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Homologies between nuclear and plastid DNA in spinach

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Summary. Homologies between spinach nuclear (n) DNA and Chloroplast (pt) DNA, have been detected with a clone bank of spinach ptDNA as hybridization probes to restriction fragments of nDNA prepared from purified root nuclei. Every cloned fragment of ptDNA showed homologies to discrete restriction fragments of nDNA, different from those of ptDNA, indicating integration of these homologies into nDNA. While most ptDNA clones were relatively large and probably contained several genes, sequence homologies were also found to the cloned plastid gene for RuBP carboxylase and the β subunit of ptATPase. Many of the homologies in nDNA occur in regions of the genome that are highly methylated and are not digested by the methylation sensitive restriction endonucleases HpaII and MspI. In contrast these enzymes cleave ptDNA into small fragments which allows the nDNA homologies to be distinguished in total root DNA. The sequence homologies observed were not due to contaminating non nuclear sequences as shown by hybridization to mitochondrial (mt) and bacterial DNAs. The total amount of homology to ptDNA in nDNA is equivalent to about five copies of the plastome per haploid nuclear genome. The homologies generally appear to be in individual segments of less than 2 kbp in length, integrated into several different places in the genome.

Key words: Spinach – DNA homologies – Nuclear DNA – Plastid DNA

Introduction

The existence of two extranuclear genetic systems in plant cells, one in the chloroplasts (plastids) and the other in mitochondria, as well as the main genetic system in the nucleus, is well known. Early studies of the ribosomal (r) RNA genes of these systems showed that chloroplast (pt) rRNA formed hybrids with both ptDNA and total DNA in *Euglena* (Scott and Smillie 1967; Scott 1973) and higher plants (Tewari and Wildman 1968; Ingle et al. 1969). Thermal denaturation studies showed that the plastid rRNA/nDNA hybrid was less specific than the homologous rRNA/DNA hybrids in *Euglena* (Scott 1973). These results may have reflected either cross-homology between the functionally similar rDNA sequences in the plastome and genome, or the existence of separate sequences in nDNA homologous to plastid rRNA genes integrated into the nucleus. The first of these two possibilities was favoured by experiments with cloned rDNA sequences of *Euglena* ptDNA and nDNA which showed two separate regions of homology between the functional genes (Curtis and Rawson 1982). Some evidence for the second possibility was obtained by restriction and hybridization studies in siwss chard (Ingle 1979) which suggest that some of the homology observed between nDNA and plastid rRNA was most likely to be due to a separate sequence in nDNA homologous to ptDNA, distinct from cross-homology.

In maize a 12 kbp region of the ptDNA, including part of a chloroplast rRNA gene has been found to be homologous to a section of the maize mitochondrial genome (Stern and Lonsdale 1982). This observation, together with the detection of homologies between mitochondrial (mt) DNA and nDNA in yeast (Farrelly and Butow 1983), locusts (Gellisen et al. 1983) and the fungus *Podospora anserina* (Wright and Cummings 1983) raises the possibility that the occurrence of homologies between organellar DNA's may be a widespread phenomenon. We recently reported the eixstence of homolo-

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gies in nDNA to sections of the ptDNA in spinach (Timmis and Scott 1983). In this report we show that a large proportion of the plastome has homologies with the nuclear genome in spinach, including the defined plastid genes for two chloroplast coded and synthesized proteins, the large subunit of RuBP carboxlase and the β subunit of ptATPase.

Materials and methods

1 Preparation of DNA

a) Total DNA. Spinach plants were grown in liquid culture (Possingham and Smith 1972) and DNA from the various tissues was prepared as previously described (Scott and Possingham 1980) except where otherwise detailed. Total DNA from roots was prepared by the method of Murray and Thomson (1980).

b) Mitochondrial DNA. Spinach leaf mitochondria were isolated (Douce et al. 1977), resuspended in 1 ml 0.3 M Mannitol, 0.001 M EDTA, 0.01 M $MgCl_2$, 0.03 M MOPS, and 0.2% bovine serum albumin per 100 g of leaves and incubated at 0 °C for 1 h with DNAse (1 mg ml^{-1}) and alkaline phosphodiesterase (500 µg ml^{-1}). After addition of EDTA to a final concentration of 0.1 M, mtDNA was purified as described for chloroplasts (Scott and Possingham 1980).

c) Nuclear DNA. Roots were extensively washed in dilute detergent, fixed in ice cold 0.5% formaldehyde in 0.01 M HEPES pH 7.0 for 1–2 h and homogenized 4×3 s in a razor blade blender in 0.025 M Tris-HCI pH 7.5, 0.275 M sorbitol, 0.005 M β-mercaptoethanol and 0.5% Triton X-100. The homogenate was filtered through one layer of cheesecloth and three of miracloth and the nuclei collected by centrifugation at 400×g for 5 min. The nuclei were resuspended in the extraction buffer, underlaid with a similar buffer containing 0.4 M sorbitol, collected by centrifugation at 400×g for 5 min, and this procedure repeated three times. Nuclei isolated in this way were free of microscopically detectable plastids or mitochondria and, as will be demonstrated later, contained barely detectable levels of ptDNA and no detectable mtDNA. The nuclei were then lysed in 0.02 M EDTA, 0.15 M NaCl, 0.015 M tri-sodium citrate pH 7.0 containing 1% sarkosyl and 100 µg ml^{-1} RNAse A. After 15 min at 37 °C, 5 mg ml^{-1} of predigested pronase was added, the sample incubated for a further 15 min and nDNA isolated as described (Scott and Possingham 1980). DNA isolated in this way was not of as high molecular weight as that obtained from roots by the direct extraction procedure of Murray and Thomson (1980) but allowed separation of clear restriction bands in the range 2–8 kbp. In experiments with a range of restriction enzymes, no difference was detected in digestion of nuclear DNA prepared after fixation compared with conventionally prepared DNA samples. Similarly, no difference was observed in the restriction enzyme digestion of ptDNA prepared from either unfixed chloroplasts or chloroplasts fixed in 4% formaldehyde.

d) Molecular cloning and preparation of plasmid DNA. Purified plasmids from a spinach ptDNA clone bank (Palmer and Thomson 1981) were provided by Drs. J. D. Palmer and W. F. Thomson and other ptDNA clones by Dr. P. R. Whitfeld. The spinach nuclear rDNA clone Sn31 was prepared from a Hind III digest of spinach root DNA cloned in the plasmid pACYC 184 (Chang and Cohen 1978) and includes the entire spinach nuclear rDNA sequence (unpublished results). Plasmids were prepared by the SDS lysis procedure of Godsen and Vapnek (1973).

2 Hybridization of DNA

a) On filters. Nuclear DNA or total root DNA preparations were digested with restriction endonucleases (Boehringer-Mannheim) under the conditions recommended by the manufacturers for 4–5 h with five units of enzyme per µg DNA. Other DNA was digested for 1–2 h with one unit of enzyme per µg DNA. In the case of double digests with Hpa II (or MspI)+EcoRI, the EcoRI digestion was second after suitable adjustment of the buffer. Following electrophoresis on 0.8% agarose gels the DNA was transferred (Southern 1975) to nitrocellulose filters (Schleicher and Schuell BA85). The filters were baked in vacuo for 3 h at 80 °C and preincubated at 65 °C for 2 h with constant shaking in 4×SSC (SSC is 0.15 M NaCl; 0.015 M Na-citrate pH 7.2), 100 µg ml^{-1} sonicated single stranded salmon testis DNA, 0.1% SDS, 0.1% gelatin, 0.1% ficoll and 0.1% polyvinylpyrrolidine-360. As much as possible of the preincubation buffer was removed and the filters hybridized at 74 °C for 16 h with constant shaking in the same buffer containing 0.04% gelatin, 0.04% ficoll and 0.04% polyvinylpyrrolidine-360 with 2×10^6 cts min^{-1} ml^{-1} of ^{32}P labelled probe added. Probes were labelled by nick translating (Schachat and Hogness 1974) each ptDNA clone with α^{32}P-dCTP (BRESA, Adelaide, South Australia) to a specific activity of 10^8–10^9 cts min^{-1} µg^{-1}. The filters were autoradiographed after four washes of 30 min in 2×SSC, 0.1% SDS at 65 °C. The molecular weights of the DNA fragments were determined by comparison with λ phage DNA digested with EcoRI and Hind III following the curve fitting procedure described by Duggleby et al. (1981). The position of bands in the DNA digests as revealed by ethidium bromide staining of the gels and autoradiography of the Southern transfers was recorded with a Hewlett Packard HP-9872 digitizer. A programme written for the HP-9845 microcomputer also used the standard curve of λ DNA fragments to calculate a normalized mobility for each DNA fragment on the gel. This enabled each gel pattern to be redrawn to a 'Master' standard curve so that any one gel could be compared directly with any other.

Following digestion of ptDNA with restriction endonucleases, the concentration of the DNA was measured by the Burton (1956) procedure. The standard errors of the mean estimates of the DNA concentrations were less than 1%. A range of loadings of the ptDNA digest fractionated by agarose gel electrophoresis was transferred to nitrocellulose filters. After hybridization with ^{32}P-ptDNA cloned probes the amount of hybridization and thus the amount of DNA on the filter homologous to the probe was estimated by densitometry of the autoradiograms. The correlation coefficient of the relationship between the amount of DNA loaded to the filter and the OD of the autoradiograms in all cases was greater than 0.97 and was significant ($P > 0.01$). Within the range of experimental conditions, this relationship was essentially linear and the amount of nDNA homologous to ptDNA was therefore estimated directly by comparing the amount of hybridization on adjacent autoradiogram tracks of known loadings of nDNA and ptDNA (see also Fig. 3).

b) In solution. The concentration of ptDNA in nuclear DNA preparations was measured by following the renaturation kinetics of ^{32}P-ptDNA in the presence of nDNA (Scott and Possingham 1980).

Results

In spinach root DNA 1–2% of the total DNA sequences are homologous with ptDNA (Scott and Possingham

1980). The majority of this homology is accounted for by ptDNA in the plastids found in the cytoplasm of root cells, and there are therefore 55–110 copies of the plastome per nuclear genome in this tissue. The renaturation kinetcis of added ^{32}P-ptDNA in the presence of excess purified ptDNA, total root DNA and nDNA are shown in Fig. 1, and the Cot½ values obtained were used to calculate the amount of ptDNA in the samples (Scott and Possingham 1980). In the experiment shown, the level of ptDNA in the root sample was 1.7% equivalent to 95 copies of the plastome per haploid genome and that of the nuclear preparation was 0.11% or 6 copies per haploid genome. The extent of renaturation of the probe was similar in all three measurements indicating that, in each DNA sample, all the plastome is able to form renatured duplex molecules. The renaturation conditions (Scott and Possingham 1980) were equivalent to the Tm-20 °C of spinach DNA as measured (unpublished results) and calculated (Wetmur 1976) from the average spinach ptDNA base composition of 37% GC (Bohnert et al. 1982). These measurements were also carried out a higher and lower stringencies of renaturation (Tm −15° and Tm −30°), yielding essentially similar results. In addition, Southern transfers of total ptDNA and nDNA digested with the restriction endonuclease EcoRI were hybridized at the same three conditions of stringency to ^{32}P-labelled cloned fragments of spinach ptDNA XhoI 3.0 kbp (Palmer and Thomson 1981) and pSocB 149 (Zurawski et al. 1981). At Tm −15 °C the amount of probe bound to either ptDNA or nDNA on the filters was significantly reduced compared with that at Tm −20 °C, while the radioactivity bound at Tm −30 °C was similar. There was no evidence of any additional sequences hybridized at reduced stringency as the hybridization bands were the same under all conditions. All subsequent hybridizations were at Tm −20 °C.

Nuclear DNA and ptDNA were digested with the restriction endonucleases EcoRI and Bam HI, and after

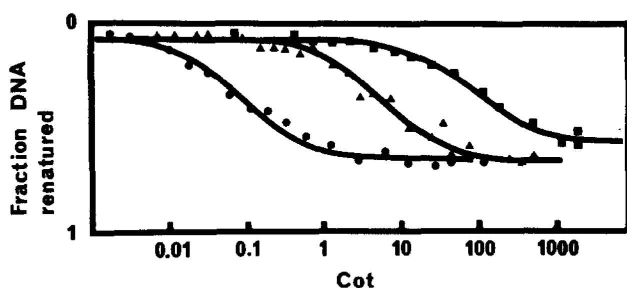

Fig. 1. Renaturation kinetics of ^{32}P-ptDNA. The renaturation kinetics of ^{32}P-ptDNA were followed in the presence of purified ptDNA (●), root DNA (▲) and nDNA (■) and gave Cot½ values of 0.10 ± 0.011, 5.9 ± 0.77 and 91 ± 8.7 mol^{-1} s^{-1} respectively

fractionation on agarose gels, transferred to nitrocellulose filters. The haploid genome size of spinach nDNA is 0.9 pg (Bennett et al. 1982) while that of spinach ptDNA (one copy of the plastome) is 1.6×10^{-16} g (Bohnert et al. 1982). The gels were loaded such that each nDNA track contained 5 µg DNA and each ptDNA track from 3–8 ng. This is the equivalent of about five copies of the plastome in ptDNA tracks relative to one copy of the genome in the nDNA tracks. The Southern filters prepared in this way were hybridized with individual radioactive probes prepared from the entire spinach ptDNA clone bank (Palmer and Thomson 1981) which includes all but 0.6% (0.9 kbp) of the plastome. The autoradiographs of these hybridizations show that all the ptDNA segments have homology with nDNA (Fig. 2). In all cases the nDNA tracks show discrete bands of hybridization to the ptDNA probes. The bands in the nDNA tracks (heterologous hybridizations) are nearly always of different mobilities to the homologous hybridization bands in the ptDNA tracks, indicating that there are several areas of homology in the nDNA for each probe that are not attributable to ptDNA contamination of nDNA. The enzyme EcoRI digests spinach nDNA extensively compared with BamHI and this is often reflected in the autoradiograms where the ptDNA fragment shows homology to discrete bands in the EcoRI digest of nDNA but to both discrete bands and undigested DNA in the BamHI digest (e.g. probes XhoI 12.8 kbp and XhoI 15.3 kbp). On the other hand the segments of nDNA homologous to the PstI 1.9 kbp probe are digested almost equally well by BamHI and EcoRI. The homologies to two adjacent ptDNA fragments show some overlap; presumably the homology in the nDNA segment overlaps the adjoining ends of the ptDNA fragments, e.g. the 7.8 kbp fragment in the EcoRI digest of nDNA hybridized to PstI 2.7 and PstI 8.1, and the 2.6 kbp fragment in the BamHI digest of nDNA hybridized to XhoI 15.3 and PstI 8.1 kbp probes. The two largest fragments of the ptDNA clone bank, PstI 17.3 kbp and PstI 25.5 kbp show the least well defined homology compared with smaller probes. This might be expected as, under our conditions, the smaller probes are at greater molar concentrations in the hybridization reaction.

The hybridization of ptDNA sequences to nDNA is a reflection of the amount, length and fidelity of base pairing of the homologous sequences. In Fig. 3 the hybridization of the PstI 7.7 kbp probe to nDNA and a series of dilutions of ptDNA is shown, Fragments of ptDNA, loaded onto gels at a rate equivalent to as little as 0.3 copies of the plastome per haploid genome were detected in the autoradiograms. Clearly the amount of homology observed between ptDNA and nDNA in Figs. 2 and 3 is of the order of several copies of the pla-

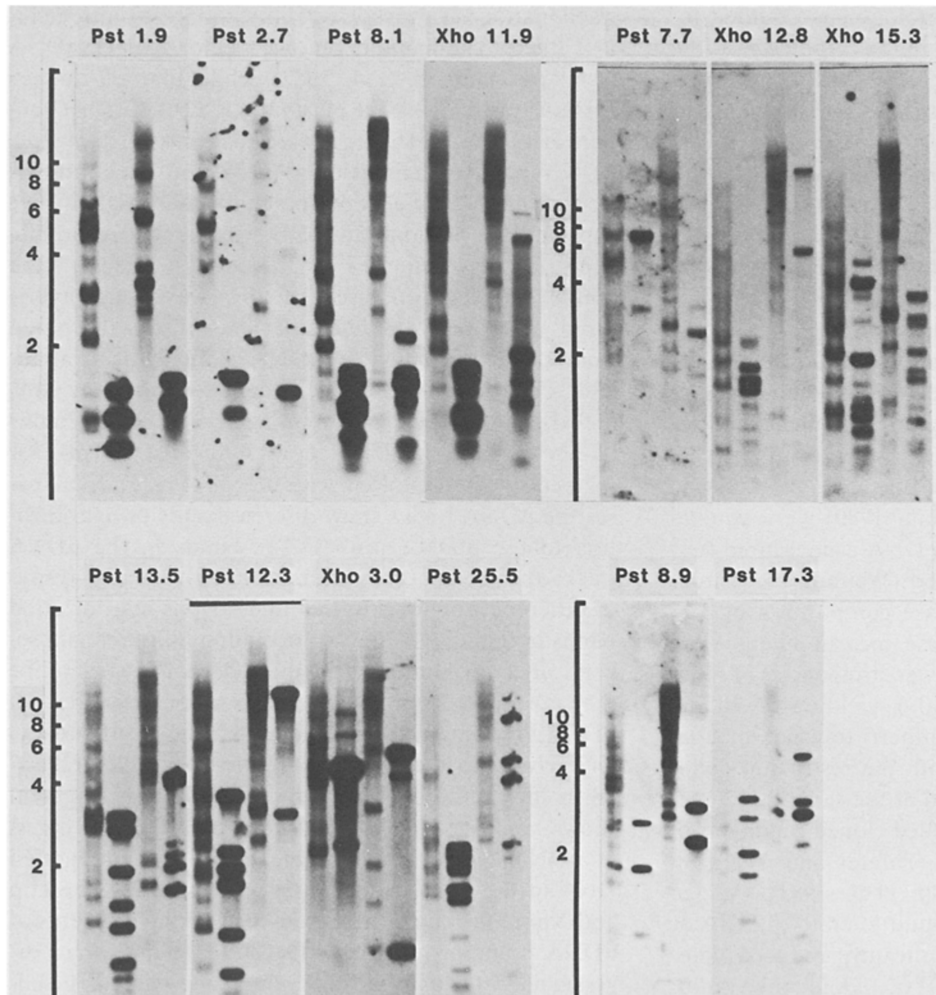

Fig. 2. Homologies between ptDNA and nuclear DNA. The hybridization of each clone from a spinach ptDNA clone bank (Palmer and Thomson 1981) to nDNA and ptDNA digested with EcoRI and BamHI is shown. Each set of four gel tracks is labelled with the ptDNA clone used as probe and comprises from *left to right*. **1** Nuclear DNA digested with EcoRI; **2** Plastid DNA digested with EcoRI; **3** Nuclear DNA digested with BamHI and **4** plastid DNA digested with BamHI. The molecular weight scale on the left of the tracks is in kbp. All the nuclear tracks contain 5 μg of nuclear DNA. The ptDNA tracks digested with EcoRI, contain 6.1 ng for those hybridized with PstI 1.9, PstI 2.7, PstI 8.1, XhoI 11.9, PstI 13.5, PstI 12.3 XhoI 3.0 and PstI 25.5, 3.0 ng for those hybridized with PstI 7.7, XhoI 12.8 and XhoI 15.3 and 3.5 ng for those hybridized with PstI 8.9 and PstI 17.3. The equivalent ptDNA tracks digested with BamHI contain 5.0, 1.9 and 8.2 ng respectively

stome per genome. Direct comparison of the amount of hybridization in adjoining nDNA and ptDNA tracks allowed an estimate of the amount of homology between each probe and nDNA (Table 1). Estimates from both EcoRI and BamHI digestions of nDNA, indicate that the nDNA contains the equivalent of about five copies of the plastome per haploid nuclear genome with all probes represented to about the same extent. These measurements include the background "smear" of hybridization in the tracks which may be due to undigested and/or randomly sheared nDNA with specific homology to ptDNA. The possibility of the "smear" of hybridization being due to less specific homologies than those appearing in bands does not seem very likely as in most cases there is digestion of the nDNA homologies into specific bands of homology with either EcoRI or BamHI (Fig. 2).

It was possible that some of the hybridization observed may have been due to bacterial contamination of the nuclear preparations, though very few bacteria could be seen in nuclear preparations by Nomarski interference microscopy. After extensive washing, unfixed roots were homogenized in sterile water and the bacterial content of the homogenate estimated by serial dilution on nutrient agar plates to be 10^7 to 10^8 microorganisms per g fresh weight of roots. A mixed cultu-

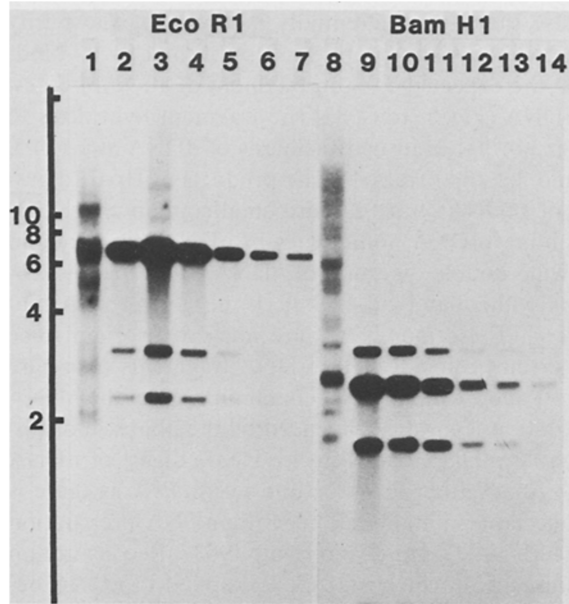

Fig. 3. Estimation of the amount of homology between ptDNA and nDNA. Spinach nDNA (5 μg) digested with EcoRI (*track 1*) and BamHI (*track 8*), and ptDNA digested with EcoRI (*tracks 2–7*) and BamHI (*tracks 9–14*) were transferred to nitrocellulose filters after agarose gel electrophoresis and hybridized with the 7.7 kbp PstI fragment of ptDNA. *Tracks 3–7* contain the equivalent of 14, 6, 1.5, 0.76 and 0.31 copies of the plastome per haploid nuclear genome respectively compared with the 5 μg of nDNA in *track 1* and *tracks 10–14* contain and 11, 5, 1.2, 0.62 copies of the plastome respectively (1 copy = 844 pg ptDNA). *Tracks 2* and *9* contained an unknown amount of ptDNA estimated to be 6 copies and 13 copies respectively by comparison with the calibrated tracks. The amount of hybridization to the EcoRI digestion of nDNA (*track 1*) is equivalent to 4 copies of the plastome and that to the BamHI digestion of nDNA (*track 8*) to 4.5 copies of the plastome per haploid nuclear genome

Table 1. Amount of hybridization of spinach ptDNA clones to spinach nuclear DNA

Spinach ptDNA fragment	No. of equivalent copies in nDNA digested with	
	EcoRI	BamHI
Xho 12.8	3.6	5.7
Xho 11.9	9.7	4.3
Xho 3.0	4.4	2.0
Xho 15.3	2.5	3.7
Pst 8.9	3.0	6.0
Pst 17.3	6.7	13.1
Pst 1.9	5.4	7.1
Pst 13.5	6.2	6.2
Pst 7.7	2.8	4.2
Pst 12.3	6.6	8.1
Pst 25.5	1.3	4.0
Pst 2.7	5.9	3.2
Pst 8.1	7.4	6.2
Mean	5.0	5.7

re of these cells was grown with ^{3}H-thymidine and added to a preparation of nuclei during the initial homogenization of the roots. The extent of contamination of the nuclei by bacteria was estimated by the yield of ^{3}H in the nuclear preparation and by direct counting of the bacteria in the preparations by Nomarski microscopy. The yield of bacteria from the preparation was 0.21% as estimated by radioactivity and 0.29% as estimated by microscopy, while the yield of nuclei estimated by DNA measurement was 12%. Assuming 2.4×10^{-14} g DNA per bacterium (four times the size of the *E. coli* genome) the maximum amount of bacterial DNA per 5 μg of nDNA would be 0.01 μg. Figure 4 shows BamHI and EcoRI digests of 10 times this amount (0.1 μg per track) of DNA from a mixed root bacteria culture and BamHI digests of an isolate of *E. coli* (maintained in this laboratory for many years) after Southern transfer and hybridization to ptDNA probes in the same bags as the relevant probes in Fig. 2. There is homology to both bacterial DNA samples, but the three homologous bands hybridized in *E. coli* DNA are the same irrespective of the probe used, as are the three homologous bands hybridized in the bacterial DNA. These bands do not correspond to any of the bands in the nDNA digests shown in Fig. 2. In the examples shown in Fig. 4 the cloned PstI 7.7 kbp ptDNA fragment was in the plasmid pBR322 while the XhoI 13.5 kbp probe includes a different vector, pACYC 177. The most likely explanation of this homology is that the plasmid part of the ptDNA clones has homology with some large endogenous plas-

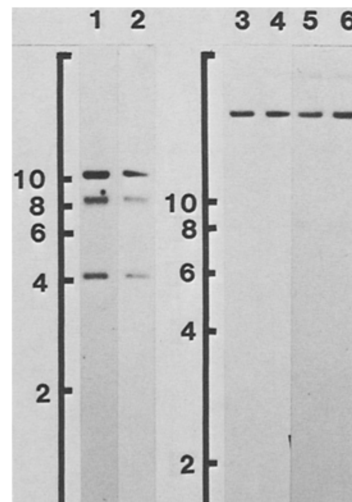

Fig. 4. Hybridization of ptDNA clones to bacterial DNA. *E. coli* DNA (*tracks 1 and 2*) or DNA from a mixed culture of bacteria from spinach roots (*tracks 3–6*) was digested with BamHI (*tracks 1, 2, 4 and 6*) or EcoRI (*tracks 3 and 5*). After electrophoresis and Southern transfer the filters were hybridized with the ptDNA fragments PstI 7.7 (cloned in pBR322; *tracks 1, 3 and 4*) and XhoI 13.5 (cloned in pACYC 177; *tracks 2, 5 and 6*)

mid(s) in the *E. coli* and the mixed bacterial culture DNA. These experiments show that it is extremely unlikely that any of the homologies we observe between nuclear and ptDNA can be due to contamination of the nuclei with bacteria from the root preparations. In addition it is likely that any bacterial DNA contamination of nDNA would be extensively reduced by the CsCl gradient purification procedure because of the high G + C content of most bacterial DNAs compared with nDNA.

The high degree of methylation of spinach nDNA (Scott and Possingham 1980) and the apparent lack of methylation in ptDNA (Smillie and Scott 1969) offers the possibility of distinguishing between sequences of different origins independently of methods using partial purification of the individual organelles. The restriction enzymes HpaII and MspI are able to cleave DNA at C/CGG but HpaII is unable to do so when the internal cytosine is methylated and MspI when the external cytosine is modified (Doerfler 1982). Spinach ptDNA is cleaved into small fragments by HpaII and MspI digestion but nDNA is relatively insensitive to MspI and HpaII (Timmis and Scott 1983). The hybridization of the XhoI 3.0 kbp ptDNA fragment to nDNA and root DNA digested with EcoRI and/or HpaII showed that nuclear and plastid sequences can be resolved because they are differentially methylated. The purity of the XhoI 3.0 kbp probe was established by its binding to the expected 3.0 kbp fragment of an XhoI digest of ptDNA (Fig. 5, track 3). The fragment hybridizes to four major bands in EcoRI digests of nDNA at 6.1, 4.5, 4.0 and 2.2 kbp (Track 1). The products of HpaII digestion of ptDNA (track 11) are smaller than the bands containing ptDNA homologies in nDNA (track 1) and following double digestion of nDNA with HpaII + EcoRI only the band at 4.5 kbp is no longer apparent (track 7). These four bands are nuclear in origin since they do not coincide with ptDNA fragments (compare tracks 1 and 2) and ptDNA contamination of the nDNA preparation is low, as evidenced by the almost indistinguishable ptDNA bands in the HpaII digest of nDNA (track 10). Neither are they due to mtDNA as there is no detectable contamination of the nDNA preparation with mtDNA (Timmis and Scott 1983). Reconstitution experiments in which ptDNA was added to nDNA before digestion (tracks 4, 9 and 12) and experiments with total root DNA (tracks 13 and 14) reinforce these conclusions. The three HpaII insensitive bands can be seen in HpaII + EcoRI digests of the mixed nDNA + ptDNA (track 9) and the total root DNA (track 13). An additional band at 1.8 kbp is apparent in HpaII digests of root

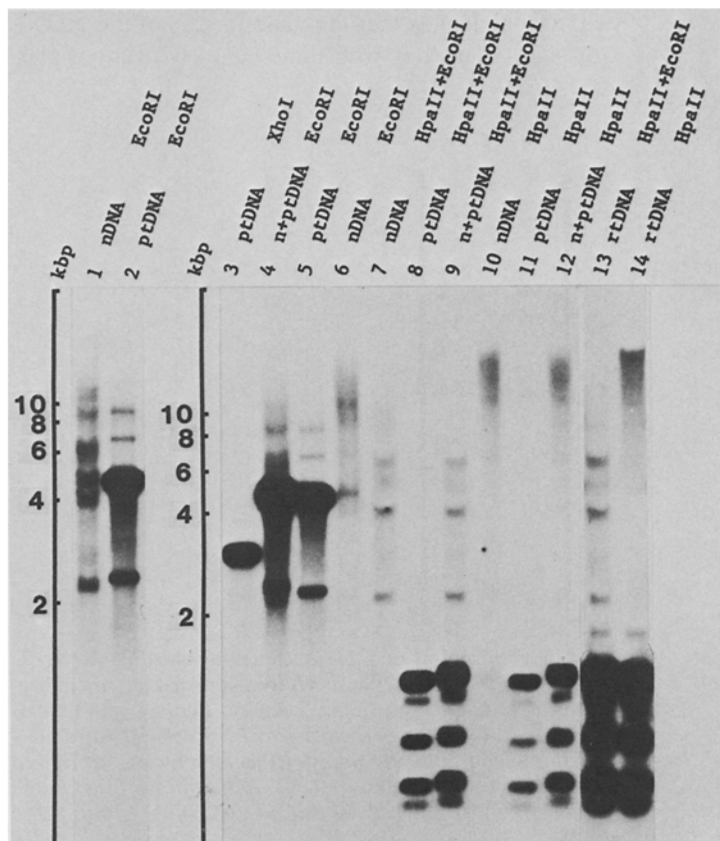

Fig. 5. Hybridization of nDNA and root (rt) DNA with the cloned XhoI 3.0 kbp fragment of ptDNA. Nuclear DNA (5 μg) and ptDNA (4 ng) were digested with the enzyme shown on the individual tracks. *Lanes 3–14* are all from the same gel and Southern transfer, while *lanes 1* and *2* are from a separate gel and are included because the nDNA EcoRI digestion in *lane 6* was only partially complete

DNA (tracks 13, 14) and this could represent an homology of the PstI 3.0 kbp fragment with mtDNA (Fig. 6 and Timmis and Scott 1983), or a low level of homology to ptDNA revealed because of the relatively high ptDNA content of root DNA compared with the amount of pure ptDNA loaded in Fig. 5, lanes 8, 9, 11 and 12.

The experiments described so far have, for the most part, used relatively large fragments of ptDNA to search for homologies with nDNA. Smaller EcoRI fragments of ptDNA contain the genes the large sub-unit of RuBP carboxylase (*pSocE48*) and the β sub-unit of ATPase (*pSocE40*) (Zurawski et al. 1981, 1982). Both of these genes show homologies to specific bands in EcoRI digests of nDNA (Fig. 6). The gene for the β subunit of ATPase shows homologies with nDNA bands of 5.7, 2.7 and 1.98 kbp (track 1) and the expected 1.98 kbp EcoRI fragment (Zurawski et al. 1982) of ptDNA (track 2). The large sub-unit of RuBP carboxylase shows homology to nDNA bands of 5.7 and 2.3 kbp (track 3) and the expected 1.75 kbp fragment of ptDNA (track 4). The nuclear sequence of 5.7 kbp (tracks 1 and 5) apparently shares homology with both genes. The nDNA bands homologous to *pSocE48* seen in track 3 can also be seen in the EcoRI digest of total root DNA (track 5) since the only ptDNA fragment to which the probe hybridizes is the low molecular weight 1.75 kbp fragment. In addition a higher molecular weight band of homology at 10.0 kbp ist visible. This band is not visible in track 3, possibly because of the poorer quality of the autoradiogram and because DNA prepared directly from roots is of higher molecular weight than that from nuclei, and therefore has better defined restriction bands, particularly those of high molecular weight. Double digestion of root DNA with either HpaII (or MspI) + EcoRI (track 6 and 8) removed the bands at 5.7 and 2.3 kbp and generated two new bands at 6.5 and 5.0 kbp, presumably by partial restriction of the band at 10 kbp (tracks 5, 6, 8). On the other hand, single digestion of root DNA with either HpaII or MspI (tracks 7 and 9) generated two additional bands of homology at 3.8 kbp and 2.8 kbp. As there is no sign of these bands in the double digests (tracks 6 and 8) it is clear that these homologies revealed by HpaII digestion of nDNA are from a different part of the genome from those generated by EcoRI digestion. The fragments resulting from EcoRI digestion of these homologies are probably obscured in tracks 5, 6 and 8 by the high level of hybridization to the 1.75 kbp ptDNA fragments in root DNA. The digestion of ptDNA with HpaII (track 11) or MspI (track 12) yielded single fragments of ptDNA larger than the EcoRI ptDNA fragments of *pSocE48* (tracks 4 and 10), and indicates that there are no HpaII sites in the gene. This is consistent with the sequence of the gene (Zurawski et al. 1981).

It is possible that some of the results observed may be due to homologies between ptDNA and mtDNA (Stern and Lonsdale 1982) contaminating the nDNA

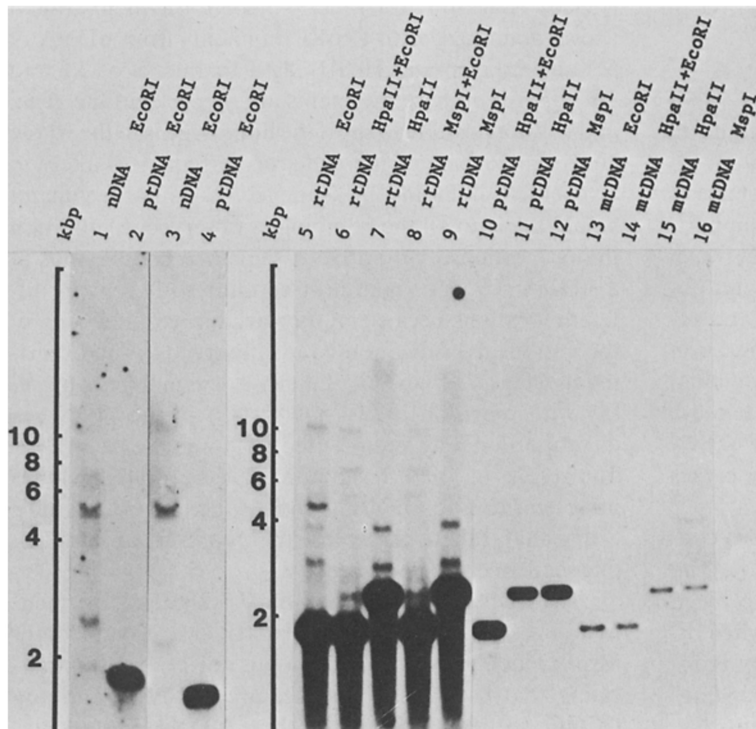

Fig. 6. Hybridization of nDNA, mtDNA and root DNA with specific cloned ptDNA genes. Nuclear DNA, rtDNA (5 µg), ptDNA (4 ng) and mtDNA (0.1 µg) were digested with the enzymes shown on the figure and, after agarose gel electrophoresis and Southern transfer, hybridized to the gene for the β subunit of ATPase (pSocE 40, *lanes 1* and *2*) or the gene for the large subunit of RuBP carboxylase (pSocE48, lanes *3–16*)

preparations or to the mtDNA undoubtedly present in the root DNA preparations. In our earlier studies (Timmis and Scott 1983) we showed that although the nDNA preparation was not contaminated with mtDNA the 7.7 kbp PstI fragment of ptDNA showed a strong homology to mtDNA, in both mtDNA preparations and in root DNA. The digestions of mtDNA shown in Fig. 6 (tracks 13–16) show that the ptDNA contamination of mtDNA is easily detected (compare the ptDNA bands in tracks 4, 10, 11 and 12 with the same bands contaminating tracks 13–16). In addition there are faint bands homologous to *PSocE48* in mtDNA not found in ptDNA, generated by digestion of mtDNA with EcoRI (track 13) and MspI (track 16) but not by HpaII (track 15). These restriction enzyme digests which reveal the presence of mtDNA/ptDNA homologies are sufficient to show that the strong sequence similarities in nDNA preparations to *pSocE48* are not of mtDNA origin. These low level homologies to mtDNA, which are barely visible even though there is 0.2 μg mtDNA per track, are not as strong as those previously reported by us for the 7.7 kbp PstI probe (Timmis and Scott 1983). The low level of mtDNA homology for *pSocE48* was not observed in studies which show widespread mt/ptDNA homologies in pea, bean, spinach and corn (Stern and Palmer, personal communication). The low level of homology also explains why mtDNA specific bands homologous with *pSocE48* are not visible in root DNA digests (tracks 5 and 9), in which homology of the 7.7 kbp PstI probe to mtDNA was easily detected (Timmis and Scott 1983).

Discussion

The ptDNA clones used in these experiments include all but 0.9 kbp of the 145 kbp spinach plastome and our results indicate that essentially all of the plastome has homologies with nuclear DNA. This conclusion is supported by the extent of the renaturation observed between ptDNA and nDNA (Fig. 1) and by the homologies observed between each cloned ptDNA fragment and restriction enzyme digests of nDNA (Fig. 2). In addition the comparative measurements made with individual ptDNA clones (Table 1) and the renaturation kinetics (Fig. 1) suggest that, on average the amount of nDNA homologous to ptDNA is the equivalent of five copies of the plastome.

This is not to say that sequences in the nucleus contain extensive unbroken homology with ptDNA. The nuclear sequences to which homologies have been found are of the order of 1.5–12 kbp in length when digested by EcoRI, though larger when digested by BamHI. In contrast, EcoRI digestion of ptDNA yields fragments no larger than 6.3 kbp, and many fragments less than 2 kbp. If long sections of homology to ptDNA were present in the nucleus we would expect to observe correspondence between at least some of the restriction bands in nDNA and ptDNA hybridizations for each probe, due to the inclusion of more that one specific restriction site in the homologies. In particular, when small probes are used, it is more likely that both nDNA and ptDNA homologies should be bounded by the same restriction enzyme sites. In these experiments the occurrence of homology bounded by the same restriction sites in nDNA and ptDNA is relatively rare and perhaps the only obvious possibilities are the fragments of 2.6 and 2.9 kbp in the EcoRI digests of nDNA and ptDNA hybridized to the PstI 13.5 kbp probe (Fig. 2). Thus, although most of the homologies observed are in nDNA fragments from 1.5 to 12 kbp it seems likely that any homology to ptDNA within these fragments is less than 2–3 kbp in length. While fragments of this length are only a small proportion of the plastome they are large enough to encompass whole genes. This distribution of homology in nDNA, together with the high degree of methylation of the cytosine residues in nDNA has been useful in facilitating the detection of the nDNA/ptDNA homologies by allowing resolution of the generally large nDNA fragments from predominantly small ptDNA counterparts. For the same reasons, however, it has not been possible to study smaller fragments of homology in nDNA because of confusion with small ptDNA fragments, as well as technical dificulties in analysing low molecular weight fragments.

The gene for the large sub-unit of RuBP carboxylase (Fig. 6) is within a 1.75 kbp EcoRI fragment, but it shows homology with EcoRI fragments from nDNA of 5.7 and 2.3 kbp and HpaII/MspI fragments of 3.8 and 2.8 kbp. All of these fragments are bigger than the gene, and it is not possible to say if the homology is to the whole gene, only a part of the gene, or perhaps less likely to a repetitive homologous sequence. A similar argument would apply to all the homologies observed. In addition the proportion of any ptDNA sequence homologous to a nDNA sequence is almost certainly different in different locations because of the variability of intensity of the various hybridization bands observed in the experiments (Figs. 2, 5 and 6). Likewise it is not possible to say with precision if any one section of the ptDNA is disproportionately represented in homologous nDNA. The results in Table 1 show that all the ptDNA clones used hybridize to nDNA to about the same extent, indicating that if there is any unequal representation of any one sequence it is not extensive.

The nDNA sites to which the 7.7 kbp PstI fragment hybridizes are not sensitive to HpaII, or its isoschizomer MspI (which can cut C/^{m}CGG but not ^{m}C/CGG), indicating that both the C residues are methylated in any CCGG sequences found in these nDNA homologies

(Timmis and Scott 1983). The same argument is true for at least three of the nDNA fragments (6.1, 4.0 and 2.2 kbp) to which the XhoI 3.0 kbp probe hybridizes, but the band at 4.5 kbp is sensitive to HpaII (compare Fig. 5, tracks 1 and 7). In the case of the gene for the large sub-unit of RuBP carboxylase (Fig. 6), HpaII generates nDNA bands homologous to the gene and also digests the nDNA EcoRI fragments homologous to ptDNA. The gene in ptDNA does not contain HpaII sites, but its homologues in nDNA are apparently located in areas of the nuclear genome where HpaII sites are not methylated. The nDNA sequences homologous to ptDNA therefore range widely in degree of methylation. There is a significant amount of evidence that DNA methylation plays an important role in the regulation of expression of DNA in many DNA functions, including transcription, recombination and repair (Doerfler 1981; Ehrlich and Wang 1981). No function can yet be ascribed to the ptDNA homologies in nDNA but it may be important that different areas of homology appear to be methylated to different extents.

The origin of plastids and of ptDNA has been the object of extensive speculation over many years, and the view that chloroplasts have an endosymbiotic origin is widely held (Stanier 1970; Taylor 1979). It has been pointed out that many of these suggestions do not explain how such a large proportion of the DNA involved in chloroplast biosynthesis came to be located in the nucleus, while relatively little of the information is located in the plastome (Scott and Possingham 1982), albeit in very high copy numbers (Scott and Possingham 1980). Our results show that the nucleus contains additional sets of ptDNA which may duplicate the information in the entire plastome. Potentially, therefore, the nucleus posesses all the genes required to make functional chloroplasts, although they may be in an interrupted, highly methylated and perhaps inactive form. These nDNA sequences may represent recent, or remnants of ancient, recombinations between nDNA and ptDNA (or ancestral ptDNA), supporting the notion of a multiorganism origin for the functionally co-operative genetic systems now extant in the plant cell. The observations do not allow us to decide in which direction the sequences have translocated and so do not support any particular one of the many hypotheses for the endosymbiont origin of plastids.

Studies of homologies between ptDNA and mtDNA in mung bean, pea and corn have schown extensive homology between mtDNA and ptDNA (Stern and Lonsdale 1982; Stern and Palmer, personal communication). Together with our results, showing homology between nDNA and ptDNA, and those showing homology between mtDNA and nDNA in a range of species (Farrelly and Butow 1983; Gellisen et al. 1983; Wright and Cummings 1982) it seems likely that nDNA may contain homologies to all organellar DNA.

Nuclear and ptDNA are separated from one another by four membranes. The nuclear membrane with its open pores offers no serious barrier to DNA movement since RNA molecules pass through it. The double chloroplast and mitochondrial membranes however are substantial barriers to communication between the nDNA and ptDNA in mature cells, but it is possible that the barrier may be breached or weakened at some particular developmental stage. Communication between organellar DNA's may involve such processes as physical contact between organelles and non-specific uptake and incorporation of DNA. Alternatively, there could be specific transfer systems involving cytoplasmic vectors analogous to plasmids, transposing elements or viruses which modify the host genome.

These observations may also bear upon the mechanisms whereby nuclear gene mutations cause temporary or permanent changes in the plastome (Kirk and Tilney-Bassett 1978) and the mechanism which maintains the homomogeneity of ptDNA in chloroplast populations within a plant species. The possibility of genetic transfer between organelles has far reaching implications for understanding plant function and for hopes of manipulating plant genotypes.

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References

Bennett MD, Smith JB, Heslop-Harrison JS (1982) Nuclear DNA amounts in angiosperms. Proc R Soc, London Ser B 216:179–199

Bohnert HJ, Crouse EJ, Schmitt JM (1982) Organization and expression of plastid genomes. In: Parthier B, Boulter D (eds) Encyclopaedia of plant physiology, vol 14 B. Springer, Berlin Heidelberg New York, pp 475–530

Burton K (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323

Chang ACY, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134:1141–1156

Curtis SE, Rawson JRY (1982) Nucleotide sequence homology exists between the chloroplast and nuclear ribosomal DNAs of *Euglena gracilis.* Plant Physiol 69:67–71

Doerfler N (1982) DNA methylation – a regulatory signal in eukaryotic gene expression. J Gen Virol 57:1–20

Douce R, Moore AL, Neuberger M (1977) Isolation and oxidation properties of intact mitochondria isolated from spinach leaves. Plant Physiol 60:625–628

Duggleby RG, Kinns H, Rood JI (1981) A computer program for determining the size of DNA restriction fragments. Anal Biochem 110:49–55

Godsen GN, Vapnek D (1973) A simple method of preparing large amounts of $\varphi\chi$ 174RF1 supercoiled DNA. Biochim Biophys Acta 299:516–522

Ehrlich M, Wang RYH (1981) 5-methyl cytosine in eukaryotic DNA. Science 211:1350–1357

Farrelly F, Butow RA (1983) Rearranged mitochondrial genes in the yeast nuclear genome. Nature 301:296–301

Gellissen G, Bradfield JY, White BN (1983) Mitochondrial DNA sequences in the nuclear genome of a locust. Nature 301:631–634

Ingle J (1979) Plant ribosomal RNA genes – a dynamic system. In: Rubinstein M (ed) molecular biology of plants. Academic Press, London New York, pp 139–164

Ingle J, Possingham JV, Wells R, Leaver CJ, Loening VE (1969) The properties of chloroplast ribosomal RNA. In: Miller PL (ed) Control of organelle development, SEB Symposium 24. Cambridge University Press, Cambridge, pp 303–325

Kirk JTO, Tilney-Bassett RAE (1978) The plastids, 2nd edn. Elsevier/North Holland Biomedical Press, Amsterdam
Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325
Palmer JD, Thompson WF (1981) Clone banks of the mung bean, pea and spinach chloroplast genomes. Gene 15:21–26
Possingham JV, Smith JW (1972) Factors affecting chloroplast replication in Spinach. J Exp Bot 23:1050–1059
Schachat FH, Hogness DS (1974) Repetitive sequences in isolated Thomas circles from *Drosophila melanogaster.* Cold Spring Harbour Symp Quant Biol 38:371–481
Scott NS (1973) Ribosomal RNA cistrons in *Euglena gracilis.* J. Mol Biol 81:327–336
Scott NS, Possingham JV (1980) Chloroplast DNA in expanding spinach leaves. J Exp Bot 123:1081–1092
Scott NS, Possingham JV (1982) Leaf development. In: Smith H, Grierson D (ed) The molecular biology of plant development. Blackwell, Oxford, pp 223–255
Scott NS, Smillie RM (1967) Evidence for the direction of chloroplast ribosomal RNA synthesis by chloroplast DNA. Biochem Biophys Res Commun 28:598–603
Smillie RM, Scott NS (1969) Organelle biosynthesis: the chloroplast. In: Progress in molecular and subcellular biology, vol I. Springer, Berlin Heidelberg New York, pp 136–202
Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
Stanier RY (1970) Some aspects of the biology of cells and their possible evolutionary significance. In: Charles HP, Knight BCJG (eds) Organization and control in prokaryotic and eukaryotic cells. 20th Symp Soc Gen Microbiol. Cambridge University Press, London, pp 1–38
Stern DB, Lonsdale DM (1982) Mitochondrial and chloroplast genomes of maize have a 12 kilobase DNA sequence in common. Nature 299:698–702
Taylor FJR (1979) Symbionticism revisited: a discussion of the evolutionary impact of intracellular symbiosis. Proc R Soc, London Ser B 204:267–286
Tewari KK, Wildman SC (1968) Function of chloroplast DNA. 1. Hybridization studies involving nuclear and chloroplast DNA with RNA from cytoplasmic (80S) and chloroplast (70S) ribosomes. Proc Natl Acad Sci USA 59:569–578
Timmis JN, Scott NS (1983) Spinach nuclear and chloroplast DNAs have homologous sequences. Nature 65–67
Wetmur JG (1976) Hybridization and renaturation kinetics of Nuclei acids. Annu Rev Biophys Bioeng 5:337–361
Wright RM, Cummings DJ (1983) Integration of mitochondrial gene sequences within the nuclear genome during senescence in a fungus. Nature 302:86–88
Zurawski G, Bottomley W, Whitfeld PR (1982) Structures of the genes for the β and ε subunits of spinach chloroplast ATPase indicate a dicistronic mRNA and an overlapping translation stop/start signal. Proc Natl Acad Sci USA 79:6260–6264
Zurawski G, Perrot B, Bottomley W, Whitfeld PR (1981) The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res 9:3251–3270