



Molecular mapping and characterization of two chloroplast-encoded chlorophyll deficient mutants of alfalfa (*Medicago sativa* L.)
by Donald John Lee

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Crop and Soil Science
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Abstract:

A molecular and structural analysis of plastids from two independently isolated cytoplasmically inherited chlorophyll-deficient mutants of alfalfa (*Medicago sativa* L.) was undertaken. This work was done in order to assess the extent of variation in the chloroplast DNA (ctDNA) from both mutants, provide molecular markers for the verification of biparental inheritance of ctDNA in alfalfa, evaluate the developmental state of the mutant plastids and determine the transcriptional and translational differences associated with each mutant phenotype.

Physical maps of chloroplast genomes from phenotypically normal alfalfa and the two mutants were developed and compared. Variation among the ctDNAs mapped in this study was limited to four small scale insertion/deletion or point mutation events that differentiated four distinct chloroplast genotypes, three from normal tissue and one from mutant tissue. While both mutants were observed as somatic sectors, each had a different phenotype; one produced albino tissue and the other yellow-green. Electron microscopy revealed distinct differences in development between the two phenotypes. Plastids from albino tissue had no internal membrane development, sparse stroma with vacuoles and osmiophyllic globuli, and envelope degradation. Plastids from yellow-green tissue had limited thylakoid membrane development, no grana, relatively dense stroma with ribosome-like particles, osmiophyllic globules, and various degrees of vacuolization.

Biparental inheritance of chloroplast DNA in alfalfa was determined by using the unique restriction fragment patterns characterized for the normal and mutant genotypes coupled with the chlorophyll deficient phenotypic marker allowed. Sexual progeny expressing the yellow-green sectorized phenotype contained paternal ctDNA in the chlorophyll deficient sectors and maternal ctDNA in the normal sectors. Heteroplasmic cells containing both normal and mutant plastids at various stages of sorting-out were observed by electron microscopy of mesophyll cells in mosaic tissue from hybrid plants. This observation confirmed the biparental transmission of plastids in alfalfa.

The characterization of chlorophyll levels in the yellow-green mutant revealed a 72% reduction in total chlorophyll and a lower chlorophyll a/b ratio relative to normal tissue. This major chlorophyll loss was accompanied by a dramatic reduction of many proteins from yellow-green chloroplasts including coupling factor 1 alpha and beta subunits, photosystem II 32 kd protein (PSII 32kd), and the light-harvesting complex proteins of photosystem I and II. Ribulose biphosphate large subunit (LSU) and small subunit, however, were found at normal levels in yellow-green chloroplasts. Northern blots detected apparently normal levels of expression of the chloroplast encoded genes for LSU and PSII 32kd. Albino tissue showed a complete loss of plastid proteins in SDS polyacrylamide gels and a total absence of chloroplast rRNA in Northern blots. Both mutants are believed to undergo arrested plastid development due to pleiotropic effects of altered plastome encoded genes critical to two distinct stages in chloroplast development.

MOLECULAR MAPPING AND CHARACTERIZATION OF TWO CHLOROPLAST-ENCODED
CHLOROPHYLL DEFICIENT MUTANTS OF ALFALFA (MEDICAGO SATIVA L.)

by

Donald John Lee

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

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in

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APPROVAL

of a thesis submitted by

Donald John Lee

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

A molecular and structural analysis of plastids from two independently isolated cytoplasmically inherited chlorophyll-deficient mutants of alfalfa (Medicago sativa L.) was undertaken. This work was done in order to assess the extent of variation in the chloroplast DNA (ctDNA) from both mutants, provide molecular markers for the verification of biparental inheritance of ctDNA in alfalfa, evaluate the developmental state of the mutant plastids and determine the transcriptional and translational differences associated with each mutant phenotype.

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CHAPTER 1

INTRODUCTION

Chlorophyll deficient mutants of higher plants have traditionally been utilized in genetic studies because of their obvious phenotypes and direct relationship to photosynthesis. The studies reported here focus on the characterization of molecular and structural aspects of two chloroplast encoded chlorophyll deficient mutants of alfalfa (Medicago sativa L.). The purpose of these studies was to test the reported biparental transmission of alfalfa plastids with a molecular analysis of the chloroplast genomes of these mutants and to evaluate mechanisms by which the mutant phenotypes could be realized.

The goals of the first part of this study were to construct physical maps of the chloroplast genomes of the mutants, determine the amount of ctDNA variation among mutant and normal plastid genotypes and compare plastid ultrastructure in mutant and normal phenotypes. The goal of the second part of this study was to utilize the ctDNA variation and mutant plastid morphology characterized in Chapter I to verify biparental plastid inheritance in alfalfa. The final part of this study focused on transcriptional and translational aspects of the two mutants. This was done in order to compare these mutants to others previously studied and to determine the level at which the plastome mutations prohibit normal chloroplast development.

CHAPTER 2

CHLOROPLAST GENOME MAPPING AND PLASTID ULTRASTRUCTURE
ANALYSIS OF CHLOROPHYLL DEFICIENT MUTANTS OF ALFALFAIntroduction

The chloroplast genome (ctDNA) of higher plants has been mapped in many species and entirely sequenced in tobacco (Nicotiana tabacum L.) and liverwort (Marchantia polymorpha L.) (Fluhr and Edelman, 1981; Palmer, 1985; Umesono and Ozeki, 1987). These studies demonstrate a high degree of gene order conservation across genera and low rates of intraspecific sequence variation indicating the ctDNA is the most highly conserved eukaryotic genome known (Lamppa and Bendich, 1981; Palmer, 1985). Variation in chloroplast genome arrangement has been utilized to establish and reevaluate phylogenetic and evolutionary relationships in plants (Gordon et al., 1982; Palmer and Jorgensen, 1985; Palmer et al., 1985; Palmer and Zamir, 1982; Shoemaker et al., 1986). Variation within genera and species has been documented but limited to small insertion/deletion events and a low frequency of point mutations (Gordon et al., 1982; Metzlaf et al., 1981; Palmer et al., 1985; Palmer and Zamir, 1985; Teeri et al., 1985; Tsunewaki and Ogihara, 1984). A higher degree of larger scale ctDNA rearrangement has been documented in species lacking the duplicated set of ribosomal RNA and ribosomal protein genes arranged as an inverted repeat sequence (Palmer and Thompson, 1981; Palmer and Thompson, 1982).

Pigment deficient mutants in many plant species are one phenotypic class where intraspecific chloroplast genome variation has been identified (Archer et al., 1987; Gordon et al., 1980; Wong-Staal and Wildman, 1973). Most mutants are a result of nuclear gene mutations (Harpster et al., 1980; Tilney-Bassett, 1984), but some cytoplasmically inherited mutants have been documented (Archer et al., 1987; Borner et al., 1972; Duesing et al., 1985; Gordon et al., 1982; Kirk and Tilney-Basset, 1967; Kutzelnigg and Stubbe, 1974; Palmer and Mascia, 1980; Stubbe and Hermann, 1982; Wildman et al., 1973).

Recently Smith et al. (1986) studied a yellow-green mutant of alfalfa that arose from tissue culture and an albino mutant of alfalfa identified in breeding populations. Genetic and developmental evidence indicated plastome mutations caused these phenotypes and plastids were biparentally inherited. Both yellow-green and albino phenotypes arise as somatic sectors in mosaic tissue. The resulting chimeric plants have normal tissue that apparently sustains the mutant sectors, rendering the mutations non-lethal.

The objectives of this study were to determine differences in the ctDNA and examine plastid ultrastructural features from normal and chlorophyll deficient tissue from these alfalfa mutants. Southern blot analysis was used to generate chloroplast genome maps from yellow-green and albino sectors of chimeric plants, normal green sectors of the same plants and their normal parents. Electron microscopy was utilized to evaluate the developmental stage of mutant and normal plastids from the same plants.

Materials and Methods

Plant Sources

Tissue sources utilized in this study were pure sectors from tetraploid Medicago sativa plants expressing either the yellow-green sectoring (YGS, a somaclonal variant) or albino sectoring (ALS, discovered in a breeding population) phenotypes described previously (Smith et al., 1986). All designations used followed those established for the plants and tissue provided by E.T. Bingham or S.E. Smith. The plants used were crosses of the original YGS and ALS plants to 6-4, a standard male-sterile tester genotype. An additional mapping analysis was performed on crosses of YGS to MAG, a second normal genotype with a multifoliate morphological trait. These chimeric plants had the pedigree [MAG x YGS] and [(MAG x YGS) x MAG] (denoted 32B-2). All plants were grown in the greenhouse under 16 hour daylength.

Total and Chloroplast DNA Extractions

Pure yellow-green, albino or normal sectors were removed from plants and freeze-dried in a VirTis freeze-dryer for 2-4 days. Total DNA was extracted from freeze-dried material using the method of Saghai-Marooof et al. (1984). Chloroplast DNA was extracted from fresh tissue using methods described by Kemble (1987).

Restriction Endonuclease Digestion, Gel Electrophoresis

DNA samples were digested with Hind III, Bam H1, Xho I and Pst I either singly or in combination according to suppliers' instructions (IBI or BRL).

Approximately 10ug of digested total DNA or 5ug of ctDNA per lane were loaded onto a 0.7%, 110 x 135 mm agarose gel and electrophoresis was carried out on a BRL horizontal apparatus at 1 V/cm for 16 hours in TBE gel buffer (Maniatis et al., 1982). Gels were then stained with ethidium bromide and photographed under UV light. Mobilities of the Lambda Hind III markers were measured from the photograph.

Southern Transfers

Transfer of restriction fragments from agarose gels to Zeta-Probe nylon membranes or nitrocellulose filters was performed according to the methods of Reed (1985) and Southern (1975) respectively. Nitrocellulose filters were baked at 80 degrees C. in a vacuum oven for 2 hours after transfer was completed.

Hybridization Probes

A library of alfalfa ctDNA PstI fragments cloned into pBR322 and transformed into DH₂B or RR1 E. coli cell lines served as hybridization probes to the filter bound DNA fragments. This library, developed by Dr. J.D. Palmer, covers the entire alfalfa chloroplast genome except for a 35 kilobase (kb) PstI fragment (Fig. 1). Three mung bean Sal I or Sal I/Pst I ctDNA fragments were ligated into either pBR322 or pUC18 that completely span this 35 kb region and complete this library (pers. comm., J.D. Palmer). Plasmids were isolated from E. coli hosts using the mini-prep procedure of Birboim and Doly (1979). Mini-prep DNA extractions were digested with Pst I and run on a 0.7% agarose minigel to verify insert size. DNA from minipreps was labeled with ³²P dNTPs using nick-translation (Rigby et al., 1977). Unincorporated labeled nucleotides were separated from labeled recombinant probes using

centrifuged sephadex G-50 1 cc columns. Prior to hybridization, the labeled probes were denatured in solution with 200 mls of 0.2 N NaOH by heating to 100 degrees C for 10 min. Salmon sperm DNA was added to the mix prior to heating.

Hybridizations

Zeta probe and nitrocellulose filters were prehybridized in 25-30 mls of a 1.5 X SSPE, 1.0% SDS and 0.5% Blotto solution or a 50% formamide, 5x Denhardt's solution, 5x SSPE and 0.1% SDS solution. Filters were prehybridized at 67 degrees C. in an water incubator with gentle shaking for 4-24 hours. Denatured probes were added to the bagged filters and prehybridization mix and the bags were resealed and incubated at 67 C for 24 hours.

Washing and Autoradiography

The hybridization mixture containing probe was recovered and reused in up to four hybridizations. Three washes at room temperature with 300 to 500 mls of 2 X SSC/ 0.1% SDS, 0.5 X SSC/ 0.1% SDS and 0.1 X SSC/ 0.1% SDS solutions successively were followed by one wash with 300 to 500 mls of 0.1 X SSC/ 1% SDS at 55-60 degrees.

Washed filters were wrapped in Saran wrap and taped to a Kodak X-ray exposure cassette with one intensifying screen. Autoradiography was performed at -70 C for 2-72 hours. To improve resolution, some autoradiographs were exposed at room temperature without the use of intensifying screens.

Filters were reused by washing in 1000 mls of 0.1 X SSC/ 0.5% SDS at 95 C for 30-60 min. to remove hybridization probe. Prehybridization and hybridization procedures were then repeated.

Fragment Size Determination

Sizes of hybridized filter bound fragments detected by autoradiography were determined by a regression analysis method described by Schaffer and Sederoff (1981). Gels showing nonuniformity in lambda HindIII fragment mobility were subdivided so that unknown fragment sizes were determined from regressions of markers no further than 4 lanes distant.

Electron Microscopy

Ultrastructural features of mesophyll cells from pure sectors of normal, yellow-green or albino leaf tissue were examined by transmission electron microscopy. The tissue was prepared for ultrathin sectioning largely according to the procedure of Carroll and Mayhew (1976). Finely chopped leaves were fixed in 3.0% glutaraldehyde in 0.01 m potassium phosphate buffer, pH 7.2, for 24 hours. Fixation in 2.0% osmium tetroxide in the same phosphate buffer followed. Tissue dehydration was in a graded concentration series of ethanol (50-100%). Ethanol was replaced with propylene oxide before infiltration of the fixed tissue with Spurr's embedment. Polymerized tissue was then thick sectioned for cytological observation under the light microscope. Selected regions of embedded tissue were thin sectioned on an ultramicrotome, mounted on grids and stained with uranyl acetate and lead citrate. Grids were then observed under the Zeiss EM 10C transmission electron microscope.

Results

Genome Sizes

Chloroplast genome sizes for all genotypes analyzed were in agreement with alfalfa chloroplast genome sizes determined by Rose et al (1986) and Palmer (personal communication). Number and sizes of fragments generated by each of the restriction endonucleases mapped are listed in Table 1.

Comparative Blots

Since total DNA from both mutant and normal tissue was used in the Southern analysis to determine restriction maps, comparative blots of chloroplast and total DNA from the normal tissue in 6-4, MAG, 6-4 x YGS and 6-4 x ALS plants were performed. With all probes, bands representing homology of the ctDNA with mitochondrial or nuclear sequences were easily distinguished from chloroplast DNA fragments. All strongly hybridized bands present in total DNA were present in ctDNA (data not shown).

Polymorphisms

Polymorphisms among mutant and normal chloroplast genomes were found with four of the alfalfa ctDNA probes, 2, 3, 6 and 7 (Figure 1). These Pst I fragment probes reveal restriction pattern differences with Hind III and Bam HI digests that define three unique normal ctDNA genotypes and one mutant ctDNA genotype.

Polymorphisms with Bam HI restriction fragments hybridized by Pst I probe 7 indicate the loss of a recognition site in both mutants, MAG

Table 1. Alfalfa chloroplast genome fragments produced by restriction endonuclease digestion.

Filter-bound ctDNA Fragments

Probes	Tissue Source	Pst I	Xho I	Hind III	Ban HI
1	all	6.2 ¹	5.3.9.0	4.0.3.0.2.1.20.0	2.3.2.1.13.0
2	a	21.0	9.0.6.8.2.9.19.1	20.0.2.6	13.0.1.4.1.5.2.2.2.4.5.5
	b,c,d	"	"	20.0.2.7	13.0.1.4.1.5.2.2.2.4.5.6
3	a	21.0.35.0	19.1.33.5	2.6.5.8	13.0.1.4.1.4.2.2.2.4.5.5.3.3
	b,c,d	"	"	2.7.5.8	13.0.1.4.1.5.2.2.2.4.5.6.3.3
4	all	35.0	33.5	5.8.7.2.9.6	5.0.4.2.23.8
5	all	35.0	33.5	9.6.1.7.2.2.11.5	23.8
6	a,c	12.7	33.5.4.9	11.5.1.4.7.7	23.8.6.7.1.6.1.9.2.8.2.2
	b	12.6	33.4.4.9	11.4.1.4.7.7	23.8.6.7.1.6.1.8.2.8.2.2
	d	12.5	33.3.4.4	11.3.1.4.7.7	23.8.6.7.1.6.1.7.2.8.2.2
7	a	18.0	4.9.3.5.16.8	7.7.1.8.4.4.1.4.2.1.11.5	3.9.9.7.6.1.5.1.6.11.0
	b,c	"	"	7.7.1.8.2.8.1.6.1.4.2.1.11.5	3.9.9.9.1.1.6.11.0
	d	"	"	7.7.1.8.2.8.1.6.1.4.2.1.11.5	3.9.9.7.6.1.5.1.6.11.0
8	all	5.1	16.8	11.5	11.0
9	all	7.0	16.7	11.5.1.4	11.0.2.9.3.4
10	all	7.1	16.7	13.1	3.4.5.0
11	all	12.5	16.7.3.6.1.2.5.3	13.1.5.5.4.0	5.0.3.4.6.5.1.7.2.3
Number of Fragments	a	9	12	21	30
	b,c	"	"	22	29
	d	"	"	22	30
Total Size (Kb)		124.6	128.6	119.3	125.9

¹ all sizes in Kb

tissue sources

a- 6-4, 6-4 x ALS normal, 6-4 x YGS normal

c- 32B-2 normal and MAG

b- yellow-green and albino

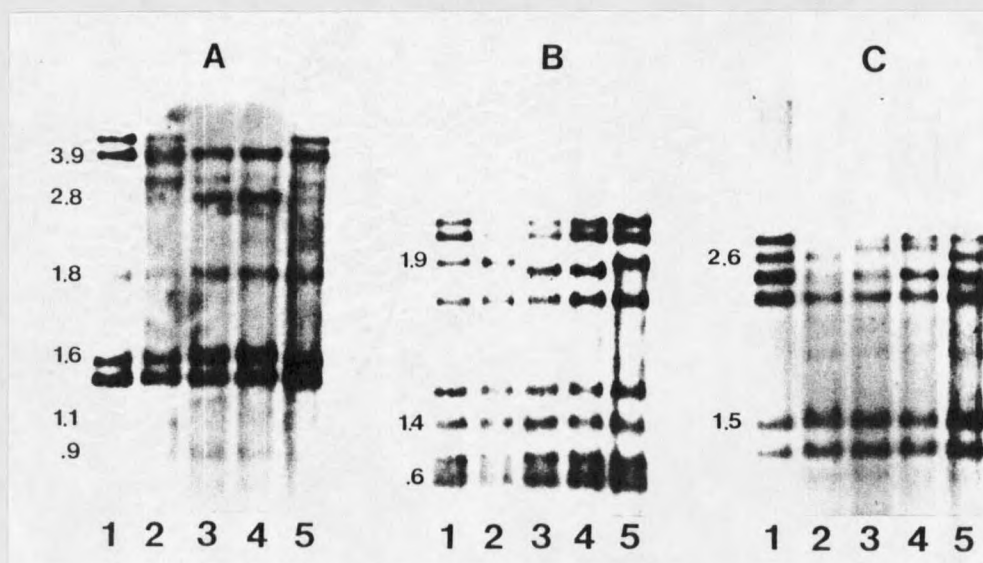
d- MAG x YGS normal

and 32B-2 normal sector ctDNA (Figure 1). This results in a 9.1 kb fragment in the mutants and a 7.6 plus a 1.5 kb fragment in the normal tissues (Table 1). This point mutation in the Bam HI recognition site is visualized on autoradiographs (Figures 3A and 3B) of Bam HI-Xho I digested fragments hybridized with probe 7. A 6.5 kb fragment in albino, yellow-green, and 32B-2 normal lanes is cleaved to 5.0 and 1.5 kb fragments in lanes containing 6-4, 6-4 x ALS normal sector, and MAG x YGS normal sector DNA. MAG ctDNA restriction fragment patterns have been compared to 6-4 and mutant ctDNAs in a subsequent study (Chapter 3).

Hind III digests show polymorphisms when hybridized by probe 7 as a result of an extra recognition site in the ctDNAs from albino and yellow-green sectors and in normal sectors from MAG x YGS and 32B-2 (Figure 1). The 6-4 ctDNA contains a 4.4 Kb fragment that consists of a 2.8 and a 1.6 Kb fragment in the two mutants (Table 1). Autoradiographs of Bam HI-Hind III double digests hybridized with probe 7 (Figure 2A) demonstrate the Hind III point mutation observed in this region. MAG, MAG x YGS normal sector and 32B-2 normal sector ctDNA have the same Hind III sites as the mutant ctDNAs in this region (data not shown).

Polymorphisms observed by Pst I fragment 2,3 and 6 hybridizations are found with all restriction endonucleases and are a result of addition/deletions rather than point mutations (Figure 1). Since the differences in fragment sizes between mutant and normals is relatively small (100 base pairs) resolution of these polymorphisms is only obvious in smaller fragments. Hybridizations with probe 6 reveal a 100 base

Figure 2. Autoradiographs of Bam HI-Hind III digest fragments hybridized by probes 7, 6, and 2. Lanes 1-5 contain the following samples of total DNA (approx. 5 ug per lane); (1) 6-4, (2) 6-4 x YGS normal, (3) 6-4 x YGS mutant, (4) 6-4 x ALS mutant, (5) 6-4 x ALS normal. Sizes of hybridized fragments were determined by regression with lambda Hind III mobilities and are noted in kb on the left of each autoradiograph.

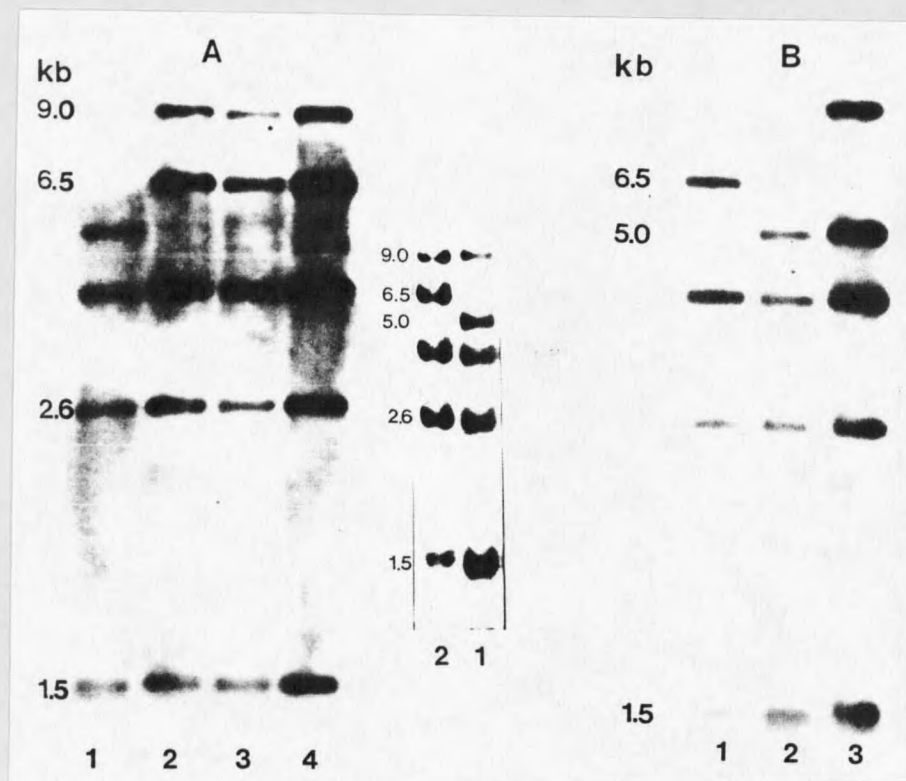


A: Probe 7 reveals a point mutation with Hind III restriction endonuclease. A 4.4 kb Hind III fragment contains an extra Hind III site in the mutants and is cleaved to a 2.8 kb and a 1.6 kb fragment (lanes 3 and 4).

B: Probe 6 hybridized with a 1.9 kb fragment (lanes 1, 2 and 5) that is slightly smaller in the mutants (lanes 3 and 4).

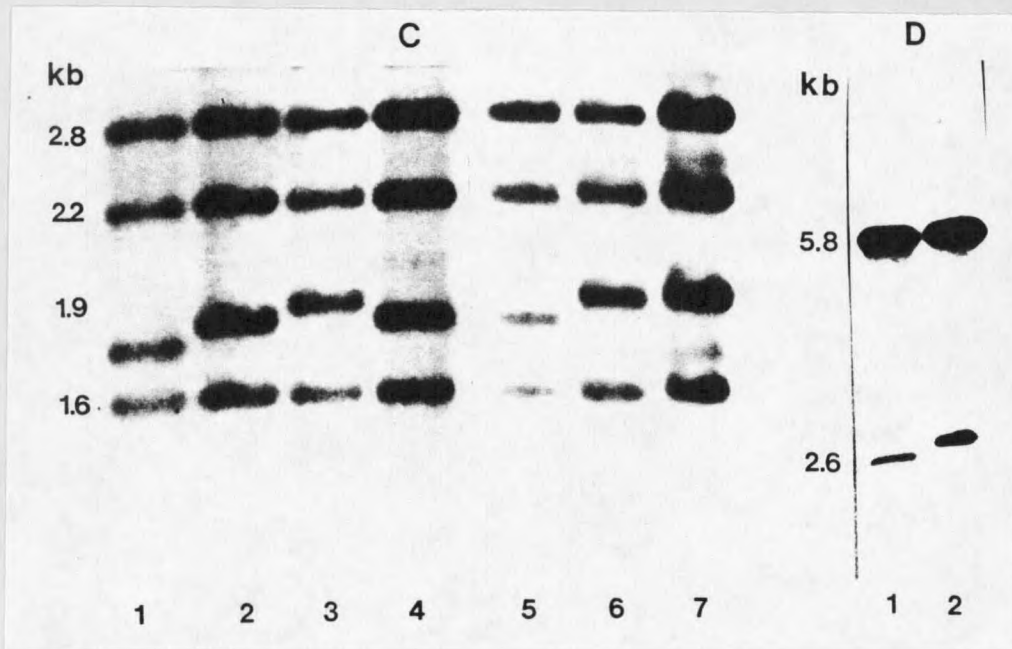
C: Probe 2 hybridizes with a 2.6 kb fragment in the normal and 6-4 lanes (1, 2 and 5) that is slightly larger in the mutants (lanes 3 and 4).

Figure 3. Autoradiographs demonstrating polymorphisms in normal and mutant ctDNA genotypes. All lanes contain approximately 10 ug of total DNA or 5 ug of ctDNA.



- A: Probe 7 hybridized to Bam HI-Xho I digested filter-bound fragments. Lanes contain the following samples of total DNA; (1) MAG x YGS normal, (2) MAG x YGS yellow-green, (3) 32B-2 normal, (4) 32B-2 yellow-green. A 6.5 kb double digest fragment in lanes 2-4 is cleaved to 5.0 and 1.5 kb fragments because of an extra Bam HI site. The extra 1.5 kb fragment is visualized more clearly in a second filter containing lane 1 and 2 samples shown in the inset.
- B: Probe 7 hybridized to Bam HI-Xho I fragments. Lanes contain the following total DNA samples; (1) 6-4 x ALS albino tissue, (2) 6-4 x ALS normal tissue, (3) 6-4. Bam HI restriction site gain in lanes 2 and 3 results in a 6.5 kb fragment cleaved to a 5.0 kb and an extra 1.5 kb fragment.

Figure 3. (continued)



C: Probe 6 hybridized to Bam HI-Xho I digested fragments. Lanes contain the following total DNA samples; (1) MAG x YGS normal, (2) MAG x YGS yellow-green, (3) 32B-2 normal, (4) 32B-2 yellow-green, (5) 6-4 x ALS albino, (6) 6-4 x ALS normal, (7) 6-4. Deletions in the 1.9 kb fragment is visualized in mutant (approx. 100 bp) and MAG x YGS normal (approx. 200 bp) lanes.

D: Probe 2 hybridized to Hind III digests of ctDNA samples from 6-4 (lane 1) and MAG (lane 2). The 2.6 kb fragment in 6-4 is approx. 100 bp larger in MAG.

pair (bp) deletion in a 1.9 Kb Bam HI fragment in the albino and yellow-green mutants (Figure 2B). Bam HI-Xho I digests (Figure 3C) show 32B-2 normal tissue has ctDNA fragments equivalent to 6-4 while MAG x YGS normal tissue contains a fragment approximately 1.7 kb compared to 1.8 kb in mutant tissue ctDNA. The fragment 2 and 3 probes reveal a 100 bp addition in a 2.6 Kb Hind III fragment in the mutants (Figure 2C), MAG (Figure 3D), and in normal tissue from MAG x YGS and 32B-2 (data not shown). No other probes revealed polymorphisms among the ctDNA from the genotypes analyzed.

Mapping

Physical maps of the alfalfa ctDNAs were constructed using the size data from both single and double digests in all combinations. Determining the serial order of all filter-bound fragments hybridized by the ctDNA probes was possible with this strategy except for three small Bam HI fragments hybridized by probe 6, two small Hind III fragments hybridized by probe 5, and four small Bam HI fragments hybridized by probes 2 and 3 (Figure 1). Direct comparison was possible with Xho I and Pst I restriction sites by comparison to the alfalfa ctDNA map of Palmer (Palmer personal communication) and no major discrepancies were found between the normal green sector ctDNAs and those genotypes mapped by Palmer.

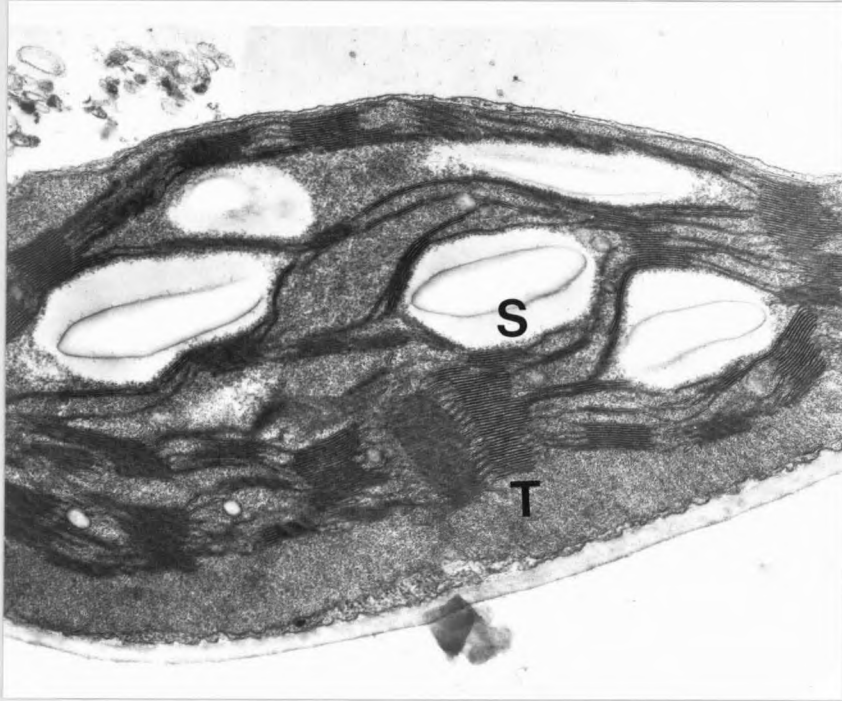
Electron Microscopy

Normal green leaf tissue contained chloroplasts with well developed thylakoid membrane systems and large starch grains indicative of high photosynthetic activity (Figure 4A). Ultrastructural features of the albino and yellow-green mutants were drastically different (Figures 4B-4D).

Plastids from yellow-green leaf tissue (Figures 4B, 4C) were slightly abnormal in shape due to an irregular envelope. In all plastids observed, however, the envelope was intact. The plastids have a low degree of lamellar development and no grana. Osmiophillic globuli are present and varying degrees of vacuolization were observed. Each of these plastids had a dense stroma containing ribosome-like particles. Mitochondria were abundant with developed cristae in the mesophyll cells.

Albino mesophyll cells, like those of the yellow-green tissue appear normal in size, shape and number under light microscopy despite an obvious absence of intracellular development. Plastids in albino tissue appear smaller in size than those in normal green tissue and have no thylakoid membranes. The stroma of each plastid were less dense, had few ribosome-like particles, and many large osmiophillic globuli. Vacuoles in the plastids are fewer in number in the albino than in the yellow-green tissue (Figure 4D). The envelopes were degraded in many of the plastids, resulting in the release of their contents into the cell lumens (Figure 4D). Mitochondria with cristae were also present in this albino tissue. Approximately 700 albino, 300 yellow-green and 150 normal plastids were examined by electron microscopy.

Figure 4. Electron micrographs of foliar tissue from chimeric plants.



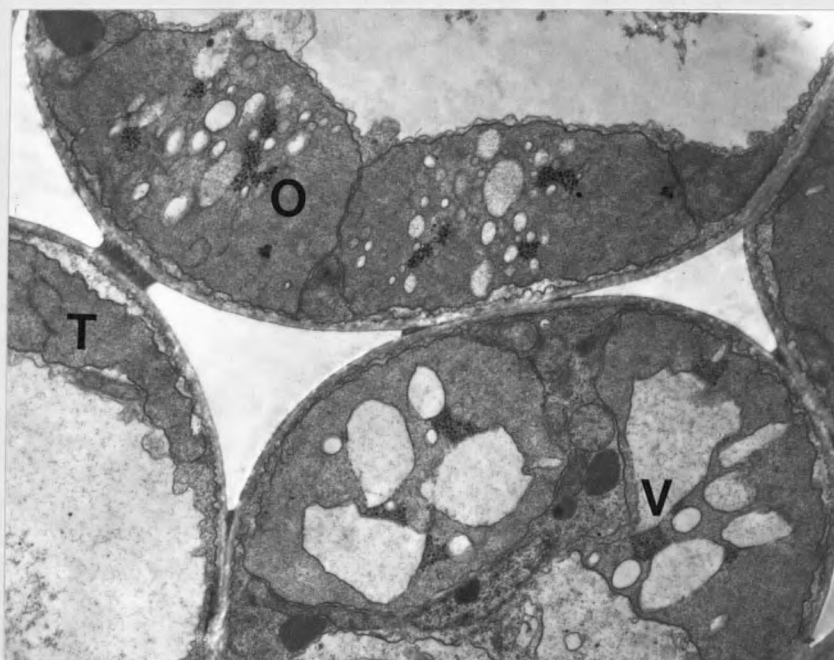
A. Normal green tissue (x30,000) showing normally developed chloroplast with extensive thylakoid membranes (T) stacked into grana and large starch grains (S).

Figure 4. (continued)

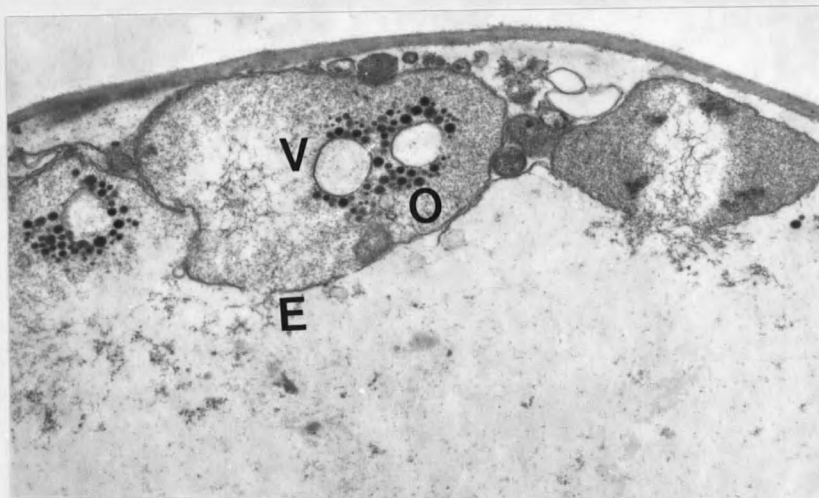


B. Yellow-green tissue plastid (x20,000) with some rudimentary thylakoid membrane development (T) and dense stroma with ribosome-like particles. Mitochondria (M) show cristae and dense matrix.

Figure 4 (continued)



C. Yellow-green tissue (x9,600) showing portions of several mesophyll cells. Plastids in these cells have poorly developed thylakoids (T) and varying degrees of vacuolization (V) as well as osmiophyllic globule accumulation (O).



D. Albino tissue plastids (x26,000) with vacuoles (V), osmiophyllic globuli (O), and disrupted envelopes (E).

Discussion

Comparison of ctDNA from pure mutant and normal tissue of albino and yellow-green sectoring plants by Southern blot analysis indicates the two mutant phenotypes possess chloroplast genomes that are different from normal ctDNAs. Four point mutation or addition/deletion events differentiate the ctDNAs examined in this study. Combinations of the polymorphisms detected in Bam HI and Hind III restriction fragments define three normal ctDNA genotypes; one found in 6-4 and normal tissue from 6-4 x YGS and 6-4 x ALS; a second found in MAG and normal tissue from 32B-2; and a third in normal tissue from MAG x YGS. The same polymorphisms distinguish the mutant ctDNA genotype found in both yellow-green and albino tissue. The mutant ctDNA genotype shows an absolute association with the albino and yellow-green phenotypes. In all observed cases, pure mutant sectors from chimeric plants utilized in this study contain the mutant ctDNA genotype.

A total of two point mutations were identified in this analysis for an estimated mutation fixation frequency of 0.5%. The alfalfa chloroplast genome is 127,000 base pairs, which provides a best estimate of 635 likely point mutations in the mutant genomes relative to 6-4. Other studies employing these techniques have found a similar frequency of point mutations and similar addition/deletion polymorphisms between closely related species (Gordon et al., 1982; Palmer, 1985; Palmer and Jorgensen, 1985) or within species (Metzlaff et al., 1981; Scowcroft, 1979; Teeri et al., 1985) while Gordon et al. (1980) have found small addition/deletion differences in plastome mutants of chlorophyll

deficient Oenothera. Palmer and Thompson (1981) have demonstrated a higher degree in large scale ctDNA rearrangements among legume species that have lost the inverted repeat. Alfalfa chloroplast genomes also lack this structure, but intraspecific variation detected in this study is not a result of major rearrangements. The variation detected among these alfalfa genotypes may be expected, however, if the instability proposed by the loss of the inverted repeat predisposes chloroplast genomes to minor as well as major alterations.

Two of the normal ctDNA genotypes observed are derived from the same clone. Normal tissue from MAG x YGS and 32B-2 chimeric plants have ctDNAs that differ by a small insertion/deletion event detected by probe 6 and a Bam HI recognition site detected by probe 7. Assuming biparental plastid inheritance, both chimeric plants should have normal plastids from MAG. Only 32B-2 normal tissue has ctDNA with the MAG genotype characterized in other studies. No evidence of restriction fragment patterns resulting from mixed cytoplasms was observed in lanes containing MAG x YGS normal tissue DNA, therefore the variant normal genotype in MAG x YGS is probably the result of its initial inheritance from MAG. Evidently, the population of plastids in MAG is mixed and gametes can be produced from this clone with different ctDNA genotypes through somatic segregation. Heteroplasmy has also been observed in alfalfa by Rose et al. (1986).

The existence of mutant plastids in albino and yellow-green tissue of these chimeric plants is verified by the ultrastructural features observed in this study. Similar features to those in the electron

micrographs of these alfalfa mutants was documented by Borner et al. (1972) in yellow-green and albino chloroplast mutants of Pelargonium. The dramatic arrest in plastid development in the albino Pelargonium mutants was correlated to an absence of plastid ribosomal RNAs. While the polymorphisms detected in these alfalfa mutants indicate specific differences between the mutant and normal ctDNAs, they are not necessarily responsible for the mutant phenotypes. The only polymorphism not found in any of the normal ctDNA genotypes is the 100 bp deletion detected by probe 6. The same mutations mapped in two unique phenotypes suggests that these differences may not have a direct physiological effect. The few observed differences indicate that the mutant and normal genomes are largely identical, there are no major rearrangements or deletions in the mutants as has been reported in another culture derived chloroplast mutants of wheat (Triticum aestivum L.) by Day and Ellis (1985).

Plastid genotypes from both mutant phenotypes are indistinguishable by these methods. Additional point mutations in the chloroplast genomes would be expected since a very small percentage of the chloroplast genome was sampled for sequence variation in this analysis. Small differences in fragment size would also be undetectable in the larger fragments generated by double digests. A more intensive study at the sequence, transcriptional, and translational levels is needed to establish cause and effect relationships between ctDNA mutations and the observed arrest in plastid development in these mutants.

Many chlorophyll deficient mutants have been reported and characterized but relatively few are a result of mutations in ctDNA

genes. Transcriptional and translational evidence has determined the level at which normal events are disrupted in many mutants but mapping of chloroplast mutations has been rare. The yellow-green and albino sectoring mutants studied here have identical insertion/deletion and point mutations mapped to their plastomes yet have distinct alterations in plastid development as evidenced by electron microscopy. While plastid development is drastically arrested in these mutants, the sorting out process resulting in chimeric plants renders them non-lethal. Because of this stability and their developmental nature they are useful systems in which to study chloroplast genome regulation and chloroplast-nuclear genome interactions.

CHAPTER 3

BIPARENTAL INHERITANCE OF CHLOROPLAST DNA AND THE
EXISTANCE OF HETEROPLASMIC CELLS IN ALFALFAIntroduction

Plastid inheritance in higher plants has been demonstrated to follow maternal, paternal and biparental modes. Chlorophyll deficiency has traditionally been the chloroplast encoded trait utilized in studies of plastid inheritance (Kirk, Tilney-Basset 1978) but recently chloroplast encoded antibiotic resistance has been used to select among large numbers of cultured cells for low frequencies of plastid transmission (Medgyesy et al. 1986).

Patterns produced following cleavage of chloroplast DNAs (ctDNA) with restriction endonucleases have provided another way with which to differentiate plastids with maternal or paternal origin in genetic studies. Variation in restriction patterns of ctDNA is limited but detectable among closely related species and among genotypes within a species (Palmer, 1986). Analysis of ctDNA restriction fragment banding patterns of parents and progeny has been used to demonstrate the pattern of plastid transmission in interspecific crosses of Triticum (Vedeĭ et al. 1981), Zea (Conde et al. 1979), Sorghum (Pring et al. 1982), Nicotiana (Medgyesy 1986), Glycine (Hatfield et al. 1985), Oenothera (Hatchel 1980), and Larix (Szmidt et al. 1987).

Two chlorophyll deficient mutants of alfalfa (Medicago sativa L.) that arise through somatic sectoring were studied by reciprocal cross analysis and found to sexually transmit their phenotypes through both parents (Smith et al. 1986). Developmental and genetic evidence suggested that the chlorophyll deficiencies represent plastid mutations and that plastids are biparentally inherited in alfalfa. Subsequent mapping of ctDNA from pure chlorophyll deficient and normal sectors from these phenotypes demonstrated the existence of a unique plastid genotype in the mutant tissue. Electron microscopy evidence also verified that development of mutant plastids is dramatically arrested the chlorophyll deficient sectors (Chapter 2). Differences in restriction fragment patterns between chlorophyll deficient and normal ctDNA could provide a definitive description of the mode of inheritance of plastids in alfalfa.

In this study ctDNA from normal and mutant tissue of parents and sexual progeny were compared by restriction fragment analysis. Tissue from sectoried (normal plus chlorophyll deficient) progeny undergoing the sorting out process were also examined by transmission electron microscopy for evidence of individual mesophyll cells containing both mutant and normal plastids.

Materials and Methods

Plant Sources

Tetraploid Medicago sativa plants used in this study were a

phenotypically normal male sterile tester line (6-4), a multifoliate fully fertile tester line that had no chlorophyll deficiency (MAG), and progeny from a cross between MAG and a sectored chlorophyll deficient (yellow-green) variant (YGS) described by Smith et al. (1986). Pure yellow-green sectors on progeny from the cross MAG x YGS (lines 33B-2 and 33B-4) produced fertile flowers that were utilized in the cross 6-4 x 33B-4 (female parents are listed first throughout). Progeny from this cross (12-1,13-2,13-4) were then selected for the yellow-green sectored phenotype. All plants were grown in the greenhouse under 16 hour daylength.

DNA Extraction, Southern Blotting, and Hybridization

Total and chloroplast DNA extractions, Southern Blotting, labeling, hybridization and autoradiography procedures used were identical to those outlined in Chapter 2. Alfalfa ctDNA fragments from the library of J.D. Palmer that detected restriction fragment polymorphisms in the Chapter 2 mapping study were utilized as hybridization probes (Table 2).

Electron Microscopy

Ultrastructural features of mesophyll cells from fine mosaic sectors of yellow-green sectored progeny were examined by transmission electron microscopy by the methods described previously.

Results and Discussion

Restriction Fragment Polymorphisms

Hybridization of filter-bound ctDNA with the alfalfa and mung bean

ctDNA probes revealed four distinct polymorphisms among the genotypes studied. In all cases, hybridization patterns of total and chloroplast filter-bound DNA in these autoradiographs were identical. Bam HI, Hind III, and Xho I restriction fragments were physically mapped (Chapter 2) and the polymorphisms were found to be due to two insertion/deletion events and two point mutations. These polymorphisms provided markers with which to distinguish the ctDNA genotypes used in this study (Table 2).

Table 2. Hybridization pattern differences detected with alfalfa ctDNA Pst I fragments.

Bam HI Fragment Polymorphisms (kb)			
<u>ctDNA</u>	<u>12.7 kb probe 6^a</u>	<u>18.0 kb probe 7</u>	<u>21.0 kb probe 2</u>
6-4	1.9	7.6 + 1.5	5.5
MAG	1.9	9.1	5.6
YGS	1.8	9.1	5.6

Hind III Fragment Polymorphisms (kb)			
<u>ctDNA</u>	<u>12.7 kb probe 6</u>	<u>18.0 kb probe 7</u>	<u>21.0 kb probe 2</u>
6-4	11.5	4.4	2.6
MAG	11.5	2.8 + 1.6	2.7
YGS	11.4	2.8 + 1.6	2.7

^a Probe designation from Chapter 2.

Autoradiographs of ctDNA fragments of parents and progeny (Figure 4) demonstrate that three distinct plastid genotypes exist in the plants studied and that plastid transmission occurs biparentally in alfalfa. Figure 5A shows that MAG (lanes 1 and 2) and 6-4 (lanes 7 and 8) have

different restriction fragment patterns with Hind III digested fragments hybridized with an 18 kilobase pair (kb) alfalfa ctDNA probe. This polymorphism is due to an extra Hind III recognition site in MAG that results in a 4.4 kb fragment being cleaved into a 2.8 kb and a 1.6 kb fragment. Hind III ctDNA fragments from 33B-4 normal sectors (lane 3) and yellow-green mutant sectors on the same plant (lane 4) were indistinguishable with this probe. Normal and mutant sectors on 13-4, however, are distinguishable. Hind III ctDNA fragments from mutant tissue (lane 5) have the same pattern as the paternal parent (33B-4 mutant sectors, lane 4) while the normal tissue fragment pattern (lane 6) is the same as 6-4, the female parent (lane 7).

Figure 5B demonstrates unique Bam HI restriction patterns of mutant ctDNA hybridized with a 12.7 kb alfalfa ctDNA probe. Yellow-green mutant tissue from both 33B-4 and 13-4 (lanes 3 and 4) contains ctDNA with a fragment that is slightly smaller than the 1.9 kb Bam HI fragment in the normal tissue ctDNAs (lanes 2 and 5). The mobility of this fragment is the same in MAG, 6-4, and normal tissue from 33B-4 and 13-4.

Figure 5C demonstrates that all yellow-green sectoring mutant progeny studied by this analysis had the same mutant ctDNA. Lane 1 contains total DNA from normal sectors on 13-2 while the remaining lanes contain total DNA from mutant sectors from five different sectoring plants from two different generations. Hind III digested ctDNA fragments from 33B-4 mutant sectors (lane 6) had the same pattern when hybridized with the 18 kb probe as its sibling, 33B-2 (lane 2), and progeny (lanes 3, 4, and 5). The stability of this genotype was confirmed by hybridization of these

filter-bound Hind III fragments with other alfalfa ctDNA probes (data not shown).

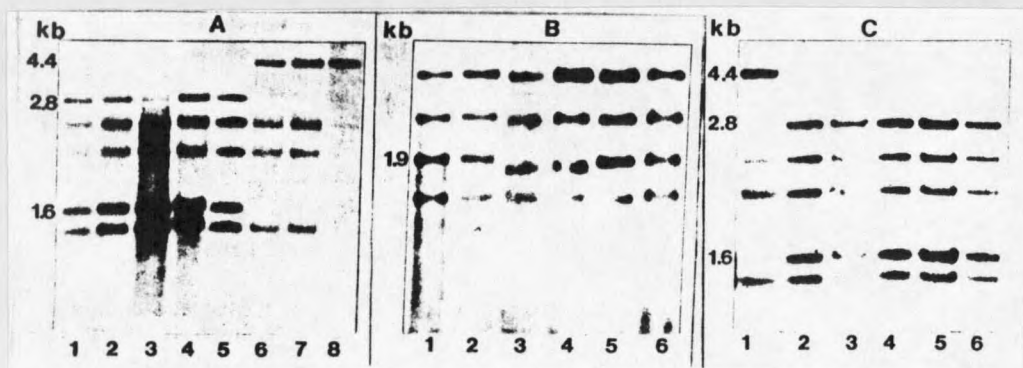


Figure 5.

Autoradiographs demonstrating ctDNA restriction fragment banding patterns of parents and progeny.

- A: Hind III digested DNA hybridized with an 18 kb alfalfa ctDNA probe. Lane 1, MAG ctDNA; Lane 2, MAG total DNA; Lane 3, 33B-4 normal sector total DNA; Lane 4, 33B-4 mutant sector total DNA; Lane 5, 13-4 mutant sector total DNA; Lane 6, 13-4 normal sector total DNA; Lane 7, 6-4 total DNA; Lane 8, 6-4 ctDNA.
- B: Bam HI digested DNA hybridized with a 12 kb alfalfa ctDNA probe. Lane 1, MAG ctDNA; Lane 2, 33B-4 normal sector ctDNA; Lane 3, 33B-4 mutant sector total DNA; Lane 4, 13-4 mutant sector total DNA; Lane 5, 13-4 normal sector ctDNA; Lane 6, 6-4 ctDNA.
- C: Hind III digested total DNA hybridized with the 18 kb alfalfa ctDNA probe. Lane 1, 13-2 normal sectors; Lane 2, 33B-2 mutant sectors; Lane 3, 12-1 mutant sectors; Lane 4, 13-4 mutant sectors; Lane 5, 13-2 mutant sectors; Lane 6, 33B-4 mutant sectors.

Electron Microscopy

Ultrastructural features of mesophyll cells from pure yellow-green mutant sectors were compared with normal tissue by transmission electron microscopy in Chapter I and the mutant phenotype had distinct features indicative of arrested plastid development. Fine mosaic sectors examined in this study revealed mesophyll cells that contained both mutant plastids and normal chloroplasts.

Electron micrographs (Figure 6) show sections of mesophyll cells from mosaic tissue in 13-4 plants. Figure 6A (x8000) shows cells containing only mutant plastids (p) or only normal chloroplasts (c) adjacent to cells with both types of plastids. Mutant plastids can be distinguished from normal chloroplasts in these mixed cells by their smaller size and absence of starch grains (s). Thylakoid membrane stacking is absent or extremely reduced in the mutant plastids (r) while large grana are present in the normal chloroplasts (g). Osmiophillic globuli (o) are found in normal chloroplasts and mutant plastids but to a much greater extent in the mutants. Vacuolization (v) is apparent in mutant plastids in Figure 6A. Figure 6B (x20,000) demonstrates the ultrastructural differences between the mutant plastids (p) and normal chloroplasts (c) with starch grains and large grana present in the normal organelle while the mutant plastid (p) has only rudimentary thylakoids (t) and many osmiophillic globuli (o). The nucleus (n) and a mitochondria (m) appear normal in this cell.

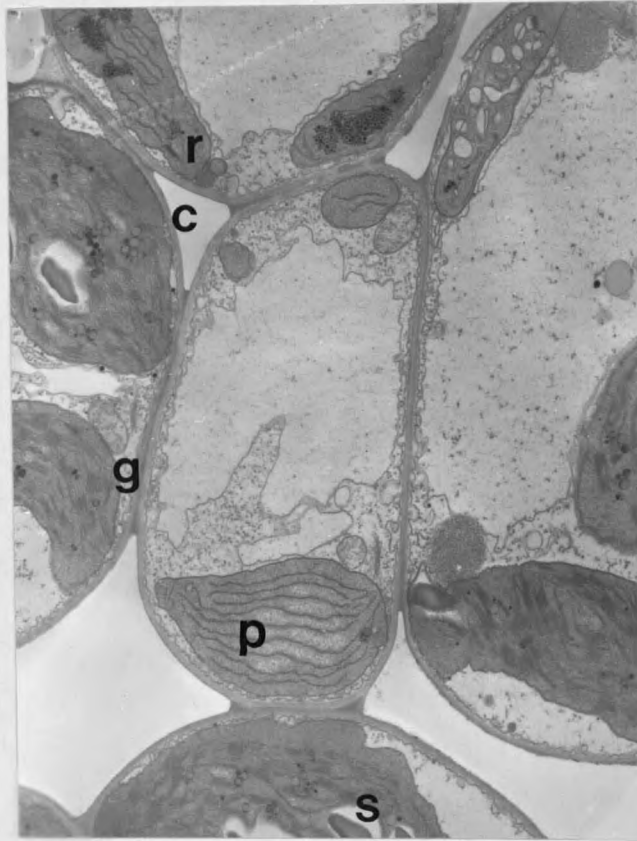
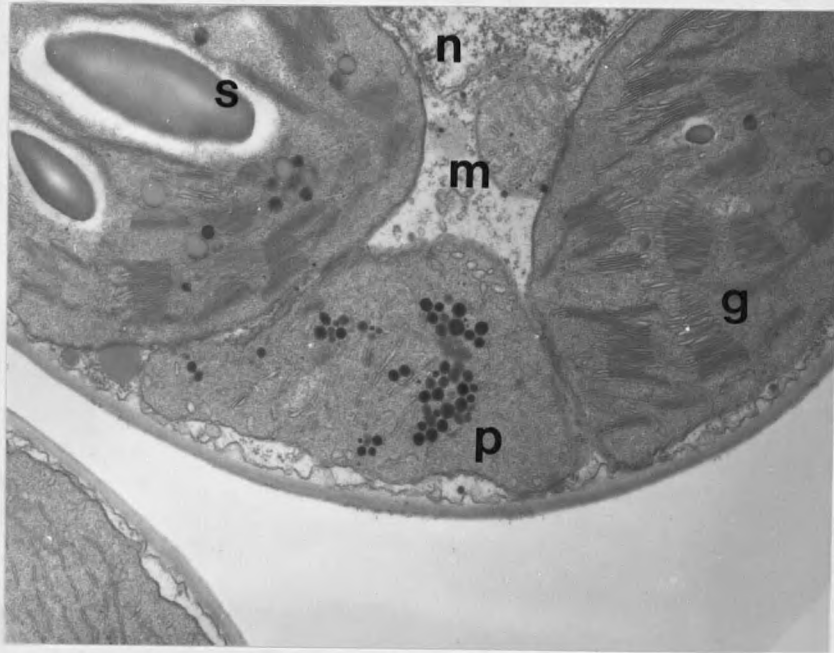


Figure 6.

Electron micrographs of mesophyll cells from 13-4 mosaic tissue.

- A: Micrograph (x8000) showing mesophyll cells containing normal chloroplasts (c), mutant plastids (p), or both. Starch grains (s) and grana (g) are present in the chloroplasts. Mutant plastids show only rudimentary thylakoid development (r).

Figure 6. (continued)



- B: Mixed cell (x20,000) with normal nucleus (n) , mitochondria (m) , chloroplasts and a mutant plastid (p). Large starch grains (s) and grana (g) are indicative of normal photosynthetic function while mutant plastids contain large numbers of osmiophilic globuli and rudimentary thylakoids.

The observation of biparental transmission of chlorophyll deficiency in alfalfa by Smith et al. (1986) provided evidence for the biparental inheritance of plastids in this species. The mutant phenotypes appeared as yellow-green sectors on otherwise normal tissue and sorted out through a fine mosaic to coarse mosaic pattern eventually giving rise to pure mutant or normal sectors.

Mapping ctDNA from normal and pure yellow-green sectors from chimeric plants has verified the existence of a mutant genotype as well as three distinct ctDNA genotypes in normal alfalfa. Utilization of the unique restriction fragment patterns of each genotype in crosses involving two normal tester lines and the yellow-green sectors on mutant plants confirms the biparental inheritance of plastids proposed by Smith et al. The ability to differentiate between the two normal plastid genotypes allowed for the determination of the maternal origin of ctDNA in normal tissue from 6-4 x 33B-4 progeny while the chlorophyll deficient phenotype provided the marker with which to select for tissue with paternal plastids. Comparison of yellow-green tissue ctDNA from several plants indicates that the mutant genotype is stable and not likely to be due to de novo events.

Biparental inheritance of plastids necessitates the existence of "mixed" cells in the sexual progeny containing plastids from both parents. As such plants develop, random sorting-out of the two plastid types will eventually lead to pure sectors having only maternal or paternal plastids. Tissue in the fine mosaic state should consist of cells in the process of sorting to pure mutant or normal types. The electron micrographs of mosaic tissue from 13-4 show cells with both

normal and mutant plastids at various stages of sorting-out, confirming the existence of biparental plastid inheritance.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF CHLOROPHYLL DEFICIENT
PLASTOME MUTANTS OF ALFALFAIntroduction

Chlorophyll deficient mutants of higher plants have traditionally been utilized in both genetic and physiological studies because of their obvious phenotypes and direct effects on photosynthesis. Mutant studies have helped in the identification of nuclear and chloroplast encoded components of the photosynthetic apparatus as well as determined a functional association between many of the proteins making up the chloroplast protein complexes.

In this study the albino and yellow-green mutants have been characterized with respect to chlorophyll levels, stable chloroplast proteins and the expression of selected chloroplast encoded genes in order to understand the mechanism by which their plastome mutations disrupt normal chloroplast development. This assessment permits their comparison to other mutants characterized in similar studies.

Materials and MethodsPlant Material

Alfalfa (*Medicago sativa* L.) plants containing pure yellow-green

sectors were utilized in this study. These chimeric plants have stems of chlorophyll deficient tissue as a result of somatic sectoring and are supported by the plants normal sectors, rendering the mutations nonlethal. Both mutants have been previously described by Smith et al (1986). All plants were grown under 16 hour daylength in the green house.

Pigment Levels

Chlorophyll a and b levels were determined using the methods of Arnon (1949) from foliar tissue extracts of pure sectors from chimeric plants.

Chloroplast Isolation

Methods used to isolate chloroplasts from mutant and normal leaf tissue were essentially identical to those outlined by Archer and Bonnet (1987). Young leaf tissue (3 to 5 grams) was briefly ground in a blender in 20 mls of a buffer containing 2.0 mM NaEDTA, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 350 mM sorbitol, 0.5% BSA (w/v), 4.0mM Na ascorbate, and 50 mM Hepes-KOH at pH 8.3. The brei was filtered through four layers of cheese cloth and one layer of miracloth. The filtrate was layered over 40% Percoll in grinding buffer and centrifuged for 2 min at 1,200g. The pellet was resuspended in a buffer containing 375 mM sorbitol, 2mM Na EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.96 mM DTT, and 35 mM Hepes-KOH (pH 8.3). Aliquots of this chloroplast suspension were stored at -70 degrees C. Thylakoid membrane enriched proteins were separated from total chloroplast proteins by an additional centrifugation of the suspension for 4 min in an Eppendorf centrifuge followed by washing and resuspension of the pellet in resuspension buffer. Stromal proteins

were collected from the pellet washings and supernatant.

Protein Electrophoresis

Total chloroplast and thylakoid membrane proteins were analyzed on 10% to 15% gradient gels. Heat denatured samples (90 degrees C for 10 minutes) from yellow-green, albino and normal tissue were loaded on an equal weight basis in a sample buffer containing 1% SDS, 0.1mM DTT, and bromphenol blue tracking dye. Gels 0.75 mm thick, 200 mm wide and 200 mm in length consisted of a 3% polyacrylamide stacking gel over a SDS 10% to 15% polyacrylamide gradient (Laemmli, 1970). Gels were run for 16 to 18 hours at 15 to 20 mamps. Gels were stained in a 0.1% Coomassie Blue, 30% methanol, 10% acetic acid solution for 3-5 hours and destained in several changes of the same solution without Coomassie Blue overnight. Destained gels were dried prior to photography.

In order to verify the identity of major chloroplast proteins two dimensional gel electrophoresis was performed (O'Farrell, 1979). Chloroplast proteins in a 1% SDS, 0.1mM DTT sample buffer were loaded on the basic end of a 8M urea, 6% polyacrylamide tube gels with amphelines to establish a pH gradient from 3.5 to 9.5. (LKB). Gels were focused at 1500 volts for 20 hours. After the focusing dimension gels were removed from tubes, treated by boiling for 10 minutes in a treatment buffer consisting of 0.0625 M tris-Cl pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and overlaid on a 1.5 mm 10-15% gradient slab gel. Second dimension electrophoresis was performed as previously mentioned on the focused proteins and additional lanes containing molecular weight markers and unfocused chloroplast proteins. Gels were stained in Coomassie Blue, destained and dried.

RNA Isolation

Total leaf RNA isolations were performed on newly expanded leaves similar in age to those used for protein isolations. Fresh leaf tissue was ground with a mortar and pestle in liquid nitrogen and transferred to an extraction tube containing equal volumes of phenol (Tris saturated, pH 7.5) and Hepes-EDTA buffer (10 mM, pH 7.5). Tubes were inverted, vortexed briefly and centrifuged 10 minutes at 5000 rpms. The aqueous layer was transferred to a fresh tube containing one tenth volume of 3 M Na acetate and isopropanol (2x volume). Tubes were again centrifuged at 5000 rpms for 10 minutes. The supernatant was discarded and the tubes dried at room temperature for one hour. Pellets contained total nucleic acids and were redissolved in Hepes-EDTA buffer and stored as aliquotes at -70 degrees C. RNA samples were denatured with glyoxal and electrophoresed on 1.3% agarose gels at 20 volts for 16 hours in a circulated .01 M NaPO_4 buffer according to the methods of Maniatis (1982). Gels were stained with ethidium bromide and distinct bands of unsheared high molecular weight DNA (greater than 30 kb) and lower molecular weight rRNA were visualized.

RNA was transferred to Zeta-probe (Bio-Rad) nylon membrane with 20x SSC for 20-24 hours. After transfer filters were baked at 80 degrees C. for 2 hours.

Northern Hybridizations

Filter-bound RNA was hybridized with chloroplast DNA (ctDNA) probes from three different species. Clones from the library of mung bean and alfalfa ctDNA fragments with specific genes mapped were utilized. Genes mapped included the 23S and 16S ribosomal RNA genes, the psbA gene

encoding the photosystem II 32 kd protein, and the *rbcl* gene encoding the large subunit of ribulose biphosphate carboxylase, (Palmer personal communication). In addition, a 1750 bp fragment of the *rbcl* gene from spinach cloned into pBR325 (pSocE48) by G. Zurawski (1981) was utilized. Plasmids were isolated from *E. coli* hosts using the mini-prep procedure of Birnboim and Doly (1979). Entire recombinant plasmids were labeled with ^{32}P dNTPs using nick-translation (Rigby et al., 1977) or isolated fragments were labeled by primer extension (Feinberg and Vogelstein, 1983). Unincorporated nucleotides were separated from labeled probes using centrifuged Sephadex G-50 1 cc columns. Prior to hybridization probes were denatured in solution with 200 mls of 0.2 N NaOH with heating to 80 degrees C. for 10 minutes. Specific activities of hybridization probes exceeded 10^8 counts per min per ug DNA.

Prehybridization, hybridization and washing procedures used were identical to those previously outlined. Autoradiography was performed at room temperature or at -70 degrees C. with the use of intensifying screens for 4-96 hours.

Electron Microscopy

Pure sectors of normal, yellow-green or albino tissue and fine mosaic tissue from yellow-green sectoring plants were examine by transmission electron microscopy as previously described.

Results

Chlorophyll Analysis

Average levels of chlorophyll a and b determined from pure normal

and YGS tissue from seven different clones of a yellow-green sectoring genotype are listed in Table 3. Young trifoliolate leaves in the process of expansion were utilized in order to reflect the developmental stage of foliar tissue used in both the protein and RNA isolations. While the seven clones represent plants with identical genotypes and were grown in the same light and temperature environment, considerable variation in chlorophyll levels could be detected visually in both normal and YGS tissue. This is reflected in the large standard deviations (Table 3). Levels of total chlorophyll in YGS tissue averaged 28.5% of normal levels and chlorophyll a/b ratios were consistently lower in the YGS tissue.

Table 3 Average Levels of Chlorophyll a, Chlorophyll b, Total Chlorophyll, and Chlorophyll a/b Ratios in Normal and YGS Tissue.*

	<u>Chl a</u>		<u>Chl b</u>		<u>Total Chl</u>		<u>a/b</u>	
	<u>mean</u>	<u>std dev</u>	<u>mean</u>	<u>std dev</u>	<u>mean</u>	<u>std dev</u>	<u>mean</u>	<u>std dev</u>
Normal	.474	.151	.261	.087	.735	.233	1.83	.28
YGS	.105	.069	.104	.058	.210	.131	.99	.19
%N	**22.2%		**39.8%		**28.5%			

*Means and standard deviations (std dev) determined from samples representing seven chimeric clones with pure normal and YGS sectors. Levels expressed in mg/gfw. Percent of normal (%N) expressed as (YGS/normal) x 100.

**Significant at the .005 level

Chloroplast Proteins

Coomassie Blue stained SDS polyacrylamide gradient gels are shown in Figure 7. Lanes representing total chloroplast or thylakoid

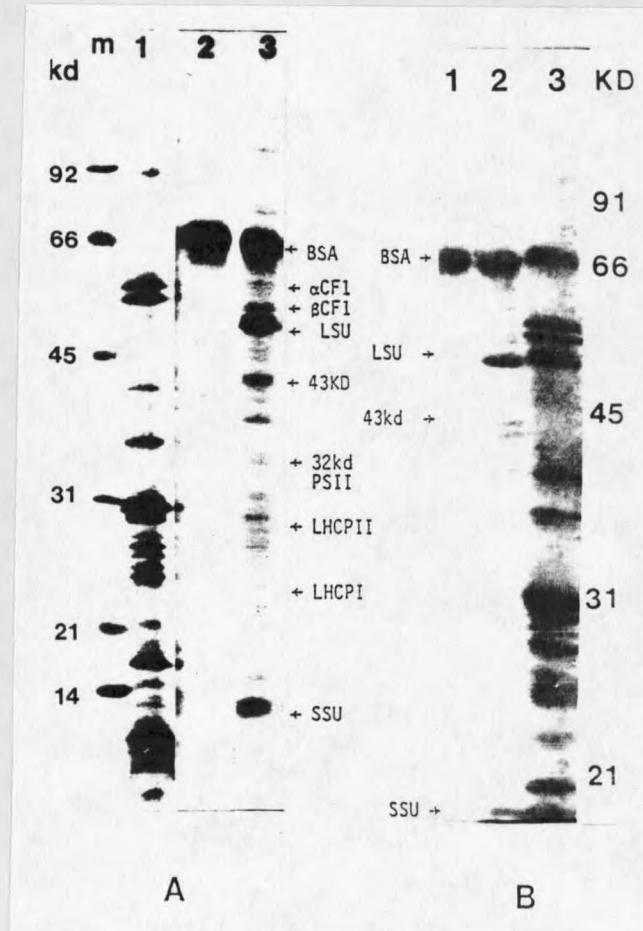


Figure 7. SDS polyacrylamide gradient gels of total chloroplast or thylakoid membrane proteins from normal, YGS, and ALS leaf tissue. All lanes were loaded on an equal tissue fresh weight basis and gels stained with Coomassie blue. A. Thylakoid membrane proteins from normal tissue (lane 1) compared to total chloroplast proteins from ALS (lane 2) and YGS (lane 3). Molecular weight markers (Bio-Rad) are shown in lane m and sizes are listed in kd. B. Total chloroplast proteins from ALS, YGS, and normal tissue (lanes 1,2 and 3 respectively). Proposed identity of major bands is shown. Heavy bands at 66 kd in all total chloroplast protein lanes is from residual BSA.

membrane proteins were loaded on an equal tissue fresh weight basis and young normal, YGS, or ALS trifoliolate leaves were used as the tissue source. Thylakoid membranes from normal chloroplasts (Figure 7A, lane 1) reflect typical profiles found in other studies (Leto and Miles, 1980; Metz et al., 1983) and major proteins with identities proposed from similar molecular weights and relative staining intensities are shown on the figure. Identification of stromal proteins from total normal chloroplast protein profiles (Figure 7B) was possible by comparison of total and membrane protein lanes. The identity of several of these major proteins (alpha and beta CF1 and PSII 32kd) was supported by comparison of their isoelectric point migration in 2D gels (data not shown) to those previously reported (Deitz and Bogorad, 1987a,b).

Profiles from YGS chloroplasts showed a major reduction or loss of specific proteins when compared to normal lanes representing equal fresh weights. Prominent thylakoid membrane proteins from the normal profile at reduced levels or missing in the YGS lanes included alpha and beta CF1 (60 kd), PS II 34 and 32 kd, light harvesting complex proteins (LHCP) of PSII (30 kd) and PSI (25-26 kd) and a PSI 20 kd protein. Stromal ribulose bisphosphate large and small subunit proteins (LSU and SSU), however, were present in nearly equal levels in YGS and normal lanes. Two additional bands not found in the normal chloroplasts were also observed in the YGS lanes at approximately 40 kd (Figure 7B).

No stainable proteins were observed in lanes representing ALS plastid extracts other than the 66 kd BSA protein (present in the extraction buffer and observed in all total chloroplast protein lanes) and several high molecular weight proteins (identity unknown).

Northern Blots

Autoradiographs of filter-bound total leaf RNA from normal, YGS and ALS tissue are shown in Figure 8. Hybridization probes used were an 18 kb alfalfa PstI fragment spanning the ribosomal RNA genes (Figure 8A), a 7.1 kb Pst I fragment that includes the entire *rbcl* gene and portions of the *psbA* and *atpBE* genes (Figure 8B), and a 1.75 kb EcoRI fragment from spinach that covers the 5 prime end of *rbcl* (pSocE48, Figure 8C).

Autoradiographs of filters hybridized by the rRNA probe show high levels of the species from normal and YGS tissue and no chloroplast rRNAs in ALS tissue. Lanes 1 through 3 in Figure 8A contain RNA from YGS, ALS and normal tissue on an equal fresh weight basis. The four hour autoradiograph revealed strongly hybridized bands from the YGS and normal lanes that likely represent the 23S, 16S, 5S and 4.5S rRNAs. A 96 hour exposure of the same autoradiograph verified the absence of chloroplast rRNAs in ALS tissue.

The *rbcl*, *psbA*, and *atpBE* containing fragment hybridized strongly to RNAs 1.6 and 1.2 kb in length from YGS and normal tissue (Figure 7B, lanes 1 and 2). No RNAs from ALS tissue were strongly hybridized with this probe (lane 3). Other studies utilizing chloroplast probes specific for the *rbcl* and *psbA* genes in Northern blots have demonstrated hybridization to 1.6 and 1.2 kb RNAs respectively, confirming our results (Kobayashi et al, 1987; Palmer et al., 1982; Rodermel and Bogorad, 1985). Northern hybridizations using pSocE48 (Figure 8C) confirmed the size of the *rbcl* transcript, it's presence in nearly equal levels in normal and YGS tissue (lanes 1 and 2) and it's absence in ALS tissue.

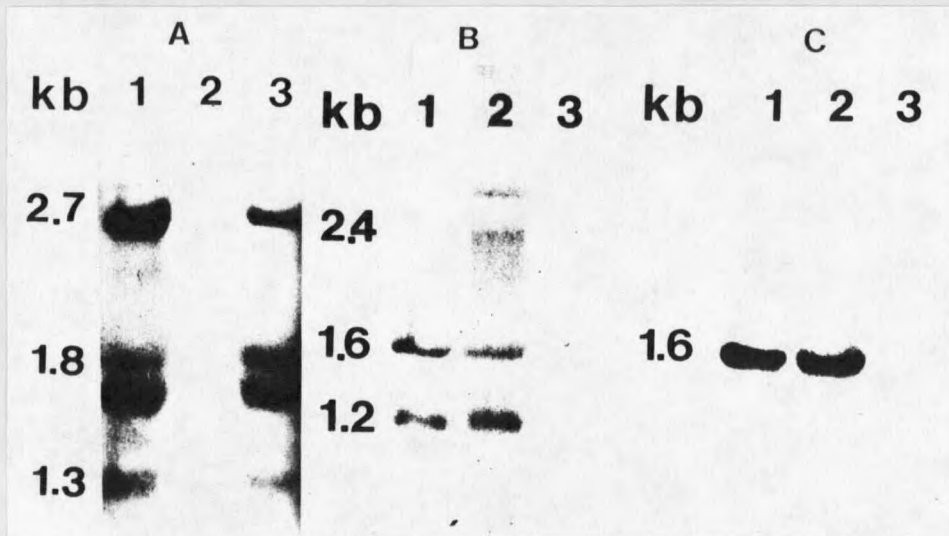
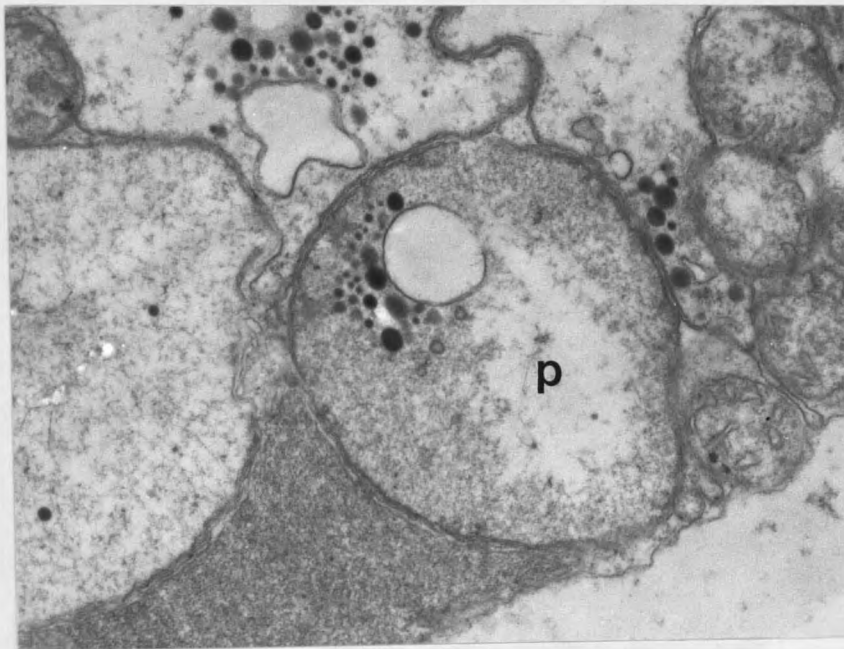


Figure 8. Northern Blots of total leaf RNA from normal, YGS and ALS tissue hybridized with specific ctDNA probes. All lanes are loaded on an equal tissue fresh weight basis. A. Filter-bound RNA hybridized with an 18 kb alfalfa ctDNA Pst I fragment encoding the rRNA genes. Lanes 1-3 contain RNA from YGS, ALS, and normal tissue respectively. B. Filter-bound RNA from normal (lane 1), YGS (lane 2), and ALS (lane 3) hybridized with a 7.1 kb alfalfa ctDNA probe containing the *rbcL*, *psbA* and *atpBE* genes. C. The *psbA* gene-specific probe pSocE48 hybridized to a filter-bound RNA from normal, YGS, and ALS leaf tissue (lanes 1-3).

Electron Micrographs

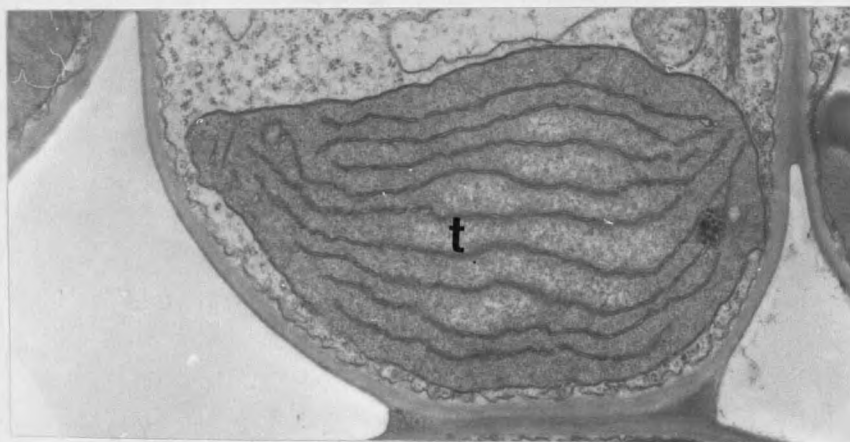
The extreme alteration in chloroplast protein profiles in YGS and ALS tissue was accompanied by a dramatic change in plastid ultrastructure. Albino plastids lacked all internal membrane development (Figure 9A) while YGS plastids had only agranal thylakoids (Figure 9B).

Figure 9. Electron micrographs of mesophyll cells from a pure ALS leaf or mosaic tissue from a YGS sectoring leaf.



A. ALS plastid (p) void of all internal membrane development (x22,000).

Figure 9 (continued)



B. YGS chloroplast (x20,000) with only agranal thylakoids (t).



C. Heteroplasmic cell (x7,500) containing fully developed chloroplasts with large grana (g) and starch grains (s), and developmentally arrested YGS chloroplast that lacks both features (p).

Discussion

Chlorophyll deficient mutants with a reduction or complete absence of chlorophyll accumulation have been widely reported (Kirk and Tilney-Basset, 1967). The levels of total chlorophyll reported here in normal tissue are comparable to those found in young leaf tissue in other species (Huffaker et al., 1970; Kyle and Zalik, 1982). An alteration of chlorophyll a/b ratios often accompanies chlorophyll level reduction with mutants having both higher (Kyle and Zalik, 1982) and lower chlorophyll a/b (Metz and Miles, 1982; Polacco et al., 1985). The YGS mutant studied here had a consistently lower chlorophyll a/b ratio than normal tissue (.99 to 1.83) and averaged only 28.5% of the normal total chlorophyll levels. A lower chlorophyll a/b ratio has been associated with a preferential reduction of chlorophyll a binding thylakoid proteins in the *hcf*-3* maize (Zea mays L.) mutants (Metz and Miles, 1982). While the lower chlorophyll a/b ratio in YGS tissue may also be associated with a preferential loss of chlorophyll a binding proteins, the extensive degree of chlorophyll reduction is indicative of a more dramatic alteration of thylakoid membrane proteins.

Chloroplast protein electrophoresis (Figure 7) demonstrated this alteration. Total and membrane proteins from normal chloroplasts had typical profiles while YGS chloroplasts showed drastic reductions of most proteins. Other mutants characterized have demonstrated a more specific loss of major chloroplast proteins but often have pleiotropic reductions of functionally associated proteins. Nonallelic *hcf*-2* and

hcf*-6 maize mutants are single locus nuclear mutants that lack the Cyt f/b-563 complex (Metz et al., 1983). The hcf*-3 maize mutant is a well characterized system demonstrating the loss or reduction of PSII proteins (Leto and Miles, 1980; Metz and Miles, 1982; Miles and Daniel, 1974). Leto et al (1985) have found this nuclear mutant has the capability to synthesize the 34.5 kd and 48 kd PSII polypeptides but shows a specific accelerated turnover of these proteins. The chloroplast encoded lut-1 mutant of tobacco is another example of a pleiotropic loss of PSII specific peptides due to accelerated turnover (Chia and Arntzen, 1985). The loss of the alpha and beta subunits of CF1 in the hcf*-38 maize nuclear mutant has been found to be due to some post-translational event by Kobayashi et al (1987).

The reduced levels of polypeptides from all chloroplast protein complexes in the YGS mutant could be a result of a similar but more extensive pleiotropic loss, lower levels of expression of chloroplast protein genes or insufficient translational capacities in the mutant. The latter possibility is unlikely because LSU and SSU proteins are found in the YGS chloroplasts at levels similar to those found in normal chloroplasts (Figure 7B). This indicates the YGS chloroplasts have the capacity to express and translate chloroplast encoded genes and import nuclear encoded proteins. Similar results were found in studies of several chlorophyll deficient maize mutants by Harpster et al (1984). Northern blots of total leaf RNA probed with the rbcL specific pSocE48 verify equal levels of expression of this gene in normal and YGS tissue (Figure 8C). Northern blots of chloroplast rRNA (Figure 8A) demonstrating normal levels of these species also provide indirect

evidence in support of the capacity of YGS chloroplasts to direct normal protein synthesis. The PSII 32 kd protein appears to be completely absent in YGS chloroplast membranes (Figure 7A and 7B). Levels of expression of this chloroplast encoded gene in YGS tissue, however, were similar to levels detected in normal tissue (Figure 8B). Absence of the stable protein in YGS chloroplasts may result from the failure of the message to be translated or accelerated protein turnover. Thus in YGS chloroplasts, levels of expression of the psbA gene cannot account for the absence of the 32kd protein. Additional studies utilizing in vivo or in organelle labeling with [³⁵S]-methionine are needed to verify the existence of accelerated turnover of YGS chloroplast proteins.

Two bands with molecular weights of approximately 43 kd in the YGS lanes (Figure 7B, lane 2) are not clearly visible in the normal lanes. Relative increases in levels of specific polypeptides is a feature found in other mutants. Metz et al (1983) observed an increased staining intensity relative to normal lanes in a 31 kd protein from thylakoid membranes of the hcf*-6 maize mutant lacking the cyt-b/f complex and Kobayashi et al (1987) have documented an increase in the LHCII proteins in hcf*-38. Thylakoid membrane profiles of developing chloroplasts from an etiolated barley (Hordeum vulgare L.) virescent mutant studied by Kyle and Zalik (1982) showed a delay in the loss of two polypeptides of 42-43 kd relative to normal barley. While this barley virescent mutant eventually developed normal thylakoid membranes, the YGS alfalfa mutant studied here does not (Figure 9B). The eventual processing of these polypeptides may be an integral part of the chloroplast development

sequence and the persistence of these proteins in the YGS plastids is a result of a block in this step.

The rudimentary thylakoid membrane system observed in YGS chloroplasts (Figure 9B) is characterized by a complete lack of grana formation. This is probably a result of the loss of PSII chlorophyl a/b binding proteins which have been directly related to thylakoid stacking (Ryrie et al., 1980).

The presence of mesophyll cells containing both normal and mutant plastids (Figure 9C) as a result of biparental plastid inheritance indicates the YGS phenotype cannot be masked by a nuclear encoded protein component. The mutation in the chloroplast encoded gene or genes in YGS causes a lack of plastid development despite the normal expression of nuclear genes within the same cell and the proper transport of the proteins they encode (as seen with SSU). These observations indicate the alteration of chloroplast derived factors necessary for proper complex assembly can initiate the pleiotropic loss or turnover that occurs in YGS. This premise is supported by the direct measure of PSII polypeptide turnover in the *lut-1* tobacco plastome mutant (Chia and Arntzen, 1985). While *lut-1* has a developmentally dependent functional phenotype and occurs only in older plants, the YGS mutant has a constitutive block in plastid development. Both mutants, however, implicate the chloroplast genome's role in directing chloroplast development and degradation. Other studies have demonstrated the role of the nuclear genome in thylakoid complex assembly and turnover (Chua and Bennoun, 1975; Kobayashi et al., 1987; Leto et al., 1982; Leto et al., 1985), thus emphasizing the complex

genome interaction that is a part of this developmental process.

The complete absence of chloroplast rRNAs in ALS tissue (Figure 8, lane 2) verified the lack of stainable proteins observed on electrophoresis gels (Figure 7B lane 2). A lack of chloroplast ribosomes has been documented in other nuclear (Walbot and Coe, 1979) and chloroplast (Borner et al, 1972) encoded albino mutants. These mutants have plastids which lack internal membranes, a central feature observed in electron micrographs of plastids from ALS tissue (Figure 8A). The chloroplast encoded ALS mutant likely has a mutation that blocks rRNA transcription or prevents the assembly of stable 70S ribosomes, resulting in rRNA degradation.

Mapping the chloroplast genomes of both the YGS and ALS plastome mutants in Chapter 2 detected only one minor deletion event in both mutants relative to normal alfalfa genotypes. Additional, undetected small scale alterations are likely but the dramatic changes in plastid phenotype documented in this study are not paralleled by large changes in the chloroplast genome of these mutants.

These mutants have a unique set of characteristics in that they are chloroplast encoded; constitutively expressed; arrest plastid development at two distinct stages; and are rendered nonlethal by their presence on chimeric plants. Further analysis of these mutants should provide insight into the role of chloroplast encoded genes at two stages of the plastid developmental sequence.

CHAPTER 5

SUMMARY

This work has focused on the molecular and structural characterization of the plastids from two chloroplast-encoded chlorophyll deficient mutants of alfalfa. The results not only support the classical genetic analysis previously performed on these mutants, but add to the current assessment of intraspecific chloroplast genome variation, biparental plastid inheritance, and chloroplast development in higher plants.

Physical mapping of the ctDNA from YGS and ALS by Southern blot analysis demonstrated an absolute association of a mutant ctDNA genotype with the mutant tissue. The genotypes of both mutants differed from normal alfalfa ctDNA by combinations of four detectable polymorphisms (two point mutations and two small scale addition/deletion events), but were indistinguishable from each other. No effort was made to select for cytoplasmic diversity in the tester lines used in these studies yet they were also distinguishable from each other utilizing combinations of these same polymorphisms. While differences between the YGS and ALS mutants were not detectable by the mapping study, the two were found to have dramatically different plastid ultrastructures. The albino mutant possessed no internal membrane development while the yellow-green mutant

was developmentally arrested short of thylakoid stacking to form grana.

The detection of ctDNA variation in the normal genotypes used in the mapping study coupled with the YGS selectable marker permitted the verification of biparental plastid inheritance in crosses between these tester lines and flowers on pure YGS sectors. Two generations of progeny from these crosses demonstrated the stable inheritance of the mutant ctDNA from the YGS male parent and normal ctDNA from two distinct normal parents. The detection of heteroplasmic cells in fine mosaic tissue in these chimeric progeny by transmission electron microscopy provide visual confirmation that plastids were biparentally transmitted.

The physiological characterization of these mutants enabled their phenotypes to be assessed on the molecular level and compared to other mutants described in similar studies. A large total chlorophyll reduction was measured in YGS relative to normal tissue from the same clones. Chlorophyll a was preferentially lost in this mutant and the large scale chlorophyll reduction was accompanied by a major reduction of most proteins in the YGS chloroplasts. This extensive loss of thylakoid membrane proteins was contrasted with normal levels of LSU and SSU in YGS chloroplasts. Northern blots verified normal *rbcl* expression and revealed normal levels of *psbA* expression despite the absence of this protein on SDS polyacrylamide gels. Turnover of this and other thylakoid membrane proteins in the YGS chloroplasts is likely the result of the pleiotropic effect of an altered plastome encoded gene.

A complete lack of plastid proteins and chloroplast rRNAs was detected in the ALS mutant. These results complemented the total lack of plastid directed development observed in electron micrographs and

implicate a plastome mutation that blocks rRNA transcription with massive pleiotropic results.

While the results presented here are descriptive in nature, they provide the prerequisite evaluation necessary for studies focused on understanding the genetic mechanisms causing the unique phenotypes of these mutants. The strategy of determining normal functions of genes by comparison to mutated versions has had a successful history and should prove fruitful in future studies utilizing these mutants.

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