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Molecular cloning of spinach chloroplast DNA isolated by alkaline lysis

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AN ABSTRACT OF THE THESIS OF Robert Gray Drager for the
Master of Science in Biology presented February 27, 1987.

Title: ^uMolecular Cloning of Spinach Chloroplast DNA Isolated
by Alkaline Lysis.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

John W. Myers, Chairman

Malcolm S. Lea

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John H. Golbeck

Chloroplast genomes of land plants show conservation of structure and gene arrangement. The spinach chloroplast genome is comprised of a covalently closed, circular DNA molecule of 150 kilobases and is typical of these plants. Approximately 20% of the proteins found in the spinach chloroplast are encoded by the chloroplast genome and translated on chloroplast ribosomes. The remainder are

encoded on chromosomes in the nucleus, translated on cytoplasmic ribosomes and transported into the chloroplast.

Spinach chloroplast DNA was isolated from crude chloroplast preparations by a new method. Chloroplasts were lysed with alkaline sodium dodecyl sulfate, contaminating macromolecules precipitated with acidified potassium acetate and plastid DNA was purified by phenol:chloroform extraction and ethanol:ammonium acetate precipitation. The yield was approximately 50 ug chloroplast DNA per 100 grams leaf material. The DNA consisted of 10% circular molecules and 90% linear molecules.

The chloroplast DNA was digested with restriction enzyme PstI and the fragments were cloned into the plasmid vector pUC9. Several recombinant plasmids were isolated and the chloroplast DNA inserts identified. The recombinant plasmid pRD105 containing the PstI #5 fragment was subjected to further investigation. The ClaI restriction sites of the PstI #5 fragment were mapped and the insert was subcloned into the plasmid vector pGEM4, which bears bacteriophage SP6 and T7 RNA polymerase promoter sequences.

MOLECULAR CLONING OF SPINACH CHLOROPLAST DNA
ISOLATED BY ALKALINE LYSIS

by

ROBERT GRAY DRAGER

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
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1987

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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INTRODUCTION

Chloroplasts of eukaryotic photoautotrophs have been extensively studied as the sites of conversion of sunlight to chemical energy, fixation of CO_2 to organic carbon and production of molecular oxygen. Investigation of the biochemical processes of the Calvin cycle and the photosynthetic apparatus continue at a rapid pace, but since chloroplasts were shown to contain their own genetic material (55) and protein synthesizing machinery (46), few areas of plant biology have been pursued as enthusiastically as chloroplast molecular genetics.

Characterization of the chloroplast genome by electron microscopy, renaturation kinetics and CsCl buoyant density centrifugation (34, 41, 42) showed the gross structure to be a supercoiled, circular, double-stranded DNA molecule with 37% G-C content and no highly repetitive sequences. This genome structure is similar to that of prokaryotes and quite different from the linear, histone-containing nuclear chromosomes. Other similarities between chloroplasts and prokaryotes are the size of the ribosomes (70S) and the antibiotic sensitivities of the ribosomes and RNA polymerases. These similarities support the endosymbiotic hypothesis of chloroplast and mitochondria evolution

supported by Margulis (48), namely that these subcellular organelles were once free-living organisms similar to modern prokaryotes, and became incorporated into cells with the characteristics of modern eukaryotes.

These organelles are not capable of autonomous survival since approximately 80% of the organelle proteins are encoded by nuclear genes, synthesized on cytoplasmic ribosomes and transported into the organelle (38). In the endosymbiotic hypothesis this situation is presumed to have arisen by the net transfer of genetic material from the organelle to the nucleus. Evidence for transfer of genetic material between cellular compartments is provided by the existence of homologous DNA sequences in chloroplasts, mitochondria and nuclei of higher plants (19, 20, 61, 62). The net transfer of DNA has probably been from the chloroplast to the nucleus as suggested by the small size of the chloroplast genome (100-300 kilobases) compared to that of modern prokaryotes (approximately 4000 kilobases). The size of the plant mitochondria genome is much more variable, 100-1000 kilobases (kb). Another hypothesis, supported by Bogorad (7), is that the organelles arose as buds from the nuclei of ancestral cells and the net transfer of genetic material has been from the nucleus to the organelle. The fact that there is a high degree of homology between chloroplast genes and proteins and those of Escherichia coli is powerful evidence for the endosymbiotic hypothesis.

Whatever the evolutionary history of chloroplasts, the further characterization of the structure and regulation of the chloroplast genome is an important and promising goal. Much of the promise is imparted by the small size of the chloroplast genome which facilitates mapping and sequencing. Chloroplast DNA can be isolated and clone banks of some species have been established (50). This allows for the detailed investigation, by sequencing and other methods, of the way genes of the chloroplast are coordinated with and controlled by the nucleus in response to environmental stimuli. The importance of understanding the biochemical processes that occur in chloroplasts cannot be overemphasized and there is potential for genetic modification of these processes (10). Examples of such modifications include increasing the efficiency of CO_2 fixation by decreasing the affinity of ribulose 1,5-bisphosphate carboxylase for O_2 , modifying proteins that are the binding sites of herbicides and introducing prokaryotic genes to perform such functions as N fixation. At this time, the level of knowledge of chloroplast molecular genetics is considerable, many ribosomal RNA, transfer RNA and protein genes have been mapped and sequenced, but the mechanisms of control remain completely undefined. Significant understanding of these control processes is necessary to implement genetic manipulation strategies that could produce benefits in terms of crop improvements, disease resistance and pest control.

The most striking feature the of chloroplast DNA of higher plants is its uniformity. Almost all chloroplasts so far examined contain circular DNA molecules from 100-300 kb in size. In any species each chloroplast has a homogeneous population of identical DNA circles characteristic of that species (reviewed in 4, 18, 24, 37, 53, 71). Land plants show a high degree of conservation in both genome size and gene arrangement. The same chloroplast genome structure has now been demonstrated in the bryophytes (Marchantia), ferns (Osmunda) and gymnosperms (Ginkgo), (52), as well as the angiosperms. The size range is 120-220 kb and nearly all show the characteristic inverted repeat containing the ribosomal RNA genes. These repeat regions separate the genome into large and small single copy regions (Fig. 1). The only exceptions are some legumes which have lost one of the repeat units and show a reduced genome size (120 kb), (51, 60).

Only in the green algae is there significant variability in chloroplast genome size (reviewed in 53). The species Acetabularia mediterranea and A. cliftonia have been shown to have complexity of 2000 kb by renaturation kinetics and form linear molecules up to 600 kb. Other algal groups seem to conform to the usual size and arrangement parameters with the possible exception of some species of brown algae which have been reported to contain heterogeneous circular DNA populations (53).

Figure 1. Structure of spinach chloroplast genome with ribosomal RNA genes.

In the angiosperms, the chloroplast genome has been best characterized in the genera Zea (29, 61), Nicotiana (21, 22, 57), Brassica (44), Pisum (12, 41, 42), Spirodela (30, 31), Vicia (27, 28, 58) and Spinacia (16, 17, 35). Spinach has been arbitrarily designated as the archetype. The arrangements of the other well characterized genera can be related to spinach by inversions, additions and deletions.

In 14-day spinach leaves, plastid DNA comprises approximately 22% of the total cellular DNA. There are approximately 100 chloroplasts per cell and 88 plastid genomes per chloroplast. In 24-day leaves, the plastid DNA is 18% of the total, there are 500 chloroplasts per cell and 24 plastid genomes per chloroplast (56). Electron microscopy suggests that chloroplast DNA replicates in a two step process (43). The first step is the formation of Cairns replivative forked structure and the second step is rolling circle replication. It was proposed that the Cairns mechanism is used for normal DNA duplication and the rolling circle mechanism dominates when rapid DNA synthesis is necessary (43).

The 150 kb spinach chloroplast genome is divided into four distinct regions. Two 24 kb inverted repeats separate the 82 kb (large) and 20 kb (small) single copy regions (Fig. 1). The restriction enzymes SalI and PstI cleave the circular molecule into 11 and 10 fragments, respectively

Figure 2. PstI (◆) and SalI (▼) restriction sites of spinach chloroplast genome.

(Fig. 2). Transfer RNA genes have been mapped (16) and show a dispersed arrangement. Twenty-one of the thirty-two tRNA species necessary to utilize all possible codons have been identified, and several have been sequenced (5). The tRNAs for Asp, Cys, Glu and Gln have not yet been found on the spinach chloroplast genome. Some identified tRNA genes have been localized only approximately. The ribosomal RNA genes are located in the inverted repeat regions (Fig. 1). The gene arrangement is similar to that of E. coli with the addition of a 4.5S species between the 5S and 23S rRNA genes (reviewed in 13, 18).

Although an estimated 80% of the proteins functioning in the spinach chloroplast are transcribed from nuclear genes, translated on cytoplasmic ribosomes and transported into the organelle (18, 23, 24) twenty-nine protein genes have been mapped in the chloroplast genome. These include functional proteins in each of the four supramolecular complexes of the photosynthetic membrane, as well as soluble proteins of the Calvin cycle and maintenance proteins. Most of these genes and their flanking regions have been sequenced, and show sequences similar to the -10 and -35 promoter sequences of prokaryotes, although the positions of the promoter regions is variable, the spacing between the promoter regions is consistently 15-19 base pairs (bp), (reviewed in 71).

Survey of Spinach Chloroplast Protein Genes

The gene psbA, coding for the 32 kilodalton (kd) herbicide binding protein of photosystem II has been localized to the SalI fragment #6 in the large single copy region adjacent to the right inverted repeat, (17), (Fig. 3). The sequence analysis of this gene (74) showed the gene was comprised of 353 codons resulting in a protein with a derived molecular weight (MW) of 38.95 kd. The nucleotide sequence shows 95% homology to that of Nicotiana debneyi and complete amino acid homology. Transcription is counter-clockwise (as the genome is conventionally displayed) and there is a prokaryotic promoter sequence approximately 90 bp upstream of the first ATG start signal. Also present in the 5' flanking region is the putative ribosome binding sequence AAGA at position -10. A sequence in the 3' flanking region is capable of forming a stable stem-loop structure with a free energy of formation (ΔG) of -34.9 kilocalories (kcal).

The psbB gene coding for the 51 kd p680 reaction center chlorophyll-a apoprotein of photosystem II is located on the SalI #7 fragment (67). The sequence data (49) show an open reading frame of 508 codons and a protein with a derived MW of 56.2 kd. The gene is transcribed toward the nearest (left) inverted repeat and thus in the same direction as the adjacent genes petB and petD. Promoter sequences are present in the 5' region and a stem-loop

Figure 3. Map of spinach chloroplast genome protein genes.

sequence ($\Delta G = -41.8$ kcal) is 300 bp downstream of the TAA stop signal of psbB.

Genes psbC and psbD coding for the 44 kd and D2 proteins of photosystem II, respectively, are located on PstI fragment #4 (67). Sequence analysis (3, 39) shows that the coding regions overlap by 50 bp, although the reading frames are different. The psbD open reading frame consists of 353 codons resulting in a derived protein of 39.6 kd. This protein is 97% homologous to that of pea and the 160 bp 5' to the ATG codon is 76% homologous to pea. The psbC gene consists of 473 codons and begins 50 bp prior to the TAA stop signal of psbD. The derived MW is 51.8 kd and the amino acid sequence is 96% homologous to pea. The genes psbC and psbD are apparently cotranscribed to a 3.5 kb RNA species. Transcription is counter-clockwise toward the PstI site between PstI #4 and #8 and 21 codons of psbC are within the PstI #8 fragment (39).

The protein of cytochrome b-559 of photosystem II is thought to consist of two polypeptides coded by genes psbE and psbF (9, 36, 70). These genes are located on the SalI #3 fragment and transcribed toward SalI #2. The sequence shows psbE to have 83 codons and a derived protein MW of 8.0 kd. Promoter and ribosome binding sequences are present, but stem-loop sequences are absent. Gene psbF has 39 codons, derived protein MW of 4.3 kd and 97% homology to Nicotiana and Oenothera. In spinach the reading frame is

shifted from that of psbE and the ATG (methionine) codon follows the psbE TAG stop signal by 8 bp.

Genes psaA and psaB code for two P700 chlorophyll-a apoproteins of photosystem I. These genes are located on PstI fragment #8 (68) and sequence data (40) show two open reading frames of 750 and 734 codons transcribed clockwise toward psbC and psbD. These open reading frames are separated by a 25 bp spacer and the proteins of derived MW of 83 kd and 82.4 kd are 95% homologous to those of Zea. There are promoter sequences approximately 150-170 bp 5' to the ATG start signal of psaA and a stem-loop sequence ($G=-9.8$ kcal) 50 bp 3' to the TAA stop signal of psaB. These genes are followed closely by the rps14 gene for ribosomal small subunit protein 14. This 100 codon open reading frame follows 121 bp 3' to psaB. This third gene is transcribed in the same direction as the other two and is followed by a very stable stem-loop sequence ($\Delta G=-41.4$ kcal). Each of the three genes is preceded by the possible ribosome binding sequence AGGAGG. This structure suggests a gene group that is transcribed as a polycistronic message.

Gene petA codes for cytochrome *f* and is located on SalI fragment #3 (2). This may be the only spinach chloroplast coded protein that is translated as a precursor with an N-terminal signal sequence to facilitate insertion of the polypeptide through the thylakoid membrane. This

conclusion was based on the observations that in vitro translation systems produce a protein of 35.3 kd, while the protein isolated from chloroplasts is apparently 4 kd or 35 amino acids smaller. The possible ribosome binding sequence GGA is just upstream from the only methionine codon and the putative signal sequence is similar to that of known signal sequences (2). This gene of 320 codons (including N-sequence) is transcribed counter-clockwise toward the psbE and psbF genes. No clear promoter or stable stem-loop sequences have been identified.

Genes petB and petD coding for cytochrome b and subunit 4 are located on the SalI #8 fragment. Sequence data (32) show the 211 codon petB gene is followed by a 1018 bp spacer prior to the 139 codon petD gene. The two genes are transcribed counter-clockwise, in the same direction as the nearby gene psbB. PetB and petD are in different reading frames and the derived protein molecular weights are 23.7 kd and 15.2 kd, respectively. Each gene is preceded by AAAG ribosome binding sequences and a stem-loop sequence ($\Delta G = -26.0$ kcal) follows the TAA stop codon of petD by 30 bp.

Genes atpB and atpE coding for the β and ϵ subunits of the ATP synthase complex are located on the SalI #3 fragment and transcribed clockwise, away from gene rbcl (66). Sequence data (75) show atpB consists of 498 codons with a derived protein MW of 53.9 kd. This open reading frame

overlaps by four codons the 153 codon gene atpE, which is read in a different frame and results in a protein of 14.7 kd derived molecular weight. The amino acid sequences of the β -subunits of the ATPase complex from E. coli and spinach chloroplast are 67% homologous.

Genes for the ATPase subunits CFo-IV, proteolipid, CFo-I and CF - α , atpI, atpH, atpF and atpA, respectively, are grouped on the SalI #10 and SalI #4 fragments (69). These genes are transcribed clockwise toward the right inverted repeat in the above order. The sequence of atpI (33) shows an open reading frame of 247 codons and a protein of 27 kd derived MW. This gene is preceded by promoter sequences at -150 and -115 and a GAGG ribosome binding site is at -9. A sequence 300 nucleotides downstream of the TGA stop codon is capable of forming a stem-loop structure. The resultant protein is 28% homologous to E. coli ATPase subunit-a. A 692 bp spacer follows the stop codon, followed by the 81 codon atpH gene for the proteolipid protein (1). This gene is immediately preceded by a GAGG sequence as well as promoter sequences at positions -100 and -50. This open reading frame encodes a protein with derived MW of 7.9 kd 100% homologous to that of wheat. Stem-loop sequences follow at 10 bp and 200 bp 3' to the TAA stop codon. Three hundred bp 3' to the stop codon of atpH, begins gene atpF, which codes for coupling factor CFo-I. This gene is preceded by the GAGG ribosome binding sequence but not by a

promoter sequence (33). The atpF gene is the only interrupted gene coding for a protein in the spinach chloroplast genome. The first 56 codons are followed by a 714 bp non-translated intron, the coding region then continues for 201 more codons and the protein has a derived MW of 20.9 kd. This intron displays all features of class II introns (33). The gene atpA begins 65 bp distal to the atpF stop codon and has not been completely sequenced.

The gene rbcL for the large subunit of ribulose 1,5-bisphosphate carboxylase is located at the junction of PstI #5 and PstI #2. The sequence (73) shows the direction of transcription is counter-clockwise toward the left inverted repeat, away from the atpB-atpE operon. Promoter sequences are present, as is the GAGG ribosome binding sequence. The open reading frame is 475 codons, resulting in a protein of 52.7 kd and is followed by a stem-loop sequence ($\Delta G = -16 \text{ kcal}$). The amino acid sequence is 90% homologous to the Zea protein.

Several ribosomal protein genes have been sequenced, rps4, rps11, rps14, rps19, rp12 and two others have been located by other techniques, rp124 and rp125. The gene for the ribosomal small subunit protein 4, rps4, is located on SalI fragment #2 near the junction with SalI #3 and transcribed clockwise. The sequence (5) shows 201 codons and a protein of 22.9 kd derived MW. Promoter and ribosome binding sequences precede the open reading frame and a

stem-loop sequence ($\Delta G = -16.6$ kcal) follows 100 bp after the TAG stop codon. The protein shows 77% homology to Zea and 39% to E. coli.

The sequence of gene rps11 (59), coding for ribosomal small subunit protein 11, is located on SalI #8 and transcribed clockwise. This gene contains 138 codons for a derived protein of 14.9 kd that is 52% homologous to a ribosomal protein of E. coli. Evidence suggests this gene is cotranscribed with infA and rpoA (see later section).

Gene rps14, located on PstI #8, consists of 100 codons and the protein has a derived MW of 12 kd (40). This gene follows the psaB stop codon by 121 bp and is apparently cotranscribed with psaA and psaB. The AGGA ribosome binding sequence precedes the open reading frame and a stem-loop sequence ($\Delta G = -41.4$ kcal) follows the TAA stop codon by 50 bp. The amino acid sequence is 37% homologous to a ribosomal protein of E. coli.

The genes for ribosomal proteins small subunit 19 and large subunit 2, rps19 and rp12, are located at the junctions of the large single copy region and the left and right inverted repeats (76). Gene rp12 is completely contained within the inverted repeat and is thus represented twice, once on each SalI #5 fragment. This gene has 286 codons and the protein has a derived MW of 31.8 kd. The stop codon TAA is 21 bp before the start of rps19. This gene spans the junction of the inverted repeat, large single

copy region and only the open reading frame on the left side has homology to Nicotiana debneyi. This 92 codon gene and the derived 10.6 kd protein is 89% homologous to that of N. debneyi and 58% homologous to the E. coli S19 protein. The gene spanning the right inverted repeat large single copy junction, dubbed rps19', is identical to rps19 for the first 48 codons (within the repeat) but the remaining 66 codons are divergent. The derived protein has a MW of 12.5 kd, while a stop codon shortens rps19' in N. debneyi to an open reading frame coding a protein of 3.1 kd. Computer search has shown no E. coli genes with significant homology to rps19' and the function of this gene remains unknown. As the 5' flanking regions for rps19 and rps19' are identical, they are presumably expressed with equal efficiency.

Two other ribosomal protein genes, rp124 and rp125, have been localized to the SalI #5 fragments of the inverted repeats by cell free transcription-translation and immunoprecipitation (14). No sequence or specific mapping has been reported for these genes. The proteins, large ribosomal subunit 24 and 25 have apparent MW of 13.5-16 kd and 13-14.5 kd, respectively, as determined by polyacrylamide gel electrophoresis.

Other proteins for which the genes have been sequenced include initiation factor I and the α -subunit of RNA polymerase (59). These genes are located on PstI #2 near the junction with PstI #9, and are cotranscribed clockwise

with rps11. The gene rps11 is located between infA and rpoA. Gene infA consists of 77 codons resulting in a derived protein of 9.1 kd, which is 43% homologous to the E. coli IF-1 protein. Gene rpoA has 335 codons and the protein a derived MW of 38.4 kd. This gene cluster, infA, rps11, rpoA is followed by a stem-loop sequence ($\Delta G = -28.2$ kcal) which could also serve as a transcription terminator for the petB-petD transcription unit.

The gene for elongation factor Tu, tufA, has been localized to PstI #4 of spinach chloroplast genome by hybridization (65), but has not been sequenced.

In addition, fourteen chloroplast thylakoid membrane protein genes and one soluble protein (ribulose 1,5-bisphosphate carboxylase small subunit) gene which reside on nuclear chromosomes have been characterized (63).

The important features revealed by detailed sequence analysis are the conservation of gene arrangement and protein homology among the chloroplasts of land plants, and the similarities to prokaryotic systems. Selective forces could constrain both the size of the chloroplast genome and gene arrangement. Amino acid sequence conservation of 100% often occurs between monocotyledons and dicotyledons and there is significant homology from the bryophytes through the angiosperms. Factors that influence this conservation must include the putative control of the chloroplast by the nucleus. This particular chloroplast genome arrangement may

be necessary for the coordinated expression of the proteins comprising the photosynthetic apparatus. The similarity of the genomes of the chloroplasts of land plants suggests the possibility of a founder effect, only a small population of organelles that assumed this particular genome arrangement survived. Alternatively, other arrangements may have developed but were at some selective disadvantage to the surviving form. That this consistency is the result of convergent evolution seems unlikely.

Evolution of these organelles from ancestors resembling prokaryotes is supported by the similarity seen in the 5' and 3' flanking sequences. The consistent presence of promoter, ribosome binding and terminator sequences in chloroplast genes similar to those of E. coli is strong evidence of a common ancestor. One of the main differences between chloroplast genes and those of eubacteria is the occurrence of intervening sequences in both tRNA and protein genes. This may be evidence against common ancestry, or it might be viewed as a secondary development resulting from the interaction between the organelle and the nucleus. Maybe as evolution continues introns will continue to develop in chloroplast genes and these genomes will become less prokaryotic in nature.

It has been shown that E. coli RNA polymerase will bind to spinach chloroplast DNA (72), although not all regions of the genome showed high affinity for the enzyme.

Spinach chloroplast RNA polymerase has been shown to be immunologically similar to the E. coli enzyme (45). Cloned chloroplast genes have been expressed in E. coli using the maxi-cell technique (21), and in vitro using a highly defined E. coli transcription/translation system (11). Not all chloroplast genes would be expected to be efficiently expressed in E. coli because of the variations in the promoter and terminator sequences. It has also been shown that not all spinach chloroplast tRNA genes require 5' promoter sequences for efficient in vitro transcription using a spinach chloroplast derived transcription system (25, 26).

This work develops a method for spinach chloroplast DNA isolation using the alkaline lysis technique of Birnboim and Doly (6). The goal of chloroplast DNA isolation is to purify high molecular weight chloroplast DNA that is suitable for restriction enzyme digestion and molecular cloning. Chloroplast DNA need not be isolated in circular form for these manipulations, but must be relatively free from nuclear and mitochondrial DNA contamination and be of high molecular weight, 100-150 kb.

Traditional methods of chloroplast DNA isolation use one of three methods to reduce nuclear DNA contamination: DNase treatment, aqueous step-gradients (sucrose or percoll), or nonaqueous gradients (CCl_4 :hexane) to purify chloroplasts. Mitochondrial DNA contamination is not a

major problem because mitochondria are easily separated from chloroplasts by differential centrifugation.

Tewari and Kolodner (41, 42) separated a crude chloroplast pellet from cellular debris by centrifugation. The chloroplast pellet was treated with DNase to eliminate nuclear DNA. DNase was inactivated by protease digestion prior to organelle lysis by SDS. Lysed chloroplasts were extracted with phenol and the DNA precipitated with ethanol. Chloroplast DNA isolated in this manner was examined by electronmicroscopy, approx. 20% of the sample consisted of circular molecules. No restriction endonuclease digestion or electrophoretic analysis was performed.

Nuclear DNA contamination is caused by DNA from disrupted nuclei adhering to chloroplasts that have lost their outer membranes (54). Separating intact chloroplasts from broken ones by step-gradient centrifugation can decrease contamination by nuclear DNA. Aqueous systems use either sucrose (54) or percoll (L. Hanely-Bowdoin personal communication) gradients and nonaqueous systems utilize CCl_4 :hexane gradients (8). Once the chloroplasts are recovered from the gradient, they are treated with DNase and/or protease and phenol extracted. These methods are followed by CsCl density gradient centrifugation to further purify the chloroplast DNA.

The method of spinach chloroplast DNA isolation used in this work differs significantly from those just

described. Chloroplasts were concentrated from a leaf homogenate by centrifugation and contaminating nuclear DNA and proteins were denatured by lysis with alkaline SDS (6) and precipitated with acidified potassium acetate. Residual proteins were removed by phenol:chloroform extraction and RNA reduced by repeated ethanol precipitation in the presence of 2.5 M ammonium acetate. Treatment with DNase, protease, RNase and CsCl centrifugation were not necessary to recover moderate to good yields (10-15 ug/ml) of high molecular weight chloroplast DNA. This DNA was suitable for restriction endonuclease digestion, electrophoretic analysis and molecular cloning.

MATERIALS AND METHODS

The following bacterial strains and plasmids were used in this work;

Bacterial Strains

Strain or Plasmid
E. coli K-12
Strains

JM 109, JM 105

Relevant Properties

thi, rpsL, endA, sbcB15,
hspR4 (lac-proAB), F',
traD36, proAB, lacI ,
lacZ M15

Plasmids

pUC8
pUC9
pGEM3
pGEM4
PRD105
PRD108
PRD110
PRG105

Amp resistance
Amp resistance
Amp resistance, T7 promoter
Amp resistance, T7promoter
pUC9+PstI #5 insert
pUC9+PstI #8 insert
pUC9+PstI #10 insert
pGEM4+PstI #5 insert

The following abbreviations are used in this work;

amp	ampicillin
bp	base pairs
BPB	bromphenol blue
CIA	chloroform:isoamyl alcohol 24:1
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
F'	carrying the <u>E. coli</u> sex factor F, with a chromosomal insertion
HEPES	N-2-hydroxyethylpiperazine-N'-2
	ethanesulfonic acid
IPTG	isopropyl- -D-thiogalactoside
kb	kilobases
kcal	kilocalories
kd	kilodaltons
lac	lactose operon
LA	Luria agar
LB	Luria broth
MCS	multiple cloning site
MW	molecular weight
SDS	sodium dodecyl sulfate
TE	10 mM Tris pH 8.0, 1 mM EDTA
TEG	10 mM Tris pH 8.0, 1 mM EDTA, 1% glucose
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indolyl- -D-galactoside

Isolation of Spinach Chloroplast DNA

Spinach leaves were purchased at a local produce market. Leaves were torn by hand into 2-5 cm pieces and the midveins removed. Leaf pieces (200-300 grams) were washed in tap water and blotted dry.

Approximately 75-100 grams of the washed spinach leaves were placed in a precooled Waring blender with 200 ml ice cold grinding buffer (330 mM sorbitol, 50 mM HEPES pH 8.0, 2 mM EDTA). Leaves were homogenized with three 5 second bursts. A glass stirring rod was used to stir the mixture briefly between bursts. The slurry was filtered through 2 layers of Miracloth (Calbiochem) and collected in a sterile 600 ml beaker over ice. The filtrate was centrifuged in a Sorvall table top centrifuge at 2000xg for one minute at room temperature. The crude chloroplast pellet was washed 2 times in 30 ml grinding buffer, resuspended in 10 ml of the same and held on ice.

Twenty ml of alkaline SDS (0.2 N NaOH, 1% SDS), (6), was added to the chloroplast suspension, which was mixed gently and kept on ice for 5 min. Ten ml of a solution of 5 M acidified potassium acetate (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H₂O), (47), was added and mixed by pouring the solution gently back and forth between two 40 ml centrifuge tubes. The mixture was placed on ice for 15 min then centrifuged in a SS-34 rotor at 12,000xg for 30 min at 4°C. The supernatant was poured

through two layers of cheesecloth into a 150 ml Corex centrifuge bottle and 0.6 volumes isopropanol was mixed in by gentle inversion. The nucleic acids were precipitated on ice for one hour and collected by centrifugation at 12,000xg for 30 min at 4°C. The pellet was resuspended in 0.5 ml TE. Spinach chloroplast DNA was either digested by restriction endonucleases or further purified by the following procedure. The DNA solution was extracted with an equal volume phenol:ClA and the phases separated by one minute centrifugation in a Beckman Microfuge-B. The aqueous phase was removed with a micropipette to a clean microfuge tube, made 2.5 M with ammonium acetate and the DNA precipitated by adding 2.5 volumes 95% EtOH on ice for 30 min. The precipitate was collected by 10 min microcentrifugation at 4°C. The ammonium acetate:EtOH precipitation was repeated and the final DNA pellet resuspended in 100 ul TE.

Restriction Analysis of Spinach Chloroplast DNA

Twenty-six ul of spinach chloroplast DNA (250 ng) was mixed with 3 ul 10x restriction buffer (salt concentration appropriate to specific enzyme) and 1 ul restriction enzyme (10 units/ul). The reaction mixture was incubated at 37°C for 2 hours and the reaction stopped by heating to 68°C for 10 min. Four ul reaction mixture or 4 ul intact DNA sample was mixed with 1 ul DNA tracking dye (40% sucrose, 1.0% SDS, 0.25% BPB). Samples were loaded into wells of a 0.7% agarose horizontal slab gel and electrophoresis carried out

at 75 volts, 40 milliampres for 2 hours in Tris-acetate buffer (47). Gels were stained in 1 ug/ml EtBr for 15-30 min and destained under running tap water for 15 min. DNA bands were visualized by placing the destained gel on an ultraviolet transilluminator. Gels were photographed with a Polaroid MP-4 Land camera using a Kodak 22A Wratten filter and Polaroid type 57 direct positive film. DNA concentration was estimated by comparison of band fluorescence with known DNA concentrations.

Cloning of Spinach Chloroplast DNA into pUC Plasmids

Approximately 0.5 ug vector DNA (pUC8 or pUC9) was digested with restriction enzyme PstI as previously described. Ten ul 10x calf intestinal alkaline phosphatase buffer (0.5 M Tris pH 9.0, 10 mM MgSO_4 , 1 mM ZnSO_4), (47), 0.5 ul calf intestinal alkaline phosphatase (19 units/ul) and 59 ul H_2O were added to the 30 ul restriction reaction for a final volume of 100 ul. This mixture was incubated at 37°C for 30 min then another 0.5 ul calf intestinal alkaline phosphatase was added and the incubation continued for an additional 30 min. The reaction mixture was heated to 68°C for 10 min and extracted with one volume phenol:ClA. After centrifugation the aqueous phase was removed to a clean microfuge tube and the organic phase re-extracted with 100 ul TE to maximize DNA recovery. The two aqueous phases were pooled, made 2.5 M with ammonium acetate and the DNA precipitated with 2.5 volumes 95% EtOH on ice for 30 min.

The precipitate was collected by 10 min centrifugation at 4°C. The supernatant was poured off and the DNA pellet allowed to drain and air dry for 30 min. The DNA was resuspended in 20 ul TE.

Restriction enzyme digested spinach chloroplast DNA (100 ng) was made up to 100 ul total volume and extracted with phenol:CIA as previously described. The DNA was precipitated with 2.5 M ammonium acetate:EtOH and resuspended in 7 ul TE. This 7 ul aliquot was mixed with 1 ul alkaline phosphatase treated plasmid DNA, the insert:vector ratio was approximately 3:1. The mixture was heated to 65°C for 5 min to free sticky ends and cooled to room temperature. One ul 10x ligation buffer (0.66 M Tris pH 7.5, 50 mM MgSO₄, 50 mM dithiothreitol, 10 mM ATP), (47), and 0.5 ul T4 DNA ligase (1 unit/ul) was added to the DNA mixture and stirred carefully with a micropipette tip. The ligation was carried out at 16°C for 12 hours followed by 4 hours at 4°C. As restriction and ligation controls, samples of alkaline phosphatase treated plasmid DNA were incubated in the same manner, with and without T4 DNA ligase.

Competent cells, E. coli JM 105, were prepared by the CaCl₂ procedure (47). Early log phase cells were held on ice for 15 min, collected by centrifugation at 3000xg for 5 min at 4°C, resuspended in 1/2 volume ice cold, sterile CaCl₂ and held on ice for 10 min. The cells were recentrifuged as above and resuspended in 1/15 volume CaCl₂. Aliquots of

100-200 μ l were pipetted into sterile, prechilled tubes and stored at 4°C for 12-24 hours.

Transformation of competent cells to ampicillin resistance was performed by adding 5 μ l ligation mixture or controls to cell aliquots. The cells were incubated on ice for 30 min, heat shocked to 42°C for 2 min, returned to ice for 2 min then allowed to warm to room temperature. One ml LB was added to each transformation mixture and the cells were incubated at 37°C for 2 hours with occasional gentle shaking.

Aliquots of each transformation mixture, 0.1-0.2 ml, were spread on ampicillin, IPTG, X-gal plates (50 μ g/ml amp, 0.003% X-gal, 40 μ M IPTG). The plates were incubated at 37°C overnight.

Identification of Cloned Spinach DNA Fragments

White transformant colonies resulting from the insertion of spinach chloroplast DNA into the MCS of the pUC plasmid and the loss of β -galactosidase activity were streaked onto LA-amp plates (50 μ g amp/ml), incubated overnight at 37°C and isolated colonies used to inoculate 150 ml LB-amp cultures. Cells were grown overnight at 37°C with vigorous shaking. The cells were collected by centrifugation and the plasmids harvested by the alkaline lysis method of Birnboim and Doly, (6). The cells (1-2 grams) were resuspended in 10 ml TEG, transferred to a 40 ml centrifuge tube and held on ice for 10 min. Cells were lysed

by the addition of 20 ml 0.2 N NaOH, 1% SDS, gentle mixing and incubation on ice for 15 min. Ten ml of 5 M acidified potassium acetate was added, mixed by gentle inversion and the mixture held on ice for 15 min. Proteins and other cellular debris were collected by centrifugation at 12,000xg for 30 min at 4°C in an SS-34 rotor. The supernatant was poured through two layers of cheesecloth into a clean 150 ml Corex centrifuge bottle, 0.6 volumes isopropanol mixed in by inversion and the nucleic acids precipitated on ice for one hour or overnight at -20°C. The precipitate was collected by centrifugation at 12,000xg for 30 min at 4°C, the supernatant was discarded and the pellet resuspended in TE to a total volume of 6 ml. The nucleic acid solution was poured into a 10 ml Beckman polypropylene ultracentrifuge tube and 6 grams CsCl was added. The tubes were sealed with parafilm and gently inverted 10 times to dissolve the CsCl. Protein debris floating to the top was removed with a sterile cotton swab. To each tube, 0.5 ml EtBr (10 mg/ml) was added and mixed by inversion. The tubes were filled to the top with mineral oil and sealed with aluminum caps.

Cesium chloride density gradient centrifugation was carried out in a Beckman L-2 65B ultracentrifuge using a Ti 50 rotor at 45,000 rpm for 36-48 hours at 20 C. The resultant DNA bands were visualized with long wavelength ultraviolet light and the lower band, representing covalently closed circular DNA, was removed by inserting a

16 gauge needle through the tube just below the band and drawing the band into a 3 ml disposable plastic syringe. The sample (1-2 ml) was placed in a 15 ml Corex centrifuge tube and the EtBr removed by repeated extraction with isopropanol saturated with 5 M NaCl and TE. Once the EtBr was removed, 2 volumes distilled H₂O and 6 volumes 95% EtOH were added and the DNA precipitated on ice for one hour or at -20°C overnight. The DNA was collected by centrifugation at 12,000xg for 30 min at 4°C, and the pellet resuspended in 200 ul TE. The DNA was further purified by 1-2 additional EtOH precipitations in the presence of 2.5 M ammonium acetate.

These samples were diluted 1:100 and digested with the enzyme used for the original cloning, PstI, and by SalI, as described previously. The restriction digests were analyzed by agarose gel electrophoresis and DNA fragments sized by comparison to the HindIII restriction fragments of bacteriophage λ DNA. In most cases the SalI digests of the recombinant plasmids facilitated the identification of the cloned spinach chloroplast DNA fragment and showed the orientation of the fragment in the PstI site of the pUC plasmid MCS.

Some spinach chloroplast DNA fragments were difficult to identify by sizing and SalI digestion. These recombinant plasmids were digested with other enzymes whose restriction sites are mapped on the spinach chloroplast genome. The

ClaI sites in PstI fragment #5 were mapped by single digestion with ClaI and sequential digestion with ClaI and then SalI.

Subcloning the PstI #5 Fragment in pGEM4

The PstI #5 fragment of spinach chloroplast DNA was subcloned into plasmid pGEM4 by the following procedure, (15). A 52 ul sample of the recombinant plasmid pRD305 (200 ng DNA) was mixed with 6 ul 10x restriction buffer and 1 ul restriction enzyme PstI (10 units/ul) and incubated at 37°C for 2 hours. The digestion was stopped by heating to 68°C for 10 min. DNA tracking dye (15 ul) was added and the sample was micropipetted into an oversized well in a 0.7% agarose gel. The oversized well was formed by taping 6 teeth of the well forming comb together and the agarose was poured containing 1 ug/ml EtBr. The sample was electrophoresed at 75 volts for one hour, the gel removed from the electrophoresis apparatus and visualized on an ultraviolet transilluminator. The band representing the spinach chloroplast PstI #5 fragment was located and an incision made with a razor blade just in front of the DNA band. A piece of Whatman DE 81 DEAE cellulose paper the same length as the oversized well and 3 mm wide was placed on edge in the incision. The gel was carefully slid back onto the gel tray and replaced in the electrophoresis tank. The voltage was raised to 100 volts for 15 min. The gel was again removed to the ultraviolet transilluminator and the

fluorescence was shown to be on the paper rather than in the gel. The paper was removed and placed in the barrel of a 3 ml disposable plastic syringe. The syringe was placed in a 15 ml Corex centrifuge tube and the paper washed by pipetting 200 μ l 0.1 M NaCl, 50 mM Tris pH 8.0, 0.1 mM EDTA into the syringe and centrifuging the tube at 2000xg for one min. This process was repeated once. The wash that was collected in the Corex tube was discarded. A microcentrifuge tube was placed over the end of the syringe and the DNA was eluted from the paper by pipetting 100 μ l 1.0 M NaCl, 50 mM Tris pH 8.0, 0.1 mM EDTA into the syringe and letting it stand at room temperature for 15-20 min. The eluate was collected in the microcentrifuge tube by centrifugation at 2000xg for one minute. This process was repeated three times. The DNA was precipitated from the 400 μ l total eluate by adding 1 ml 95% EtOH and placing the tube on ice for 30 min or at -20°C overnight. The DNA was pelleted by 10 min microcentrifugation at 4°C and the sample resuspended in 100 μ l TE, extracted with phenol:ClA and precipitated with EtOH in the presence of 2.5 M ammonium acetate. The DNA was resuspended in 7 μ l TE and the ligation into pGEM4 was done as described previously.

RESULTS

Spinach Chloroplast DNA Isolation

High molecular weight spinach chloroplast DNA was obtained by alkaline lysis and precipitation in isopropanol. The yield was approximately 50 ug chloroplast DNA per 100 grams starting leaf material. The DNA consisted of 90% linear molecules (100-150 kb) and 10% open circular molecules, as determined by agarose gel electrophoresis, (Fig. 4). Upon digestion with restriction enzyme Sall, the circular and high molecular weight bands disappear and bands representing 10 of the 11 restriction fragments are visible, (Fig. 4). The band representing the smallest fragment, (0.50 kb) could not be seen. A large amount of RNA is also present in the preparation.

After further purification by repeated EtOH:ammonium acetate precipitation, most of the RNA was removed (Fig. 5). There was also a significant loss of DNA during this procedure. The yield was reduced to approximately 20 ug chloroplast DNA per 100 grams starting leaf material.

Cloning of Spinach Chloroplast DNA into pUC Plasmids

Transformation of E. coli JM 105 cells to ampicillin resistance with a PstI digested chloroplast DNA-pUC9 ligation mixture typically resulted in 100-200 resistant colonies per 0.1 ml cell suspension spread on amp, IPTG,

Figure 4. Spinach chloroplast DNA isolated by alkaline lysis, digested with SalI (lane a), undigested (lane b). Fragment molecular weights shown at left in kb.

Figure 5. Agarose gel electrophoresis of spinach chloroplast DNA isolated by alkaline lysis and purified by repeated EtOH:ammonium acetate precipitation, undigested (lane a), digested with SalI (lane b) and digested with PstI (lane c).

X-gal LA plate. Twenty percent of these colonies were white, showing insertion into the MCS of pUC9 and loss of -galactosidase activity. The rest of the colonies were blue. Control plates showed 100-200 blue colonies and no white colonies when ligation mixtures containing pUC9 digested by PstI and treated with alkaline phosphatase were incubated with T4 DNA ligase and no transformants if the T4 ligase was omitted.

Identification of Cloned Chloroplast DNA Fragments

All white colonies screened contained plasmids larger than pUC9 (data not shown). Several clones were selected for identification of the inserted chloroplast DNA fragment. Digestion of recombinant plasmids with PstI showed bands corresponding to the pUC plasmid and inserts of 1.9kb, 7.7kb and approximately 12.0-13.0kb (Fig #6). These inserts were tentatively identified as spinach chloroplast DNA PstI fragments #10, #8 and #5, respectively, on the basis of molecular weight. The recombinant plasmids were designated pRD110, pRD108 and pRD105 according to the convention established by Alt, et al (1). When the recombinant plasmids were digested with SalI, pRD110 showed a single band of 3.3kb and pRD108 a single band of 10.1kb (Fig. 6). This shows that neither of these inserts has a SalI site, the single site in the recombinant plasmids represents the site in the MCS of the pUC plasmid, and supports the identification assigned. Recombinant plasmid

pRD105 shows two bands of 5.0 kb and 10 kb upon SalI digestion (Fig. 6). Therefore, there is one SalI site in the insert. Both PstI fragment #4, 13.5 kb and PstI fragment #5, 12.3 kb have one SalI site in the same relative position (Fig. 3). Sequence data of these fragments show PstI #4 has 3 BamHI sites, (39), and PstI #5 has only one (67). Digestion of pRD105 resulted in fragments of 9.5 kb and 5.5 kb and demonstrates that the insert has only one BamHI site and therefore must be PstI #5 (Fig. 6, lane h).

Subcloning of PstI #5 into pGEM4

Transformation of JM 109 to ampicillin resistance with PstI #5 ligated into pGEM4 on amp, IPTG, X-gal plates resulted in 20-30% white colonies. Examination of the recombinant plasmids by electrophoresis after PstI and SalI confirmed the insertion of PstI #5 into pGEM4 (data not shown). The recombinant plasmid was designated pRG105.

Mapping of ClaI Sites in PstI #5

Digestion of pRD105 with ClaI resulted in fragments of 9kb and 6kb (Fig. 7). There are no ClaI sites in pUC9, thus there are two recognizable ClaI sites in PstI #5. The ClaI recognition sequence, ATCGAT, contains three (GAT) of the four (GATC) bases of the dam DNA methylation sequence and it is possible that there are other ClaI sites, preceded by G or followed by C that are methylated in a dam⁺ strain such as JM 109. These sites cannot be recognized by ClaI. Sequential digestion of pRD105 with ClaI and SalI resulted in

Figure 6. Electrophoretic analysis of recombinant plasmids pRD110, pRD108 and pRD105. pUC9 digested with PstI (lane a). pRD110 digested with PstI (lane b) and SalI (lane c). pRD108 digested with PstI (lane d) and SalI (lane e). pRD105 digested with PstI (lane f), SalI (lane g) and BamHI (lane h). λ DNA digested with HindIII (lane i), molecular weights shown at right in kb.

Figure 7. Electrophoretic analysis of ClaI digestion of pRD105. HindIII digestion of λ DNA (lane a), with molecular weights shown at left in kb. pRD105 digested with SalI (lane b), ClaI (lane c) and SalI+ClaI (lane d). Molecular weights of double digestion fragments shown at right in kb.

four fragments of 1.8 kb, 2.1 kb, 2.9 kb and 8.6 kb (Fig. 7). The positions of the recognized *Cla*I sites in *Pst*I #5 have been mapped (Fig. 8).

Figure 8. Positions of *Cla*I restriction sites in spinach chloroplast genome *Pst*I fragment #5.

DISCUSSION

Isolation of spinach chloroplast DNA by alkaline lysis is a quick and convenient method. The 2-3 hour preparation time is much less than most methods because no step gradients are necessary to isolate intact chloroplasts and no CsCl centrifugation is used to purify the DNA. No plants need to be grown ahead of time as commercially available spinach leaves give reasonable yields. This method probably works because alkaline conditions (pH 12.0-12.5) denature the linear nuclear DNA. The nuclear DNA consists of large linear molecules after cell homogenization and organelle lysis, while much of the chloroplast DNA is still circular. When the lysate is neutralized by acidic potassium acetate, most of the nuclear DNA aggregates to form an insoluble network while the circular chloroplast DNA stays in solution. The high potassium acetate concentration also causes the precipitation of the protein-SDS and chlorophyll-protein-SDS complexes. Therefore, most of the contaminating macromolecules may be removed in one centrifugation. The remaining proteins and RNA are effectively reduced by the phenol:ClA extraction and repeated EtOH:ammonium acetate precipitations.

Most of the chloroplast DNA does not survive as covalently closed circular molecules. This might be due to

mechanical shear forces during cell homogenization and subsequent handling or the presence of 10-20 ribonucleotide substitutions found in spinach chloroplast DNA (reviewed in 4). Alkaline conditions would be expected to degrade DNA at these ribonucleotide sites, but Maniatis et al report that alkaline lysis results in less than 5% nicked plasmid molecules when ribonucleotide substitution is induced by plasmid amplification by chloramphenicol, (47 p. 91). The chloroplast DNA shows consistently good restriction patterns, so there must be very few breaks per DNA molecule. The ability of these fragments to be cloned into plasmid vectors attests to their integrity. The disadvantage of this procedure is that the yield is less than the 100ug/100g starting leaf material reported by Palmer (54).

The JM 105 pUC cloning system uses a histochemical assay of β -galactosidase activity to indicate insertion of a DNA fragment into the MCS of the plasmid. The pUC plasmid carries a portion of the lacZ gene whose product complements the defective β -galactosidase encoded by the F' borne in JM 105 (64). If the pUC plasmid is intact, a functional β -galactosidase results. This is demonstrated by induction with IPTG and cleavage of X-gal to an insoluble blue dye. If there is an insertion into the MCS of the plasmid, no functional β -galactosidase is formed and X-gal is not cleaved. Therefore, blue colonies show an intact plasmid

and white colonies signify an insertion into the MCS of the pUC plasmid.

The cloning procedures showed a high background of blue colonies, this could be caused by incomplete digestion of the plasmid or by recirculaization of the digested plasmid. No colonies were observed on plates spread with cells transformed by digested plasmid DNA incubated without T4 DNA ligase, so incomplete digestion can be ruled out. The only other explanation of the numerous blue colonies is that the dephosphorylation was ineffective and there was recircularization of digested plasmid DNA. Several different buffers were tried and the most likely explanation is the calf intestinal phosphatase was defective or inactivated in some way.

The PstI #5 spinach chloroplast DNA fragment was subcloned into the plasmid pGEM4 because this plasmid has promoters for bacteriophage SP6 and T7 RNA polymerases. This construction is a preparation for in vivo or in vitro transcription/translation studies. Experiments of this type might be used to study the mechanisms that control expression of chloroplast genes or possibly to express open reading frames that have not yet been identified. Expression of these open reading frames and production of the coded polypeptide might be one method of identifying the protein when there is insufficient homology to E. coli proteins for identification.

In summary, a new method of spinach chloroplast DNA isolation has been developed. This method is quicker and easier than methods currently in use and yields reasonable quantities of DNA that is suitable for cloning and amplification. Several recombinant plasmids have been constructed and identified and the PstI #5 fragment has been inserted into plasmid pGEM4 in preparation for transcription/translation studies.

REFERENCES

1. Alt, J., Winter, P., Sebald, W., Moser, J.G., Schedel, R., Westoff, P. and Herrmann, R.G. (1983). Localization and Nucleotide Sequence of the Gene for the ATP Synthase Proteolipid Subunit on the Spinach Plastid Chromosome. *Curr. Genet.* 7:129-138.
2. Alt, J. and Herrmann, R.G. (1984). Nucleotide Sequence of the Gene for Pre-apocytochrome f in the Spinach Plastid Chromosome. *Curr. Genet.* 8:551-557.
3. Alt, J., Morris, J., Westoff, P. and Herrmann, R.G. (1984). Nucleotide Sequence of the Clustered Genes for the 44kd Chlorophyll Apoprotein and the "32kd"-like Protein of the Photosystem II Reaction Center in the Spinach Plastid Chromosome. *Curr. Genet.* 8:597-606.
4. Bedbrook, J.R. and Kolodner, R. (1979). The Structure of Chloroplast DNA. *Ann. Rev. Plant Physiol.* 30:593-620.
5. Ben Tahar, S., Bottomley, W. and Whitfield, P.R. (1986). Characterization of the Spinach Chloroplast Genes for the S4 Ribosomal Protein, tRNA^{Thr} (UGU) and tRNA^{Ser} (GGA). *Plant Mol. Biol.* 7:63-70.
6. Birnboim, H.C. and Doly, J. (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. *Nuc. Acids Res.* 7(6):1513-1523.
7. Bogorad, L., Regulation of Intracellular Gene Flow in the Evolution of Eukaryotic Genomes. In On the Origins of Chloroplasts, Schiff, J.A., ed. Elsevier North Holland, Inc., New York, 1982.
8. Bowman, C.M. and Dyer, T.A. (1982). Purification and Analysis of DNA from Wheat Chloroplasts Isolated in Non-aqueous Media. *Analytical Biochem.* 122:108-118.
9. Carrillo, N., Seyer, P. Tyagi, A. and Herrmann, R.G. (1986). Cytochrome b-559 Genes from Oenothera hookeri and Nicotiana tabacum Show a Remarkably High Degree of Conservation as Compared to Spinach. *Curr. Genet.* 10:619-624.

10. Cocking, E.C., Davey, M.R., Pental, D., and Power, J.B., (1981). Aspects of Plant Genetic Manipulation. *Nature* 293:265-270.
11. Cohen, B.N., Coleman, T.A., Schmitt, J.J. and Weissbach, H. (1984). In vitro Expression and Characterization Start Site of the psbA Gene Product (QB Protein) from Higher Plants. *Nuc. Acids Res.* 12(15):6221-6230.
12. Courtice, G.R.M., Bowman, C.M. Dyer, T.A. and Gray, J.C. (1985). Localisation of Genes for Components of Photosystem II in Chloroplast DNA from Pea and Wheat. *Curr. Genet.* 10:329-333.
13. Crouse, E.J., Bohnert, H.J. and Schmitt, J.M. Chloroplast RNA Synthesis. In Chloroplast Biogenesis, Ellis, R.J., ed. Cambridge University Press, Cambridge, 1984.
14. Dorne, A.M., Heizman, D., Alt, J. and Mache, R. (1985). Spinach Chloroplast Genes Coding for Three Ribosomal Proteins. *Mol. Gen. Genet.* 198:521-524.
15. Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981). A Reliable Method for the Recovery of DNA Fragments from Agarose and Acrylamide Gels. *Anal. Biochem.* 112:295-298.
16. Driesel, E.J., Crouse, E.J.K., Gordon, K., Bohnert, H.J. and Herrmann, R.G. (1979). Fractionation and Identification of Spinach Chloroplast Transfer RNAs and Mapping of their Genes on the Restriction Map of Chloroplast DNA. *Gene* 24:285-306.
17. Driesel, A.J., Speirs, J. and Bohnert, H.J. (1980). Spinach Chloroplast mRNA for a 32000 Dalton Polypeptide. Size and Localization on the Physical Map of the Chloroplast DNA. *Biochim. Biophys. Acta* 610:297-310.
18. Dyer, T.A., The Chloroplast Genome: Its Nature and Role in Development. In Topics in Photosynthesis vol.5 Chloroplast Biogenesis, Baker, N.R. and Barber, J., eds. Elsevier, Amsterdam, 1984.
19. Ellis, J. (1982). Promiscuous DNA--Chloroplast Genes Inside Plant Mitochondria. *Nature* 299:185-186.
20. Ellis, J. (1983). Mobile Genes of Chloroplasts and the Promiscuity of DNA. *Nature* 304:308-309.

11. Cohen, B.N., Coleman, T.A., Schmitt, J.J. and Weissbach, H. (1984). In vitro Expression and Characterization Start Site of the psbA Gene Product (QB Protein) from Higher Plants. Nuc. Acids Res. 12(15):6221-6230.
12. Courtice, G.R.M., Bowman, C.M. Dyer, T.A. and Gray, J.C. (1985). Localisation of Genes for Components of Photosystem II in Chloroplast DNA from Pea and Wheat. Curr. Genet. 10:329-333.
13. Crouse, E.J., Bohnert, H.J. and Schmitt, J.M. Chloroplast RNA Synthesis. In Chloroplast Biogenesis, Ellis, R.J., ed. Cambridge University Press, Cambridge, 1984.
14. Dorne, A.M., Heizman, D., Alt, J. and Mache, R. (1985). Spinach Chloroplast Genes Coding for Three Ribosomal Proteins. Mol. Gen. Genet. 198:521-524.
15. Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981). A Reliable Method for the Recovery of DNA Fragments from Agarose and Acrylamide Gels. Anal. Biochem. 112:295-298.
16. Driesel, E.J., Crouse, E.J.K., Gordon, K., Bohnert, H.J. and Herrmann, R.G. (1979). Fractionation and Identification of Spinach Chloroplast Transfer RNAs and Mapping of their Genes on the Restriction Map of Chloroplast DNA. Gene 24:285-306.
17. Driesel, A.J., Speirs, J. and Bohnert, H.J. (1980). Spinach Chloroplast mRNA for a 32000 Dalton Polypeptide. Size and Localization on the Physical Map of the Chloroplast DNA. Biochim. Biophys. Acta 610:297-310.
18. Dyer, T.A., The Chloroplast Genome: Its Nature and Role in Development. In Topics in Photosynthesis vol.5 Chloroplast Biogenesis, Baker, N.R. and Barber, J., eds. Elsevier, Amsterdam, 1984.
19. Ellis, J. (1982). Promiscuous DNA--Chloroplast Genes Inside Plant Mitochondria. Nature 299:185-186.
20. Ellis, J. (1983). Mobile Genes of Chloroplasts and the Promiscuity of DNA. Nature 304:308-309.

21. Fluhr, R., Fromm, H. and Edelman, M. (1983). Clone Bank of Nicotiana tabacum Chloroplast DNA: mapping of the alpha, beta and epsilon subunits of the ATPase coupling factor, the large subunit of ribulosebisphosphate carboxylase, and the 32-kDal membrane protein. *Gene* 25:271-280.
22. Frankel, R., Scowcroft, W.R, and Whitfield, P.R. (1979). Chloroplast DNA Variation in Isonuclear Male-Sterile Lines of Nicotiana. *Mol. Gen. Genet.* 169:129-135.
23. Gray, J.C., Phillips, A.L. and Smith, A.G., Protein Synthesis by Chloroplasts. In Chloroplast Biogenesis, Ellis, R.J., ed. Cambridge University Press, Cambridge, 1984.
24. Groot, G.S.P., Chloroplast DNA of Higher Plants. In Chloroplast Biogenesis, Ellis, R. J., ed. Cambridge University Press, Cambridge, 1984.
25. Gruissem, W., Greenberg, B.M., Zurawski, G., Prescott, D.M. and Hallick, R.B. (1983). Biosynthesis of Chloroplast Transfer RNA in a Spinach Chloroplast Transcription System. *Cell* 35:815-828.
26. Gruissem, W. Elsner-Menzel, C., Latshaw, S., Narita, J.O., Schaffer, M.A. and Zurawski, G. (1986). A Subpopulation of Spinach Chloroplast tRNA Genes does not Require Upstream Promoter Elements for Transcription. *Nuc. Acid Res.* 14(19):7541-7556.
27. Hachtel, W. (1982). Biosynthesis and Assembly of Thylakoid Membrane Proteins in Isolated Chloroplasts from Vicia faba L: The P700-Chlorophyll a-Protein. *Z. Pflanzenphysiol.* 107:383-394.
28. Hachtel, W. (1983). Biosynthesis and Membrane Insertion of a Chlorophyll a/b-binding Protein by Isolated Vicia faba L. Chloroplasts. *Z. Pflanzenphysiol.* 108:381-390.
29. Hanley-Bowdoin, L., Orozco, E.M. and Chua, N-H. (1985). In Vitro Synthesis and Processing of a Maize Chloroplast Transcript Encoded by the Ribulose 1,5 Bisphosphate Carboxylase Large Subunit Gene. *Mol. and Cell. Biol.* 5(10): 2733-2745.
30. de Heiji, H.T., Lustig, H., Moeskops, D. M., Bovenberg, W.A. and Bisanz, C. (1983). Chloroplast DNAs of Spinacia, Petunia Spirodela Have a Similar Gene Organization. *Curr. Genet.* 7:1-6.

31. de Heij, H.T., Jochemsen, A-G., Willemsen, P.T.J. and Groot, G.S.P. (1984). Protein Synthesis During Chloroplast Development in Spirodela oligoriza. Eur. J. Biochem. 138:161-168.
32. Heinemeyer, W. Alt, J. and Herrmann, R.G. (1984). Nucleotide Sequence of the Clustered Genes for Apocytochrome b6 and Subunit 4 of the Cytochrome b/f Complex in the Spinach Plastid Chromosome. Curr. Genet. 8:543-549.
33. Hennig, J. and Herrmann, R.G. (1986). Chloroplast ATP Synthase of Spinach Contains Nine Nonidentical Subunit Species, Six of which are Encoded by Plastid Chromosomes in Two Operons in a Phylogenetically Conserved Arrangement. Mol. Gen. Genet. 203:117-128.
34. Herrmann, R.G., Bohnert, H-J., Kowallik, K.V. and Schmitt, J.M. (1974). Size, Conformation and Purity of Chloroplast DNA of Some Higher Plants. Biochim. Biophys. Acta 378:305-317.
35. Herrmann, R.G., Whitfield, P.R. and Bottomley, W. (1980). Construction of a SalI/PstI Restriction Map of Spinach Chloroplast DNA Using Low Gelling Temperature Agarose Electrophoresis. Gene 8:179-191.
36. Herrmann, R.G., Alt, J., Schiller, B., Widger, W.R. and Cramer, W.A. (1984). Nucleotide Sequence of the Gene for Apocytochrome b-559 on the Spinach Plastid Chromosome: Implications for the Structure of the Membrane Protein. FEBS Lett. 176(1):239-244.
37. Herrmann, R.G., Seyer, P., Schedel, R., Gordon, K., Bisanz, C., Winter, P., Hildebrandt, J.W., Wlaschek, M., Alt, J., Driesel, A.J. and Sears, B.B., The Plastid Chromosomes of Several Dicotyledons. In Biological Chemistry of Organelle Formation, Bucher, T., Sebald, W. and Weiss, H., eds. Springer-Verlag, Berlin, 1980.
38. Highfield, P.E. and Ellis R.J. (1978). Synthesis and Transport of the Small Subunit of Chloroplast Ribulose Biphosphate Carboxylase. Nature 271:420-424.
39. Holschuh, K., Bottomley, W. and Whitfield, P.R. (1984). Structure of the Spinach Chloroplast Genes for the D2 and 44 kd Reaction-Centre Proteins of Photosystem II and for tRNA^{Ser} (UGA). Nuc. Acids Res. 12(23):8819-8834.

40. Kirsch, W., Seyer, P. and Herrmann, R.G. (1986). Nucleotide Sequence of the Clustered Genes for Two P700 Chlorophyll a Apoproteins of the Photosystem I Reaction Center and the Ribosomal Protein S14 of the Spinach Plastid Chromosome. *Curr. Genet.* 10:843-855.
41. Kolodner, R. and Tewari, K.K. (1972). Molecular size and Conformation of Chloroplast Deoxyribonucleic Acid from Pea Leaves. *J. Biol. Chem.* 29:6355-6364.
42. Kolodner, R. and Tewari, K.K. (1975). The Molecular Size and Conformation of the Chloroplast DNA from Higher Plants. *Biochim. Biophys. Acta* 402:372-390.
43. Kolodner, R. and Tewari, K.K. (1975). Chloroplast DNA from Higher Plants Replicates by Both the Cairns and the Rolling Circle Mechanism. *Nature* 256:708-711.
44. Lebacqz, P. and Vedel, F. (1981). SalI Restriction Enzyme Analysis of Chloroplast and Mitochondrial DNAs in the Genus Brassica. *Plant Sci. Lett.* 23:1-9.
45. Lerbs, S. and Mache, R. (1985). Immunological Comparison of the Polypeptides of DNA-dependent RNA Polymerase of Spinach Chloroplasts and E. coli. *Biol. Chem. H-S.* 366:819.
46. Lyttleton, J.W. (1962). Isolation of Ribosomes from Spinach Chloroplasts. *Exp. Cell Res.* 26:312-317.
47. Maniatis, T., Fritsch, E.F. and Sambrook, J. Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, 1982.
48. Margulis, L. Symbiosis In Cell Evolution. Walt Freeman and Co., San Francisco, 1981.
49. Morris, J. and Herrmann, R.G. (1984). Nucleotide Sequence of the Gene for the P680 Chlorophyll a Apoprotein of the Photosystem II Reaction Center for Spinach. *Nuc. Acids Res.* 12(6):2837-2850.
50. Palmer, J.D. and Thompson, W.F. (1981). Clone Banks of the Mung Bean, Pea And Spinach Chloroplast Genomes. *Gene* 15:21-26.
51. Palmer, J.D., Singh, G.P. and Pillay D.T.N. (1983). Structure and Sequence Evolution of Three Legume Chloroplast DNAs. *Mol. Gen. Genet.* 190:13-19.

52. Palmer, J.D. and Stein, D.B. (1986). Conservation of Chloroplast Genome structure among Vascular Plants. *Curr. Genet.* 10:823-833.
53. Palmer, J.D. (1985). Comparative Organization of Chloroplast Genomes. *Ann. Rev. Genet.* 19:325-354.
54. Palmer, J.D. (1986). Isolation and Structural Analysis of Chloroplast DNA. *Methods in Enzymology* 118:167-185.
55. Ris, R. and Plaut, C. (1962). Ultrastructure of DNA-Containing Areas of the Chloroplast of Chlamydomonas. *J. Cell Biol.* 13:383-391.
56. Scott, N.S. and Possingham, J.V. (1983). Changes in Chloroplast DNA Levels During Growth of Spinach Leaves. *J. Exp. Bot.* 34:1756-1767.
57. Seyer, P., Kowallik, K.V. and Herrmann, R.G. (1981). A Physical Map of Nicotiana tabacum Plastid DNA Including the Location of Structural Genes for Ribosomal RNAs and the Large Subunit of Ribulose Bisphosphate Carboxylase/Oxygenase. *Curr. Genet.* 3:189-204.
58. Shinozaki, K., Sun, C-R. and Sugiura, M. (1984). Gene Organization of Chloroplast DNA from the Broad Bean Vicia faba. *Mol. Gen. Genet.* 197:363-367.
59. Sijben-Muller, G., Hallick, R.B., Alt, J., Westoff, P. and Herrmann, R.G. (1986). Spinach Plastid Genes coding for Initiation Factor IF-1, Ribosomal Protein S11 and RNA Polymerase β -subunit. *Nuc. Acids Res.* 14(2):1029-1044.
60. Spielmann, A., Ortiz, W., and Stutz, E. (1983). The Soybean Chloroplast Genome: Construction of a Circular Restriction Site Map and Location of DNA Regions Encoding the Genes for rRNAs, the Large Subunit of the Ribulose-1,5-Bisphosphate Carboxylase and the 32 KD Protein of the Photosystem II Reaction Center. *Mol. Gen. Genet.* 190:5-12.
61. Stern, D.B. and Lonsdale, D.M. (1982). Mitochondrial and Chloroplast Genomes of Maize Have a 12-kilobase DNA Sequence in Common. *Nature* 299:698-702.
62. Timmis, J.N. and Scott, N.S. (1983). Sequence Homology Between Spinach Nuclear and Chloroplast Genomes. *Nature* 305:65-67.

63. Tittgen, J., Hermans, J., Steppuhn, J., Jansen, T., Jansson, C., Andersson, B., Nechushtai, R., Nelson, N. and Herrmann, R.G. (1986). Isolation of cDNA Clones for Fourteen Nuclear-Encoded Thylakoid Proteins. *Mol. Gen. Genet.* 204: 258-265.
64. Vieira, J. and Messing, J. (1982). The pUC Plasmids, an M13mp7-Derived System for Insertion Mutagenesis and Sequencing with Universal Primers. *Gene* 19:259-268.
65. Watson, J.C., Palmer, J.D. and Thompson, W.F. (1983). Chloroplast Genes for the Translational Apparatus. *Carnegie Inst. Yearb.* 82:24-26.
66. Westoff, P., Nelson, N., Bunemann, H. and Herrmann, R.G. (1981). Localization of Genes for Coupling Factor Subunits on the Spinach Plastid Chromosome. *Curr. Genet.* 4:109-120.
67. Westhoff, P., Alt, J. and Herrmann, R.G. (1983). Localization of the Genes for the Two Chlorophyll a-Conjugated Polypeptides (mol. wt. 51 and 44 kd) of the Photosystem II Reaction Center on the Spinach Plastid Chromosome. *EMBO J.* 2(12):2229-2237.
68. Westhoff, P., Alt, J., Nelson, N., Bottomley, W., Bunemann, H. and Herrmann, R.G. (1983). Genes and Transcripts for the P700 Chlorophyll a Apoprotein and Subunit 2 of the Photosystem I Reaction Center Complex from Spinach Thylakoid Membranes. *Plant Mol. Bio.* 2:95-107.
69. Westhoff, P., Alt, J. and Herrmann, R.G. (1985). Genes and Transcripts for the ATP Synthase C_{fo} Subunits I and II from Spinach Thylakoid Membranes. *Mol. Gen. Genet.* 199:200-209.
70. Westhoff, P., Alt, J., Widger, W.R., Cramer, W.A. and Herrmann, R.G. (1985). Localization of the Gene for Apocytochrome b-559 on the Plastid Chromosome of Spinach. *Plant. Mol. Bio.* 4:103-110.
71. Whitfield, P.R. and Bottomley, W. (1983). Chloroplast DNA. *Ann. Rev. Plant Physiol.* 34:280-310.
72. Zech, M., Hartley, M.R. and Bohnert, H.J. (1981). Binding Sites of *E. coli* DNA-Dependent RNA Polymerase on Spinach Chloroplast DNA. *Curr. Genet.* 4:37-46.

73. Zurawski, G., Perrot, B., Bottomley, W. and Whitfield P.R. (1981). The Structure of the Gene for the Large Subunit of Ribulose 1,5-Bisphosphate Carboxylase from Spinach Chloroplast DNA. Nuc. Acids Res. 9(14):3251-3270.
74. Zurawski, G., Bohnert, H.J., Whitfield, P.R. and Bottomley, W. (1982). Nucleotide Sequence of the Gene for the Mr 32,000 Thylakoid Membrane Protein from Spinacia oleracea and Nicotiana debneyi Predicts a Totally Conserved Primary Translation Product of Mr 38,950. PNAS 79:7699-7703.
75. Zurawski, G., Bottomley and Whitfield, P.R. (1982). Structures of the Genes for the and Subunits of Spinach Chloroplast ATPase Indicate a Dicstronic mRNA and an Overlapping Translation Stop/Start Signal. PNAS 79:6260-6264.
76. Zurawski, G., Bottomley, W. and Whitfield, P.R. (1984). Junctions of the Large Single Copy Region and the Inverted Repeats in Spinacia oleracea and Nicotiana debneyi Chloroplast DNA: Sequence of the Genes for tRNA^{His} and the Ribosomal Proteins S19 and L2. Nuc. Acids Res. 12(16):6547-6558.