *P. linearis* ($2n = 18$) and *P. integrifolia* ($2n = 14$), probably because their copy number was greatly reduced. The Southern hybridization pattern proved to be useful for the prediction of the FISH pattern. For all of the wild species and *P. hybrida* genotypes, when a RAPD clone revealed one, two or three major Southern bands, their number was correlated with the number of the observed FISH signals. The smeared and/or the multiple-band pattern, corresponded to multiple FISH sites, indicating that the amplified repeated sequence was dispersed throughout the genome and present on every chromosome. This dispersed physical localization suggested that the RAPD clones studied belong to a multilocus family. Their genetic mapping at a unique location on a particular chromosome would concern only one member of this multilocus family, while in situ hybridization revealed all or at least the majority of the other loci. Similar results have been found in wheat chromosomes for homoeologous group 7 when RFLP markers were used as in situ hybridization probes (Chen and Gustafson 1995).

A multilocus in situ localization was obtained for all the RAPD markers used with the exception of OPJ18-250. This amplified sequence, which was probably tandemly repeated, was located by FISH at a unique site about the middle of the long arm of chromosome VI in *P. axillaris* and *P. hybrida* ST40. It has been previously mapped to linkage group VI at 5 cM from the color gene *An2*, which has a terminal position on the short arm (Peltier et al. 1994; Maizonnier and Moessner 1979). The low recombination level within the segment *An2*-OPJ18-250 including the centromere, as shown by FISH, can be a consequence of an inhibition process. In addition, this constitutes a good illustration of the discrepancy between genetic and physical distance on chromosome VI. Such suppression of recombination has been reported for another segment, *An2-RT*, in chromosome VI (Robert et al. 1991), and in chromosome II by FISH localization of T-DNA insertions (Ten Hoopen et al. 1996). Evidence for low recombination rates came also from RAPD analysis in the wild parental species and in *P. hybrida* where RAPD markers were clustered in blocks without any rearrangement (Peltier et al. 1994). The regulation of recombination is probably complex in most higher plants, like *Petunia*, and this generate difficulties in genetic mapping due to bias in the segregation of markers, consequently also justifying the benefit of physical mapping by FISH.

In our study, it appeared that obtaining a FISH signal was affected by the nature and the level of the sequence repetition in the genome. We know that the size of the target sequence is a limiting factor in the optimal detection of the hybridization sites (Jiang and Gill 1996). The sequences used as probes were repeated and have a size between 250 and 1100 bp. In the FISH analysis, they were observed as single spots instead of both chromatids being detected as two spots. Such labeling of one chromatid only has already been

reported in plant as well as in animal FISH studies (Clark et al. 1989; Gustafson and Dillé 1990). The probes showing FISH signals on both chromatids were generally highly repeated and longer than 7 kb (Gustafson and Dillé 1992).

The in situ hybridization patterns revealed species specificity for 3 RAPD clones, OPJ18-250 and OPV20-350 being detected only in the white-flower species and OPV08-600 in the colored-flower species. The OPJ18-250 clone, which revealed sequence homology by Southern analysis in most of the genotypes studied, showed a FISH signal on chromosome VI only in *P. axillaris* and *P. parodii*. Moreover, only 1 of the 2 homologous chromosomes VI was labeled in the F_1 hybrid (*P. inflata* × *P. axillaris*), while the 2 chromosomes VI were labeled in *P. hybrida* line ST40, indicating their origin from the white-flower species. Therefore, we assumed that this RAPD clone can be considered to be a chromosome marker for following the introgression of *P. axillaris* and/or *P. parodii* chromosome VI-mediated characters. This conclusion is also supported by genetic data showing that, on chromosome VI, RAPD markers appeared to segregate as blocs without further rearrangements between blocs inherited from the two groups (Peltier et al. 1994).

The present study showed the potentialities of RAPD markers in physically labeling chromosomes. The FISH technique that we used was sensitive and reproducible, and it revealed additional information about the physical localization as compared to genetic methods. The RAPD markers were efficient in physically discriminating between the two closely related groups constituting the genus *Petunia*. The rapidity and low cost of such markers offer new possibilities for genome labeling and chromosome tagging and identification. Their possible conversion into sequence characterized amplified regions (SCARs, Parran and Michelmore 1992) may probably increase their discrimination potential by reducing the number of in situ hybridization sites corresponding to common sequences shared by closely related species.

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