

NUCLEOTIDE SEQUENCE ANALYSIS OF CHLOROPLAST DNA
FROM A LIVERWORT, *MARCHANTIA POLYMORPHA* L.

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ABBREVIATIONS

DNA	deoxyribonucleic acid
<u>E. coli</u>	<u>Escherichia coli</u>
IPTG	isopropyl- β -D-thiogalactopyranoside
IR	inverted repeat
LSC	large single copy
<u>M. polymorpha</u>	<u>Marchantia polymorpha</u> L.
OD	optical density
ONPG	o-nitrophenyl- β -D-galactopyranoside
ORF	open reading frame
RNA	ribonucleic acid
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SSC	small single copy
Tris	tris(hydroxymethyl)aminomethane
bp	base pairs
kb	kilobase pairs
kd	kilodaltons
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA

Gene symbols

atp	genes for subunits of H ⁺ -ATP synthase
infA	gene for initiation factor 1
ndh	ORFs homologous to human mitochondrial NADH dehydrogenase
pet	genes for photoelectron transfer polypeptides
psa	genes for photosystem I chlorophyll <u>a</u> apoproteins
psb	genes for photosystem II chlorophyll <u>a</u> apoproteins
rbcl	gene for the large subunit of Rubisco
rpl	genes for 50S subunit of ribosomal proteins
rpo	genes for subunits of RNA polymerase
rps	genes for 70S subunit of ribosomal proteins
rrn	genes for ribosomal RNAs
trn	genes for transfer RNAs

INTRODUCTION

The chloroplast is the organelle of plants and green algae that has the photosynthetic apparatus and its own house keeping machinery. Chloroplasts and other plastids contain their own autonomously replicating DNA genome that is composed by double stranded covalently closed circular DNA. In 1960s, DNA molecules were visualized in chloroplasts by electronmicroscopic analysis (Ris and Plaut 1962, Sager and Ishida 1963). In 1976, maize chloroplast DNA was analyzed by restriction enzymes and physically mapped to be a circle of about 140,000 nucleotide pairs (Bedbrook et al. 1976). This included two large inverted repeat regions (IR_A and IR_B) of 22 kb coding for a set of ribosomal RNA genes. These IR regions were separated by large and small single copy regions (LSC and SSC regions). Chloroplast DNA of most plants and green algae has chromosomes of this general structure except for a few legumes (Pisum sativum and Vicia faba) and Euglena gracilis. Pea chloroplast DNA has no large repeat regions and Euglena gracilis chloroplast DNA has tandemly repeated regions coding for ribosomal RNA operons. Among chloroplast DNAs of the inverted repeat type from green plants, one of the smallest size is that of Marchantia polymorpha (121 kb) with 10 kb IR sequences (Ohyama et al. 1983), whereas that of Chlamydomonas reinhardtii is the largest (195 kb) with 21 kb IR sequences (Rochaix and Malnoe 1978).

The majority of proteins present in chloroplasts are encoded by nuclear DNA, but the rest are encoded by chloroplast DNA and synthesized by the chloroplast transcription-translation machinery. The nucleotide sequences of many chloroplast genes from various plant species have been determined (Crouse et al. 1985). The first chloroplast gene sequence to be determined for a chloroplast protein was that for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) (McIntosh et al. 1980). Since that time, a sizable number of plastid genes for proteins have been sequenced as summarized in Table 1. However, the complete sequence has not been determined as yet for any species of plants.

Table 1. Genes for proteins coded by chloroplast genome.

Gene	Protein product	Plant source	Reference
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase	Maize	McIntosh <i>et al.</i> 1980
		Spinach	Zurawski <i>et al.</i> 1981
		Tobacco	Sinozaki & Sugiura 1982
		Anabaena	Curtis & Haselkorn 1983
		<u>Chlamydomonas reinhardtii</u>	Dron <i>et al.</i> 1983
		Synechococcus	Reichelt & Delaney 1983
		<u>Anacystis nidulans</u>	Shinozaki <i>et al.</i> 1983
		<u>Rhodospirillum rubum</u>	Nargang <i>et al.</i> 1984
		Barley	Zurawski <i>et al.</i> 1984
		<u>Euglena gracilis</u>	Gingrich & Hallick 1985
		Maize	Fisch <i>et al.</i> 1985
psaA	PS I, P700 apoprotein	Spinach	Kirsch <i>et al.</i> 1986
psaB	PS I, P700 apoprotein	Maize	Fisch <i>et al.</i> 1985
psbA	PS II, "32 kd" protein	Spinach	Kirsch <i>et al.</i> 1986
		<u>Nicotiana debneyi</u>	Zurawski <i>et al.</i> 1982
		Spinach	Zurawski <i>et al.</i> 1982
		<u>Amaranthus hybridus</u>	Hirshberg & McIntosh 1983
		Soybean	Spielmann & Stutz 1983
		Anabaena	Curtis & Haselkorn 1984
		<u>Chlamydomonas reinhardtii</u>	Erickson <i>et al.</i> 1984
		Pea	Oishi <i>et al.</i> 1984
		<u>Solanum nigrum</u>	Goloubinoff <i>et al.</i> 1984
		<u>Euglena gracilis</u>	Keller & Stutz 1984
		<u>Nicotiana tabacum</u>	Sugita & Sugiura 1984
		Mustard	Link & Langridge 1984
psbB	PS II, P680 "51 kd" protein	Spinach	Morris & Herrmann 1984
psbC	PS II, "44 kd" protein	Spinach	Alt <i>et al.</i> 1984
psbD	PS II, "D2 polypeptide"	Spinach	Holschuh <i>et al.</i> 1984
		<u>Chlamydomonas reinhardtii</u>	Rochaix <i>et al.</i> 1984
		Pea	Rasmussen <i>et al.</i> 1984
		Spinach	Alt <i>et al.</i> 1984
		Spinach	Holschuh <i>et al.</i> 1984
psbE	PS II, cytochrome b-559	Spinach	Herrmann <i>et al.</i> 1984
		<u>Oenothera hookeri</u>	Carrillo <i>et al.</i> 1986
		<u>Nicotiana tabacum</u>	Carrillo <i>et al.</i> 1986
		Wheat	Hird <i>et al.</i> 1986
		Spinach	Herrmann <i>et al.</i> 1984
psbF	PS II, cytochrome b-559 (URF39)	<u>Oenothera hookeri</u>	Carrillo <i>et al.</i> 1986
		<u>Nicotiana tabacum</u>	Carrillo <i>et al.</i> 1986
		Wheat	Hird <i>et al.</i> 1986
		Maize	Steinmetz <i>et al.</i> 1986
psbG	PS II, G-protein		

Table 1 (continued).

Gene	Protein product	Plant source	Reference
petA	Cytochrome f preprotein	Pea	Willey <i>et al.</i> 1984
		Spinach	Alt <i>et al.</i> 1984
		wheat	Willey <i>et al.</i> 1984
		<i>Oenothera hookeri</i>	Tyagi & Herrmann 1986
petB	Cytochrome b6	Spinach	Heinemeyer <i>et al.</i> 1984
petD	Cytochrome b6/f complex, subunit 4	Pea	Phillips & Gray 1984
		Spinach	Heinemeyer <i>et al.</i> 1984
atpA	H ⁺ -ATP synthase, alpha subunit	Tobacco	Deno <i>et al.</i> 1983
atpB	H ⁺ -ATP synthase, beta subunit	Maize	Krebbers <i>et al.</i> 1982
		Spinach	Zurawski <i>et al.</i> 1982
		Tobacco	Shinozaki & Sugiura 1983
		Barley	Zurawski & Clegg 1984
atpE	H ⁺ -ATP synthase, epsilon subunit	Maize	Krebbers <i>et al.</i> 1982
		Spinach	Zurawski <i>et al.</i> 1982
		Tobacco	Shinozaki <i>et al.</i> 1983
		Barley	Zurawski & Clegg 1984
atpF	H ⁺ -ATP synthase, subunit I	Wheat	Bird <i>et al.</i> 1985
		Tobacco	Shinozaki <i>et al.</i> 1986
atpH	H ⁺ -ATP synthase, subunit III	Wheat	Howe <i>et al.</i> 1982
		Spinach	Alt <i>et al.</i> 1983
		Tobacco	Deno <i>et al.</i> 1984
atpI	H ⁺ -ATP synthase, subunit IV	Pea	Cozens <i>et al.</i> 1986
rp12	50S ribosomal protein 2	<i>Nicotiana debneyi</i>	Zurawski <i>et al.</i> 1984
		Spinach	Zurawski <i>et al.</i> 1984
rp16	50S ribosomal protein 16	<i>Spirodela oligorhiza</i>	Posno <i>et al.</i> 1986
rps4	30S ribosomal protein 4	Maize	Subramanian <i>et al.</i> 1983
rps7	30S ribosomal protein 7	<i>Euglena gracilis</i>	Montandon & Stutz 1984
rps11	30S ribosomal protein 11	Spinach	Muller <i>et al.</i> 1986
rps12	30S ribosomal protein 12	<i>Euglena gracilis</i>	Montandon & Stutz 1984
rps14	30S ribosomal protein 14	<i>Marchantia polymorpha</i>	Umesono <i>et al.</i> 1984
		Spinach	Kirsch <i>et al.</i> 1986
rps16	30S ribosomal protein 16	Tobacco	Shinozaki <i>et al.</i> 1986
rps19	30S ribosomal protein 19	Tobacco	Sugita & Sugiura 1983
		<i>Nicotiana debneyi</i>	Zurawski <i>et al.</i> 1984
		Spinach	Zurawski <i>et al.</i> 1984
infA	initiation factor I	Spinach	Muller <i>et al.</i> 1986
tufA	Elongation factor Tu	<i>Euglena gracilis</i>	Montandon & Stutz 1983
rpoA	RNA polymerase α subunit	Spinach	Muller <i>et al.</i> 1986
rpoB	RNA polymerase β subunit	Tobacco	Ohme <i>et al.</i> 1986

The chloroplast DNAs from common bean, soybean, the fern Osmunda and the algae Chlamydomonas reinhardtii have been shown to exist as a 50:50 mixture of two genetically identical but physically distinct molecules called isomer differing only for in the relative orientation of their single copy regions (Palmer 1983). In the case of M. polymorpha, isomers have been identified by fragmentation using PstI, and southern hybridization analysis of the chloroplast DNA with cloned DNA fragments as shown in Fig. 1. ³²P-Labeled DNA fragments involved in either single copy region (probe 1) or inverted repeat (probe 2) hybridized to the chloroplast DNA segments generated by BamHI or PstI restriction enzyme (Fig. 2). Probe 1 containing a DNA segment of an inverted repeat hybridized with the four largest fragments (P1, P1', P2 and P2') produced by PstI. On the other hand, probe 2 containing a DNA segment of a single copy region only hybridized with two fragments (P1 and P1'). So in the M. polymorpha chloroplasts two types of DNA molecules exist in an equal amount. It is interesting to know whether the events on DNA replication have any relationship to the formation of isomers. But the mechanisms of flip-flop recombination have not been clarified yet.

The chloroplast biogenesis depends on the coordinative gene expression of both the chloroplast genome and the nuclear genome. But the interaction between chloroplast and nuclear genetic systems has not been clarified. In this study, the author has intended to reveal the mechanisms of gene expression of the chloroplast genome on the basis of the molecular biological methods as follows.

Chapter I; Chloroplast transcriptional promoters were cloned and characterized using the E. coli gene fusion method. Chapter II; The primary structure of the M. polymorpha chloroplast DNA was determined and genes for transfer RNAs, photosynthetic polypeptides, ribosomal proteins, and α subunit of RNA polymerase were identified. Chapter III; An unique system for gene expression that may be called trans-splicing was found in the biogenesis of ribosomal protein S12.

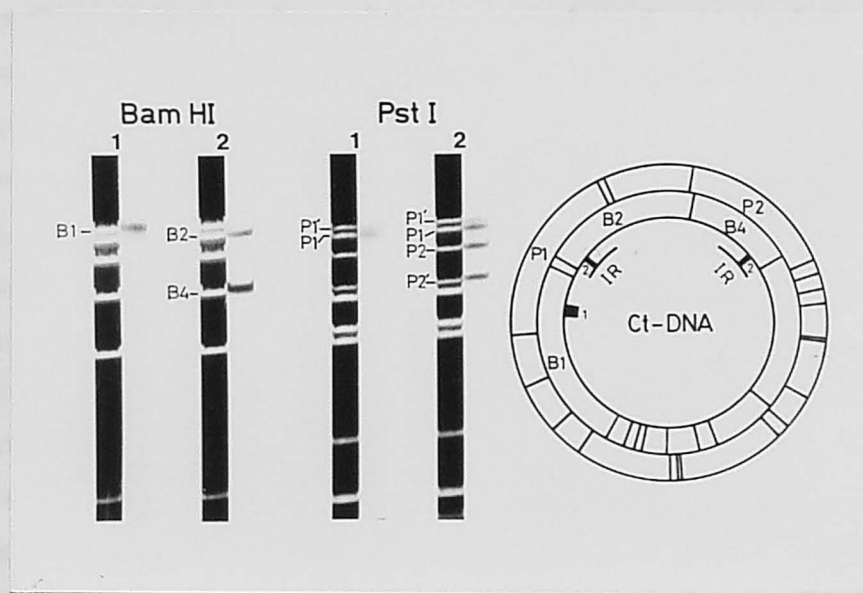
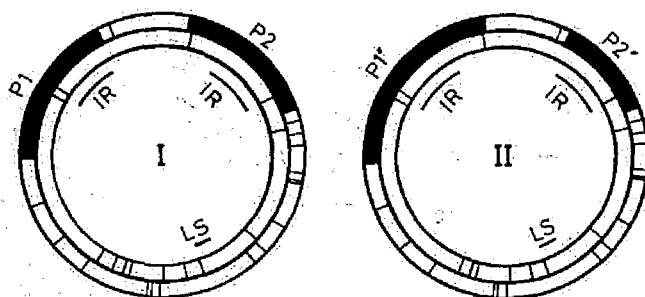


Figure 1. Southern hybridization of the probes containing DNA segments of an inverted repeat region and a single copy region. Chloroplast DNA fragments digested by BamHI and PstI restriction enzymes were electrophoresed in 0.6% agarose gels (each left lane), transferred to nylon membrane filters and hybridized with ^{32}P -labeled probes. Filters were hybridized with the probe 1 containing the Bg16 segment of a single-copy region (black box in the right figure and Lane 1) and probe 2 containing the Bg21 fragment of an inverted repeat (Lane 2). Autoradiograms are shown right side of each electrophoretic pattern.



Isomers in chloroplast DNA
from M. polymorpha

Figure 2. Isomers and their restriction maps of the chloroplast DNA. Two kinds of molecules (form I and II) of the chloroplast DNA are shown. IR and LS indicate inverted repeat sequences containing ribosomal RNA genes, the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, respectively.

CHAPTER I Molecular cloning of promoters functional in Escherichia coli from chloroplast DNA

Chloroplasts have their own transcriptional and translational systems including unique transfer RNAs, ribosomal RNAs, RNA polymerase and ribosomal proteins different from those of the cytoplasm. Determination of the locations of transcriptional and translational regulatory regions on the chloroplast genome would lead to a basic understanding of the mechanisms of gene expression in chloroplasts. It has been suggested that mechanisms of gene expression in chloroplasts are similar to those in E. coli (Kidd and Bogorad 1979, Whitfeld and Bottomley 1983, Gruissem et al. 1983, Gruissem and Zurawski 1985). If this is the case, E. coli plasmid vectors can be used to clone chloroplast DNA fragments containing transcriptional start signals which can function in E. coli. Casadaban et al. constructed a plasmid in which β -galactosidase activity was expressed by insertion of exogenous DNA fragments having promoter sequences, ribosomal binding sites, and translation start codons in the right frame, and showed that the strength of promoters and ribosome binding activity can be assayed by measuring the enzyme activity (Casadaban et al. 1980).

In this chapter, the author shows an evidence that some of the chloroplast promoters can be expressed in E. coli by using gene fusion techniques with the β -galactosidase gene. The nucleotide sequence of the DNA fragment with the highest enzyme activity in E. coli was determined and the location of the promoter was mapped on the chloroplast genome. To find out whether the chloroplast DNA fragment in fact initiates transcription in E. coli as well as in chloroplasts, S1 nuclease mapping experiments were done. The mechanisms of gene expression in chloroplasts are discussed.

MATERIALS AND METHODS

DNA isolation, restriction endonuclease digestion, and hybridization

Chloroplast DNA was isolated from liverwort, M. polymorpha suspension culture by the method describes previously (Ohyama et al. 1982) with some modifications.

Chloroplasts were isolated from cultured cells by using blender containing sea sand (20-25 mesh). Restriction digestion, hybridization and plasmid DNA isolation were carried out by the procedure described by Maniatis et al. (1982).

Cloning of chloroplast DNA fragments

Chloroplast DNA was cleaved by EcoRI, Sau3A, BglIII, SmaI and BamHI restriction endonucleases. Restricted chloroplast DNA fragments were ligated into dephosphorylated EcoRI, BamHI and SmaI sites of plasmid pMC1403 (Casadaban et al. 1980). The ligated DNA was used to transform competent cells of *E. coli* strain MC1061 (araD139, Δ (ara, leu)7697, Δ lacX74, gal^U-, gal^K-, hsr-, hsm-, strA) (Casadaban and cohen 1980). Transformed cells were selected on lactose MacConkey agar plates containing 100 µg/mg of ampicillin. Red colonies using lactose (lac⁺) and resistant to ampicillin were picked up and cultured in L-broth in the presence of ampicillin.

β -Galactosidase assay

E. coli cells harboring recombinant plasmids were grown to $2-5 \times 10^8$ cells/ml and β -galactosidase activities were assayed as described by Platt et al. (1972).

DNA sequencing

The nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al. 1977) using M13 phages mp10 and mp11 (Messing 1983). For size markers in S1-nuclease mapping, a HincII-EcoRI fragment (see Fig. 3) was labeled with [γ ³²P]-ATP (3000 Ci/mmol, Amersham) at the 5' terminus by T4 polynucleotide kinase after alkali phosphatase treatment, and sequenced by the method of Maxam and Gilbert (1977).

RNA isolation and S1-nuclease mapping

Chloroplast RNA was extracted from chloroplast pellets by phenol chloroform

extraction described by Yamano *et al.* (1984). *E. coli* total RNA was extracted by the SDS-hot phenol method and S1 mapping was performed by the procedure described by Aiba *et al.* (1981) using 5' end labeled EcoRI-Sau3A fragment (10^{5-6} cpm/ μ g) and 100 μ g of RNA.

RESULTS

Cloning and characterization of chloroplast DNA fragments having promoter function

Eleven recombinants were selected on lactose MacConkey agar plates containing ampicillin as red colonies showing β -galactosidase production in *E. coli*. The β -galactosidase assays of recombinants showed a variety of levels of the activities summarized in Table 1. The recombinants can be grouped in three categories by comparing with IPTG induced β -galactosidase activity in *E. coli* wild strain W3110: 1, a group with a slightly higher level of the enzyme activity such as recombinants harboring plasmids pMP901-903; 2, a group with a rather low level of the activity such as recombinants carrying plasmids pMP921, pMP954-956; and 3, a group with a very low level of the activity such as recombinants carrying plasmids pMP910-912, pMP953.

Restriction analysis of recombinant plasmids (pMP901-903) with high levels β -galactosidase activity showed that they had the same 5.1 kb DNA fragment, which corresponded to Ec8 fragment (see Fig. 3). To confirm the inserted DNA fragment in the plasmid pMC1403 was generated from *M. polymorpha* chloroplast DNA and to find its site on the chloroplast physical maps, plasmid pMP903 was labeled with [α^{32} P]-dCTP using the nick translation reaction and hybridized with chloroplast DNA fragments on nitrocellulose filter (Fig. 1). 32 P-Labeled pMP903 hybridized to the chloroplast DNA fragments Ba2, Ba4, Ba6 and Ba11 (Fig. 1B). The 1.1 kb BamHI fragment derived from the 5.1 kb EcoRI fragment with an additional 10 bp of pMC1403 was recloned into pMC1403 and the recombinant plasmid was named pMP904 (Fig. 2). Southern hybridization with chloroplast DNA fragments showed that the inserted DNA fragment

Table 1. β -Galactosidase activity and copy numbers in clones containing recombinant plasmids.

Host strains	Plasmids	β -Galactosidase activity (units) ^a	Relative amounts of plasmids ^b
W3110 (-IPTG)	—	<11	—
W3110 (+IPTG)	—	344 $\pm$ 28	—
MC1061	—	<5	—
MC1061	pMC1403	<6	1.0
MC1061	pMP901	361 $\pm$ 20	0.9 $\pm$ 0.1
	pMP902	328 $\pm$ 27	0.9 $\pm$ 0.1
	pMP903	360 $\pm$ 23	0.9 $\pm$ 0.1
	pMP904 ^c	408 $\pm$ 39	1.0 $\pm$ 0.1
	pMP905 ^c	365 $\pm$ 20	0.9 $\pm$ 0.1
	pMP906 ^c	308 $\pm$ 33	1.0 $\pm$ 0.1
MC1061	pMP954 ^d	200 $\pm$ 14	1.0 $\pm$ 0.1
	pMP955 ^d	267 $\pm$ 11	0.9 $\pm$ 0.1
	pMP956 ^d	233 $\pm$ 24	0.8 $\pm$ 0.2
	pMP921	253 $\pm$ 20	1.1 $\pm$ 0.1
MC1061	pMP953 ^c	49 $\pm$ 7	0.7 $\pm$ 0.2
	pMP910	17 $\pm$ 3	0.9 $\pm$ 0.1
	pMP911	73 $\pm$ 8	0.9 $\pm$ 0.1
	pMP912	56 $\pm$ 11	1.0 $\pm$ 0.1

a) Numbers are averages of three experiments and expressed as units per mg of total protein (Platt et al. 1972).

b) Plasmid copy numbers were measured by the method of Projan et al. (1983) and expressed as the number relative to pMC1403 in E. coli strain MC1061 as 1.0.

c) Derived from pMP903 (see Figure 2)

d) Containing *rbcl* gene.

e) Containing β subunit gene of H^+ -ATP synthase.

(1.1 kb) of pMP904 hybridized to Ec4 (located on the other IR region), Ec8, Ba6 and Ba11 (see Fig. 1C). These results and no cleavage site of EcoRI on the Ba11 fragment indicate that the cloned DNA fragment originates from the Ba6 fragment and is located on the large single copy region close to the inverted repeat (IR) (Fig. 3). As the clone harboring plasmid pMP904 still expressed the high level of the enzyme activity, the internal HincII-EcoRI fragment was further subcloned into pMC1403 (Fig.2). The recombinant plasmid, named pMP905, kept its high level of β -galactosidase production. However, the recombinant plasmid pMP906, which was constructed by deletion of HincII-AluI fragment (position at 3 to 348) from pMP905 (see Fig. 4), gave slightly less activity than plasmids pMP904 and pMP905 (Table 1).

Nucleotide sequence of a promoter and its downstream region in chloroplast DNA

As the HincII-EcoRI chloroplast DNA fragment inserted pMP905 appeared to have a transcriptional and translational start signals, the nucleotide sequence between two HincII sites was determined by the strategy shown in Fig. 3. The nucleotide sequence of the 1192 bp chloroplast DNA fragment and junction region between the chloroplast DNA fragment and the lacZ gene of pMP905 is shown in Fig. 4A and 4B, respectively.

S1 nuclease mapping

The position in the sequence corresponding to the 5' end of the mRNA was determined by an S1 nuclease mapping procedure. The 533 bp HincII-EcoRI fragment (position at 4 to 536 in Fig. 4A) was labeled at the 5' ends with [γ - 32 P]-ATP and digested by Sau3A restriction enzyme, generating two fragments, 66 nucleotides long and 467 nucleotide long. The 32 P-labeled 467 bp fragment was hybridized with either E. coli RNA or chloroplast RNA at 37°C. Hybridized DNA-RNA molecules were digested by S1 nuclease and the length of the S1 nuclease-resistant DNA fragment was measured by electrophoresis with a Maxam-Gilbert sequence ladder generated from the 467 bp fragment as shown in Fig. 5. The 5' end of the mRNA from both E. coli and



Figure 1. Southern hybridization of chloroplast DNA fragments with plasmids pMP903 (B) and pMP904 (C). Panel A shows agarose gel electrophoresis stained by ethidium bromide. Lanes 1, 2, 3, 4 and 5 correspond to the electrophoretic patterns of digests by EcoRI, EcoRI/BamHI, BamHI, BamHI/BglIII and BglIII restriction enzymes, respectively.

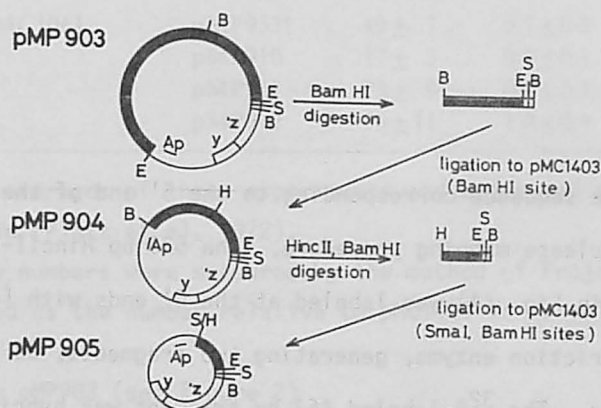


Figure 2. Construction of plasmids pMP904 and pMP905 from pMP903 containing promoter regions. Heavy lines indicate chloroplast DNA segments. The structural gene of β -galactosidase is shown as 'Z'.

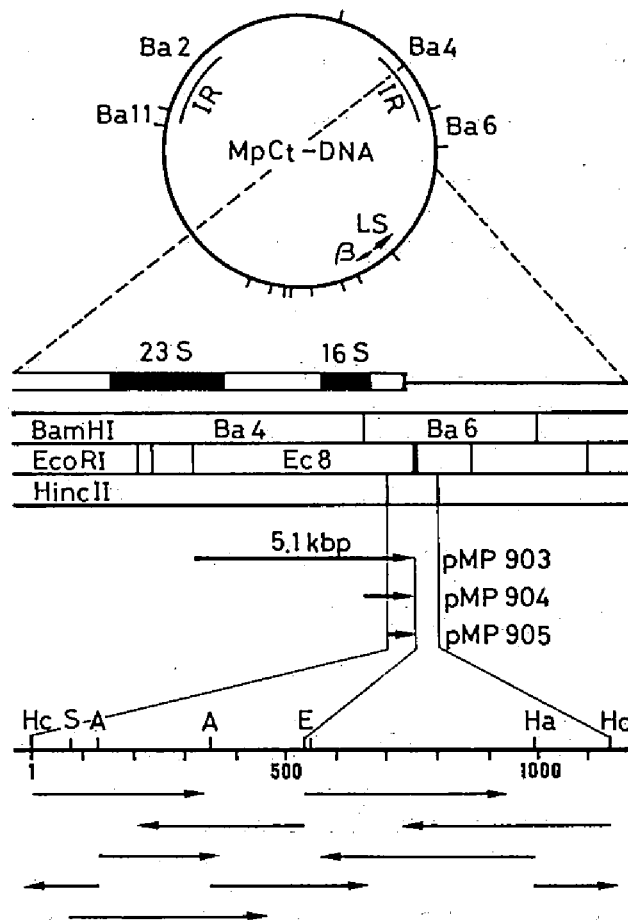
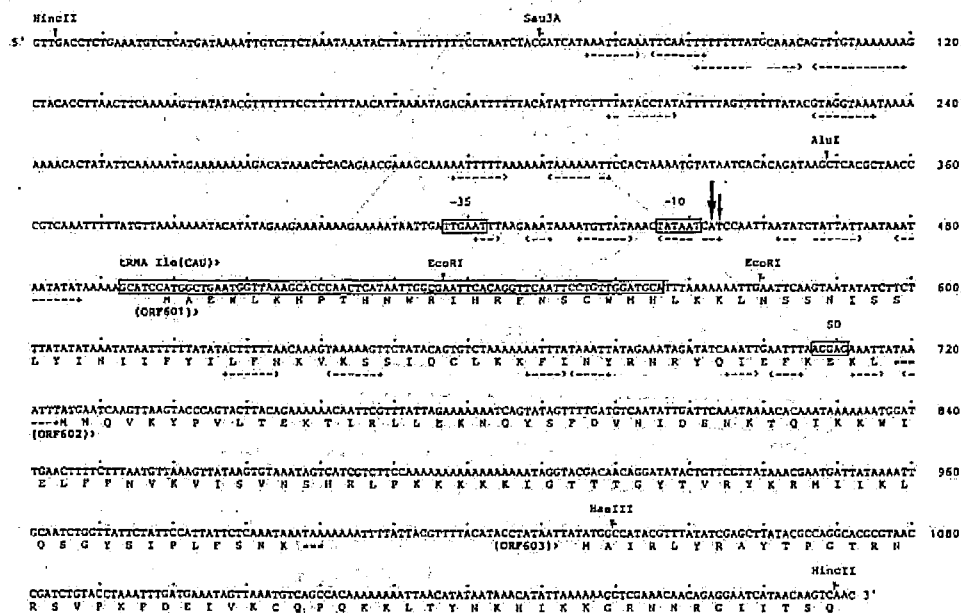


Figure 3. Location of the DNA fragments having promoter function on the physical map of chloroplast DNA, and the sequencing strategy. IR indicates inverted repeat region containing ribosomal RNA operons including 16S and 23S rRNA genes. LS and β indicate the genes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and for the β subunit of H^+ -ATP synthase, respectively. A, E, Ha, Hc and S indicate cleavage sites of the restriction enzymes AluI, EcoRI, HaeIII, HincII and Sau3A, respectively. Bottom arrows indicate sequencing strategies using the M13 phages mp10 and mp11.

A



B

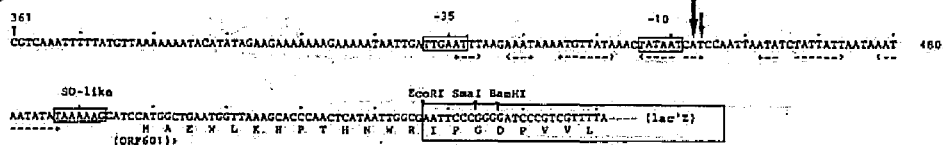


Figure 4. Nucleotide sequence of a DNA fragment having promoter function derived from liverwort chloroplast DNA. Nucleotide sequence of the 1192 bp HincII fragment (Fig. 3) is shown in (A). Possible stem and loop structures are shown by broken lines with arrows. Bold vertical arrows indicate the start sites of mRNA transcription in *E. coli* as well as in chloroplasts. The regions of "-35", "-10", Shine-Dalgarno sequence (SD), and genes for tRNA^{Ile}(CAU) and β -galactosidase are boxed. Deduced amino acid sequences of ORF601, 602 and 603 are shown under the nucleotide sequences by single letter symbols. Double underlines indicate termination codons. N-Terminal region of the fused protein between ORF601 and β -galactosidase coded by pMP903-906 is shown in (B).

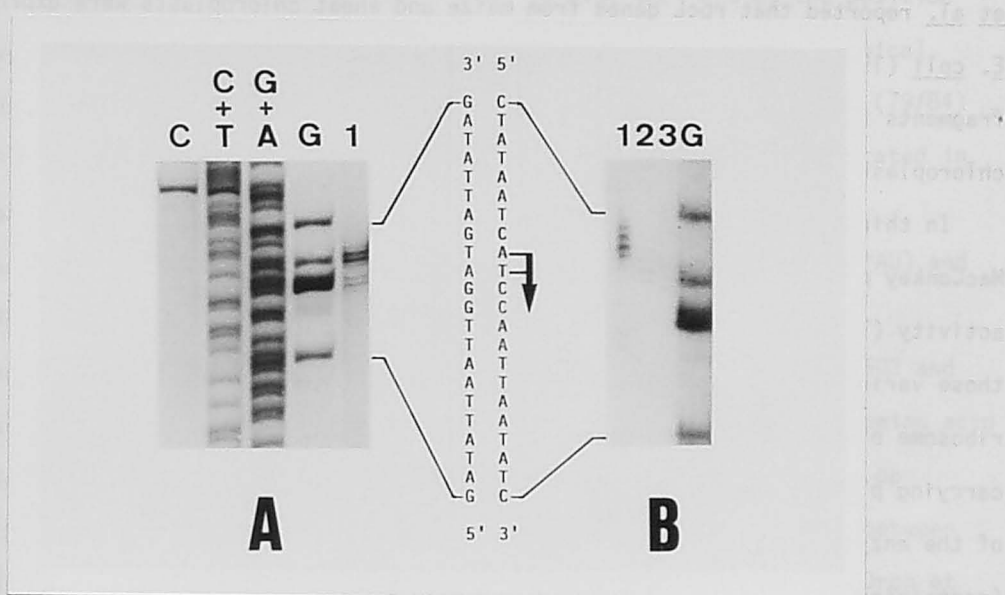


Figure 5. S1 nuclease mapping by *E. coli* RNA (A) and chloroplast RNA (B) of the promoter region. The S1 nuclease protected DNA fragments (lanes 1, 2 and 3) were electrophoresed in parallel with the Maxam-Gilbert sequence ladders. Lane 1 (A), and lanes 1, 2 and 3 (B) correspond to the concentration of S1 nucleases, 5000, 50, 500 and 5000 units per reaction, respectively. S1 mapping in lane 1 (B) should read to be A, because of the smiling pattern of the gel electrophoresis. An arrow indicates the direction of the transcription.

chloroplast was thus mapped on the sequence to be 45-46 nucleotides upstream from the ATG translational start codon of ORF601 (Fig. 4).

DISCUSSION

From *M. polymorpha* chloroplast DNA, DNA fragments were cloned functional in *E. coli* transcription and translation system. Several chloroplast genes have been cloned into *E. coli* plasmids. With the *rbcl* gene, its expression was observed in an *in vitro* coupled transcriptional translational system derived from *E. coli*. Gatenby

et al. reported that *rbcl* genes from maize and wheat chloroplasts were expressed in *E. coli* (1981). Kong et al. reported the cloning of promoter-containing restriction fragments from *Nicotiana* chloroplast DNA and location of the fragments on the chloroplast genome (1984).

In this study 11 recombinants were obtained which were selected on lactose MacConkey plates as red colonies. These clones, however, varied in their enzyme activity (Table 1). As plasmid copy numbers were not much different in each clone, those variation may reflect the efficiency of the transcriptional start signals and ribosome binding activities in the chloroplasts. For instance, a recombinant carrying plasmid pMP954-956 (containing the *rbcl* promoter) had a rather high level of the enzyme activity as expected. A recombinant harboring plasmid pMP953 (containing the promoter region of the β subunit gene of H^+ -ATP synthase) showed quite a low level of the activity. These results coincide with the fact that the mRNA synthesis of the β subunit gene is considerably lower than that of *rbcl* gene (Shinozaki et al. 1983). Therefore, the efficiency of *E. coli* transcriptional system may reflect that of the transcription in chloroplasts.

The plasmid pMP905, carrying a promoter region of an unidentified open reading frame named ORF601, gave the highest level of enzyme activity in *E. coli*. Analysis of the nucleotide sequence of the promoter and its downstream region revealed that a translational initiation codon (ATG) of ORF601 was found 38 bp upstream from the *EcoRI* site and its open reading frame was fused to the *lacZ* gene in the right frame (see Fig. 4B). And 12 bp upstream from ATG codon, a sequence (TAAaaAG) partially complementary to the 3' end of *E. coli* 16S rRNA (Shine-Dalgarno (SD) sequence) (Shine and Dalgarno 1974) was found. There was a typical sequence for the transcriptional promoter signal (TATAAT), called the Pribnow-box (Pribnow 1975) or "-10" region at 53 bp upstream from the ATG codon. There was an unique sequence (aTTGAat) at 82 bp upstream, called the "-35" region which is thought to be a RNA polymerase recognition site in *E. coli* (Takanami et al. 1976). In addition, three possible stem-loop structures can be formed between the "-35" region and SD-like

sequence as indicated by underlining with arrows in Fig. 4A. But at the position 493-566 a tRNA gene, whose anticodon was CAU, was identified by forming typical secondary structure as shown in Fig. 6. This tRNA gene has 94.6% homology (79/84) with spinach chloroplast isoleucine tRNA gene (Francis and Dudock 1982) located in IR regions. So this tRNA gene was confirmed to code isoleucine tRNA in M. polymorpha chloroplasts. This highly active promoter may be for tRNA^{Ile}(C*AU) and for the downstream ORFs in the LSC region. Three open reading frames were identified downstream from the promoter region, and designated ORF601, ORF602 and ORF603. Their possible gene products were estimated to be 73, 91 and >52 amino acid residues, respectively. A typical SD sequence (AGGAG) was found seventeen bp upstream from the ATG codon of ORF602, and a stem structure can be formed between the SD sequence and ATG codon as reported in the Chlamydomonas rbcl gene (Dron et al. 1983). This stem structure may have a role to let the SD sequence close to the ATG codon. ORF602 and ORF603 were identified to be putative genes for proteins corresponding to E. coli ribosomal protein L23 and L2, respectively. Detail data are presented in chapter II.

Nucleotide sequence analysis revealed that the organization of this chloroplast promoter was similar to that of E. coli promoters. Furthermore, these results of S1 mapping using in vivo transcripts from both E. coli and chloroplasts showed that the transcription starts at almost the same position downstream from the promoter region. So the gene fusion method described here could be a powerful technique to clone and characterize the promoters on the chloroplast genome.

Chapter II Structure and gene organization of the chloroplast genome

To understand the genetic system in the chloroplast, the nucleotide sequence of the liverwort, *M. polymorpha* chloroplast DNA was determined. The *M. polymorpha* chloroplast DNA has been physically mapped previously (Ohya *et al.* 1983). The gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase has been mapped on the chloroplast genome by heterologous hybridization with tobacco *rbcL* gene. The genes for ribosomal RNAs; 23S, 16S, 5S and 4.5S have been also localized in the inverted repeat regions (Ohya *et al.* 1983, Yamano *et al.* 1984, Yamano *et al.* 1985). The overall gene organization deduced from the complete nucleotide sequence is described by Ohya *et al.* (1986). In this study, properties and characterization of genes on the LSC region (from *psbG* to 16S rRNA gene; 30,600 bp) deduced from the nucleotide sequence are presented and discussed. The region analyzed in this study is shown as a black box with physical maps in Fig. 1. In the region described here, putative genes for seven tRNAs, ten photosynthetic polypeptides, thirteen ribosomal proteins and α subunit of RNA polymerase were identified. In addition, an open reading frame (ORF) was found to show significant amino acid sequence homology to a subunit of NADH dehydrogenase in human mitochondria.

MATERIALS AND METHODS

Chloroplast DNA was isolated from cell suspension culture of *M. polymorpha* as described previously (Ohya *et al.* 1982). Chloroplast DNA was cloned into *E. coli* plasmid vectors; pBR322, pKC7, pUC13, pUC18 and pUC19. Recombinant plasmids used for the nucleotide sequencing were summarized in Table 1. The locations of chloroplast DNA fragments used in sequence determination are shown in Fig. 2. Each plasmid was sonicated (Deininger 1983) by TOMY handy sonicator and randomly cloned into *Sma*I or *Hinc*II sites of phage mp18 and mp19 (Perron *et al.* 1981). Recombinant phages containing chloroplast DNA fragments were screened by dot-hybridization with the chloroplast DNA (Hu and Messing 1982). Obtained shotgun libraries were used for

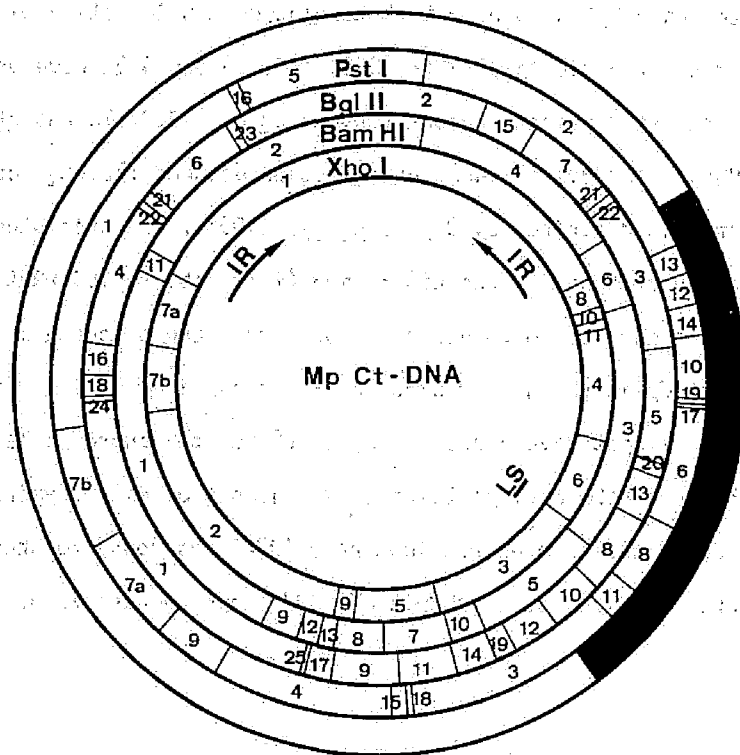


Figure 1. Restriction map of the *M. polymorpha* chloroplast DNA. Narrow lines with arrow heads inside the circular map indicate inverted repeat regions. LS indicates the site of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL). The sequenced region in this study is shown as a black box.

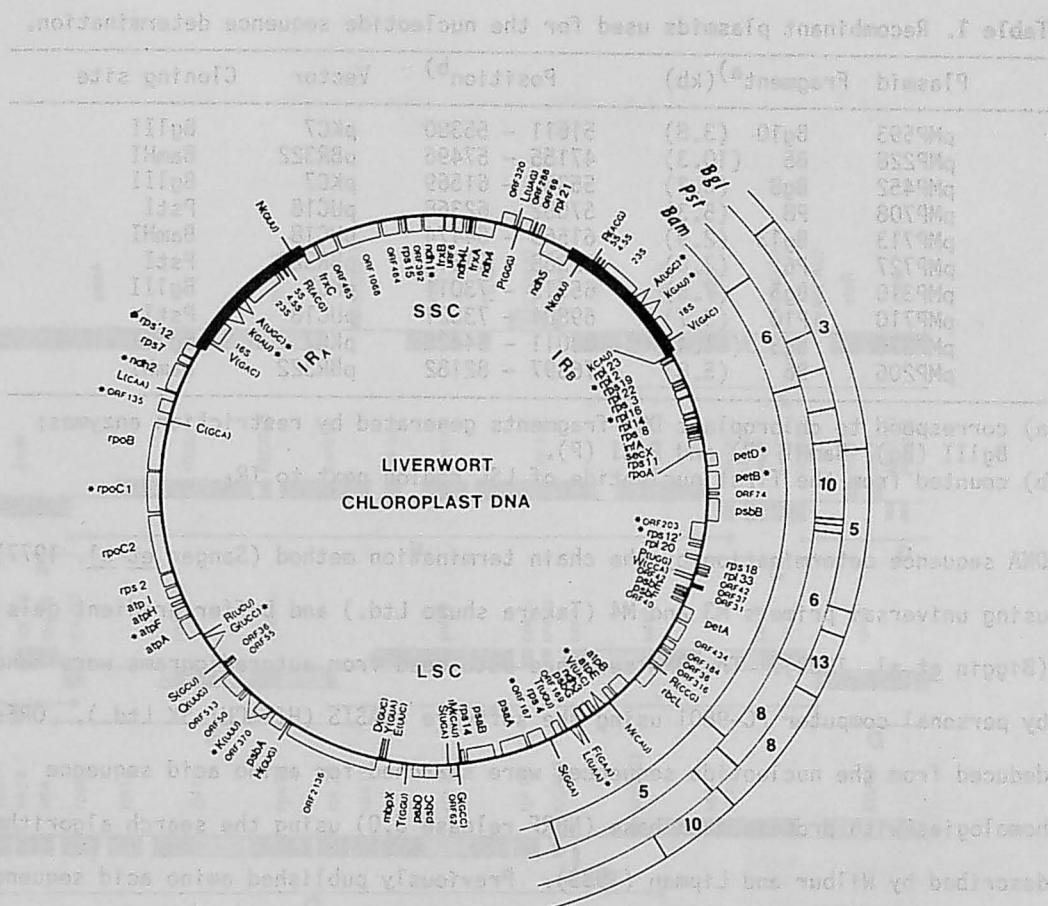


Figure 2. Gene organization of the chloroplast genome from a liverwort, *M. polymorpha* and sequenced restriction fragments. Thick lines indicate the inverted repeats (IR_A and IR_B). SSC and LSC indicate the small single-copy region and large single-copy region, respectively. Genes shown outside the map are transcribed anticlockwise, and those inside are transcribed clockwise. The tRNA genes are identified by the one-letter amino acid code with their anticodons given in parentheses. Asterisks indicate genes having introns in their sequences (Ohyama *et al.* 1986). Restriction fragments used in sequence determination are shown outside the genetic map.

Table 1. Recombinant plasmids used for the nucleotide sequence determination.

Plasmid	Fragment ^{a)} (kb)	Position ^{b)}	Vector	Cloning site
pMP593	Bg10 (3.8)	51611 - 55380	pKC7	BglII
pMP228	B5 (10.3)	47155 - 57496	pBR322	BamHI
pMP452	Bg8 (6.2)	55380 - 61569	pKC7	BglII
pMP708	P8 (5.3)	57082 - 62368	pUC18	PstI
pMP713	Bg13 (2.9)	61569 - 64474	pUC18	BamHI
pMP727	P6 (7.0)	62368 - 69315	pBR322	PstI
pMP310	Bg5 (7.5)	65513 - 73011	pKC7	BglII
pMP710	P10 (3.7)	69804 - 73521	pUC18	PstI
pMP376	Bg3 (11.4)	73011 - 84425	pKC7	BglII
pMP206	B6 (5.8)	76397 - 82182	pBR322	BamHI

a) correspond to chloroplast DNA fragments generated by restriction enzymes; BglII (Bg), BamHI (B) and PstI (P).

b) counted from the first nucleotide of LSC region next to IR_A.

DNA sequence determination by the chain termination method (Sanger *et al.* 1977) using universal primers M3 and M4 (Takara shuzo Ltd.) and buffer gradient gels (Biggin *et al.* 1983). The DNA sequence data read from autoradiograms were handled by personal computer PC-9801 using the software DNASIS (HITACHI SK Ltd.). ORFs deduced from the nucleotide sequences were searched for amino acid sequence homologies with protein data base (NBRF release 6.0) using the search algorithm described by Wilbur and Lipman (1983). Previously published amino acid sequences of polypeptides in other plant species were also used to homology search.

RESULTS

Gene organization in the LSC region (psbG-16S rRNA gene)

The nucleotide sequence (30,600 bp) from the BglII site (position 51611) to BamHI site (82188) covering the junction (J_{LB}) between LSC and IR_B regions was determined. A computer search of the DNA sequence led to the identification of ORFs that begin with the translation initiation codon AUG and end at an either termination codon (UAA, UAG, and UGA). The gene organization deduced from the nucleotide sequence are shown schematically in Fig. 3. The LSC region sequenced in this study was divided into seven blocks (Fig. 3 A-G) depending on the directions of the translational orientation (shown by horizontal arrows in Fig. 3). The

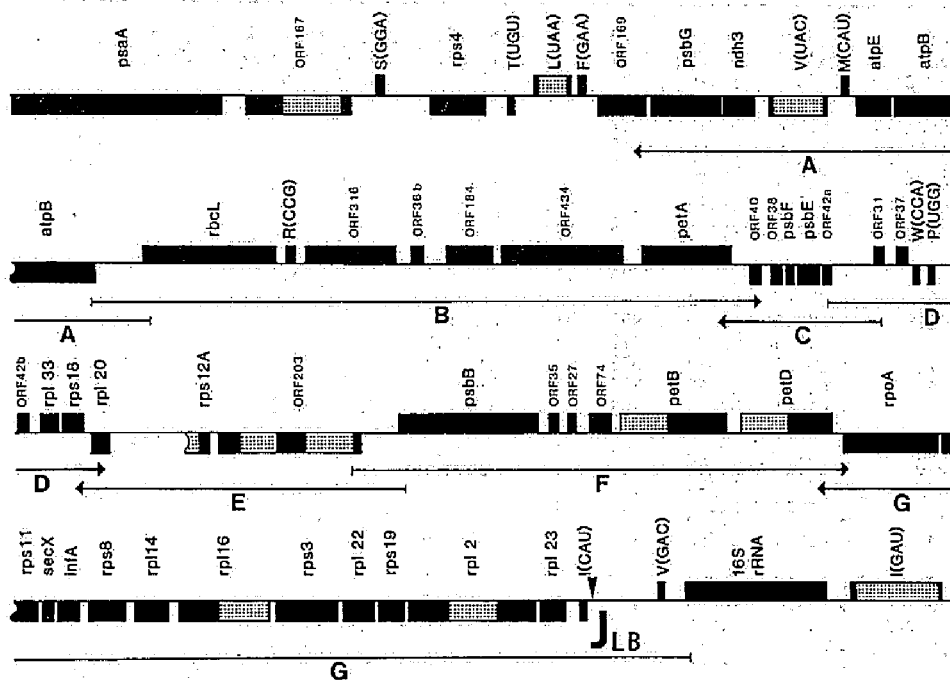


Figure 3. Detail gene organization of the region sequenced in this study.

The coding regions of genes are indicated by bold lines. Introns (intervening sequences) are shown as hatched box. Genes shown on lines are transcribed to the right side, and those under lines are transcribed to the left side. The sequence files are indicated by arrows with the names of sequence files. *J_{LB}* indicates the junction site between LSC region and *IR_B* region.

Each line indicates 10 kb long.

Table 2. List of identified genes and open reading frames, and their loci on the chloroplast genome.

Gene	From	To	Length (bp)	Amino acid residue	M.W.	Comments
(A)						
atpB	- 55846	54368	1479	492	53179.3	88.4% (Spi) 62.8% (Eco)
atpE	- 54362	53955	409	135	15054.3	63.0% (Spi) 22.2% (Eco)
trnM(CAU)	+ 53801	53874	74	-	-	94.6% (Tob)
*trnV(UAC)	- 53652	53051	602	-	-	91.7% (Tob)
(Exon 1)	53652	53616	37	-	-	
(intron)	53615	53086	530	-	-	
(Exon 2)	53085	53051	35	-	-	
ndh3	- 52877	52515	363	120	14188.7	30.8% (mit)
psbG	- 52524	51793	732	243	27609.6	62.1% (Mz)
ORF169	- 51742	51233	510	169	20084.8	
(B)						
rbcL	+ 56355	57782	1428	475	52790.0	90.5% (Spi)
trnR(CCG)	+ 57877	57950	74	-	-	
ORF316	+ 58065	59015	951	316	35826.3	
ORF36b	+ 59193	59303	111	36	4017.8	
ORF184	+ 59525	60079	555	184	21533.1	
ORF434	+ 60151	61455	1305	434	51866.2	
petA	+ 61641	62603	963	320	33482.6	78.8% (Spi)
(C)						
ORF40	- 62916	62794	123	40	4101.8	72.6% (Cya)
ORF38	- 63152	63036	117	38	4479.1	
psbF	- 63293	63174	120	39	4468.3	89.7% (Spi)
psbE	- 63554	63303	252	83	9493.5	89.2% (Spi)
ORF42a	- 63684	63556	129	42	5101.9	
(D)						
ORF31	+ 64152	64247	96	31	3466.4	
ORF37	+ 64370	64483	114	37	4075.9	
trnW(CCA)	- 64626	64553	74	-	-	93.4% (Spi)
trnP(UGG)	- 64788	64715	74	-	-	93.4% (Spi)
ORF42b	+ 65027	65155	129	42	4746.6	
rp133	+ 65273	65470	198	65	7782.1	36.9% (Eco)
rps18	+ 65498	65725	228	75	8879.5	34.7% (Eco)
(E)						
rp120	- 66157	65807	351	116	12773.0	46.6% (Eco)
*rps12A	- 67057	?	?	123	13797.0	70.2% (Eco) 91.9% (Tob)
(Exon 1)	67057	66944	111	38	-	trans-split
(intron ?)	66943	?	?	-	-	(Exon 2,3 coded opposit strand)
*ORF203	- 68640	67130	1511	203	22685.0	(87.0% homologous to spinach X-gene)
(Exon 1)	68640	68570	71	-	-	
(intron)	68569	68052	518	-	-	
(Exon 2)	68051	67760	292	-	-	
(intron)	67759	67379	381	-	-	
(Exon 3)	67378	67130	249	-	-	
(F)						
psbB	+ 69026	70552	1527	508	56191.5	88.2% (Spi)
ORF35	+ 70669	70776	108	35	3958.8	
ORF27	+ 70872	70955	84	27	3268.0	
ORF74	+ 71092	71316	225	74	7928.3	
*petB	+ 71424	72566	1143	215	24306.5	86.0% (Spi)
(Exon 1)	71424	71429	6	-	-	
(intron)	71430	71924	495	-	-	
(Exon 2)	71925	72566	642	-	-	
*petD	+ 72715	73690	976	160	17413.5	95.6% (Spi)
(Exon 1)	72715	72722	8	-	-	
(intron)	72723	73215	493	-	-	
(Exon 2)	73216	73690	475	-	-	

Table 2 (continued).

Gene	From	To	Length (bp)	Amino acid residue	M.W.	Comments
(6)						
rpoA	- 74824	73802	1023	340	39240.2	25.6% (Eco), 54.1% (Spi)
rps11	- 75249	74857	393	130	14172.5	51.5% (Eco), 72.3% (Spi)
secX	- 75413	75300	114	37	4521.5	62.2% (Eco), 86.5% (Spi)
infA	- 75686	75450	237	78	8978.4	56.4% (Eco), 60.3% (Spi)
rps8	- 76171	75773	399	132	14921.4	45.5% (Eco)
rpl14	- 76621	76253	369	122	13496.6	58.2% (Eco)
*rpl16	- 77685	76719	967	143	16149.8	53.8% (Eco), 72.0% (Spir)
(Exon 1)	77685	77677	9	-		
(intron)	77676	77142	535	-		
(Exon 2)	77141	76719	423	-		
rps3	- 78396	77743	654	217	25055.0	40.6% (Eco)
rpl22	- 78804	78445	360	119	13580.8	37.8% (Eco)
rps19	- 79100	78822	279	92	10553.3	63.0% (Eco), 83.7% (Spi)
*rpl2	- 80514	79137	1378	277	31162.9	48.4% (Eco), 58.5% (Spi)
(Exon 1)	80514	80118	397	-		
(intron)	80117	79574	544	-		
(Exon 2)	79573	79137	437	-		
rpl23	- 80825	80550	276	91	10768.5	29.7% (Eco)
trnI(C*AU)	- 81057	80984	74	-		93.2% (Spi) C*; modified base
trnV(GAC)	+ 81814	81885	72	-		95.8% (Spi) in 1R region

Amino acid sequence homologies are calculated as $\frac{(\text{identical residue number})}{(\text{residue number of liverwort product})}$.

Homology percentages with gene products of Spinach (Spi), *E. coli* (Eco), Tobacco (Tob), human mitochondria (mit), Maize (Mz), Cyanella (Cya), and *Spirodela oligorhiza* (Spir) are shown in comments.

[illegible]

— 26 —

[illegible]

-31-

[illegible]

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 IR ← J_{LB} → LSC
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 K X I G T T T G Y T V R Y K R M I I K L Q S G Y S I P L F S N K ---
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 N K I G T T T G Y T V R Y K R M I I K L Q S G Y S I P L F S N K ---
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Figure 4G (continued).

[illegible]

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Y L ---

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Figure 4G (continued).

Figure 4. Nucleotide sequence of the chloroplast DNA. The nucleotide position numbers are counted from the 5'-terminal nucleotide next to the inverted repeat IR_A. Dots are put on every ten nucleotide. Amino acid sequences deduced from the nucleotide sequences are shown under the DNA sequence by one letter symbols. Stop codons are shown by double underlines. Putative stem-loop structures are shown by broken lines with arrow heads. Predicted promoter sequences and Shine-Dalgarno sequences are shown under the DNA sequence. Transfer RNA sequences are shown under the DNA sequence. Introns are shown by dots under the DNA sequence with 5' and 3' terminal consensus sequences (gagyg and ragccg.augaa..gaaa..uucaugu.cgguuy; r represents a or g, and y represents c or u).

nucleotide sequences of each block are shown in Fig. 4 A-G. The amino acid sequences of ORFs and the nucleotide sequences of transfer RNAs deduced from the DNA sequence are shown below the nucleotide sequences. Introns in ORFs are predicted in one tRNA gene (valine-UAC) and six protein coding sequences for petB, petD, rp12, rp116, and rps12 genes and ORF203 by the presence of the 5' consensus sequence (GUGYG; Y represents C or T), and 3' consensus secondary structures with the common sequences (RAGCCG.AUGAA..GAAA..UUCAUGU.CGGUUY; R represents A or G) characteristic to group II introns found in fungal mitochondrial genes and *Euglena gracilis* chloroplast genes (Michel and Dujon 1983, Keller and Michel 1985). Identified genes and open reading frames (ORFs), and their loci on the chloroplast genome are summarized in Table 2. Genes are categorized into three groups: II-1 transfer RNA genes; II-2 genes for photosynthetic polypeptides; II-3 genes for ribosomal proteins and subunits of RNA polymerase. In the section II-4, unidentified open reading frames are discussed. Detail characterization of these genes are described following sections.

II-1 Transfer RNA genes

As previously mentioned, chloroplasts contain genes for their own rRNAs. They probably also contain genes for all of their tRNAs. They show high homology with the corresponding genes of *E. coli*. Genes for numerous tRNAs have been sequenced and mapped on chloroplast chromosomes (Crouse *et al.* 1985).

RESULTS

From the DNA sequence, tRNA genes were predicted as regions that have higher GC content than spacer regions between ORFs as shown in Fig. 5. Seven tRNA genes were located by searching for the T- ψ loop consensus sequence (GTTTCRA) and identified by constructing the clover-leaf structures as shown in Fig. 6.

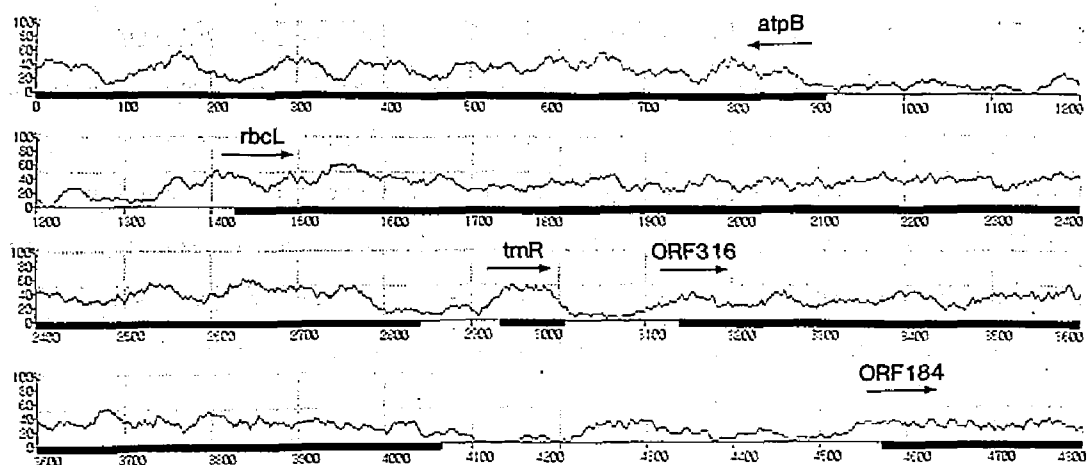


Figure 5. GC content and corresponding genes. GC content was plotted by calculation in average 30 nucleotides. The coding sequences are shown by bold lines with names of genes.

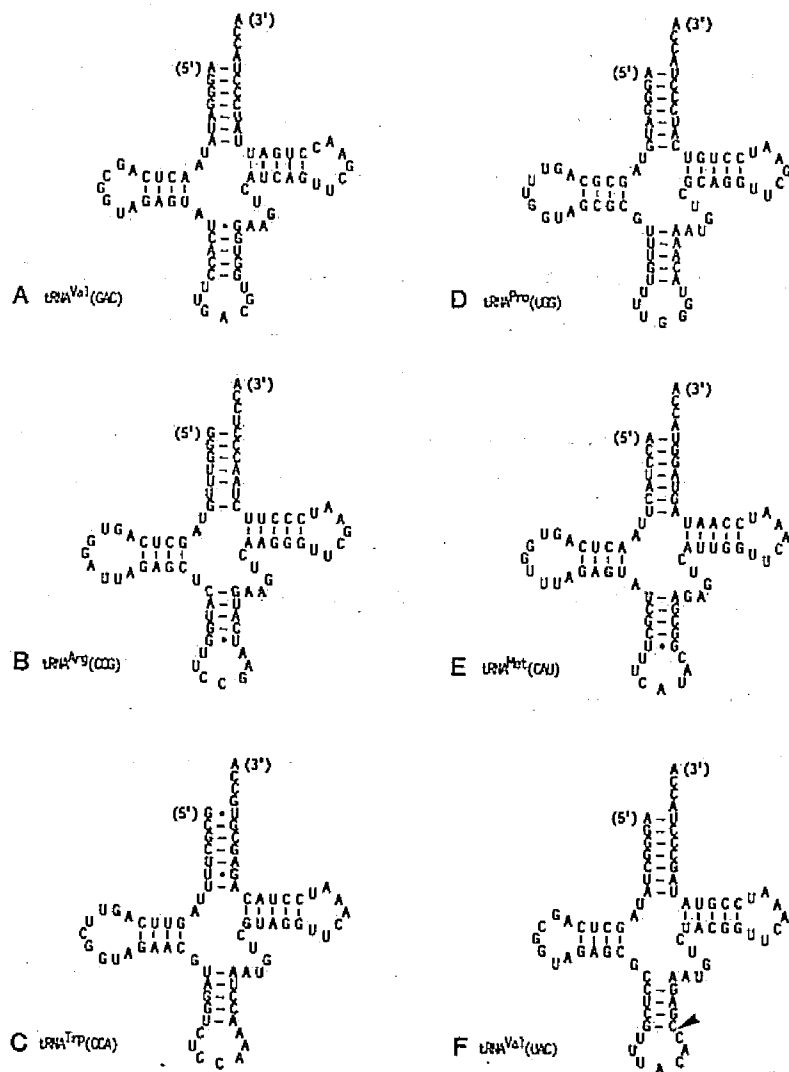


Figure 6. Secondary structures of tRNAs deduced from the DNA sequences. The 3'-terminal CCA nucleotides are not coded by the chloroplast genome. The insertion site of an intron in the $tRNA^{Val}(UAC)$ is shown by an arrow head.

Valine and isoleucine tRNA genes (trnV-GAC and trnI-C*AU)

A gene for tRNA^{Val}(GAC) was found at position 81814 to 81885. The 5' terminus of 16S ribosomal RNA gene was mapped at the position 82109 by comparing with maize and tobacco chloroplast 16S rRNA genes (Schwarz and Kossel 1980, Tohdoh and Sugiura 1982). It was shown that the primary transcript of the 16S rRNA does not include tRNA^{Val}(GAC) (Strittmatter *et al.* 1985). In the liverwort putative promoter sequences were individually located upstream from 16S rRNA gene and valine tRNA gene. A tRNA gene (81057-80984) is located on the opposite DNA strand 756 bp apart from the 5' terminal end of the trnV-GAC gene. The unmodified anticodon is CAU complementary to the methionine codon AUG. The nucleotide sequence shows 43.2% and 54.1% homologies with the spinach initiator methionine tRNA(CAU) (Calagan *et al.* 1980) and elongator methionine tRNA(CAU) (Pirtle *et al.* 1982), respectively, but exhibits 93.2% homology with the spinach chloroplast isoleucine tRNA(C*AU). In addition, the tRNA gene in liverwort has extra mismatching within the anticodon stem as seen in the case of spinach isoleucine tRNA(C*AU) (Kashdan MA *et al.* 1982 and Francis *et al.* 1982). Therefore, coding sequence for this tRNA can be tRNA gene (trnI-C*AU) highly modified in the first nucleotide of the anticodon.

Arginine tRNA gene (trnR-CCG)

A tRNA gene (57877-57950) was found 94 bp downstream from the termination codon of the rbcL gene. The tRNA has the anticodon of CCG that can recognize CGG arginine codon. A pair of mismatching nucleotides (U-U) was found in the amino acyl stem (see Fig. 6B). The liverwort trnR-ACG gene in the IR region also have two mismatching nucleotides (U-U and U-U) in its amino acyl stem (Kohchi *et al.* 1986).

Tryptophan and proline tRNA genes (trnW-CCA and trnP-UGG)

Coding sequences for two tRNAs were found at positions 64626-64553 and 64788-64715 near the psbE gene in Fig. 4D. Their secondary structures showed the anticodon triplets, CCA and UGG, pairing with codons for tryptophan UGG and proline

CCA, respectively (Fig. 6C and 6D). Therefore these tRNAs were identified to be tryptophan tRNA and proline tRNA. The tRNA^{Trp}(CCA) and tRNA^{Pro}(UGG) show 93.4% and 93.4% sequence homologies with spinach chloroplast tRNA^{Trp}(CCA) and tRNA^{Pro}(UGG) (Canaday *et al.* 1981 and Francis *et al.* 1982), respectively. The genes for these tRNAs were designated trnW-CCA and trnP-UGG. Their coding sequences were separated by 88 bp spacer region. A significant promoter sequence for these genes is present 20 bp upstream from the 5' end of the proline tRNA gene, but not in the spacer region between two tRNA genes. Two tRNA genes must be co-transcribed in a primary transcript and processed into mature tRNA molecules, although 42 bp stem structure in the spacer region can be formed as described in tRNA^{Arg}(CCG).

Elongator methionine and valine tRNA genes (trnM-CAU and trnV-UAC)

In the BglII fragment Bg10, 83 bp apart from atpE coding region, a tRNA gene (53801-53874) was found on the opposite strand. The anticodon of the tRNA was AUG. The tRNA showed sequence homology with spinach elongator methionine tRNA (94.8%), and with initiator methionine tRNA (46.8%). Therefore this tRNA gene was confirmed to be elongator methionine tRNA gene. A tRNA gene (53652-53051), whose anticodon was UAC was found 148 bp apart from trnM-CAU. This putative valine tRNA gene was split by 530 bp intron at the junction between anticodon stem and loop as shown in Fig. 6F.

DISCUSSION

Thirty two species of tRNA genes have been identified and mapped on the liverwort chloroplast genome (Ohyama *et al.* 1986). Those identified tRNAs are listed in the codon table as shown in Table 3. The tRNAs encoded by chloroplast genome are sufficient to read all codons taking into account an extended wobbling and modification in the anticodons. No tRNA gene would encode a 3'-terminal CCA nucleotides. Five species of tRNA genes (trnI-GAU, trnV-UAC, trnA-UGC, trnK-UUU and trnG-UCC) have been found to be split by group II introns. A tRNA gene (trnL-UAA)

Table 3. Codon table and unmodified anticodons of tRNAs coded by *Marchantia polymorpha* chloroplast genome.

codon	anticodon	codon	anticodon	codon	anticodon	codon	anticodon
UUU } Phe	GAA	UCU } Ser	GGA	UAU } Tyr	GUA	UGU } Cys	GCA
UUC }		UCC }		UAC }		UGC }	
UUA } Leu	UAA*	UCA }	UGA	UAA Ter		UGA Ter	
UUG }	CAA	UCG }		UAG Ter		UGG Trp	CCA
CUU }		CCU }		CAU } His		CGU }	ACG
CUC } Leu		CCC } Pro (GGG)		CAC }	GUG	CGC } Arg	
CUA }	UAG	CCA }	UGG	CAA } Gln	UUG	CGA }	
CUG }		CCG }		CAG }		CGG }	CCG
AUU }		ACU }		AAU } Asn		CGU } Ser	
AUC } Ile	GAU*	ACC } Thr	GGU	AAC }	GUU	AGC }	GCU
AUA }	CAU	ACA }	UGU	AAA } Lys	UUU*	AGA } Arg	UCU
AUG } Met	CAU	ACG }		AAG }		AGG }	
	fMet						
GUU }		GCU }		GAU } Asp		GGU }	
GUC } Val	GAC	GCC } Ala	UGC*	GAC }	GUC	GGC } Gly	GCC
GUA }	UAC*	GCA }		GAA } Glu	UUC	GGA }	UCC*
GUG }		GCG }		GAG }		GGG }	

AUG codon is an initiation codon. Termination codons (UAA, UAG and UGA) are indicated by Ter. Asterisks indicate presence of introns in the coding sequences.

is interrupted by group I intron. Five species of tRNA genes (trnV-GAC, trnI-GAU, trnA-UGC, trnR-AGC, trnN-GUU) have been identified in each inverted repeat region (see Fig. 2).

Three kinds of arginine tRNA genes have been identified on the chloroplast genome. Duplicated genes, trnR-ACGs are localized in IR regions (Kohchi *et al.* 1986) and a trnR-UCU gene was mapped near the 3' side of the atpA gene coding for α subunit of H^+ -ATP synthase (Umesono *et al.* 1986, see Fig. 2). In liverwort chloroplast genome, however, the trn^{Arg}(CCG) gene, which has not been found in the chloroplasts of any species of plants, was identified near the 3' end of the rbcL gene. Arginine codons in the codon table are separated into two boxes including AGR and CGN codons (see Table 3). Codons, AGA and AGG, can be read by trn^{Arg}(UCU) using G/U wobbling of the third letter. The CGU and CGG codons can also be recognized by trn^{Arg}(ACG) and trn^{Arg}(CCG), respectively. However, CGC and CGA codons could not be read by trn^{Arg}(ACG) and trn^{Arg}(CCG) without modification of the first letter of ACG and CCG anticodons. In *E. coli* there are also three species of arginine tRNAs having anticodons ACG, CCG and UCU. Therefore these results indicate that the mechanisms of the codon-anticodon recognition in chloroplasts is similar to those in *E. coli*. In addition, unpaired nucleotides in amino acyl stem were also reported in chloroplast trn^{Arg}(ACG) molecules of liverwort (Kohchi *et al.* 1986), *Euglena gracilis* (Hallick *et al.* 1984), *Spirodela oligorhiza* (Keus *et al.* 1984), *Pelargonium zonale* (Hellmund *et al.* 1984), and tobacco (Kato *et al.* 1985). These incomplete structures of amino acyl stems may alter the codon-anticodon recognition. A promoter sequence is not detected in the 94 bp spacer region between rbcL and trnR coding regions. Instead, there are two stem structures consist of 8 and 32 bp long ($dG = -44.9$ kcal) that may function as intracistronic termination or RNA processing signals (Fig. 4B). If it is so, the trnR gene may be co-transcribed with rbcL gene and processed into mature tRNA.

II-2 Genes for photosynthetic polypeptides

The most important role of chloroplasts is photosynthesis. Liverwort chloroplast genome is smaller (121 kb) than those in other higher plants (Palmer et al. 1985). Nevertheless it is interesting to know whether the liverwort chloroplast genome has necessary sets of genes for photosynthetic polypeptides. Events in photosynthesis take place on the thylakoid membrane as well as in stroma of the chloroplasts. The thylakoid membrane contains reaction centers (called photosystems I and II), cytochrome b6/f complex and coupling factors. A cytochrome b6/f complex in chloroplasts operates in an electron transfer chain of a plastoquinol-plastocyanin oxidoreductase between photosystem II and I. The complex has four major polypeptides. Three of them, cytochrome f, cytochrome b6 and 17 kd subunit 4, are synthesized in the chloroplasts, whereas the Rieske FeS protein is synthesized in the cytoplasm (Hauska 1985). The genes for cytochrome b6 and the 17 kd subunit 4 are separated by 1 kb on the chloroplast genome and are transcribed as a common precursor mRNA (Alt et al. 1983). The gene for cytochrome f is located distantly from others and translated into a preprotein larger than mature cytochrome f, suggesting that processing occurs during insertion from the ribosomes in the stroma into the thylakoid membrane (Alt et al. 1983, Whilley et al. 1984, Alt and Herrmann 1984).

RESULTS

Genes for photosynthetic polypeptides were identified by comparing amino acid sequences of the ORFs with those of photosynthetic proteins reported previously in other species of plants. The amino acid sequence alignments of the identified photosynthetic proteins are shown in Fig. 7 A-K. On the LSC region sequenced in this study, there are ten genes for photosynthetic proteins; the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL), 51 kd P-680 chlorophyll a apoprotein (psbB), cytochrome b-559 polypeptides (psbE and psbF), cytochrome f preprotein (petA), cytochrome b6 (petB), cytochrome b6/f complex subunit 4 (petD)

[illegible]

Liverwort MGLPMYRVIIIVLNDPGRLLIAVILMHITALVSGVAGSMALEYLAVFDPSPVLDPMWGHFVFPFMTRLGITSKMGWSITGETVTNAGIWSYEGYAAVH
Spinach MGLPMYRVIIIVLNDPGRLLSVIIIMITALVAGVAGSMALEYLAVFDPSPVLDPMWGHFLIPFMTRLGITSKMGWSITGGITDPSIWSYEGVAGAI

IVLSGLLLFAAIHWVYMDLELFRDERTGKPSLDLPKIFGIHLFSGVLCFAGFAHIVTGLFGPGIWSDPYGLTKGVQVPAPANGAEQFPFVPGGIAS
IMFSGLCFAAIHWVYMDLEISDERTGKPSLDLPKIFGIHLFSGVACFGFGAHVITGLYGGIIVWSDPYGLTKGVQVPCSANGVEQFPFVPGGIAS

HHTAAGILGILAGLFHLSVRPPQRLYKGLRHGIVETLVSSIAAVFAAFVVGATMNYGSAATPIELFGPTRYQWDQGFQOEIDRRIRSSKAHLSLS
IHIAAGLTGILAGLFHLSVRSPQRLYKGLRHGIVETLVSSIAAVFAAFVVGATMNYGSATPIELFGPTRYQWDQGFQOEIYRRVSAGLAENQFSSE

AWSKIPKLAFYDYGHNIPAKGGLFRAGAVDNGOGIAVGLGIADVFXDGHGILFYRRMPTFETFPVVLVDQGGIVRADVPFRRAESKYSVEQVGVTVE
AWSKIPKLAFYDYGHNIPAKGGLFRAGSDHGGIAVGLGIADPFXDGHGILFYRRMPTFETFPVVLVDQGGIVRADVPFRRAESKYSVEQVGVTVE

FYGGELQGVFSDPATVKKYARRAQLGEIFELDRATLKSOGVFRSSPRGWFTHGIAATFALLFFGHIMHGARTLFRQVFIAGIDPOLDAQVFEFGAQKLGQ
FYGGELNEVSYDPATVKKYARRAQLGEIFELDRATLKSOGVFRSSPRGWFTHGIAATFALLFFGHIMHGARTLFRQVFIAGIDPOLQVQVFEFGAQKIGQ

Liverwort	M S G N T G E R F A D I I T S I R Y V H I S T I P S L F I A G W L F V S T G L A Y O V G F S P R P N E Y F T E I R Q E V P L I T G R F H S L E Q I D E F T K S F	83
Spinach	::S::S::S::I::T::S::R::Y::V::H::I::S::T::P::S::L::F::I::A::G::W::L::F::V::S::T::G::L::A::Y::O::V::G::F::S::P::R::P::N::E::Y::F::T::E::I::R::Q::E::V::P::L::I::T::G::R::F::H::S::L::E::Q::I::D::E::F::T::K::S::F::	83 89.27
Nobacco	::S::S::S::S::I::T::S::R::Y::V::H::I::S::T::P::S::L::F::I::A::G::W::L::F::V::S::T::G::L::A::Y::O::V::G::F::S::P::R::P::N::E::Y::F::T::E::I::R::Q::E::V::P::L::I::T::G::R::F::H::S::L::E::Q::I::D::E::F::T::K::S::F::	83 88.02
Oenothera	::S::G::S::S::I::T::S::R::Y::V::H::I::S::T::P::S::L::F::I::A::G::W::L::F::V::S::T::G::L::A::Y::O::V::G::F::S::P::R::P::N::E::Y::F::T::E::I::R::Q::E::V::P::L::I::T::G::R::F::H::S::L::E::Q::I::D::E::F::T::K::S::F::	83 88.02

Liverwort	MTIDRTYPIFTVRVLAVHGLAVPTVFLGASAMQFIQR	39	
Spinach	:::::RTPYIFTVRVLAHVGLAVPTVFLLGASAMQFIQR	39	89.7%
Tobacco	:::::RTPYIFTVRVLAHVGLAVPTVFLLGASAMQFIQR	39	92.3%
Oenothera	:::::RTPYIFTVRVLAHVGLAVPTVFLLGASAMQFIQR	39	92.3%

Liverwort: MYLNFKFFKFCNSLEDNSTTLMKHSIESSFINKLTNHSILTTTFNDFSIMARLSSWLPLLYGTSCCFIEFASLIGSRFDFDRYGLVPRSSPRQADLITAA
 Matze: HVLTEYSEKKKKEGKDSJETIM-SLIEFPFLQDTSSNSVSTTPNDLSMSRLSSWLPLLYGTSCCFIEFASLIGSRFDFDRYGLVPRSSPRQADLITAA
 GTVTMKHAPSRLVRLYEQMPKPEKYVIAMGACTITGGFSTDSYTYRGVDKLIPVDIYLPGGPPKPEATIDAIILKRKKAQETIYECKKILKKGTRFFTLN
 GTVTMKHAPSRLVRLYEQMPKPEKYVIAMGACTITGGFSTDSYTYRGVDKLIPVDIYLPGGPPKPEAVIDALTKLRKKAARETIEDRTLQSQKKHRSFT
 HQEFHFSNLNDPKLT-SSHFFQFSKSTSKVLLTSLTFXKEKNL 243
 TRHKLYVRSTHTGTGEEQLLYOSPTLDSSETFTFKSKSSVSYSKLVN 248 62.1%

Figure 7 (continued).

[illegible][illegible]

SHUTVYHASTOKVSKJFRKEKGVEYJTDJDSGHKWVOTSAAPGLLJSEGLVKVDPDLTHPHVGGFGQDAEVLQDPLRIQGLLFFGCSYLAO
:::STA:::I::K::V:::V:::A:::A:::A:::RE:::TPR:::LV:::SI::L:::L:::S:::F::A:::A:::
:::M::S:::TA::V::KQ::I:::V:::A:::SE::I:::IPP::LV::L:::L:::V:::V:::V:::A:::T:::A:::I:::
:::TAA::I:::L:::L:::L:::T:::A:::RQ:::IPS::LV::ST::L:::S:::F::LQ::A:::
:::TS:::I::R::L:::L:::S::V::A::RQ::I:::IPP::LV::SI::L:::S:::F::A:::A:::

IFLVKKKQFEKVQLAEMNF	320	
.....S.....	320	78.8%
.....S.....	320	75.9%
.....S.....	318	78.1%
V.....Y.....	320	79.1%

Liverwort	MGKVYDWFEEERLEIQADDDITSKYVPPHVNIFCYLGGITLTCLFLVQVATGFAMTFYYRPTVTAEFSSVQYIMTEVNFHGLIRSVHRMSASMMWLHMLH
Spinach	MIGSKNVSFRRLRLMIITSKYVPPHVNIFCYLGGITLTCLFLVQVATGFAMTFYYRPTVTAEFSSVQYIMTEVNFHGLIRSVHRMSASMMWLHMLH

**FVRVYLGGGKKPRELWTWGTGVILAVLTVSFGVTGYSLPHDQIGYHAVKIVTGVP^AEALPIVGSPVELLRGSASVEQSGLTRFYSLHTFVLPLTIAIFML

FVRVYLGGGKKPRELWTWGTVLGTLTSFGVTGYSLPHDQIGYHAVKIVTGVPDAIPVIGSPVELLRGSASVEQSGLTRFYSLHTFVLPLTTAIFMFL**

MIIFLMIRKQGISGPL	215	
.....		
MIIFLMIRKQGISGPL	211	86.0%

Liverwort: MGVTKKPDLSDPILRAKLAKGHHIYYGGEAPWPHDLLYIPFVYILGTIACTVGLAVLEPSMIGEPANPFATPLEILPEUYFFPVFOILRTVPNKLGLVL
 Spinach:N:.....N:.....D:.....
 Pea:N:.....N:.....D:.....

```
MAAVPAGLLTVPFLENVHKQMPFRPPVATTVFLLIGTVVALWLGIGAAPIDKSLTLGLF      160
::S::::::::::::::::::::::::::V::::::::::::::::::T::::::::::::::::::139 (160)   95.6%
::VS::::::::::::::::::::::::::T::E::::::::::::::::::139                93.1%
```

[illegible]

GEATLGRIFHVLGPVDNLGPVEVTTTFPIHRAAPFTQLTKLSIFEIGKVVLDLAPYRGGKILGLFGGAGYGKTVLIMELINHLKANGVSVFQGV
 :GP::S:::R:I:DTR::S:::S:::N:::A:::
 :G::DTS::S:::I::E:::
 :G::DTSA::S::T:::
 :K::N:::MK:EIGEERMA::SYEE:SWSOELL:::I:MC:FAL::V:::NM:::R:AIE:S:Y::A:

[illegible][illegible]

DIATLGLDELSEEDRLTVARAKIERFLSQPFVAEVFTGSPGKYVSLRIETKGFOMLSGELOSLPEQAFYLVGNIOEATAKAAATLOVES	492
.....G:A:-R:.....MHLE:-K:KK	498 88.4%
.....G:A:-R:.....MHLE:-H:KK	498 87.4%
.....G:A:-R:.....G:-G:-ST:-MHLE:-K:KK	498 86.2%
.....M:-K:V:-Q:-R:-K:D:-K:G:-M:-Y:-H:-M:-S:-E:-K:KL	460 82.8%

—46—

(J) atpE

Liverwort: HLNLRIHAPRRIVVNSDIOEIIILSTHSGQIGILPHNASVLTALDIGIVKIRL-IDQHSYHALHGGFAMIDHHLTILVHDAEKASEIDYQEAQETFOKAR
 Spinach: HT::CVLT::S::EVK::V::PTA::V::LR::L::L::R::G::D::P::Q::LEI::E
 Tobacco: HT::SVLT::D::EVC::V::PTA::V::LR::L::L::R::G::D::P::Q::LEI::E
 Maize: HK::YVLT::K::I::DCEVK::V::PIN::V::M::PLR::L::L::AV::MS::P::N::V::EII::G::L::D::PE::QALEI::C
 E. coli: HMTYH:DVVSAEQHFSGLVK:QVTGSE:EL::Y::G::PL::IKP::NIR::VKQHGHEEFTY::S::ILEVQPG::V::V::ADT::IRGQDL::EAR::MEAKR::E

THLEEAEGNKKKEIEALLVFKRAKARLEAIIHASKL 135
 A::RK::KRQ::N::ALR::RT::S::TI::S 134 63.0Z
 A::VKK::RRQ::N::ALR::RT::S::PI 133 63.7Z
 A::SK::Y::EL::V::K::ALR::RT::I::V::WIPPSH 137 57.9Z
 EHISSSH:DVGY-AQ:SAEL:A:I:Q:RLSS 133 22.2Z

(K) ndh3

ORF120: MFLQKYDYFFVLLIISFFSILFSLSKMIAPINKGPEKFTSYESGIEPHGEACIQFIYYMFALVFVIFDVEIVFL--YPMHMSFYHFGISSFIEAL
 Hu. mt. ORF3: MIFALILHMITLLALLMIITFHLPLNGYMEKSTPYECGFDPMSPARVPFSMKFELVAITFLLEIALLLPLPALQTTNL-PLMVHSSL

IFICILIIGLVYAMR-KGALEHS 120
 LLIIILALSAYENLQKG-LDWE 114 30.8Z

Figure 7. Amino acid sequence alignments of photosynthetic polypeptides.

The amino acid sequences are shown by one letter codes. Identical amino acid residues are shown by colons, and deleted residues are shown by dashes. The amino acid residue numbers and sequence homologies with liverwort gene products are indicated at the end of sequences.

and β and ϵ subunits of H^+ -ATP synthase (atpB and atpE). The amino acid sequences of the photosynthetic proteins in liverwort chloroplasts exhibit high homologies (78.8%-95.6%) to those of spinach chloroplasts except for atpE (63.0% homologous to spinach atpE) and psbG (62.1% to maize).

Gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcl)

The rbcl gene was previously mapped in the Bam5 fragment by heterologous hybridization with the tobacco chloroplast rbcl gene (Ohyama et al. 1983). An ORF475 (56355-57782) shows high amino acid sequence homology with rbcl of spinach (90.5%) (Zurawski et al. 1981), tobacco (90.5%) (Shinozaki and Sugiura 1982), maize (87.6%) (McIntosh et al. 1980) and *A. nidulans* (80.6%) (Shinozaki and Sugiura 1983) (Fig. 7A). The rbcl gene was localized 508 bp apart on the opposite strand to the atpB gene coding for β subunit of H^+ -ATP synthase. In the spacer region between atpB and rbcl genes, typical stem-loop structures can be formed including 45 bp AT-rich stem structure ($\Delta G = -44.9$ kcal).

Genes for photosystem II P-680 chlorophyll *a* apoprotein (psbB), and for cytochrome b-559 polypeptides (psbE and psbF)

The amino acid sequence of an ORF508 (69026-70552) shows 88.2% homology to that of the spinach 51 kd photosystem II P-680 chlorophyll *a* apoprotein (Morris and Herrmann 1984). The amino acid sequence alignment with that of spinach is shown in Fig. 7B.

ORF83 (63554-63303) and ORF39 (63293-63174) are located close together (10 bp apart). The ORF83 shows 89.2% amino acid sequence homology to that of the spinach apocytochrome b-559 polypeptide, which is psbE gene product (Herrmann et al. 1984). The ORF39 shows 89.7% amino acid sequence homology to the b-559 ORF39 gene product of spinach (Herrmann et al. 1984). The psbE and psbF genes are preceded by the typical Shine-Dalgarno-like sequences (GGAGG and AGGAGG, respectively). The psbF gene terminates by a TAG stop codon that overlap to two nucleotides of the SD

sequence for psbE. Their sequence alignments are shown in Fig. 7C and 7D.

Gene for photosystem II G-protein (psbG)

Recently Steinmetz et al. identified a new protein (248 amino-acid residues long) associated with the photosystem II complex and analyzed the fine structure of its gene, psbG, on the maize chloroplast genome (1986). The M. polymorpha counterpart of psbG gene (52524-51793; encoding 243 amino acids, 27.6 kd) was identified and the predicted amino acid sequences were compared as shown in Fig. 7E. Unlike other photosystem II polypeptides, the psbG proteins are significantly diverse in N- and C-terminal portions; in average they are only 62.1% homologous, whereas the central portions (maize, amino acid residue number 36-182; M. polymorpha 37-183) are 91.9% identical. In M. polymorpha, the psbG gene overlaps with the last seven nucleotides of the preceding ndh3 gene.

Gene for cytochrome f preprotein (petA)

An ORF320 (61641-62603) shows high degree of amino acid sequence homologies to those of the cytochrome f preprotein of spinach (78.8%) (Alt et al. 1984), pea (75.9%) (Willey et al. 1984), Oenothera hookeri (78.1%) (Tyagi et al. 1986), and wheat (79.1%) (Willey et al. 1984). The ORF320 can be liverwort cytochrome f preprotein gene (petA) whose molecular weight is 33.4 kd. The amino acid sequence alignments compared with those of other plant species are shown in Fig. 7F. The N-terminal 35 amino acid sequence of liverwort cytochrome f polypeptides shows relatively lower homology (40.0%) to that of spinach. On the other hand, the remaining sequence of 285 residues gives high homology (83.5% with spinach cytochrome f mature protein). However, the hydrophobic characteristics of the N-terminal region are conserved in petA gene products indicating that N-terminal 35 amino acid residues may be functional as a signal peptide. The molecular weight of liverwort mature cytochrome f polypeptide is 31.3 kd.

Genes for cytochrome b6 (petB) and for cytochrome b6/f complex subunit 4 (petD)

ORF162 (72078-72566) can be postulated by using initiation codon AUG (72078-72080). The C-terminal amino acid sequence was found to be homologous to that of the spinach cytochrome b6 polypeptide (petB) (Heinemeyer *et al.* 1984). However the N-terminal portion including initiation codon is quite different from that of spinach petB gene. Instead, a consensus sequence for the 3' end of the intron (RAGCCG.AUGAA..GAAA..UUCAUGU.CGGUUY) was found in the beginning (71859-71924) of the ORF162. A consensus sequence specific to the 5' end of the intron (GUGYG) was also located further upstream of the ORF162. This indicates that the liverwort petB gene (71424-72566) is interrupted by 495 bp intron (71430-71924) and codes a polypeptide of 215 amino acid residues (Fig. 4F and 7G).

The amino acid sequence of ORF139 (73271-73690) shows 95.6% and 93.1% homologies to cytochrome b6/f complex subunit 4 of spinach (Heinemeyer *et al.* 1984) and pea (Phillips *et al.* 1984), respectively (Fig. 7H). However, the consensus sequences for an intron (493 bp: 72723-73215) are also present in petD gene as in the petB gene. The putative secondary structures of introns in petB and petD genes are shown in Fig. 8. The initiation codon of the petD gene can be extended to the upstream methionine codon (72715) as shown in Fig. 4F. The molecular weight of the liverwort petD gene product can be estimated to be 17.4 kd (160 amino acid residues) considering presence of an intron in the coding sequence. On the other hand, intron specific sequences can also be identified on the DNA sequences of the spinach and pea genes, although they do not describe the presence of introns in the coding sequences for petD gene. If this is true, the amino acid sequences of the 5' extended region of the petD genes were highly conserved in the three species of plants (95.6% homologous to spinach petD protein, see Fig. 4F). A sequence motif (AGGA) for the ribosome binding sites are present 9 bp upstream from initiation codons of each petD and petB gene.

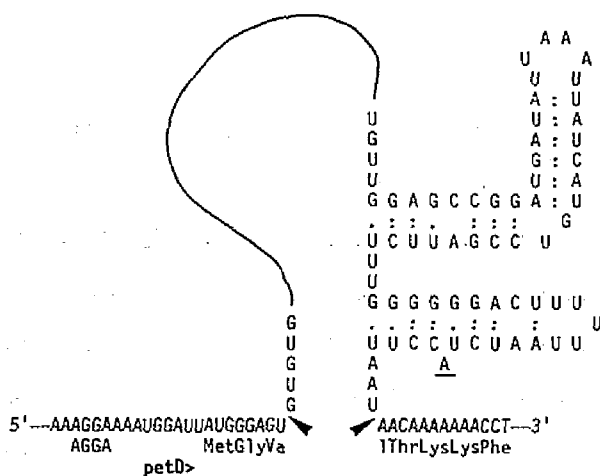
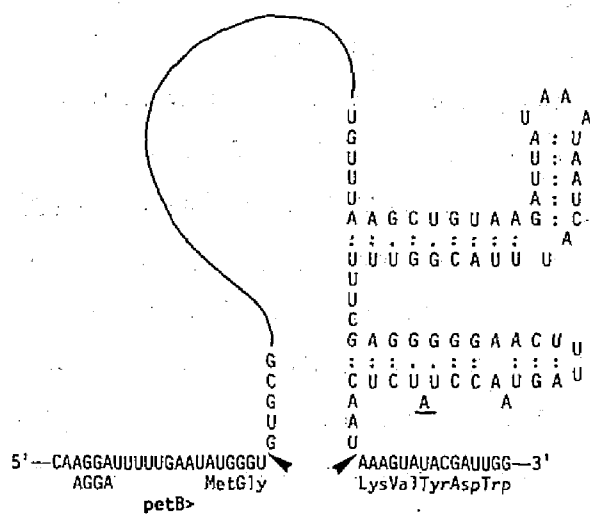


Figure 8. Secondary structures of introns in *petB* and *petD* precursor mRNA molecules. The Shine-Dalgarno sequences and amino acid sequences are shown under the messenger RNA molecules. Underlined nucleotide A is putative branch point in the lariat form. The junction sites between exons and introns are indicated by arrow heads.

Genes for β and ϵ subunits of H^+ -ATP synthase (atpB and atpE)

A gene cluster, atpB (55846-54368) - atpE (54362-53955), was localized 508 bp apart from rbcL in head to head on the opposite DNA strand. The nucleotide-binding subunit β encoded for by atpB gene contains 492 amino acids (Higgins *et al.* 1985). The *M. polymorpha* atpB amino acid sequence showed 88.4% homology to spinach atpB gene product (Zurawski *et al.* 1982), 86.2% to maize (Krebbers *et al.* 1982), 62.8% to *E. coli* (Saraste *et al.* 1981). In contrast, *M. polymorpha* ϵ subunit (135 amino acid residues), product of atpE, is less homologous to that of spinach (63.0%, Zurawski *et al.* 1982), maize (51.9%, Krebbers *et al.* 1982) and *E. coli* (22.2%, Saraste *et al.* 1981). Although the Shine-Dalgarno sequence of atpE gene was overlapped with the atpB coding region in spinach and maize, the coding sequences of these two genes of *M. polymorpha* were not overlapped as shown in Fig. 9.

DISCUSSION

The comparison of amino acid sequences of photosynthetic polypeptides between liverwort and other higher plant chloroplasts reveals the highly conserved amino acid sequences (78.8-95.6% with spinach photosynthetic polypeptides) during the events of chloroplast evolution. In contrast, it is interesting to note less amino acid sequence conservation in transcriptional and translational polypeptides including α subunit of RNA polymerase (54%), ribosomal proteins (46-68%) and initiation factor 1 (65%). These results suggest that chloroplast ribosomal proteins and RNA polymerases may have changed faster than photosynthetic polypeptides in the event of evolution.

Previous studies showed that several plants have two rbcL mRNA transcripts (Erion 1985). It has been shown that the smaller mRNA transcripts are produced by processing of the primary rbcL transcripts (Mullet *et al.* 1985). Although the spacer region between rbcL and atpB coding regions show less sequence homology with those of other plant species, several regions are conserved in land plant

E. coli AAAAGCCAAAACCTTTAAGCCTTAATCGAGGGTGATATGGCAATGACTTACCACCTGGACGCTGTCAGCGCAGAGCAACAAATGTTCTCT
 K A K K L --- GGAGG M A M T Y H L O V V S A E Q Q H F S
 (atpB)> (atpB)>

Liverwort GCTACTGCAAAAGCAGCTACTTTACAAGTCGAGAGTTAAAATATGCTAAATCTTCGTATCATGGCTCTTAATCGAATTCGTTGGAATTCG
 A T A K A A T L Q V E S --- M L N L R I M A P N R I V W N S
 (atpB)> GGAG (atpB)>

Spinach GCTACCGCGAAAGCTATGAAGCTAGAAATGGAGAGCAAAATTAAGAAATGACCTTAAATCTTTGTGTACTGACTCCGAATCGAAGTATTTGGAATCA
 A T A K A M N L E M E S K L K K --- M T L N L C V L T P N R S I W N S
 (atpB)> GGAG (atpB)>

Figure 9. Comparison of nucleotide sequence of spacer region between *atpB* and *atpE* genes. The identical amino acid residues between *M. polymorpha* *atpBE* and spinach *atpBE* proteins are underlined. Shine-Dalgarno sequences are shown under the nucleotide sequences by bold letters.

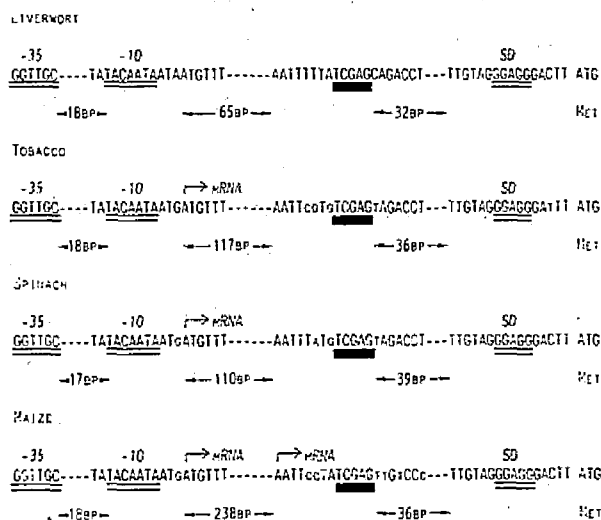


Figure 10. Comparison of the leader sequences of *rbcl* gene. The promoter sequences referred to "-10" and "-35", and Shine-Dalgarno sequences are double underlined. The conserved sequences in the 5' leader regions of plant chloroplast *rbcl* gene are indicated by thick underlines.

chloroplasts. The promoter sequences for *rbcl* and *atpB* referred to "-10" and "-35" regions, and the Shine-Dalgarno sequence (GGAGG) for *rbcl* were conserved. In addition, a sequence motif (TCGAG) was found 38-45 bp apart from the SD sequence of *rbcl* by comparing the DNA sequences as shown in Fig. 10. Interestingly, one of the 5' terminal sites of processed *rbcl* mRNAs are located 8-12 bp upstream from this conserved sequence motif in maize and spinach. The distance from the *rbcl* promoter to SD sequence in liverwort *rbcl* is shorter than those in other plant chloroplast *rbcl*, but the distances between promoter regions and this newly identified sequence motifs were not conserved in land plants. This sequence motif may have some roles on the translational regulation of *rbcl* gene expression.

In the liverwort cytochrome *f* preprotein (*petA*), the N-terminal region (1-35 amino acid residue) shows rather low homology (40%) with corresponding regions of those from pea and spinach chloroplasts. The N-terminal 35 amino acid residues of the pea and spinach cytochrome *f* proteins have been shown to be removed as a signal peptide for localization into the thylakoid membrane (Willey *et al.* 1983).

The liverwort chloroplast *petB* and *petD* genes were found to be interrupted by introns classified as group II intron in fungal mitochondria (Keller and Michel 1985). Introns were not reported in the spinach chloroplast *petB* and *petD* genes and it has been suggested that the *psbB*, *petB* and *petD* genes are co-transcribed in a primary transcript following intercistronic splicing (Westhoff 1985). A similar gene organization as in the liverwort genome can be interpreted in the spinach nucleotide sequence presented by Heinemeyer *et al.* (1984) by application of intron consensus sequences (Keller and Michel 1985, Ohyama *et al.* 1986). The molecular weight calculated from the deduced amino acid sequences of the *petD* (17.4 kd) agrees reasonably with that (17.5 kd) of the spinach subunit 4 polypeptide determined by SDS-polyacrylamide gel electrophoresis (Alt *et al.* 1983). Findings of introns in liverwort *petB* and *petD* genes and absence of promoter sequences and termination signals in the spacer regions between *psbB*, *petB* and *petD* genes suggest that these three genes are transcribed in a 7.3 kb common precursor mRNA. Further study to

identify the splicing junction between their exons is in progress.

II-3 Genes for ribosomal proteins and α subunit of RNA polymerase (rpoA)

Gene expression in the chloroplasts shows many similarities with those in prokaryotic cells such as E. coli. Ribosomal RNAs (rRNA), transfer RNAs (tRNA) and ribosomal proteins involving the transcription and translation in chloroplasts have prokaryotic features (Whitfield and Bottomley 1983, Kozak 1983). Chloroplast ribosomes play a fundamental role not only in chloroplast biogenesis but also in photosynthesis, since several photosynthetic proteins are made on chloroplast ribosomes (Ellis 1981). Chloroplast ribosomes share many structural and functional similarities in common with prokaryotic ribosomes including subunit sedimentation coefficients, mode of action, size and sequences of rRNAs (Bartsch et al. 1982, Schmidt et al. 1985, Bartsch 1985). The similarities in the translational systems are so extensive that initiation and elongation factors, even ribosomal subunits of E. coli and chloroplast can be interchanged (Graves and Spremulli 1983, Schmidt et al. 1983). Some of the cDNA coding for the chloroplast ribosomal protein have been obtained from pea (Gantt and Key 1986).

The genes for chloroplast ribosomal proteins; S4 (Subramanian et al. 1983), S7 (Montandon and Stutz 1984), S11 (Muller et al. 1986), S12 (Montandon and Stutz 1984), S16 (Shinozaki et al. 1986), S19 (Sugita and Sugiura 1983), L2 (Zurawski et al. 1984), S14 (Umesono et al. 1984, Kirsch et al. 1986), and L16 (Posno et al. 1986) have been identified by amino acid sequence homology with the corresponding E. coli ribosomal proteins. One third of chloroplast ribosomal proteins are thought to be encoded by chloroplast genome (Filho et al. 1981) but some of the rests are shown to be encoded by nuclear genome as a precursor polypeptides (Schmidt et al. 1985). It is not so far clarified how many genes for ribosomal proteins are encoded by chloroplast genome.

Two different types of RNA polymerase activities appear to be present in chloroplasts (Greenberg et al. 1984). One is associated with a transcriptionally

active chromosome and is preferentially active in rRNA synthesis (Briat and Mache 1980). The other is readily extracted in soluble form active in tRNA and mRNA transcription (Gruissem et al. 1983). The site of synthesis of these RNA polymerases have not proved to be either in cytoplasm or in chloroplasts (Lerbs et al. 1985, Muller et al. 1986). The soluble RNA polymerase from maize (Kidd and Bogorad 1979) and pea (Tewari and Goel 1983) has been shown to have subunit structure.

RESULTS

Thirteen ORFs shows significant amino-acid sequence homologies with respective *E. coli* ribosomal proteins (L2, L14, L16, L20, L22, L23, L33, S3, S8, S11, S12, S18, and S19). The amino acid sequence alignments with corresponding *E. coli* ribosomal proteins are shown in Fig. 11 A-M. Percentages of the amino acid sequence homology of liverwort ORFs to *E. coli* ribosomal protein genes range from 25.3% (L23) to 70.2% (S12).

A large cluster of ribosomal protein genes was found on the LSC region next to the Junction site (J_{LB}) (Fig. 3G and 4G). The organization of the gene cluster was (trnI)-L23-35bp-L2-36bp-S19-17bp-L22-48bp-S3-57bp-L16-97bp-L14-81bp-S8-86bp-(infA)-36bp-(secX)-50bp-S11-32bp-(rpoA). The putative infA gene product exhibits 56.4% and 60.3% amino acid sequence homologies to the *E. coli* initiation factor 1 (Pon et al. 1979) and the spinach infA product (Muller et al. 1986), respectively (Fig. 11-O). The secX is the putative gene for unknown polypeptide expressed in *E. coli* (Ceretti et al. 1983). The amino acid sequence shows 62.2% homology to the *E. coli* secX protein and also shows 86.5% homology to the spinach open reading frame corresponding to the liverwort secX protein (Muller et al. 1986) (Fig. 11P). The rpoA is the putative gene for α subunit of RNA polymerase whose amino acid sequence shows homologies to those of *E. coli* (25.6%, Meek et al. 1984) and spinach (54.1%, Muller et al. 1986) (Fig. 11N). Spacer regions between their genes in the cluster appears to be less than 97 bp long. No significant prokaryotic promoter sequences

(A) GP12 (L2)

[illegible]

(B) rp114 (L14)

Liverwort MIQPQTYLHVADNSGARKLHCIRVIGTSHRKYANIGDI IIAVVKEAVPNMIPKKSEIVRAVIVRTCKEFRNIIGSI IKFDDHAAVVVINQEG-HPXGTRVF
:
:
E. coli HIQEQTMLNVADNSGARVRVCIKVLGGSHRRYAGVGIIKITIKEAIPRGVKKGDOVLKAVVVRTKGVRRPBGSVIRFDGHACVLNNNSEQPIGRIF
:
:
GIPIARELSNFTXIVSLAPEVL 122
:
:
GPVTRELRSKFMIISLAPEVL 121 58.2%

(C) rp116 (L16)

<u>Liverwort</u>	MLSPKRTKFRKQHCNGLKGISTRGNVICFGKFPLQALEPSWTSRQIEAGRAITRYARRGGKLWIRIFDPDKPITIRPAETRMGSGKGSPEYVAVVKPG	
<u>Spirodela</u>	:::::R::::R:M::K::Y::H:S:RYA::V:A::A:::S:::I::V:L:::V:L:::S:::	
<u>E. coli</u>	:::Q:::M::K:RHR:LAQGT:D::S::S:G:K:VGRRGL:A:::S:::AVK::Q::I::V::EK:LAV::K::NV:::LIQ:::	
	KILYEISGVSENIAARAAMKIAAYKKPIRTQFITSSLNKKQE:I	143
	R::::G::::TV::T::LL::S:::::IEE	135 72.0Z
	V::::MD::P:EL::E:F:L:A:L::K:T:V:KTVM	136 53.8Z

(D) rp120 (L20)

[illegible]

(E) rp122 (L22)

Liverwort: HQNTNTSHKKIRAVAXHHM---SPHKVRRVVSQIGRSYEQALMILEFMPYRACNPILQLSSAAAHNHNFGLSKTNLFISEIQVNGKTFKRFQPRAQ
 E. coli: METIAXKRHRHARSSAQKVRVLADLRGKKVSQALDILTYTNKKAAYLVKKVLESAIANAHHWDGADIDDLVKVTIFVDEGSPMKRIMPRAK
 RGYPIHKPTCHITIVLNLPK 119
 RADRLKRTSHITVVVSDR 110 37.82

(F) mp123 (L23)

Liverwort	MIHQYQVPLTEKIRLLEK-HQYSFOVNI ¹ DSNKTQIKK ¹ WELFFHWK ¹ VISVHSHRLPKKKKIGTTGGTVRYXRMIK ¹ LQSGYSIPLFSNK	91
<i>E. coli</i>	MI REERLLKVL RAPHVSEKASTAMEKSH ¹ TVLKAKDATKAE ¹ TKAAVOKLFEVEVEVNTLVYKGVKVRKHGR ¹ IGRRSD-KKAYVTILKEGONLDFVGGAEK	100 25.3%

(G) rp133 (L33)

Liverwort MAKSKDIRVTINLECNCAQNDKRRKGISRYTTQKNRRNTPIRLELKKFCVCYCNKHTIHKEIKK 65
 ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: ::::: :::::
E. coli MAK—GTREKIKL———VSSAGTGHFYTTTKNRKTPEKLELKKFDPPVRQHVVYIKEAKIK 54 36.9%

(II) rps3 (S3)

```

Liverwort  MGQKIMPLGRLGITQHRHSYWFANKKYSKVFEEDKKIRDCI---ELYQKHINKSSHYGGIARVEIKRKTDLIQVEIYTGFPALLVESRGGGIEQLKLN
E. coli     MGQKVHPNGIRLGIKVPWISTHFWANTKEFADNLOSDFKVRYLTKE LAKASVS-----RIVIERPAKSIRVTIHTARPGIYIGKKG--EDVEKL-
VQNILSSEDRRLRLMTLIEIAKPYGEPKILAKKIALKLESVAFRRTHKKAIELAKKGNIGKIKIQIAGRLNGAEIARVEWAREGRVPLQTIARIRINYCY
RKVVADIAGVPAQINIAEYRKPELDAKLVAOSITSQLERRVHFRRAHKRAVQNHMLRGAKGKIVEVSGRLGGAEIARTEWYREGRVPLHLTRADIDYNTS
AAQTIYGVLGIKVWIFQDEE
FAHTTYGVIGVKVWIFKGEILGGMAAEOPEKPAAPAKKQORGRK

```

Figure 11 (continued).

(Q) ORF184
 Livewort ORF184 MNLQVDHIRVDFIGSRRI SHFCWAFILLLFGALGFFVFGSSYLQKDLIPFLSAEQILFIPQGIWMCFYGIAGLFISFYI
 Euglena ORF149 MNLRDINIMTL SKNHNIAKQKQIMLPKILRQIEKNHKIKWFIY-NIVMLGGIGFLVIGISSYIGNNLIYFLDASEIIFPPQGITMCFYGTGILFSINQ
 WCTICWVGSGYNKFDKQKQIFSIIRWFGPGKNRRIFIQFLIKDIQSIRMEVQEGFLSRRLVYIKIGQDPDPLSRIEEYFTLREMEDKAAELARFLKVSIEGI 184
 ISIILNGVGEGYHEFNKELNMTIYRKQKQKNSDINITYSLKDIVKE 149 38.32

Liverwort ORF203 (Exon 1) MPIGVPKVPFRLPGEDAVWIDV-(intron)
 Spinach X-gene (Exon 1) MPIGVPKVPFRSPGEDASHVDV-(intron) 87.0%

Liverwort MANTTGRVPLWLIGTVAGILVLGLVGIFFFYGSYSGLGSSL 40
::: :::
Cyanella MANTGGRIPLWLVATVAGLAAIGVLGIFFYGGYSGLGSSI 40 72.6%

-59-

were observed between them. Another ribosomal protein gene cluster is localized between *trnP*-UGG and *psbB* as the order of *trnP*-124bp-L33-27bp-S18-81bp-L20-786bp-S12(exon1)-72bp-ORF203-385bp-*psbB*. The *rps12* gene (Exon1) is localized 72 bp downstream from TAG stop codon of ORF203. The rest of the exons are located far apart on the different DNA strand indicating trans-splicing mechanism for the gene expression (Fukuzawa *et al.* 1986). Detail discussion on the trans-split gene *rps12* are in Chapter III. The coding sequences for ribosomal protein L2 and L16 are interrupted by 545 bp and 534 bp group II introns, respectively (Fig. 4F).

DISCUSSION

In the liverwort chloroplast genome, nucleotide sequence revealed a large cluster of genes coding for ribosomal and related proteins (*trnI*-L23-L2-S19-L22-S3-L16-L14-S8-*infA*-*secX*-S11-*rpoA*) on the LSC region near the *J*_{LB}. Upstream from the gene for L23 ribosomal protein, there is an isoleucine *tRNA*(C*AU) gene whose promoter highly functioned in *E. coli* as well as in chloroplasts (Fukuzawa *et al.* 1985). The length of the gene cluster from the 5' end of isoleucine *tRNA* gene to 3' end of *rpoA* gene is approximately 7.3 kb. No promoter sequence can not be found in the short (less than 97 bp) spacers between ribosomal protein genes indicating that the gene cluster may be transcribed into a single precursor RNA from the *trnI* to *rpoA* genes. Furthermore, this cluster has similar order to the clusters reported in the *E. coli* ribosomal protein operons such as the S10 operon (Zurawski and Zurawski 1985), *spc* operon (Cerretti *et al.* 1983), and *alpha* operon (Bedwell *et al.* 1985) (S10-L3-L4-L23-L2-S19-L22-S3-L16-L29-S17, L14-L24-L5-S14-S8-L6-L18-S5-L30-secY-secE, and S13-S11-S4-*rpoA*-L17, respectively). Two additional clusters of ribosomal protein genes are seen in the orders of S12(Exon1)-L20 and S18-L33 between *trnP*-UGG and *psbB* genes. The genes for S12 (from the 39th amino acid residue to C-terminal end) and S7 ribosomal protein also have been clustered as seen in *E. coli* *str*-operon (S12-S7-EF_G-EF_{TU}, Post *et al.* 1978, Post and Nomura 1980). In contrast, the chloroplast *rps2*, *rps4*, *rps14*, *rps15* and *rpl21* genes are scattered throughout the

liverwort chloroplast genome.

The *rpl2* genes of spinach and *Nicotiana debneyi* has been located in the inverted repeat regions. In *N. debneyi*, but not in spinach, *rpl2* is interrupted by a 666 bp intron (Zurawski *et al.* 1984). However, liverwort *rpl2* gene is located on the LSC region just outside of the *IR_B* region and has 544 bp intron. Introns are also present in the *rpl16* gene of *Spirodela oligorhiza* (interrupted by 1411 bp intron) (Posno *et al.* 1986). The first exon of genes for L16 ribosomal protein encodes for the first three amino acid residues (Met-Leu-Ser) of N-terminal. It is interesting that the products of ribosomal protein genes encoded in the chloroplast genome are important components in the initial stage of ribosome and rRNA assembly (Dorne *et al.* 1984).

A putative gene for α subunit of RNA polymerase is located at the 3' end of the gene cluster for ribosomal proteins. Genes for β and β' subunits of RNA polymerase (*rpoB*, *rpoC1* and *rpoC2*) are located as a single operon at 50 kb apart from *rpoA* gene (Umesono *et al.* 1986). An ORF homologous to the *E. coli* sigma subunit of RNA polymerase is not found on the liverwort chloroplast genome.

II-4 The putative gene *ndh3* and unidentified open reading frames

It is reported that the *M. polymorpha* chloroplast genome contained a set of homologues of mammalian mitochondrial "URF" genes (Ohyama *et al.* 1986). In this region, a gene named *ndh3* corresponding to human mitochondrial URF3 was identified by amino acid sequence comparison (Fig. 7K). The mitochondrial URF3 gene code for the component of respiratory-chain NADH dehydrogenase complex (Chomyn *et al.* 1985). An ORF120 (52877-52515) is located at the upstream region of *psbG* with an overlap of seven nucleotides. This ORF dose not contain an intron and the product (120 amino acid residues, 14.2 kd) is similar in size to a human mitochondrial URF3 protein (114 amino acid residues, Anderson *et al.* 1981) sharing 30.8% homology. Actually proteins of chloroplast *ndh* genes are not yet identified, but a 3' half of *ndh3* gene would be also conserved in maize chloroplast genome; the published sequence of maize

psbG gene contains its 5' flanking region of 158 nucleotides (Steinmetz et al. 1986). If two G residues (located at -68 and -96 in their numbering) are deleted, the region from -157 to +7 will encode a polypeptide similar to the last 54 amino acids of the M. polymorpha ndh3 product (85.2% identical) as well as an overlapping to the downstream psbG gene. There has been no previous report of the presence of ndh genes in chloroplast genome. However, an NADH-plastoquinone-(PQ) oxidoreductase activity has been detected in the chloroplasts of Chlamydomonas reinhardtii (Bennoun 1982), thus it is possible that this ORF encodes one of subunits of the NADH-PQ oxidoreductase.

Fifteen significant ORFs, which do not show any homologies with previously reported genes, were located on the sequenced region in this study (see Table 2).

Amino Acid sequences of two unidentified open reading frames in liverwort chloroplast genome show significant homologies with those of unidentified frames reported in other kinds of chloroplast genome. An ORF184 (59525-60079) shows 38.3% local homology to the ORF149 located at the next to the gene for elongation factor Tu of Euglena chloroplasts (Fig. 11Q). The Euglena ORF149 does not terminate of its stop codon but follow intron sequence (Montandon and Stutz 1983). It is interesting to compare the C-terminal region of liverwort ORF184 (position 128 to 184 amino acid residue) with the corresponding Euglena ORF in the Exon 4 described by them. The first exon (68640-68570) of the ORF203 shows 20 out of 23 amino acid identity with an reading frame in spinach X-gene on the opposite strand of psbB gene as shown in Fig. 11R. It is reasonable to believe that the open reading frames conserved in two kinds of chloroplasts would code polypeptide having an unknown function. In addition, an ORF40 (62916-62794) showed 72.6% homology with cyanella Cyanophora paradoxa ORF40, which is not proved to have any function (Fig. 11S, Bryant personal communication).

CHAPTER III The split gene for chloroplast ribosomal protein S12

Introns (intervening sequences) in a chloroplast RNA gene have been reported; the 23S rRNA gene of *Chlamydomonas reinhardtii* (Rochaix and Malone 1978, Rochaix *et al.* 1985); the tRNA genes, trnI-GAU and trnA-UGC, in the 16S-23S rDNA spacer region of *Zea mays* (Koch *et al.* 1981) and *Nicotiana tabacum* (Takaiwa and Sugiura 1982); as well as the chloroplast tRNA genes trnL-UAA (Steinmetz *et al.* 1983a, Bonnard *et al.* 1984), trnK-UUU (Sugita *et al.* 1985), trnG-UCC (Deno *et al.* 1984a, Quigley and Weil 1985) and trnV-UAC (Deno *et al.* 1982, Krebbers *et al.* 1984, Zurawski and Clegg 1984). Introns within chloroplast protein genes also have been reported in several genes of *Euglena gracilis*; for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcl) (Koller *et al.* 1984), the elongation factor Tu (tufA) (Montandon and Stutz 1983), and the 32-kd protein (psbA) (Karabin *et al.* 1984, Keller and Michel 1985). The gene for the 32-kd protein of *C. reinhardtii* also has introns (Erickson *et al.* 1984) as does the gene for the H⁺-ATP synthase subunit I (atpF) of wheat (Bird *et al.* 1985). Zurawski *et al.* (1984) reported that the chloroplast ribosomal protein L2 (rpl2) in *Nicotiana debneyi* has a single intron. Several genes in the chloroplast DNA from the liverwort, *M. polymorpha* are shown to have introns in their coding sequences (see Fig. 2 in chapter II).

Hallick *et al.* (1985) and Fromm *et al.* (1986) reported that the reading frame of the ribosomal protein S12 in *N. tabacum* is interrupted by two introns, but they described only the second one. During nucleotide sequencings of the chloroplast DNA from the liverwort, *M. polymorpha*, however, the first exon with the 5' intron boundary sequence was found on the opposite strand of the chloroplast DNA. In this chapter, the complex structure of the putative gene for chloroplast ribosomal protein S12 from the liverwort, *M. polymorpha* is presented, which has three exons split into different DNA strands. The mechanisms of the expression of this unusually organized gene will be discussed.

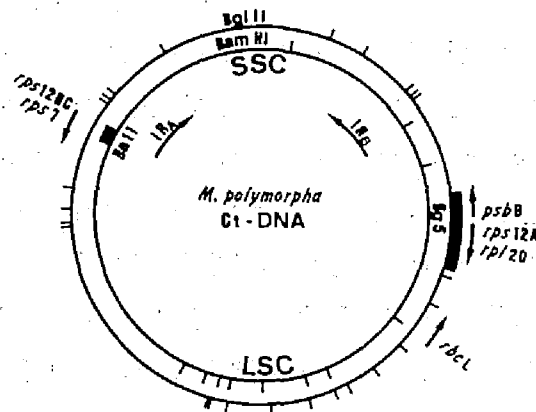


Figure 1. Locations of coding regions for chloroplast ribosomal protein S12 on physical maps of chloroplast DNA from a liverwort, *M. polymorpha*. Exon 1 (rps12A) was located on the BglIII fragment (Bg5); and the direction of its transcription was clockwise indicated by an arrow. By contrast, exons 2 and 3 (rps12B and C) were found on the BamHI fragment (Ba11), and their transcription being in the opposite direction from that of exon 1 (counter clockwise). The abbreviations rps12, rps7 and rpl20 are for genes of ribosomal proteins S12, S7 and L20. The site of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) is shown. IR_A and IR_B indicate a set of inverted repeats, and SSC and LSC indicate the small single copy and large single copy regions, respectively.

MATERIALS AND METHODS

Chloroplast DNA fragments, the BamHI (Ba11) and BglIII (Bg5) fragments, were cloned into the respective plasmids, pBR322 and pKC7, as described by Maniatis et al. (1982). A physical map of the chloroplast DNA for BamHI fragments has been described previously (Ohyama et al. 1983, Umesono et al. 1984). The location of the Bg5 fragment on that map was determined by restriction analysis and Southern hybridization (Fig. 1). Methods for the sequence determination are described in chapter II.

RESULTS AND DISCUSSION

At first a coding region for ribosomal protein S12 on the BamHI fragment (Ba11) was identified using Southern hybridization with an E. gracilis probe (provided by Drs. Montandon and Stutz). The Ba11 fragment was mapped at the junction (J_{LA}) between the inverted repeat (IR_A) and the large single copy (LSC) region (Fig. 1). DNA sequence analysis of the Ba11 fragment revealed that the coding sequence for the ribosomal protein S12 was found, however, the N-terminal 38 amino acids of the protein was missing in this coding region.

By amino acid homology search between E. coli S12 (Post et al. 1978) protein and open reading frames deduced from the nucleotide sequence data files, the missing N-terminal 38 amino-acid sequence was found on the BglIII fragment (Bg5) approximately 60 kb away on the opposite DNA strand (Fig. 1). Complete nucleotide sequences of the coding regions for ribosomal protein S12, rps12A and rps12B-C, including the flanking regions, are shown in Fig. 2. Exons 1 and 2 were followed by a consensus sequence (GTGCG) of the 5' boundary regions found in fungal mitochondrial group II introns and E. gracilis introns (Michel and Dujon 1983). In addition, much conserved sequences, RAGCCG.AUGAA..GAAA..UUCAUGU.CG GUUY, were found in the introns 75 nucleotides upstream from exon 2 and 61 nucleotides upstream from exon 3. This consensus sequence has been present in all the introns found so far in chloroplast genes of E. gracilis (Keller and Michel 1985). The secondary structures of introns

A

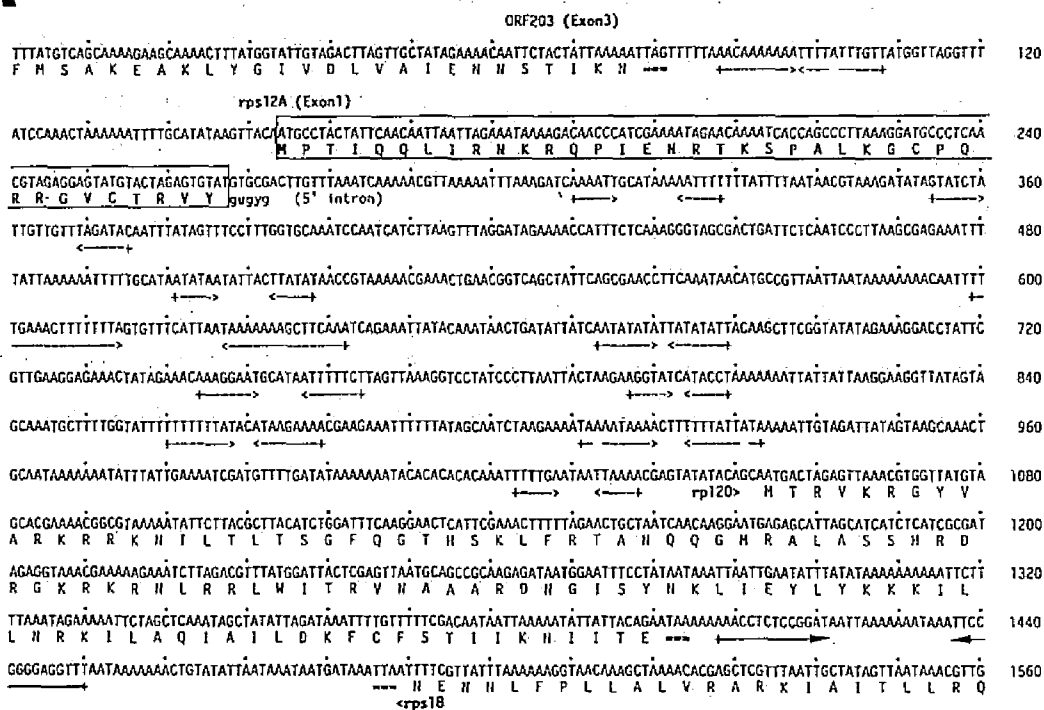


Figure 2. Complete nucleotide sequences near exon 1 (A), and exons 2 and 3 (B) of the *rps12* gene. The exons of *rps12* gene are boxed, and the consensus sequences of their 5' intron boundary regions (gugyg, y represents C or T residues), as well as those of the near 3' intron boundary regions (ragccg-augaa--gaaa--uucaugu-cgguuy, and cuayy(-)y-ay, r represents A or G residues) are shown under the nucleotide sequences. J_{LA} stands for a junction of an inverted repeat (IR_A) and a large single copy region (LSC). Amino acids are expressed as one letter symbols under the nucleotide sequences. Possible stem structure for the transcription termination are shown by bold arrows.

B

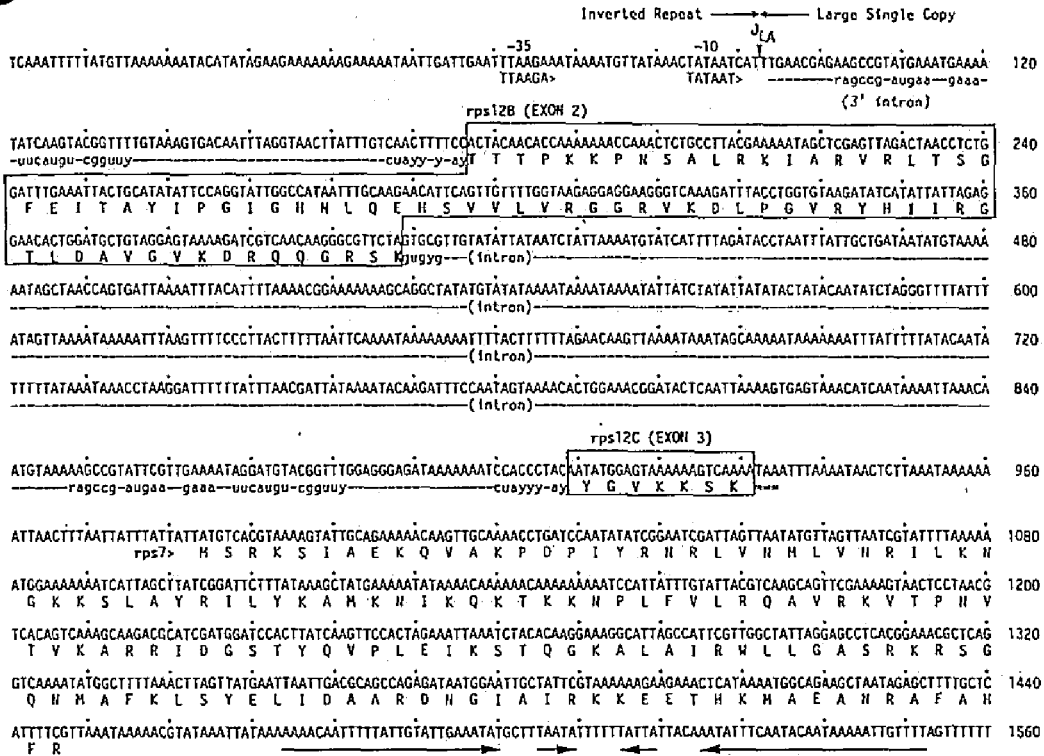


Figure 2 (continued).

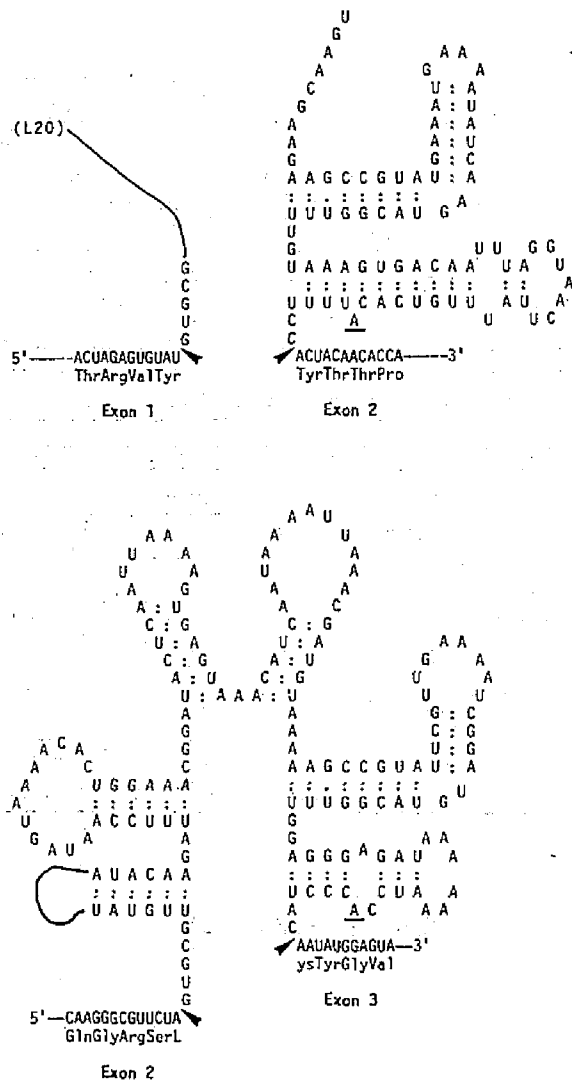


Figure 3. Possibly secondary structures of introns in *rps12* gene. Intron-exon boundaries are shown by arrow heads. Two distinct RNA molecules containing exon1 and exon2 are placed close together according to group II introns specific stem-loop structure. Amino acid sequence are shown under the RNA sequences by three letter codes.

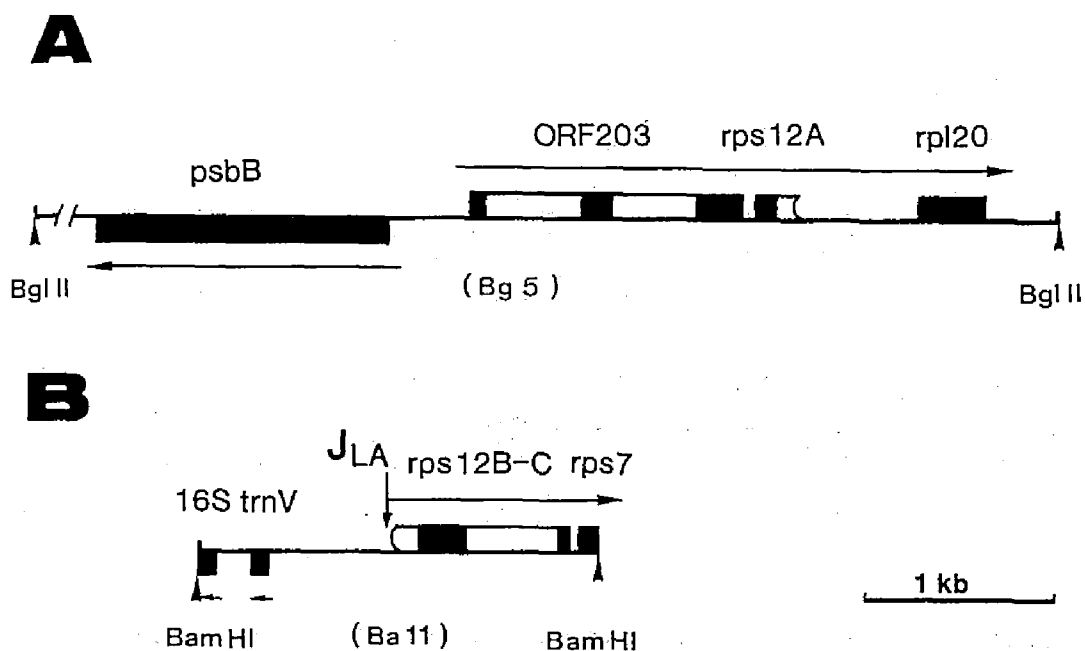


Figure 4. Gene organizations near the *rps12* gene in exon 1 (A) and exons 2 and 3 (B). Arrows indicate the direction of transcription. The abbreviations *rps12*, *rps7* and *rpl20* are the same as in Fig. 1. The symbol, *psbB*, represents the gene for the P680 chlorophyll *a* apoprotein in photosystem II. J_{LA} stands for a junction of an inverted repeat (IR_A) and a large single copy region (LSC).

	10	20	30	40	50	60
<i>E. gracilis</i>	MP	TL	EL	TR	SP	RK
<i>M. polymorpha</i>	MP	TL	EL	TR	SP	RK
<i>E. coli</i>	MP	TL	EL	TR	SP	RK
	70	80	90	100	110	120
<i>E. gracilis</i>	LE	VT	AY	IP	GI	GH
<i>M. polymorpha</i>	LE	VT	AY	IP	GI	GH
<i>E. coli</i>	LE	VT	AY	IP	GI	GH

Figure 5. Amino acid sequences for the ribosomal protein S12 from *M. polymorpha* compared with those from *E. coli* and *E. gracilis*. Vertical arrow heads indicate sites of splicing junctions in the ribosomal protein S12 from *M. polymorpha*. Asterisks denote amino acids that are identical between the two proteins. Amino acids are expressed as one letter symbols.

between exons are shown in Fig. 3. Splice junctions are indicated by arrow heads deduced from the conserved sequences and secondary structures. The putative branching nucleotide (adenine) is shown by underline in Fig. 3.

The gene organizations deduced from DNA sequences near coding regions for ribosomal protein S12 are shown in Fig. 4. ORFs corresponding to the ribosomal proteins S7 and L20 were identified by their amino acid sequence homologies, 42.6% and 46.6%, for the respective *E. coli* ribosomal proteins (Post and Nomura 1980, Wittmann and Seib 1979). In case of the ribosomal protein S14 of *M. polymorpha* amino acid homology was 45% to that of *E. coli* (Umesono et al. 1984). By contrast, the amino acid sequence of chloroplast ribosomal protein S12 from *M. polymorpha* showed markedly higher homologies, 73.6% to that of *E. gracilis* (Montandon and Stutz 1984) and 70.2% to that of *E. coli* (Post et al. 1978) (Fig. 5). The amino acid sequence of the ribosomal protein S12 from *M. polymorpha* near the splice junctions (arrow heads in Fig. 5) showed an even higher homology to sequences from *E. gracilis* and *E. coli*, both of which have no intron. This highly conserved amino acid sequence suggests that the chloroplast ribosomal protein S12 may have an essential role in the ribosomal function during protein synthesis in chloroplasts and that rps12 gene may have a regulating function in the coordinative biogenesis of chloroplast ribosome.

The ORF203, which was interrupted by two introns (518 bp and 380 bp), was detected further upstream from exon 1 of rps12 gene (Fig. 3A). An reading frame from the ATG codon (67372) to the TAG stop codon (67130) in the third exon of ORF203 was previously called ORF80 because of assuming no intron. Although the exon-intron boundary sequences of ORF203 are a little diverged from the consensus sequences, introns are correctly excised from the precursor messenger RNA including rps12A and rpl20 coding sequences (Kohchi and Umesono unpublished data). In addition, first exon of ORF203 (68640-68570) shows 20 out of 23 amino acid residues identity (87.0%) with an reading frame in spinach X-gene next to psbB gene, which is shown to be transcribed in vivo.

Exon 1 of ribosomal protein S12 with the 5' intron boundary sequence was followed by a coding sequence of ribosomal protein L20 (Fig. 2A) 786 bp apart. Following the coding region of ribosomal protein S12 in exon 3 is a ribosomal protein S7 coding region (Fig. 2B). Close linkage of ribosomal protein S7 and S12 genes also exists in *E. gracilis* (Montandon and Stutz 1983) and *E. coli* str-operon (Post et al. 1978, Post and Nomura 1980).

Transcription for exons 2 and 3, as well as for the ribosomal protein S7 gene, is initiated by a typical prokaryotic promoter sequence (-35 and -10 regions) found upstream (Fig. 2B). S1 mappings showed that this promoter was highly active in chloroplasts as well as in *E. coli* (Fukuzawa et al. 1985). Northern hybridizations also showed the active transcription for exon 1. If the split gene described here provides active mRNA that could be translated into mature S12 protein, there must be a rejoining of exons at the RNA or DNA level. The DNA rearrangement of these coding regions is not observed in chloroplast genome by restriction analysis. Results of S1 mappings and Northern hybridizations suggest that transcription units for exons 2 and 3 are independent of the unit for exon 1. Therefore, active mRNA for ribosomal protein S12 was thought to be formed post-transcriptionally by a mechanism such as that of trans-splicing described by Solnick (1985) and Konarsk et al. (1985). An further investigation of the transcription and splicing mechanisms for the split gene rps12 would clarify this complex gene organization and expression in the chloroplast.

After the publication of these results, the nucleotide sequences of the tobacco chloroplast ribosomal protein S12 was reported to be trans-split as in the case of liverwort (Torazawa et al. 1986). This suggest that trans-split rps12 gene on the chloroplast genome may be a general feature in plant chloroplasts and has some regulatory function in chloroplast biogenesis.

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SUMMARY

CHAPTER I Molecular cloning of promoters functional in Escherichia coli from chloroplast DNA

DNA fragments cloned from chloroplast DNA of a liverwort, Marchantia polymorpha, functional in E. coli as transcriptional promoters using gene fusion to the E. coli lac' gene. A recombinant plasmid gave as high a level of β -galactosidase activity as when it was induced by IPTG in E. coli wild type strain W3110. The inserted chloroplast DNA fragment was sequenced and mapped at the terminus of the inverted repeat region upstream from the 16S ribosomal RNA gene. The direction of the transcription from this promoter was opposite from that of 16S ribosomal RNA gene. This highly active promoter was for trnI-C*AU gene and clustered genes for ribosomal proteins. S1 nuclease mappings using both chloroplast and E. coli RNAs showed that the transcription starts at almost the same position downstream from the consensus Pribnow-box-like region. This clone also had a higher activity of β -galactosidase in E. coli than those containing promoters of rbcl and the β subunit gene of H^+ -ATP synthase. Two clusters of genes for ribosomal proteins were identified downstream from this highly active promoters.

CHAPTER II Structure and gene organization of the chloroplast genome

The nucleotide sequence of the large single copy region (psbG-16S rRNA gene; 30,600 bp) of the chloroplast DNA from a liverwort, M. polymorpha was determined. This region encodes genes for seven tRNAs; $tRNA^{Val}(GAC)$, $tRNA^{Ile}(C*AU)$, $tRNA^{Arg}(CCG)$, $tRNA^{Pro}(UGG)$, $tRNA^{Trp}(CCA)$, $tRNA^{Met}(CAU)$, $tRNA^{Val}(UAC)$, ten photosynthetic polypeptides; the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcl), 51 kd photosystem II chlorophyll a apoprotein (psbB), apocytochrome b-559 polypeptides (psbE and psbF), cytochrome f preprotein (petA), cytochrome b6 polypeptide (petB) and cytochrome b6/f complex subunit 4 polypeptide (petD), β and ϵ subunits of H^+ -ATP synthase (atpB and atpE), photosystem II G-protein (psbG), and ribosomal proteins (L2, L14, L16, L20, L22, L23, L33, S3, S8,

S11, S12, S18, S18 and S19), initiation factor 1 (infA) and α subunit of RNA polymerase (rpoA). Interestingly, functionally related genes are clustered as follow: (1) A ribosomal protein gene cluster involving transcriptional and translational machinery, trnI-L23-L2-S19-L22-S3-L16-L14-S8-infA-secX-S11-rpoA, was found at the terminus of the large single copy region next to the inverted repeat region (IR_B). (2) A cluster of photosynthetic genes, psbB-ORF35-ORF27-ORF74-petB-petD is located next to the ribosomal protein gene cluster. (3) A cluster including photosynthetic genes rbcL-trnR-ORF316-ORF36b-ORF184-ORF434-petA, was also found in large single copy region. Introns (intervening sequences) were found in coding sequences for ribosomal protein genes (rp12, rp16 and rps12), tRNA^{Val}(UAC) gene and photosynthetic genes (petB and petD). Interestingly, an open reading frame was found to show significant amino acid sequence homology to a subunit of NADH dehydrogenase in human mitochondria.

CHAPTER III Split gene for chloroplast ribosomal protein S12

A coding sequence corresponding to the E. coli ribosomal protein S12 gene (rps12) was found to be split into three exons. Strikingly, the first exon with the 5' intron boundary sequence was located on the opposite strand of the chloroplast DNA (121 kb, circular molecule) approximately 60 kb away from the rest of the exons. The amino acid sequence deduced from the DNA sequence was highly homologous to the sequences of the S12 ribosomal protein of E. coli (70.2%), and Euglena gracilis chloroplasts (73.6%). As the DNA rearrangement of these coding regions is not observed, the active messenger RNA for ribosomal protein S12 is thought to be formed post-transcriptionally such as that of trans-splicing. This may be the first identification of an example for in vivo trans-splicing.

LIST OF PUBLICATIONS

- (a) Ohyama K, Wetter LR, Yamano Y, Fukuzawa H, Komano T (1982)
"A simple method for isolation of chloroplast DNA from Marchantia polymorpha L. cell suspension culture."
Agric Biol Chem 46:237-242
- (b) Ohyama K, Yamano Y, Fukuzawa H, Komano T, Yamagishi H, Fujimoto S, Sugiura M (1983)
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- (d) Yamano Y, Kohchi T, Fukuzawa H, Ohyama K, Komano T (1985)
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- (e) Fukuzawa H, Uchida Y, Yamano Y, Ohyama K, Komano T (1985)
"Molecular cloning of promoters functional in Escherichia coli from chloroplast DNA of a liverwort Marchantia polymorpha."
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"Structure and organization of Marchantia polymorpha chloroplast genome (III) LSC region (rbcL-J_LB) having gene clusters of ribosomal proteins and photosynthetic polypeptides."
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(h) Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki S, Takeuchi M, Chang Z, Aota S, Inokuchi H, Ozeki H (1986)

"Chloroplast gene organization deduced from complete sequence of liverwort Marchantia polymorpha chloroplast DNA."

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Chapter I is described in reference (e).

Chapter II is described in reference (f).

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