Theor Appl Genet (1990) 79: 807–812

Homologies to plastid DNA in the nuclear and mitochondrial genomes of potato

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Received January 2, 1990; Accepted February 9, 1990
Communicated by R. Hagemann

Summary. Potato plastid DNA clones, representing one-fourth of the potato plastome complexity and containing sequences of the *16SrRNA*, *rps16*, *atpA*, *atpE*, *psaA*, *psaB*, *trnK*, *trnV*, and *trnG* genes, were used as hybridization probes on nuclear- and mitochondrial-enriched DNAs. Each probe hybridized to multiple nuclear restriction fragments distinct from the plastid cleavage products generated by the same endonucleases. The nuclear hybridizable fragments are highly methylated at their HpaII target sequences (C/CGG). In some instances, the transfer seemed to involve plastid regions of several kilobase pairs, as reflected by the co-integration in the nucleus of restriction sites that are distant in the plastome. Three clones hybridized additionally to distinct mitochondrial fragments. These results indicate that extensive DNA transfers did occur between plastids and other organelles in potato.

Key words: Plastid DNA – Subcellular genomes – DNA homologies – Potato

Introduction

In plants, DNA transfer between the subcellular genomes left different imprints in the present organization of the genetic information inside the cell.

The loss of autonomy of the endosymbiotic ancestors of plastids and mitochondria was a direct consequence of gene transfer to the nuclear genome. Though the deletion of the cytoplasmic gene copy is assumed to be the rule, pseudogenes have been found in the chloroplast DNA of higher plants for which it is suggested, but not demonstrated, that their active copies reside in the nucleus (Zurawski and Clegg 1987; Wolfe and Sharp 1988). To our knowledge, pseudogenes have not yet been reported in plant mitochondrial DNA. Another situation has been shown to be widespread in Chenopodiaceae and to involve large-scale transfer of plastid DNA to the nucleus: here the active copy of the gene resides in the plastid, and the nucleus contains highly methylated, probably rather short, and thus probably inactive plastid DNA stretches (Timmis and Scott 1983; Scott and Timmis 1984; Ayliffe et al. 1988). The existence of similar events in Solanaceae was proved by the fortuitous finding of two short chloroplast DNA stretches in an intron of the cloned *Cab-7* gene of tomato (Pichersky and Tanksley 1988).

In fact, the existence of identical sequences in more than one subcellular genome, a situation termed 'intracellular promiscuity' (Ellis 1982), was first highlighted by the discovery in the mitochondrial genome of maize of a 12-kbp plastid gene cluster (Stern and Lonsdale 1982). Numerous cpDNA-homologous mtDNA sequences were later shown to occur in different plant taxa by hybridization, DNA sequencing and immunological characterization of the in vitro transcription-translation products of cloned mitochondrial DNA sequences (Lonsdale et al. 1983; Stern and Palmer 1984; Iams et al. 1985; Schuster and Brennicke 1987; Makaroff and Palmer 1987; Moon et al. 1988).

The extent of the intracellular DNA promiscuity had never been investigated in *Solanum tuberosum*. In this work, potato plastid DNA clones were used to probe the nuclear and mitochondrial genomes of *Solanum tuberosum* ssp. *tuberosum*. Numerous homologies were found in both subcellular compartments. Some sequences are common to the three genomes.

Materials and methods

Plant material, organelle purification and DNA extraction

The cultivar Desiree was used throughout this work. Total DNA was extracted by scaling up the method of Dellaporta et al.

(1983). For the isolation of chloroplast DNA, young plants were grown in a greenhouse, kept in the dark 1 day before use, and the fresh leaflets were harvested. Chloroplasts were purified on sucrose step-gradient and the DNA (abbreviated 'ptDNA') was recovered essentially as described by Hosaka (1986).

The nuclear- and mitochondrial-enriched fractions were co-extracted from mature cold-stored tubers, by differential centrifugation as follows. Tuber cores (250 g) were vacuum infiltrated for 30 min in the homogenization buffer (A buffer: 0.3 *M* mannitol, 50 m*M* TRIS-HCl, 3 m*M* EDTA, 5 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol, 0.1% bovine serum albumin, pH 8.0), sliced and blended in a standard mixer equipped with razor blades, with 250 ml of ice-cold A buffer. The lysate was filtered on nylon netting (two layers of 1,000 µm and two layers of 250 µm) and centrifuged for 2 min at 40 *g* (4 °C) to remove most of the starch grains. After a 1,500 *g* × 10 min centrifugation at 4 °C, the crude nuclei pellet was resuspended in A buffer (10 ml) with a soft brush and diluted with 2 vol. of the same buffer. Triton X-100 was added to 0.5% (v/v) and the nuclei were re-pelleted (1,500 *g* × 10 min, 4 °C). Two additional washes were performed in the same way, and the final pellet was resuspended in 5 ml of B buffer (B buffer: 50 m*M* TRIS-HCl, 20 m*M* EDTA, pH 8.0). Nuclei were lysed with 0.5% SDS and 100 µg/ml of proteinase K (Boehringer Mannheim) at 37 °C for 30 min with agitation. The lysate was extracted once with TE-buffered phenol (TE is 10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0) by repeated inversions (30 min) using a vertical rotative shaker. After centrifugation (3,500 *g* × 10 min, 4 °C), the aqueous phase was re-extracted in the same way with phenol : chloroform (1 : 1; v/v) and precipitated by adding 1/10 vol. of sodium acetate 3 *M*, pH 5.2, and 1 vol. of isopropanol. The pellet (12,000 *g* × 3–20 min, 0 °C) was washed with 70% ethanol, dried and solubilized in TE buffer. Further purification was achieved by isopycnic centrifugation in a cesium chloride gradient using a standard protocol (Maniatis et al. 1982).

For the isolation of the mitochondrial-enriched DNA, the first 1,500 *g* supernatant from above was centrifuged twice at 2,500 *g* × 10 min, 4 °C and the pellets were discarded. A crude mitochondrial pellet was obtained by centrifuging the last super natant at 18,800 *g* × 10 min, 4 °C and was resuspended in 10 ml of A buffer. The suspension was brought to 13 m*M* $MgCl_2$, DNAse I (Boehringer Mannheim) was added to 150 µg/ml and incubation on ice extended to 1 h. After centrifugation (18,800 *g* × 10 min, 4 °C), the mitochondria were resuspended in 10 ml of 0.3 *M* mannitol, 50 m*M* TRIS-HCl, 20 m*M* EDTA, pH 8.0, diluted with an equal volume of the same buffer and re-pelleted. Two additional washes were performed in the same way. The lysis of the mitochondria, and the recovery and purification of the DNA were as described for the nuclei.

Southern analysis

All DNA extracts were digested with 5–10 units of restriction endonuclease (BRL) per microgram of DNA, for approximately 5 h, as recommended by the supplier. For the double digestions, the DNA was ethanol-precipitated after the first digestion and resolubilized in TE. In order to monitor the completeness of the digestions with an internal control, 5 ng of pBR322 were added to aliquots of the digestion mixes and the plasmid digests were Southern-analyzed with a pBR derivative as probe.

Agarose gel electrophoresis and Southern blotting on nylon membranes (Hybond-N, Amersham) were performed using standard methods (Maniatis et al. 1982). Lambda DNA/HindIII and PhiX174 RF DNA/HaeIII restriction fragments (BRL) were used as molecular weight standards.

The blots were prehybridized for at least 5 h in 3 × SSC (1 × SSC = 0.15 *M* NaCl, 0.015 *M* tri-sodium citrate), 5 × Denhardt (1 × Denhardt = 0.2 g/l polyvinylpyrrolidone, 0.2 g/l bovine serum albumin, 0.2 g/l Ficoll), 0.1% SDS, 100 µg/ml of denatured herring sperm DNA (Sigma), at 65 °C. The hybridization solution included additionally $>10^6$ cpm/ml of heat-denatured probe, prepared by random-primed Klenow extension using the 'Multiprime' kit from Amersham and [α-^{32}P]dCTP (Amersham, ~3,000 Ci/mmol). Hybridization extended to 16–20 h. The blots were washed at 65 °C three times for 20 min ea. in 2 × SSC, 0.1% SDS and three times for 20 min ea. in 0.1 × SSC, 0.1% SDS. They were exposed to autoradiographic films (Kodak X-OMAT AR) for 2–5 days at −70 °C.

Table 1. Plastid DNA clones: physical and genetical data

Clone	BamHI insert[a]	Insert size (kbp)	Genes: partial data (Ref.)
pPBm58	B3	12.3	*atpE* (Heinhorst et al. 1988) *trnV(UAC)* (P. du Jardin, unpublished results)
pPBm7	B4	9.2	*atpA* (Heinhorst et al. 1988) *trnG(UCC)* (P. du Jardin, unpublished results)
pPBm98	B14c	3.0	*16SrRNA*[b] (Heinhorst et al. 1988)
pPBm131	B15	2.5	*rps16-trnK(UUU)*[b] (P. du Jardin, unpublished results)
pPBm13	B16	2.4	*psaA-psaB*[b] (P. du Jardin, unpublished results)

[a] Fragment numbers refer to Heinhorst et al. (1988)
[b] The cloned fragment is specific to this (these) gene(s)

Plastid DNA clones

A potato plastid DNA clone bank was previously constructed in the pUC9 plasmid vector after complete BamHI digestion of purified chloroplast DNA. The *E. coli* recipient strain was JM101. The map positions of the inserts were determined by restriction enzyme analysis and/or hybridization on cpDNA, by reference to the physical map of Heinhorst et al. (1988; and unpublished results). The genic content of some of the cloned fragments has been specified by Heinhorst et al. (1988) (fragments B3, B4, B14c). By Southern hybridization on tobacco DNA and sequencing, additional data were obtained for B3, B4, B15, B16 (unpublished results). The available data concerning the five cloned fragments used in this work are given in Table 1.

Plasmid DNA amplification and purification by alkaline lysis were performed as described in Maniatis et al. (1982).

Results

Methylation status of the plastid and nuclear DNAs in tubers

We first characterized the restriction patterns of the plastid and nuclear DNAs from tubers with the 4 bp-recognizing methylation-sensitive endonucleases HpaII and MspI (Fig. 1). Both enzymes cut total DNA to a very

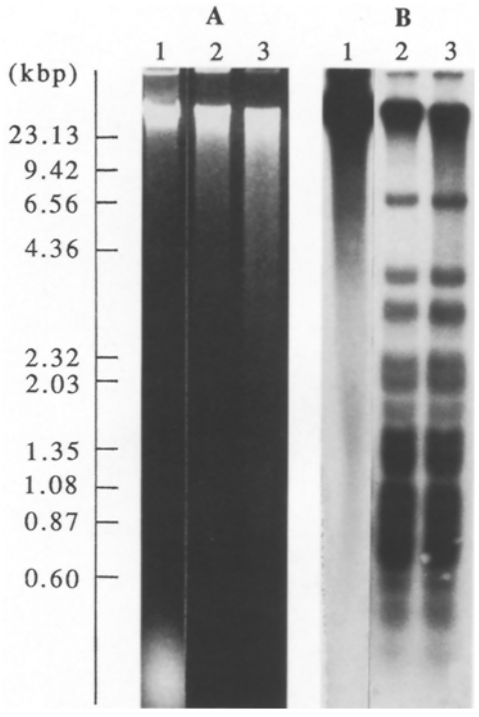

Fig. 1 A and B. Southern analysis of the HpaII and MspI restriction patterns of tuber plastid DNA. **A** Ethidium bromide staining of total DNA from tuber, undigested (1), digested by HpaII (2), and digested by MspI (3). **B** The gel from A was blotted and hybridized with ^{32}P-labeled chloroplast DNA. The gel is 0.8% agarose

limited extent (Fig. 1 A) due to the hypermethylation of nuclear DNA, which accounts for more than 85% of total DNA. A Southern analysis of these DNAs was performed using ^{32}P-labeled purified chloroplast DNA as probe (Fig. 1 B). When the restriction profiles of total DNA hybridized with ^{32}P-chloroplast DNA are compared with equivalent EtBr-stained patterns of purified chloroplast DNA, both profiles match perfectly (data not shown). Thus, the restriction patterns of plastid DNA may be characterized following this hybridization approach in organs where plastid purification is delicate, as in tubers. Such Southern analysis of the HpaII and MspI restriction patterns of tuber ptDNA (Fig. 1 B) highlights the contrast between its methylation status and that of nDNA. The ptDNA is efficiently cut by both enzymes, the bulk of the fragments being below 2.0 kbp.

Nuclear homologies to plastid DNA

This difference in methylation allows an efficient electrophoretic separation in agarose gel of nuclear and plastid DNAs, after digestion with the appropriate restriction enzymes. Here we characterized nuclear sequences homologous to ptDNA from DNA samples that are mixtures of nuclear and plastid DNAs. The nuclear-enriched fractions prepared from tubers indeed contained plastids, and the DNA was abbreviated 'n+ptDNA'. Upon hybridization of these DNAs with plastid clones (Fig. 2), the strongest signals on the 'n+ptDNA' profiles corresponded to ptDNA, due to its high ploidy level in the tuber cells. The nuclear homologies to plastid DNA are revealed by the lower intensity signals corresponding to restriction fragments with mobilities different from those of the ptDNA fragments. On the single digestion patterns, hybridization to ptDNA obscures most of the profile but some faint signals of nuclear origin are detected above the smear. On double digests, the HpaII cleavage lowers the ptDNA fragments and allows the identification of additional nuclear fragments.

If we take the probe pPBm13 as an example, we see that it hybridizes to a 3.9-kbp plastid DNA fragment after HindIII digestion (Fig 2, lane 1) and to a 6.5-kbp ptDNA fragment after EcoRI digestion (Fig. 2, lane 3). Small amounts (2.5 ng) of purified chloroplast DNA were loaded on these lanes to avoid overexposure. As expected, these plastid fragments are observed in the lanes containing n+ptDNA digested by the same enzymes (Fig. 2, lanes 5 and 8 respectively), and hybridization to each of these fragments gives rise to an intense signal that obscures all the region underneath. When the HindIII-digested ptDNA is further cleaved by HpaII, the 3.9-kbp fragment described is cleaved into 1.35- and 0.7-kbp fragments (Fig. 2, lane 7). The 6.5-kbp EcoRI fragment is also cut by HpaII (Fig. 2, lane 10). Lanes of Fig. 2 with n+ptDNA digested by HindIII, contains faint signals above 3.9 kbp that are of nuclear origin. After HpaII digestion (Fig. 2, lane 6), these fragments are still observed with identical signal intensities, and the region between 1.35 and 3.9 kbp that was obscured in Fig. 2, lane 5 by the 3.9-kbp signal is now shown to contain at least three additional fragments. The comparison of the EcoRI digests (Fig. 2, lane 8) and EcoRI+HpaII digests (Fig. 2, lane 9) of n+ptDNA leads to similar observations. Equivalent situations are encountered with the five probes tested.

Each probe hybridizes to several HpaII-resistant fragments, their number (up to >10) not being correlated with the length of the probes. A wide range of mobilities is noticed. As typically observed in the plastome of potato, EcoRI cuts more frequently than HindIII. After electrophoresis, some nuclear HpaII-resistant fragments co-migrate with EcoRI or HindIII fragments of ptDNA. This is observed for at least one fragment per probe tested (except for probe pPBm131) and involves fragments up to 13.5 kbp (the largest HindIII+HpaII fragment hybridizing to pPBm58).

All nuclear fragments revealed after the first digestion are still observed after the second digestion by HpaII. As it is extremely improbable that all these fragments are

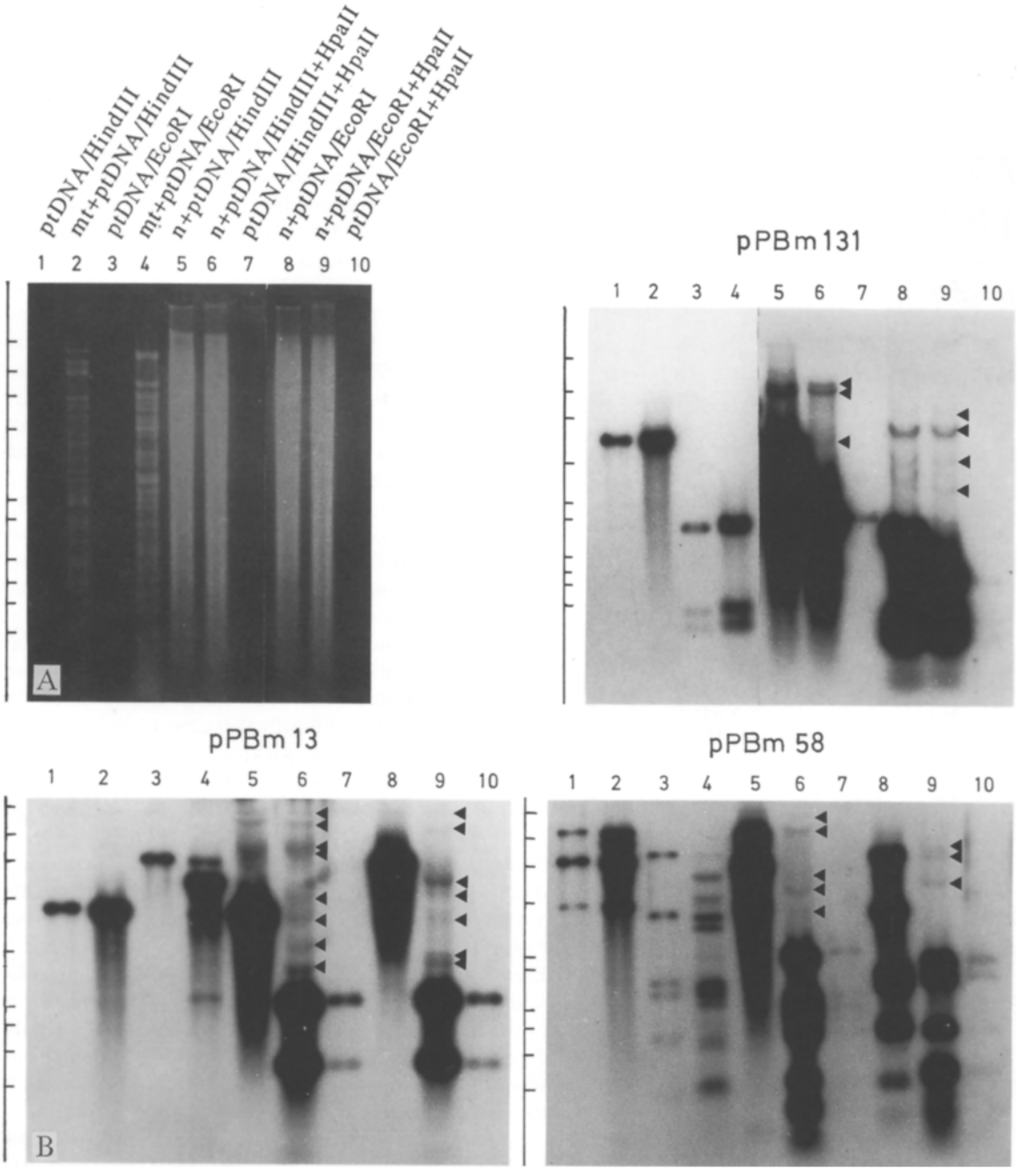

Fig. 2. Southern hybridization of the three subcellular DNAs with selected plastid probes. A representative example of the gels blotted is given in the *upper left*. The probes used are indicated above each set of lanes. The markers indicate the main homologies of nuclear origin in the double digestion patterns. The molecular weight scales are positioned at *left*; from *top* to *bottom* (kbp): 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87, 0.60. DNA amounts were 10 µg for n + ptDNA, 2 µg for mt + ptDNA, 0.5 ng for ptDNA in the lanes adjacent to the n + ptDNA lanes, and 2.5 ng in those adjacent to the mt + ptDNA lanes. The gels are 0.8% agarose

devoid of CCGG recognition sequence, we assume that at least one of the cytosine residues in the target tetranucleotide is methylated. As an identical pattern is obtained with the isoschizomer MspI (data not shown), it is highly probable that at least the external cytosine is methylated.

The intensity of the signals obtained with each of the probes is variable. Alongside the 'n + ptDNA' lanes containing 10 µg of DNA, 0.5 ng of chloroplast DNA was run, which corresponds approximately to a ratio of one plastid genome copy per tetraploid nuclear genome, assuming a 4C value of 3.6 pg (Jacobsen et al. 1983). This

was done to ensure that a perfectly homologous sequence present once in the nuclear genome could be detected according to our protocol. The comparison of the intensities of the nuclear and chloroplast signals in order to estimate the copy number per nucleus of the plastid inserts, as attempted by some authors, suffers from a major drawback, as both copy number and hybrid stability can equally contribute to the signal intensity. Hybrid stability depends on the length of pairing and on the percentage of mismatched base pairs. As these data are not available for the detected hybrids, direct estimation of the copy number per nucleus is hazardous.

The completeness of the digestions was monitored with an internal control (data not shown).

Mitochondrial homologies to plastid DNA

Each chloroplast DNA restriction fragment is present in the mitochondrial-enriched DNA digested by the same enzyme (compare lanes 1 and 2, and lanes 3 and 4 in Fig. 2), reflecting the presence of plastid DNA in the mitochondrial extracts. Plastid DNA-homologous mitochondrial DNA sequences are revealed by the hybridized fragments which have distinct mobilities and should represent chimerical sequences including ptDNA and the mitochondrial flanking region(s). Such fragments are observed with probes pPBm13, pPBm58 (Fig. 2), and pPBm98 (data not shown).

Discussion

Nuclear homologies to plastid DNA

Numerous nuclear DNA sequences homologous to the cloned plastid fragments have been detected by our Southern hybridizations. The hybridized fragments displaying the relatively weak signals must be of nuclear origin as (i) they have mobilities different from those of the ptDNA digestion products generated by the same endonucleases, (ii) they do not represent partial digestion products of ptDNA, (iii) they have mobilities different from those of the hybridized mitochondrial DNA digestion products generated by the same endonucleases, and (iv) they are hypermethylated at cytosine residues, which is a typical feature of plant nuclear DNA. The natural occurrence of a population of plastid subgenomic molecules generated by inter- or intramolecular recombination, as described for mitochondrial DNA, has never been reported in the plastids of any system. It is thus highly improbable that the observed homologies reflect the presence of a subpopulation of altered plastid DNA molecules.

The plastid-to-nucleus transfers could have involved relatively large sequences of ptDNA. Nuclear double-digestion products are noted with mobilities identical to those of plastid EcoRI or HindIII cleavage products. These probably represent HpaII-resistant fragments internal to plastid inserts, in which the EcoRI or HindIII restriction sites were conserved upon transfer, integration, and subsequent evolution. As it cannot be ruled out that such co-migration is merely fortuitous, this statement should be more reliably restricted to situations where such conserved internal fragments are noted with each tested endonuclease (EcoRI and HindIII in our case), and such is the case with at least two out of the five probes we used (pPBm58, pPBm98).

The case of the homologies to the 12.3-kbp fragment B3 from pPBm58 is particularly interesting. Two presumed internal fragments resistant to HpaII are noted with estimated sizes of 13.5 kbp (HindIII + HpaII) and 8.0 kbp (EcoRI + HpaII). This would indicate that the length of the transferred ptDNA exceeded 13.5 kbp. As the other plastome fragments homologous to B3 are not detected in the nuclear DNA, it is probable that the plastid insert is not significantly larger than this, though post-integration rearrangements could have suppressed additional transferred restriction sites. In spinach, the occurrence of relatively large inserts (>2 kbp) with conserved internal fragments was shown to be very exceptional (Scott and Timmis 1984). The nuclear-plastid chimerical sequence cloned from tomato (Pichersky and Tanksley 1988) comprises two very short inserts (<150 bp). However, short inserts should not be the rule. The occurrence of a large insertion (>6 kbp) has been proposed in two Chenopodiaceae (Ayliffe et al. 1988). Our data suggest that a significant part of the ptDNA-homologous fragments in potato includes plastid restriction sites that were retained in the nucleus after transfer and integration of regions of several kbp (and, as we showed, of more than 10 kbp in one case).

The occurrence of plastid inserts of several kbp raises the question of their expression from the nuclear background. Their prokaryotic nature does not preclude their recognition by the nuclear transcription and cytoplasmic translation systems (Cornelissen and Vandewiele 1989), but it should be noted that frameshifts occur in the small-sized plastid inserts cloned from spinach (Cheung and Scott 1989) and tomato (Pichersky and Tanksley 1988) nuclei. In addition, considering that methylation is proposed to silence plastid genes in tomato (Ngernprasirtsiri et al. 1988a, b), it might be that the hypermethylation of these sequences efficiently impedes their expression from the nuclear background.

Mitochondrial homologies to plastid DNA

A single case of triple subcellular location of a plastid DNA sequence has been reported, in spinach (Whisson and Scott 1985). Our hybridization data demonstrate that homologies with the *16SrRNA* gene, with the

psaA-B operon, and with unspecified regions of the 12.3-kbp insert of pPBm58 (including *atpE* and *trnV*) are present in both the mitochondrion and the nucleus of potato.

In the case of the homologies to the *16SrRNA*, it is not clear whether the cross-hybridization actually reflects DNA transfer between both genetic compartments or sequence homology between functionally identical genes in these organelles. Indeed, extensive homologies exist between the mitochondrial and plastid genes coding for ribosomal RNAs (reviewed in Gillham et al. 1985; Gray 1988) and for the (C) F_1-ATPase α subunits (Braun and Levings 1985). The plastid probe pPBm7 containing *atpA* did not hybridize to mitochondrial DNA, indicating that both organellar *atpA* genes of potato do not cross-hybridize to a detectable level in such stringent conditions. The mitochondrial homology with the probe containing *atpE* (and flanking regions, in pPBm58) cannot be attributed to sequence conservation between two active organellar *atpE* genes, as the F_1-ε subunit of plant mitochondria is encoded by the nucleus. This case and the detected homologies to the *psaA-psaB* operon may thus reasonably be viewed as the consequence of interorganellar gene transfer.

Acknowledgements. I wish to thank Dr. R. Kettmann for his critical comments on the manuscript, J.-C. Martiat for his skilful technical assistance and for the photographs, and V. Sorce for typing the manuscript. This work was supported by grants from the F.R.F.C. (2.4549.87).

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