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Restriction Endonuclease Analysis of Chloroplast DNA in Interspecies Somatic *Hybrids* of *Petunia*

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Summary. Restriction endonuclease cleavage pattern analysis of chloroplast DNA (cpDNA) of three different interspecific somatic hybrid plants revealed that the cytoplasms of the hybrids contained only cpDNA of *P. parodii.* The somatic hybrid plants analysed were those between *P. parodii* (wild type) + *P. hybrida* (wild type); *P. parodii* (wild type) + *P. inflata* (cytoplasmic albino mutant); *P. parodii* (wild type) + *P. parviflora* (nuclear albino mutant). The presence of only *P. parodii* chloroplasts in the somatic hybrid of *P. parodii* + *P. inflata* is possibly due to the stringent selection used for somatic hybrid production. However, in the case of the two other somatic hybrids *P. parodii* + *P. hybrida* and *P. parodii* + *P. parviflora* it was not possible to determine whether the presence of only *P. parodii* chloroplasts in these somatic hybrid plants was due to the nature of the selection schemes used or simply occurred by chance. The relevance of such somatic hybrid material for the study of genomic-cytoplasmic interaction is discussed, as well as the use of restriction endonuclease fragment patterns for the analysis of taxonomic and evolutionary inter-relationships in the genus *Petunia.*

Key words: Somatic hybrid – Petunia – Chloroplast DNA – Plastid segregation – Restriction endonucleases

Introduction

Somatic hybridization by induced protoplast fusion, with subsequent selection of viable heterokaryons capable of plant regeneration, provides a means of obtaining heterozygosity of extra-chromosomal genes (Gleba 1978; Maliga 1980, Cocking 1981).

With the increasing availability of biochemical and physical markers on DNA it has been possible to analyse in recent years the inheritance pattern of cytoplasmic organelles in greater detail (Kung 1976; Gatenby et al. 1980; Vedel et al. 1976; Quétier et al. 1977; Wettstein et al. 1978; Gillham 1978). Characterization of chloroplast organelles in somatic hybrids suggests that in most cases so far analysed there is a rapid, apparently random, sorting out until homogeneity for one or the other type of parental chloroplasts is reached (Chen et al. 1977; Melchers et al. 1978; Belliard et al. 1978; Aviv et al. 1980; Iwai et al. 1980; Poulsen et al. 1980; Scowcroft and Larkin 1981; Douglas et al. 1981). By contrast there are also other reports where the chloroplast sorting out is always unidirectional to one parental type (Kung et al. 1975; Evans et al. 1980; Maliga et al. 1980; Kumar et al. 1981). Hence, these results indicate that chloroplast segregation is to homogeneity for each hybrid plant but the entire hybrid population produced can contain individuals homogenous for either parental type or homogenous, entirely for one parental chloroplast type. This sorting out process of the parental chloroplasts in the somatic hybrid plant population is likely to be greatly influenced by many factors encountered during protoplast fusion and subsequent selection and regeneration of somatic hybrid cells.

We report here the fate of chloroplast DNA (cpDNA) in three different somatic hybrid combinations; the somatic hybrid between *Petunia parodii* and *P. hybrida, P. parodii* and *P. inflata* and *P. parodii* and *P. parviflora.* These somatic hybrids were produced using a stringent selection (see Materials and Methods) and combine sexually compatible, unidirectional sexually compatible and sexually incompatible species of *Petunia* respectively. These three different somatic hybrid combinations have previously been characterized for their hybrid nature on the basis of their growth habit, morphology, floral pigmentation and chromosome number. Additionally, the hybrid of *P. parodii* and *P. hybrida,* and *P. parodii* and *P. parviflora,* has been analysed for isoenzyme and Fraction 1 protein profiles respectively (Power et al. 1976; Kumar et al. 1981). However, it was not possible to determine

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the amount of chloroplast heterozygosity from the Fraction 1 protein profiles because the large subunit polypeptides (coded by chloroplast DNA) of the Fraction 1 protein are indistinguishable by isoelectric focussing in these four *Petunia* species (Gatenby and Cocking 1976). Restriction endonuclease cleavage patterns of chloroplast DNA were therefore analysed in order to characterize the composition of the chloroplast genomes in these three types of somatic hybrids.

Materials and Methods

Production of Somatic Hybrids

i) Somatic hybrid plants were regenerated following fusion between leaf mesophyll protoplasts of *P. parodii* and *P. hybrida.* The selection scheme involved a differential growth response to actinomycin D, coupled with an inability of wild-type protoplasts of *P. parodii* to develop beyond the small colony stage in certain media (Power et al. 1976).

ii) Somatic hybrid plants were regenerated following the fusion of leaf mesophyll protoplasts of *P. parodii* with cell suspension protoplasts of a cytoplasmic albino mutant of *P. inflata* (Power et al. 1979). 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 the cytoplasmic albino *P. inflata,* produced calluses. Green somatic hybrid calluses were visible against a background of albino cells/callus and could therefore be picked up and transferred to shoot regeneration media.

iii) A similar selection scheme has been used to produce somatic hybrid plants using leaf mesophyll protoplasts of *P. parodii* and cell suspension culture protoplasts of a nuclear albino mutant of *P. parviflora* (Power et al. 1980).

Sufficient cloned plant material for chloroplast DNA analyis was produced by vegetative propagation from the many somatic hybrid plants regenerated from a single hybrid callus produced from each of the three fusion experiments (i) (ii) (iii). Plants were grown in the greenhouse at 27 °C with daylight supplemented with mercury vapour lamps (10,000 lux). Plants of the four parental species, *P. hybrida, P. parodii, P. inflata, P. parviflora* were grown from seeds (produced by self-fertilization of inbred lines).

Preparation of Chloroplast DNA

Chloroplast DNA was prepared according to the method described by Bovenberg et al. (1981) with some modifications: about 40 g of young, fully expanded leaves were washed in ice-cold water containing 20 mM KCl and homogenized in HM-1 buffer (0.05 M Tris-HCl pH 8.0, 0.35 M sucrose, 7 mM EDTA and 5 mM 2-mercaptoethanol) by using a Braun blender. The homogenate was filtered through nylon gauze (30 μm). Chloroplasts were isolated from the filtrate by centrifugation at 1,500×g. Chloroplasts were lysed in 2% sarkosyl and cpDNA was purified from the lysate by two different procedures: i) The lysate was made 5 M to cesium chloride and subjected to CsCl-EtBr equilibrium density gradient centrifugation. Chloroplast DNA was recovered from the gradients, dialyzed and concentrated as described by Bovenberg et al. (1981); ii) For the rapid screening of cpDNA genotypes, cpDNA was prepared by a second, simpler method. This method involved the isolation of cpDNA from the lysate by two phenol and two phenol-chloroform (1:1) extractions. Nucleic acid was precipitated from the resulting water layer using cold ethanol and then pelleted by centrifugation and dissolved by soaking in 5 mM Tris-HCl pH 8.0 and 0.5 mM EDTA.

Digestion of cpDNA with Restriction Endonucleases and Agarose Gel Electrophoresis

Chloroplast DNA was digested with restriction endonucleases (Boehringer, Mannheim) and subjected to electrophoresis using horizontal slab gels (25×20×0.4 cm) containing 0.5–1.5% agarose. Molecular weights of cpDNA restriction fragments were estimated using Hind III fragments of λ phage DNA (Bovenberg et al. 1981).

Densitometric Tracing

Polaroid photographic negatives (Polaroid 4×5 land film type 55) of ethidium bromide stained agarose gels were scanned with a Kipp densitometer, type DD2, equipped with a Kipp BD8 multirange recorder.

Results

From cesium chloride-ethidium bromide gradient centrifugation of the chloroplast lysate, two fractions of cpDNA were obtained: the lower band in the gradient contained pure covalently closed circular cpDNA and the upper band contained open circular and linear cpDNA. This upper band usually contains a small amount of nuclear DNA (nDNA). The presence of this nuclear DNA, however, does not interfere with the restriction endonuclease analysis of cpDNA. DNA prepared by the second, simplified method is contaminated with some nDNA. Upon restriction enzyme analysis of these preparations of cpDNA, the presence of nDNA results in a slight smear when the digested DNA is analyzed by electrophoresis on agarose gels. In most cases, however, the presence of nDNA in these preparations does not inferfere with the analysis of cpDNA with restriction endonucleases. Therefore, this second method was used to obtain cpDNA for the rapid screening of the parental cpDNA genotypes with various restriction endonucleases. The restriction endonuclease cleavage patterns of cpDNA isolated by CsCl-ethidium bromide density gradient centrifugation did not differ from those obtained from the phenol extracted DNA.

Chloroplast DNA isolated from the parental plants was analyzed with the restriction endonucleases: Sal I, Bam HI, Eco RI, Hpa I, Bgl I, Xho I, Pst I, Xba I, Sac I, Hind III, Hpa II, Kpn I, and Sau 3A to determine which enzymes were suitable to distinguish the parental cpDNA species from each other. Upon digestion with

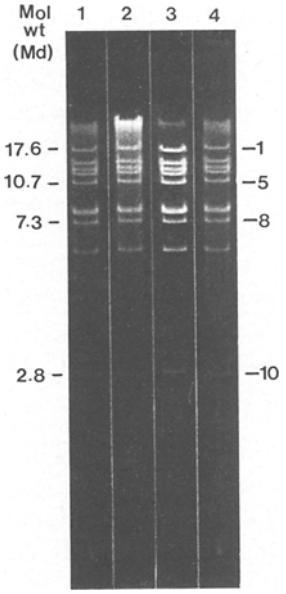

Fig. 1. Cleavage pattern of chloroplast DNA from parental plants with restriction endonuclease Sal I: lane 1, *P. inflata;* lane 2, *P. parviflora;* lane 3, *P. parodii;* lane 4, *P. hybrida;* on the right hand side of the figure the DNA restriction fragments are numbered according to Bovenberg et al. 1981

Sal I (Fig. 1) the cleavage patterns of the cpDNAs from parental and hybrid plants appeared to be similar, indicating that the molecular weight of the cpDNAs were the same and that major differences among the cpDNAs of the parental plants do not exist. With most of the other enzymes mentioned above, the cleavage patterns of cpDNA from *P. parviflora* and from *P. parodii* differed. Differences distinguishing the cpDNAs of *P. parodii, P. hybrida* and *P. inflata* could only be detected with the enzymes Bam HI and Hpa II. These results are presented in the next sections.

Restriction Endonuclease Analysis of cpDNA from P. parodii, P. parviflora and Their Somatic Hybrids

The cleavage patterns of cpDNA from *P. parodii* and *P. parviflora* with the endonucleases Hpa I, Eco RI (not shown), Bgl I (Fig. 2A), Bam HI (Fig. 2B) and Hpa II (Fig. 4) were species specific. Using Bgl I endonuclease, the cleavage pattern of cpDNA from *P. parviflora* contained one high molecular weight fragment of 25 Md which was absent from the *P. parodii* pattern. The *P. parodii* pattern, however, contained three other fragments: B3 (14.4 Md), B7 (5.3 Md) and B8 (5.3 Md) that were missing from the *P. parviflora* pattern. Using Bam HI endonuclease, the cpDNA pattern of *P. parviflora* contained fragments of molecular weights 13.5 Md, 6.4 Md and 2.7 Md. These fragments were missing from the *P. parodii* pattern but instead at least four other fragments with molecular weights 8.7 Md, 4.9 Md, 4.1 Md and 4.0 Md were present. With Hpa II endonuclease, *P. parviflora* contained two fragments of molecular weights 3.7 Md and 1.2 Md that were not present in the *P. parodii* pattern. The *P. parodii* pattern, however, contained two fragments of molecular weights 1.7 and 1.3 Md that were missing in the *P. parviflora* pattern. Digestion of cpDNA from somatic hybrid plant material sampled from approximately 10 plants of an original somatic hybrid population, maintained by vegetative propagation (see Materials and Methods) resulted in only the *P. parodii* type of cleavage pattern, indicating that in the hybrid plant, only *P. parodii* chloroplasts are present.

Restriction Endonuclease Analysis of cpDNA from P. parodii, P. hybrida, P. inflata and their Somatic Hybrids

Among the cleavage patterns of cpDNA from *P. parodii, P. hybrida* or *P. inflata* with restriction endonucleases Sal I (Fig. 1), Bgl I (Fig. 2A), Eco RI, Hpa I, Xho I,

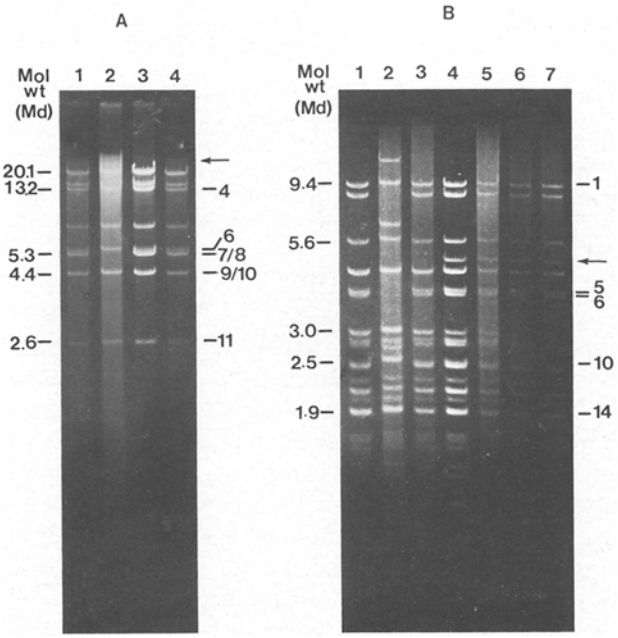

Fig. 2A and B. Cleavage pattern of chloroplast DNA from parental plants and somatic hybrid plants with Bgl I and Bam HI restriction endonucleases. **A** Bgl I cleavage pattern: lane 1, *P. parviflora* + *P. parodii* hybrid; lane 2, *P. parviflora;* lane 3, *P. parodii;* lane 4, *P. hybrida;* the arrow indicates the additional Bgl I fragment of mol. wt. 25 Md in the cpDNA of *P. parviflora* (lane 2). **B** Bam HI cleavage pattern: lane 1, *P. inflata;* lane 2, *P. parviflora;* lane 3, *P. hybrida;* lane 4, *P. parodii;* lane 5, *P. inflata* + *P. parodii* hybrid; lane 6, *P. parviflora* + *P. parodii* hybrid; lane 7, *P. hybrida* + *P. parodii* hybrid; the arrow indicates the additional Bam HI fragment in the cpDNA of *P. parodii* (lane 4)

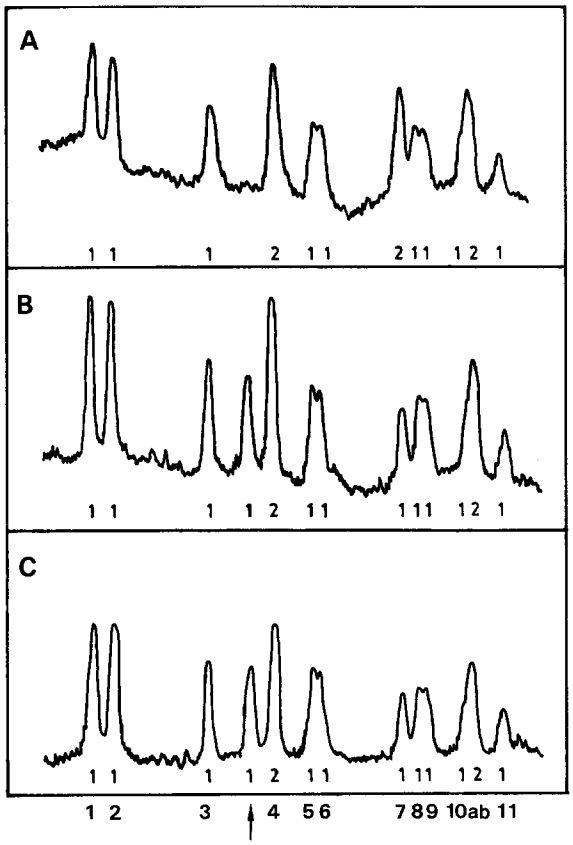

Fig. 3A–C. Densitometric tracing of the restriction endonuclease Bam HI cleavage pattern of cpDNA from: **A** *P. hybrida;* **B** *P. parodii;* **C** *P. hybrida* + *P. parodii* hybrid; the additional Bam HI fragment in the cpDNA of *P. parodii* and the hybrid is indicated by an arrow. The numbers under the various peaks indicate the relative molar ratio of the corresponding Bam HI fragment

Pst I, Xba I, Sac I, Hind III and Sau 3A (not shown) no differences could be detected. However, a difference in the cleavage patterns was observed when the enzyme Bam HI was used: the Bam HI cleavage pattern of cpDNA from *P. parodii* contained one fragment of molecular weight 4.9 Md that was absent in the cleavage pattern of cpDNA from *P. hybrida* or *P. inflata* (Fig. 2B). The cpDNA of the somatic hybrid plant materials sampled from approximately 10 plants of each individual somatic hybrid population also contained this additional Bam HI fragment, indicating the presence of *P. parodii* chloroplasts in these hybrids. The Bam HI cleavage pattern of cpDNA from *P. hybrida* and *P. inflata* contained no other fragments that distinguish these cpDNAs from *P. parodii* cpDNA. Therefore, these results do not exclude the possibility that *P. hybrida* or *P. inflata* chloroplasts are also present in the hybrids, together with the *P. parodii* chloroplasts. To test this possibility densitometric scanning of the Bam HI cleavage patterns of cpDNA from the somatic hybrids and *P. parodii* was performed to determine the molar ratio of the 4.9 Md Bam HI fragment versus the other Bam HI fragments. This ratio appeared to be the same for the Bam HI pattern of *P. parodii* and the hybrids *P. hybrida* + *P. parodii* and *P. inflata* + *P. parodii* (Fig. 3). Therefore, these scannings did not give any indication for the presence of *P. hybrida* or *P. inflata* chloroplasts in the somatic hybrids.

This result was confirmed by analysis of the Hpa II cleavage patterns of cpDNAs from the somatic hybrid and parental plants (Fig. 4). The Hpa II cleavage pattern of *P. parodii, P. hybrida* and P. inflata differed with respect to at least two fragments: a fragment with a molecular weight of about 1.9 Md present in the *P. hybrida* and *P. inflata* patterns was missing in the *P. parodii* pattern (Fig. 4, arrow b) and a fragment with a molecular weight of about 2.1 Md (Fig. 4, arrow a) present in the *P. parodii* pattern, was missing in the *P. hybrida* and *P. inflata* patterns. Digestion of the cpDNA, sampled from approximately 10 plants of each individual somatic hybrid population, yielded the *P. parodii* type cleavage pattern, indicating that in the somatic hybrids only the *P. parodii* chloroplasts are present.

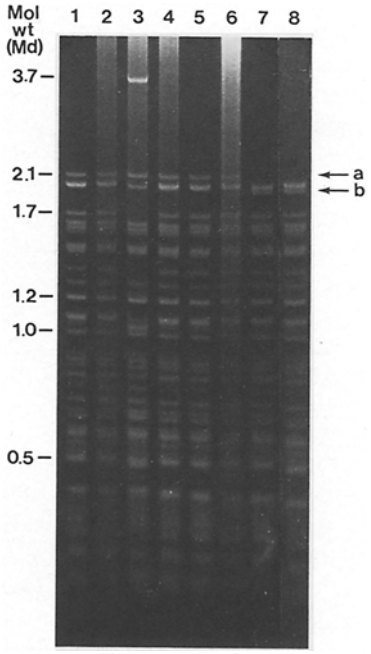

Fig. 4. Cleavage pattern of chloroplast DNA from parental plants and somatic hybrid plants with restriction endonuclease Hpa II: lane 1, *P. parodii;* 2, *P. parviflora* + *P. parodii* hybrid; 3, *P. parviflora;* 4, *P. hybrida* + *P. parodii* hybrid; 5, *P. parodii;* 6, *P. inflata* + *P. parodii* hybrid; 7, *P. inflata;* 8, *P. hybrida;* arrow a: additional Hpa II fragment in the cpDNA cleavage pattern of *P. parodii* and hybrids; arrow b: additional Hpa II fragment in the cpDNA cleavage pattern of *P. hybrida* and *P. inflata*

Discussion

Characterization of the Chloroplasts in the Cytoplasm of the Somatic Hybrid Plants

The three different inter-species somatic hybrid plants, initially characterized for their hybrid nature by nuclear markers and morphological characters (Power et al. 1976, 1979, 1980; Kumar et al. 1981), were shown in the present investigation to contain only *P. parodii* chloroplasts in their cytoplasms on the basis of cpDNA restriction cleavage pattern analysis. The presence of only *P. parodii* chloroplasts in the somatic hybrid between *P. inflata* and *P. parodii* (wild type) is possibly due to the use of a cytoplasmic albino mutant of *P. inflata.* The available evidence for an absence of recombination between cpDNAs (Belliard et al. 1978; Aviv et al. 1980; Scowcroft and Larkin 1981) and for an absence of chloroplasts with two different genotypes co-existing in a common cytoplasm at the plant level (Chen et al. 1977; Iwai et al. 1980; Poulsen et al. 1980; Douglas et al. 1981) suggested that it would be unlikely for albino plastids of *P. inflata* to become genetically corrected or to green due to complementation. This is further supported by our own results where we found only the restriction cleavage pattern of *P. parodii* cpDNA; there was no mixed cpDNA or any change in cpDNA restriction cleavage pattern in the somatic hybrid plants analysed. It is important to note, however, that as the selection was based on a visual selection of green colonies, only colonies containing solely *P. parodii* chloroplasts or both *P. parodii* and *P. inflata* chloroplasts or genetically corrected *P. inflata* chloroplasts could be picked up. As we mentioned above, our results suggest that only the *P. parodii* chloroplasts persist – there is no co-existence of two chloroplasts types and no cpDNA recombination. However, by this method of selection we miss out the chance to detect instances where the callus is a nuclear hybrid but has only *P. inflata* albino mutant chloroplasts. From our available data then we cannot say that this type of hybrid callus and therefore, pattern of chloroplast segregation does not occur.

In the case of the *P. hybrida* (wild type) and *P. parodii* (wild type) somatic hybrid, a possible explanation for the presence of only *P. parodii* chloroplasts could be due to the use of actinomycin D for selection. Actinomycin D is an intercalating compound which inhibits both DNA synthesis and DNA dependent RNA synthesis in nuclei and organelles, and *P. parodii* is known to be less sensitive to actinomycin D than *P. hybrida* (Power et al. 1976).

In the somatic hybridization between *P. parodii* and *P. parviflora,* the *P. parviflora* used for fusion with the wild type *P. parodii* was a nuclear albino mutant and the hybrid was selected as a green colony. Since the hybrid contained only *P. parodii* chloroplasts it remains unknown if *P. parodii* contains a nuclear gene which can substitute for the *P. parviflora* albino gene, but this is likely. The possibility that *P. parviflora* chloroplasts cannot be stably maintained in the hybrid of *P. parviflora* + *P. parodii* cannot be investigated in sexual hybrid plants because *P. parviflora* and *P. parodii* are sexually incompatible.

We have investigated the possibility that *P. parodii* plastids survive in the hybrids by protection of the *P. parodii* cpDNA by specific methylation, as occurs with cpDNA in *Chlamydomonas* during zygote formation (Royer and Sager 1979; Sager et al. 1981). For this purpose we have analysed the degree of methylation of the cpDNAs by using the enzyme Hpa II. This enzyme cuts only at non-methylated sequences. Consequently, if the cpDNA of *P. parodii* is heavily methylated, then the Hpa II will cut the DNA less frequently than non-methylated cpDNA. Results showed (Fig. 4) that the Hpa II cleavage pattern of *P. parodii* cpDNA is almost identical with that of *P. hybrida* and *P. inflata,* and differs only slightly from that of *P. parviflora.* This indicates that major differences in the degree of methylation between *P. parodii* cpDNA and the other *Petunia* cpDNAs, which could protect *P. parodii* cpDNA from being degraded in the somatic hybrids, are not present. Since these experiments were performed at the full grown plant level, we cannot exclude the possibility that methylation occurred only during, or just after, hybrid cell formation, and disappeared during subsequent callus formation and regeneration of plants. However, since in an entire somatic hybrid population, a hybrid plant can contain either of the parental chloroplasts, it would appear that the degradation of one particular cpDNA is not species specific in the higher plant. It should also be noted that cpDNA degradation as is known in *Chlamydomonas* may not be occurring at all, instead, segregation of the two genotypes chloroplasts may be responsible for the presence of one or the other but not both parental chloroplasts in the somatic hybrid plant.

Several other explanations for unidirectional sorting out of chloroplasts in favour of *P. parodii* in the three different interspecies somatic hybrids are possible. By chance, the plants analysed may have been sampled from calluses containing only *P. parodii* chloroplasts since only a single hybrid callus was regenerated into plants for each fusion experiment. It may also be possible that only plants with *P. parodii* chloroplasts were capable of being regenerated from the hybrid calluses. Alternatively, a selective advantage of *P. parodii* chloroplasts over those of the other three *Petunia* species may be involved, paralleling those known in *Oenothera* and *Pelargonium* (Tilney-Bassett and Birky 1981). Therefore, it is not possible to unambiguously

determine the reason why only *P. parodii* chloroplasts are present in the three types of interspecies somatic hybrid plants. It may not be possible to obtain a phototrophic somatic hybrid containing only *P. inflata* fully functional chloroplasts for the reasons explained earlier. However, it might be possible to obtain a somatic hybrid plant containing either *P. hybrida* or *P. parviflora* chloroplasts if more hybrids were available for study. Other studies where comparatively similar selection schemes have been used to produce somatic hybrid plants have shown an approximately equal number of somatic hybrid plants expressing one or the other but not both parental chloroplast genes (Melchers et al. 1978; Poulsen et al. 1980; Iwai et al. 1980; Douglas et al. 1981). Furthermore, in the intraspecific somatic hybrid plants of *Nicotiana debneyi* produced in the absence of any strong selection, a similar situation has been reported by using cpDNA cleavage pattern analysis methods (Scowcroft and Larkin 1981). However, it should be interesting to produce a somatic hybrid/cybrid callus/plant by using single heterokaryon isolation and culture technique (Patnaik et al. 1981) with two different fluorescent markers for identification of interspecific heterokaryons formed by fusion of mesophyll cell protoplasts or cell suspension protoplasts or both mesophyll cell and cell suspension protoplasts (Galbraith and Mauch 1980). The available somatic hybrid/cybrid material would provide a good opportunity to study in great detail the fate of both parental chloroplasts by using a combination of Fraction 1 protein and cpDNA analysis methods in the somatic hybrid callus/plant. Mitochondrial DNA recombination has been reported in tobacco somatic hybrid plants (Belliard et al. 1979) and we are currently attempting to examine the inheritance pattern of mitochondrial DNA of these three interspecific somatic hybrids in *Petunia*.

Evolution of cpDNA in the Genus Petunia

Analysis of the cleavage patterns of cpDNA from the various *Petunia* species with a number of restriction endonucleases revealed no major differences among these species except in the case of *P. parviflora*. This gives evidence for rather close conservation of chloroplast DNA among the genus *Petunia* beyond that suggested by identical isoelectric focusing profiles of the primary structure of Fraction I protein large subunit polypeptides in the genus *Petunia* (Gatenby and Cocking 1977).

The origin of some of the differing cpDNA restriction fragments could be determined. In the Bgl I cleavage pattern of *P. parviflora* cpDNA, a Bgl I fragment of molecular weight 25 Md was observed (indicated by an arrow in Fig. 2 A lane 2). The presence of this fragment is accompanied by the absence of the Bgl fragments B3 (14.4 Md), B7 (5.3 Md) and B8 (5.3 Md) in DNA of *P. hybrida/inflata/parodii* (Fig. 2 A lane 1, 3 and 4). The fragments B7 and B8 are part of the inverted repeat region of the cpDNA (Bovenberg et al. 1981; Fig. 5). Most likely a small modification in both inverted repeat regions of the cpDNA has resulted in the disappearance of the two recognition sites indicated by arrows in Figure 5. This leads to the fusion of the B7, B3 and B8 fragments and the appearance of the observed fragment B* of molecular weight 25 Md (Fig. 5). Recently Gordon, Bohnert, Crouse and Herrmann obtained evidence for similar modifications occurring in both inverted repeat regions of *Oenothera* cpDNA (personal communication). Since Fraction 1 analysis of the genus *Petunia* suggests that probably *P. parviflora* is the most recently evolved species (Kumar et al. 1981) we assume that the *P. parviflora* cpDNA originated from *parodii/hybrida/inflata* cpDNA group. The fact that these modifications are located on the same position in both inverted repeats suggest that this modification occurred at a time in evolution when the chloroplast DNA contained only one copy of this region. Subsequently, when duplication of this region occurred during the development of the species both contained the modified Bgl I restriction site region. A second, and in our opinion less likely, possibility is that the modification occurred in only one of the two inverted repeats. Subsequently, this modification was duplicated e.g. by intermolecular recombination between inverted repeat regions or because heterologies occurring in the stem structure formed between the two inverted repeat regions were recognized and removed. The origin of the additional Bam HI fragment in the cleavage pattern of *P. parodii* cpDNA is under current investigation. This fragment is most likely the result of a small modification just in, or close to one of the inverted repeat regions leading to the joining of two Bam HI fragments and to the presence of the additional Bam HI fragment of molecular weight 4.9 Md.

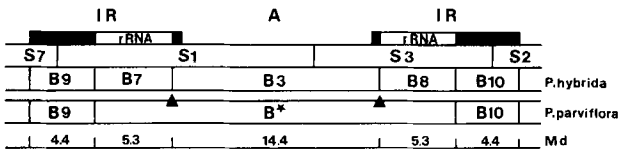

Fig. 5. Part of the Sal I (S) and Bgl I (B) restriction endonuclease cleavage site map of the cpDNA from *P. hybrida*, showing the small copy region (A) and the inverted repeat (IR) regions (from Bovenberg et al. 1981). In the Bgl I cleavage pattern of *P. parviflora*, fragments B3, B7 and B8 have disappeared and one large fragment B is formed (see also Fig. 2 A). Molecular weights are given in Md

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