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FACTORS AFFECTING CHLOROPLAST NUMBER
IN DIPLOID AND TETRAPLOID ALFALFA

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MORPHOLOGICAL AND GENETIC FACTORS AFFECTING CHLOROPLAST NUMBER IN DIPLOID AND TETRAPLOID ALFALFA

By

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ABSTRACT

MORPHOLOGICAL AND GENETIC FACTORS AFFECTING CHLOROPLAST NUMBER IN DIPLOID AND TETRAPLOID ALFALFA

By

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Two theories have been proposed concerning the control of chloroplast number per cell. One suggests that cell size is the primary regulating factor, the other proposes that genome size plays the predominant role. Chloroplast and cell face area were analyzed in cells of diploid and tetraploid genotypes and a chimeric plant.

There was significant variation in plastid density at both ploidy levels, and there was overlap in the mean number of plastids in the diploid and the tetraploid genotypes. Plastid density values in reciprocal populations appeared to be maternally influenced, although plastid numbers in the chimeric tissues were not significantly different.

Chloroplast number may be partially controlled by nuclear genes independent to those regulating cell size, and genome size does not appear to be as important a regulating factor as progenitor genotype. Chloroplast density is largely controlled by the nucleus, but cytoplasmic factors separate from the plastid may be involved.

DEDICATION

To Mollie, Sam and Marty and

to the memory of my grandfather, Pete McMullen.

ACKNOWLEDGMENTS

I would like to give my heartfelt thanks to my major professor, Jim Hancock. Jim's wealth of enthusiasm, good ideas and sense of humor were a tremendous help to me and kept me going through the many times I felt I would never finish this project.

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INTRODUCTION

Many scientists believe that plastids evolved from phototrophic prokaryotic cells. An endocytotic event between a phototrophic prokaryote and a primitive eukaryote may have resulted in a symbiotic relationship between the two cell types. The offspring of this organism inherited DNA not only from the eukaryotic cell but from the bacterial endo-symbiont as well. Margulis (1971) has characterized endo-symbiosis as "swallowing without digesting". This type of relationship exists in present day organisms. For example, Paramecium bursaria has a symbiotic relationship with the green algae Chlorella (Margulis, 1971, Karakashian et al. 1968). However, this is not an obligatory endosymbiosis, as both organisms can survive alone. When the Paramecium and Chlorella are separated and then reconstituted, the alga will multiply until a certain threshold is reached and thereafter any Chlorella that are ingested are digested with no apparent harm done to the existing algal cells (Margulis, 1971).

Several lines of evidence support an endosymbiotic origin for plastids. The chloroplast genome is circular like that of bacteria (Sears, 1983). The chloroplast ribosomal RNA is sensitive to inhibitors of prokaryotic translation such as chloramphenical, streptomycin, and tetracycline (Bottomley and Bohnert 1982, Alberts et al. 1983 and Von Wettstein, 1981). Ribosomal RNA of the chloroplast has sedimentation coefficients (16s, 23s and 5s) that are similar to prokaryotes (Hoober, 1984). Protein synthesis in chloroplasts begins with n-formylmethionine,

as in bacteria, and not with methionine as in the cytosol of eukaryotic cells (Alberts et al. 1983, Von Wettstein, 1981). Chloroplast ribosomes and bacterial tRNAs can be used together in protein synthesis, and chloroplast mRNAs can be translated by a protein synthesizing extract from E.coli (Alberts et al. 1983, Von Wettstein, 1981). There is also a 70-75% homology at the nucleotide level between cyanobacteria and higher plants with respect to the gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase (RUBPC/O) (Hoober, 1984).

PLASTID AUTONOMY. Plastid development and physiology may have initially been autonomous, but chloroplast functions are now dominated largely by the nucleus (Ellis, 1984). This is most strongly supported by the Mendelian segregation of most mutations influencing chloroplast development (Gillham, 1978). Also, the plastid genome is too small to carry all the genes associated with its metabolism. Genes in the chloroplast encode components of plastid transcription, translation and proteins involved in photosynthesis (Taylor, 1989). However, photosynthesis and the other plastid functions require the products of several hundred genes of which only about 120 are present in the approximately 150 kb chloroplast genome (Gruissem, 1989).

When Scott and Timmis (1984) used restriction enzymes to produce spinach plastid DNA fragments that were subsequently made into hybridization probes, they found that every cloned fragment of plastid DNA showed homologies to the spinach nuclear genome. Many of these homologies occurred in regions of the nuclear DNA that were highly methylated. They concluded that essentially all of the plastome has homologies with the nuclear DNA, and that potentially, the nucleus possesses all the genes

required to make functional chloroplasts, although they may be in an interrupted, highly methylated and perhaps inactive form.

While the majority of the plastid constituents are nuclear-encoded, there are reports of a possible feedback mechanism from the chloroplast to the nucleus that regulates levels of cytosolic mRNA for some chloroplast proteins. In Sinapsis alba L., it has been hypothesized that a signal from the plastid is required to allow the phytochrome mediated appearance of translatable mRNA for the small sub-unit (SSU) gene of RUBPC/O and the light harvesting chlorophyll a/b binding protein (LHCP) of photosystem II (Oelmuller and Mohr, 1986). The authors observed that phytochrome-mediated expression of both nuclear genes (or gene families) is only possible if the plastids are intact. If the plastids are severely damaged, expression of the genes for SSU and LHCP are almost completely inhibited even though nuclear genes not related to the plastid are not adversely affected. Similar results have been observed in maize, mustard and tomato with respect to LHCP, glutamine synthase, nitrate reductase and NADP-glyceraldehyde-3-phosphate dehydrogenase (Taylor, 1989, Edwards and Coruzzi, 1989, Deane-Drummond and Johnson, 1980, Feierabend and Schubert, 1978 and Reiss et al. 1983).

PLASTID DIVISION AND GENOME REPLICATION. Many unicellular plants contain only one or two chloroplasts (Possingham and Lawrence, 1983), while higher plant cells contain many (Hoober, 1984). Chloroplast division occurs immediately before cytokinesis in most unicellular organisms (Barlow and Cattolico, 1981, Slankis and Gibbs, 1972 and Cattolico et al. 1976). In the mono-plastidic, primitive vascular plant Isoetes, plastid division occurs during a number of different stages of the cell cycle (Whatley,

1974). In higher plants, chloroplast replication occurs at the time of new cell formation and continues for two to three cycles after cell division has stopped (Rose et al. 1975, Whatley, 1980 and Boffey et al. 1979).

Scott and Possingham (1980, 1982) have identified three phases of plastid development and division in intact leaves of spinach. The first phase occurs in young leaves that are growing primarily by cell division; here plastid division and plastome replication keep pace with cell division. The second phase occurs when growth changes from cell division to cell expansion. As the cell expands, plastids continue to divide but there is a twofold increase in plastome numbers per plastid. Chloroplast DNA synthesis continues until plastome copy number per cell increases from approximately 1500 to 5000. The third phase occurs when cell division ceases. Chloroplast division continues for a few more cycles, but chloroplast DNA synthesis stops. This overall pattern of plastid division and chloroplast DNA replication has been observed in pea (Possingham, 1980), beet (Possingham, 1980) and wheat (Lamppa et al. 1980, Boffey and Leech, 1982).

Several hypotheses have been presented concerning the control of chloroplast division. Many feel that division is linked to cell expansion and plastids simply multiply to cover a constant proportion of the cell surface area (Pyke and Leech, 1987). This conclusion has come from numerous studies where chloroplast number was significantly correlated with cell size (Asahi and Toyama, 1982, Chaly et al. 1980, Ellis and Leech, 1985, Pyke and Leech, 1987). However, there may also be nuclear genes that directly regulate plastid division. Frandsen (1968) found several genotypes of Petunia hybrida that had significantly different

chloroplast number per cell but a similar cell size. DeMaggio and Stetler (1971) described lines in Todea barbara where chloroplast number was not significantly related to cell size or ploidy level.

Differences in chloroplast number per cell between diploid and tetraploid plants should be expected. Ploidy level has a strong effect on nuclear size which influences cell size (Pyke and Leech, 1987). Nucleus and cell size are positively correlated with DNA content (Ramachandran and Narayan, 1985). Over time, the polyploid plant may undergo 'dosage compensation', where some of these effects are diminished due to selective disadvantage, but at least some difference in cell size is usually maintained (Hancock, 1992).

When Bingham (1968) determined chloroplast number in guard cells of diploid, triploid, tetraploid and hexaploid alfalfa, he concluded that ploidy level has a greater influence on chloroplast number than does genome source, and suggested that chloroplast number per cell could be used as a method to determine ploidy level. Butterfass (1973, 1979, 1980, 1983 and 1991) suggested that nuclear DNA amount itself regulates chloroplast number, basing his argument on the observation that polyploidy usually results in increases in plastid number per cell. He also suggested a similar system may operate within genotypes due to endopolyploidy.

While it is likely that ploidy level influences plastid numbers via its effects on cell size, some specific genic effects have been noted. Molin et al (1982) found that there were twice as many chloroplasts per cell in isogenic lines of tetraploid alfalfa as compared to the diploids. However, there was also considerable variation among two different tetraploid genotypes in chloroplast number per cell, indicating a genic

effect. Standring et al. (1990) found no consistent relationship between ploidy level and chloroplast number in Solanum muricatum (pepino) and Cyphomandra betacea (tamarillo).

Ellis and Leech (1985) found that chloroplast number was inversely proportional to chloroplast size in Triticum monococcum and Triticum aestivum indicating that plastid size may play a role in regulating plastid numbers. Pyke and Leech (1991) found mutants in Arabidopsis that had aberrant numbers of chloroplasts per cell plane area. One mutant with a significantly higher number of chloroplasts per cell had unusually small chloroplasts, and two mutants with significantly lower numbers of chloroplasts per cell had unusually large chloroplasts. Also, a mutant of Arabidopsis with a deficiency in an n-3 desaturase had unusually small chloroplasts but significantly more chloroplast numbers per cell than the wild type (McCourt et al. 1987).

In this study, a diverse array of alfalfa genotypes was evaluated to determine if nuclear genes exist which influence plastid division independent of those affecting cell expansion. Chloroplast numbers and cell face areas were measured in a broad range of diploid and tetraploid genotypes of alfalfa maintained in common environments. Reciprocal crosses of high and low chloroplast lines were also examined to determine if there were cytoplasmic factors controlling chloroplast number per cell.

MATERIALS AND METHODS

Plastid numbers and cell size were measured in guard and mesophyll cells of both greenhouse and growth chamber grown plants. Seeds were obtained from the U.S.D.A. North Central Regional Plant Introduction Station, Ames, Iowa, or from Dr. E. T. Bingham, University of Wisconsin, Madison (Table 1). We will refer to individual genotypes by the accession numbers of their donor population. Accessions with the same number, but with a hyphenated suffix number were different genotypes from the same seed population. The same genotypes from each population were not always used in each experiment. Chromosome counts were verified by Feulgen and acetocarmine squashes of root tips (Schumann, 1988). All plants were initially grown for three years in a 1:1:1 (soil, peat and sand) mixture in 10 cm^3 plastic pots in a completely randomized design in a single greenhouse at Michigan State University, E. Lansing, Michigan. Seasonal conditions ranged from day temperatures of 18-40° C, night temperatures of 18-27° C and a photosynthetic photon flux (PPF) of 85-225 $\mu moles/sec/m^2$ in winter to 750-1320 $\mu moles/sec/m^2$ in summer.

Counts of guard cell chloroplasts were made in September from seven diploid and twelve tetraploid genotypes held in the greenhouse. The source material was fully expanded leaves from the third to sixth node. A section of the lower (abaxial) epidermis was peeled with forceps and placed in a saturated potassium iodide-iodine (I_2KI, which causes starch

Table 1. Mean number of chloroplasts per cell and guard cell length in different accessions of diploid and tetraploid alfalfa grown in the greenhouse.

Ploidy	Accession	Origin	Source	Mean number chloroplasts/ cell	Mean guard cell length (μ)
Diploid	172989	Turkey	USDA	3.5a[z]	12.0a
	251689	USSR	USDA	3.6a	12.0a
	262532	Israel	USDA	3.8ab	15.5ab
	W70-22	Mixed	Bingham (Wisc)	3.9ab	16.5ab
	251830	Austria	USDA	4.0ab	18.5b
	DDC 2X	Mixed	Bingham (Wisc)	4.1b	19.3b
	235021	Germany	USDA	4.2b	12.a
Tetraploid	299049	USSR	USDA	4.2a	20.0b
	172983	Turkey	USDA	4.4ab	20.4b
	239953	Algeria	USDA	4.8ab	20.5b
	299051-2	USSR	USDA	4.9ab	20.0b
	299051-1	USSR	USDA	3.8ab	12.a
	299048	USSR	USDA	5.1ab	19.0b
	W71-42-2	Mixed	Bingham (Wisc)	5.4b	20.0b
	DDC 4X	Mixed	Bingham (Wisc)	5.7bc	19.3b
	Vernal	Cultivar	USDA	5.9bc	19.5b
	W71-42-1	Mixed	Bingham (Wisc)	5.9bc	20.9b
	253443	Yugoslavia	USDA	6.7c	19.5b
	251205	Yugoslavia	USDA	6.7c	21.0b

[z]Means within columns sharing the same letter are not significantly different at the 5% level using the Duncan's Multiple Range Test. Mean comparisons are within ploidy.

grains in the chloroplast to turn red) solution for five minutes. Chloroplasts were counted and cell length measured using a Zeiss micrometer, in ten randomly selected guard cells per three trifoliate leaves. Chloroplast counts were made using a Zeiss microscope with phase optics.

Counts of spongey mesophyll cell chloroplasts were also made from five diploid and ten tetraploid genotypes held in the greenhouse and six diploid and nine tetraploid genotypes grown in a growth chamber for three months. The growth chamber plants were maintained at 25± 2° C at PPF between 600 and 700 μmoles/sec/m^2. All plants were cut back to crown level three weeks prior to analysis. Fully expanded leaves from the fifth node of each plant were used to determine chloroplast number. The lower epidermis of forty to fifty leaves were peeled with forceps or rubbed with carborundum (320 grit). The leaf tissue was then floated for one to two hours on an enzyme solution with 5% pectinase (Sigma Chemical Co., St. Louis, Mo.), 2% cellulysin (Calbiochem, La Jolla, Ca.), 2% driselase (Kogyo co., Tokyo, Japan), 9% mannitol (Lesney et al. 1986) and cell protoplast wash solution (Frearson et al. 1973). Chloroplasts were counted in 50 randomly selected cells using a Zeiss microscope with phase optics, and the length and width of cells were measured. The enzyme solution yielded a high number of cells with intact walls. Only cells with walls were measured to get an accurate representation of cell size in vivo.

To test if differing light intensities had an effect on chloroplast number per cell, three diploid and tetraploid genotypes with high (2N:235021, 4N:251205), intermediate (2N:172989, 4N:172983) and low (2N:262532, 4N:239953) chloroplast densities in the previous analysis were

grown for several weeks in a growth room at 26°C, 16 hour photoperiod and under PPF of 400 or 900 μmoles/sec/m^2. Three weeks after being cut back to crown level the fifth trifoliate leaf was removed from several stems and immediately placed in a solution of 1.5 to 2.5% glutaraldehyde in a 0.1M potassium phosphate buffer for one hour. The leaves were then placed in a 0.1M solution of NaEDTA ([ethylene dinitrilo] tetra acetic acid-disodium salt) at 60°C for three to eight hours (Pyke and Leech, 1987). The plant tissue was macerated on a slide and viewed with a Zeiss universal microscope using Nomarski differential interference optics. Plastids in twenty five randomly selected cells were counted and plastid and cell sizes were determined. Total plastid area per unit cell face area (TPA/CFA) was also determined.

To determine if there was a cytoplasmic effect on chloroplast division, chloroplast numbers per cell, cell face area and plastid face area were measured in twelve self and reciprocal progeny of two parent lines previously shown to have distinct plastid numbers (299049-1, or S cytoplasm and W71-42-2, or F cytoplasm, Schumann and Hancock, 1989). Rooted shoots from a chimeric individual (SF-11) were also examined that were previously determined to contain different plastid types (SxF-11d, S cytoplasm and SxF-11a, F cytoplasm, Schumann and Hancock, 1990). All these plants were grown in the greenhouse at Michigan State University under the previously described conditions. Ten cells were evaluted from five leaves taken from the fifth node from the apex of each plant using the methods described above.

RESULTS

GENOTYPE SCREENS

Mean number of chloroplasts per guard cell ranged from 3.5±0.8 to 4.2±0.5 among diploid and 4.1±0.8 to 6.7±1.4 among tetraploid lines (Table 1). There was a positive correlation between chloroplast number and cell length among both diploid ($r=0.470$, $df=5$) and tetraploid genotypes ($r=0.20$, $df=10$), but neither was significant at the $P<0.05$ level. The tetraploids averaged more chloroplasts per guard cell than the diploids (5.3 vs. 3.9), although tetraploid line 299051-1 fell within the range of the diploids (Figure 1). The diploid guard cells appeared to reach a threshold in chloroplast number per cell which was not strongly associated with cell length, whereas the tetraploid genotypes seemed to reach a threshold in cell length but not chloroplast numbers (Figure 1).

Spongey mesophyll cells of tetraploids generally had larger face areas and more chloroplasts per cell than diploids (Tables 2 and 3; Figures 2 and 3). There was not a significant correlation between spongey mesophyll cell size and chloroplast number among the greenhouse grown genotypes (2N: $r=0.093$, 4N: $r=0.501$). However, cell sizes and plastid numbers were significantly correlated among plants grown in the growth chamber (2N: $r=0.829$, 4N: $r=0.949$). Chloroplast numbers and cell face areas within individual genotypes of spongey mesophyll cells of both greenhouse

Figure 1. A plot of guard cell length (μ) and number of chloroplasts per cell for seven diploid and twelve tetraploid alfalfa genotypes. Each point represents the mean of thirty cells. Diploids, r=0.470, not significant; tetraploids, r=0.20, not significant.

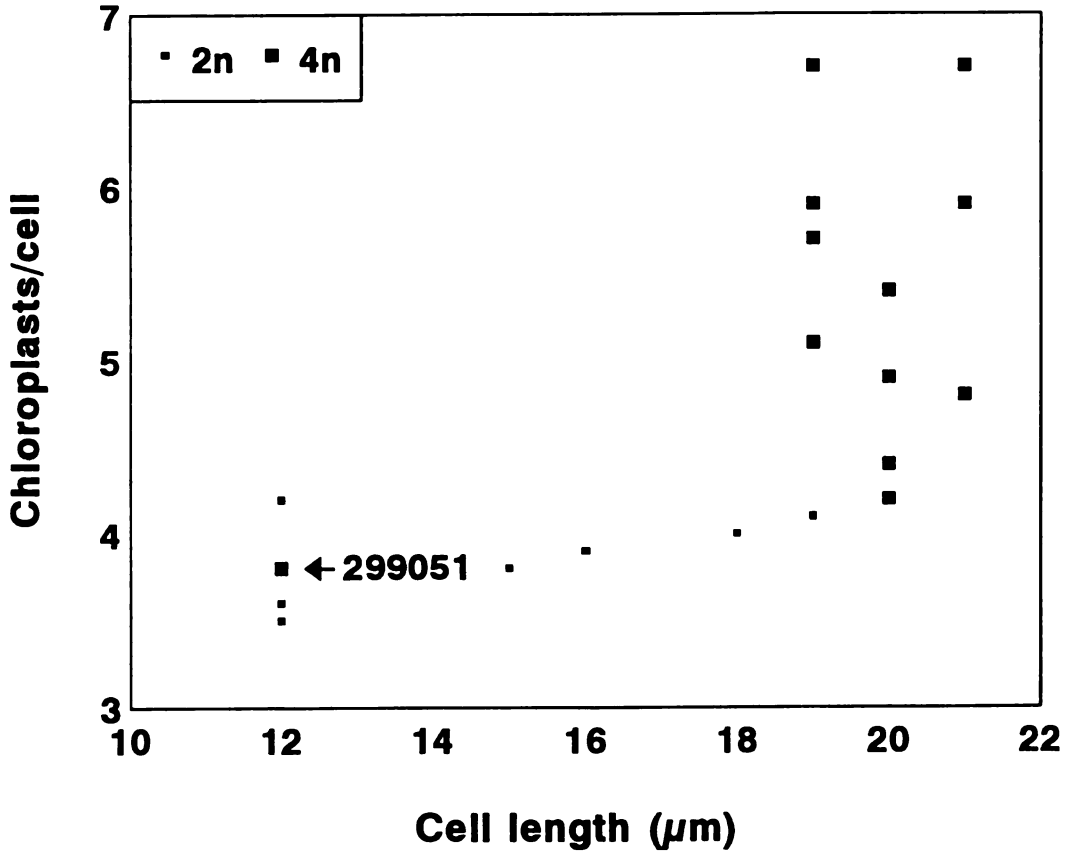
2n
4n
Chloroplasts/cell
7
6
5
4
3
299051
10
12
14
16
18
20
22
Cell length (μm)

Figure 2. A plot of mesophyll cell face area (μm^2) and number of chloroplasts per cell for five diploid and ten tetraploid alfalfa genotypes grown in a greenhouse (see text for details). Each point represents the mean of fifty cells. Diploids, r=0.093, not significant; tetraploids, r=0.501, not significant.

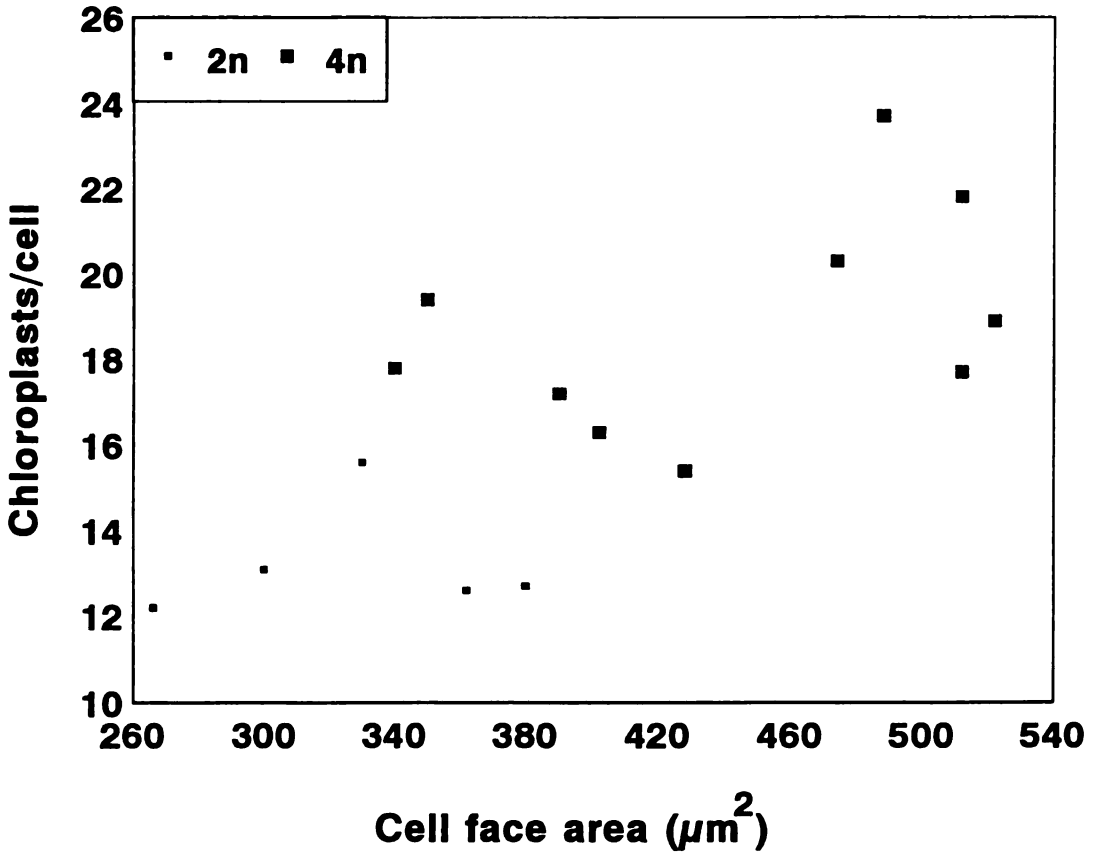
2n
4n
Chloroplasts/cell
26
24
22
20
18
16
14
12
10
260
300
340
380
420
460
500
540
Cell face area (μm²)

Figure 3. A plot of mesophyll cell face area (μm^2) and number of chloroplasts per cell for six diploid and nine tetraploid alfalfa genotypes grown in the growth chamber (see text for details). Each point represents the mean of fifty cells. Diploids, r=0.829; tetraploids, r=0.949, ($P<0.05$).

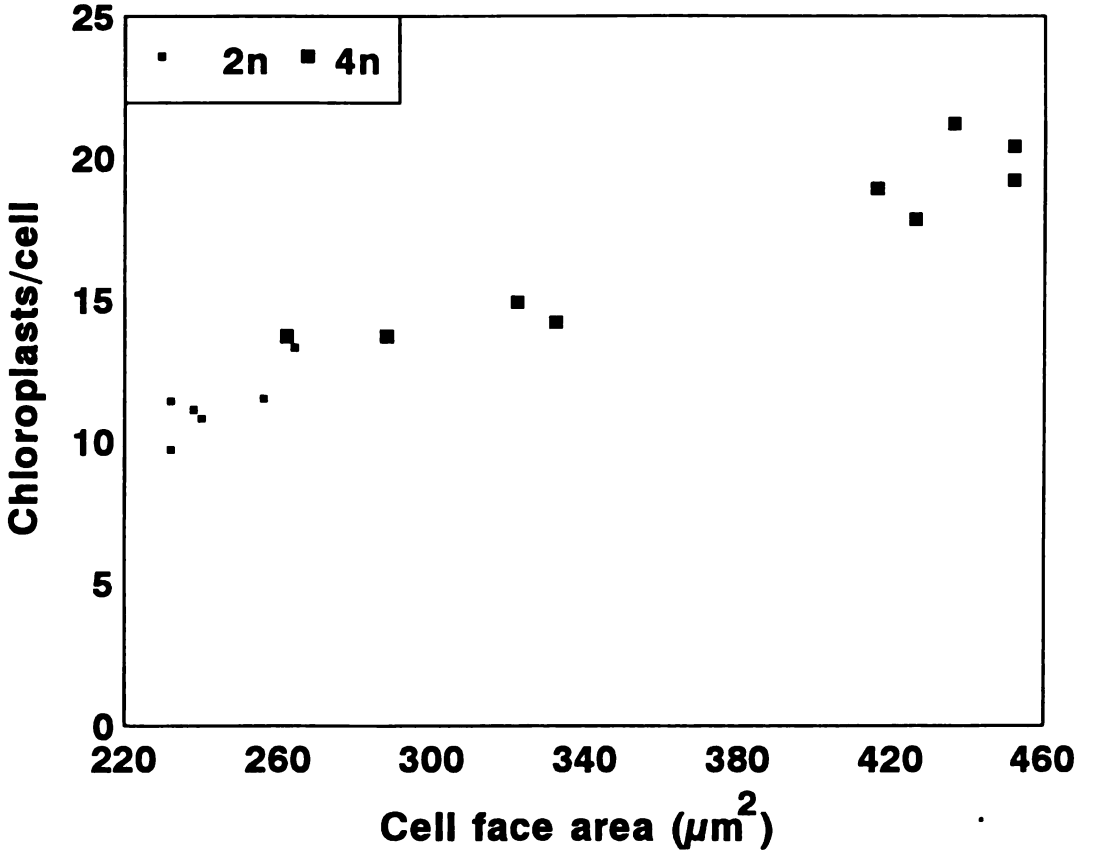
2n
4n
Chloroplasts/cell
25
20
15
10
5
0
220
260
300
340
380
420
460
Cell face area (μm²)

Table 2. Mean cell face area and chloroplast number in spongy mesophyll cells of different genotypes of alfalfa grown in a greenhouse (see text for details).

Ploidy	Genotype	Face area (μ^2)	Chloroplasts	
			No. per cell	Density[y]
2N	235021	330ab[z]	15.6c	21.1a
	DDC2x-5	266a	12.2a	21.8a
	172989	300ab	13.1b	22.9a
	262532	362b	12.6ab	28.7b
	W70-22-5	380b	12.7ab	29.9b
4N	299055	350a	19.4ab	18.0a
	239953	340a	17.8ab	19.1a
	W71-42-2	488bc	23.7b	20.6ab
	172983	390a	17.2a	22.7b
	299049	473bc	20.3b	23.3b
	DDC4x-1	512c	21.8b	23.5b
	299051-2	402ab	16.3a	24.7bc
	251205	522c	18.9ab	27.6c
	Vernal	428ab	15.4a	27.8c
	253443	512c	17.7ab	28.9c

[y]Density is cell face area divided by chloroplast number.

[z]Means within columns sharing the same letter are not significantly different at the 5% level using the Duncan's Multiple Range Test. Mean comparisons are within ploidy.

Table 3. Mean cell face area and chloroplast number in spongy mesophyll cells of different genotypes of alfalfa grown in the growth chamber.

Ploidy	Genotype	Face area (μ^2)	Chloroplasts	
			No. per cell	Density[y]
2N	235021	263a[z]	13.3a	19.8a
	172989	232a	11.4abc	20.3ab
	251689	237a	11.1bc	21.3ab
	DDC2x-5	255a	11.5abc	21.4ab
	W70-22-5	238a	10.8bc	22.0ab
	262532	231a	9.7c	23.8b
4N	299051-1	262c	13.7c	19.2a
	DDC4x-1	436a	21.2a	20.4a
	239953	288bc	13.7c	21.4ab
	W71-42-1	321abc	14.9bc	21.8ab
	253443	415ab	18.9a	22.0ab
	251205	452a	20.4a	22.1ab
	299048	332abc	14.2bc	23.3b
	299049	452a	19.2a	23.5b
	172983	425a	17.8ab	23.8b

[y]Density is cell face area divided by chloroplast number.

[z]Means within columns sharing the same letter are not significantly different at the 5% level using the Duncan's Multiple Range Test. Mean comparisons are within ploidy.

and growth chamber grown plants were all significantly correlated ($P< 0.05$).

In general, the spongey mesophyll cells of the diploids and tetraploids had distinct chloroplast numbers, but there were some overlaps. In the greenhouse grown plants, diploid genotype 235021 had 15.6 chloroplasts per cell while two tetraploid genotypes, 'Vernal' and 299051-2 had 15.4 and 16.3 respectively. In the growth chamber grown plants, diploid genotype 235021 had 13.3 chloroplasts per cell, while tetraploid genotypes 299051-1 and 239953 both had 13.7 chloroplasts per cell (Table 3).

There was significant variation among genotypes in plastid density and in many cases, genotypes with similar sized cells had very different plastid numbers (Tables 2 and 3). For example, in the greenhouse grown diploid genotypes 235021 and 262532, chloroplast number per cell was 15.6 and 12.6 even though their cell face areas were very similar (330 μm^2 and 362 μm^2). The tetraploid genotypes 253443 and DDC 4x-1 both had a cell face area of 512 μm^2, yet their chloroplast numbers per cell were 17.7 and 21.8 respectively. Among the growth chamber grown tetraploids, genotype 299051-1 had a chloroplast density (19.2) that was significantly different from genotypes 172983 (23.8), 299048 (23.3) and 299049 (23.5). Chloroplast numbers per cell also varied significantly among growth chamber grown diploids even though their cell sizes were generally similar. For example, genotype 235021 had 13.3 chloroplasts per cell and 262532 had 9.7.

The plastid density of individual genotypes was not differentially affected by light levels (Table 4), however, the genotypes did show significant variations in their means (Table 5). The same relative

Table 4. Comparison of diploid and tetraploid genotypes at low light levels (LL), 400 μmoles/sec/cm^2, photosynthetic photon flux (PPF), and high light levels (HL), 900 μmoles/sec/cm^2 PPF. Chloroplast number = CPT No., cell face area = CFA, chloroplast face area = CPFA, chloroplast density (CFA/CP No.) = CPD and total plastid area per cell area = TPA/CFA.

2N Genotypes	CPT No.	CFA	CPFA	CPD	TPA/CFA
262532 (LL)	13.7	383.7	21.4	28.1	0.76
(HL)	13.9	405.6	20.7	29.9	0.71
235021 (LL)	17.0	376.7	15.0	22.6	0.68
(HL)	21.0*[z]	454.4*	15.3	22.2	0.71
172989 (LL)	13.4	350.2	18.7	26.0	0.71
(HL)	15.6*	405.8	20.2	26.9	0.77
4N Genotypes					
239953 (LL)	16.0	350.8	20.0	22.4	0.91
(HL)	18.3*	457.5*	21.8	25.2*	0.87
251205 (LL)	19.8	530.8	20.9	27.3	0.78
(HL)	22.7*	591.6	22.8	26.7	0.87
172983 (LL)	14.9	389.1	19.5	25.8	0.75
(HL)	15.0	382.4	20.5	26.0	0.80

[z]Significant at $P<0.05$, n=25.

Table 5. Mean cell face area (CFA), chloroplast number (CPT No.), chloroplast face area (CPFA), chloroplast density (CPD) and total plastid area per cell area (TPA/CFA) in spongy mesophyll cells of different genotypes of alfalfa grown under artificial light. Values are averages of replicates grown at 400 and 900 μmoles/sec/cm^2 PPF.

Ploidy	Genotype	CFA (μ^2)	CPT No.	CPFA	D	TPA/CFA
2N	235021	415a[z]	19.0b	15.1a	21.8a	0.69a
	172989	378a	14.5a	19.4b	26.0b	0.74a
	262532	396a	13.8a	21.1b	28.7b	0.73a
4N	239953	404a	17.1ab	20.8ab	23.6a	0.88b
	172983	385a	14.9a	20.0a	25.8b	0.77a
	251205	561b	21.2b	21.8b	26.5b	0.82ab

[z]Means within columns sharing the same letter are not significantly different at the 5% level using the Duncan's Multiple Range Test.

rankings were displayed under the varying light conditions that were previously observed in the greenhouse and growth chamber experiments. The diploid 235021 had a significantly lower plastid density than 172989 and 262532, and thetetraploid 239953 had a significantly lower plastid density than 172983 or 251205. Plastid face area was significantly correlated with chloroplast number in the tetraploid population (r=0.883), although it was negatively correlated in the diploid population (r= -0.871). The tetraploid genotypes had a greater total plastid area per unit cell face area (TPA/CFA) than the diploids (4N=0.82, 2N=0.72). There was significant variation among the tetraploid genotypes with respect to TPA/CFA but not among the diploid genotypes (Table 5).

RECIPROCAL CROSSES

Mean chloroplast number per cell in 299049-1 was 20.9, and in W71-42-2 was 24.1 (Table 6). Chloroplast density was significantly different between the two parents (28.0 vs. 22.1, $P<0.05$) These differences were mirrored in the selfed crosses (S self=29.7, F self=22.5). Chloroplast numbers in both reciprocal crosses were not significantly different; however, chloroplast density was significantly associated with cytoplasmic source (SxF=25.8, FxS=23.4). In all cases, progenies with S cytoplasms had higher means than those with F (Table 6).

Chloroplast number, cell face area, chloroplast density, plastid face area and total plastid area per cell face area were not significantly different in the shoots from the chimeric plants that contained different plastid types (SxF 11d and SxF 11a, Table 7).

Table 6. Mean cell face area (CFA), chloroplast number (CPT No.), chloroplast face area (CPFA) and density (CPD) in spongy mesophyll cells of the accessions 299049-1 (S), W71-42-2 (F) and their self and reciprocal progeny.

Parent or cross	CFA (μ^2)	CPT No.	CPFA	CPD
299049-1 (S)	576	20.9	26.8	28.0
W71-42-2 (F)	528	24.1	23.4	22.1*
S self	615	20.9	26.4	29.7
F self	545	24.2*[z]	21.4	22.5*
SxF	598	23.7	28.2	25.8
FxS	573	24.2	25.0	23.4*

[z] Significant at P=0.05

Table 7. Mean chloroplast number (CPT No.), cell face area (CFA), chloroplast face area (CPFA), and chloroplast density (CPD) of shoots from chimeric sectors of cross SxF 11. SxF 11d has chloroplasts containing the S plastome and SxF 11a has chloroplasts containing the F plastome.

Sector plastome	CPT No.	CFA	CPFA	CPD
SxF 11d (S)	21.8	567.6	19.7	26.3
SxF 11a (F)	22.7	623.0	21.3	27.3

DISCUSSION

Factors regulating plastid numbers. Many feel that cell size is the primary factor that determines chloroplast number per cell. They believe chloroplasts simply divide until they fill a constant proportion of the cell surface (Pyke and Leech 1987). Others have proposed that cell size may set the threshold for a particular number of chloroplasts per cell, but the tendency to realize that potential is controlled by other factors (Paolillo and Kass, 1977; Frandsen, 1968; De Maggio and Stetler, 1971).

In this study, we did observe a number of significant, positive correlations between cell face area and chloroplast number. However, significant differences were observed in chloroplast densities, and a number of outlier genotypes were observed with similar cell sizes, but significantly different plastid densities. This genotypic variation in plastid density indicates that there may be genes influencing chloroplast number per cell that are independent of cell size.

In the crosses, F_1 hybrids displayed chloroplast number per cell values intermediate to their parents indicating nuclear control; however, the individual reciprocal crosses still varied significantly in the direction of their maternal parent. Normally this would imply that the plastids themselves exert control over their ultimate densities due to maternal inheritance of plastids, but Schumann and Hancock (1989) previously showed that plastids are inherited from the paternal parent in these populations. It is possible that other cytoplasmic factors are

regulating plastid densities or our limited population sizes led to sampling errors. Shoots originating from the distinct sectors of the chimeric plant displayed no significant differences for any of the parameters measured. This indicates that control of chloroplast number, size, density and total chloroplast area must lie within the nucleus or maternal environment (excluding the chloroplasts).

Ellis and Leech (1985) found that total chloroplast area was positively correlated with cell size in Triticum. They suggested that variation in chloroplast number per cell is due to variation in chloroplast size and that chloroplast number is inversely regulated by chloroplast size (Ellis and Leech, 1985). We did find highly significant (P=0.01) correlations between cell size and chloroplast surface area in our alfalfa populations, but we only observed a significant inverse relationship between chloroplast number and size among the diploids.

Light was probably not an important factor influencing plastid density in this study. When three genotypes (high, medium and low chloroplast number per cell) of both ploidies were grown under two different levels of PPF of the greenhouse (900 μmoles/sec/m^2) and growth chamber (400 μmoles/sec/m^2), the plastid densities of individual genotypes were significantly different in each of the enviroments, but they themselves varied little across environments.

While light can induce movement and influences growth and development of chloroplasts, other work has shown that it has little direct effect on chloroplast division. Chaly et al. (1981) found that proplastids and etioplasts grow and divide in roots and shoot apices where they may receive little or no light. When spinach leaf discs were precultured in darkness chloroplasts did divide after exposure to high intensity light,

however it was suggested that chloroplast division may depend on high energy compounds produced from photosynthesis or mitochondrial respiration rather than direct light (Possingham and Lawrence 1983, Possingham et al. 1988). In our light experiments, cell sizes were generally larger under high light, but this had little effect on chloroplast density.

Polyploidy and plastid numbers. Bingham (1968) found significantly different numbers of chloroplasts in diploid and tetraploid guard cells of alfalfa, and as a result, suggested that chloroplast counts could be used to determine ploidy level. Based on our results, we do not share Bingham's confidence in determining level of ploidy for individual genotypes, since we found overlap in plastid numbers between the two ploidies. Chloroplast numbers were more variable among spongy mesophyll cells of diploids and tetraploids than among guard cells, but there was still considerable overlap between some 2N and 4N genotypes.

Butterfass (1980) proposed that ploidy level controls chloroplast number per cell and that a doubling of the ploidy level should result in a 60-80% increase in chloroplast number per cell. In our comparisons of cell face area and chloroplast number, there was a smooth transition between the chloroplast numbers of diploids and tetraploids, rather than a distinct gap as Butterfass would predict. Such an overlap would not be observed if nuclear DNA mass alone regulates chloroplast number per cell. A similar overlap was seen by by Ellis and Leech (1985) in wheat. Likewise, Strandring et al. (1990) found no relationship between genome size and chloroplast number in tamarillo.

Therefore, the number of chloroplasts found in a polyploid may be more dependent on the cell size and genotype of the progenitor species than on ploidy level per se. Molin et al. (1982) found cell size and

chloroplast number to coordinately double in isogenic diploid and tetraploid lines of alfalfa, but Standring et al. (1990) found some trisomics of pepino to have higher chloroplast numbers than disomics while others did not. They concluded that the chromosomes exert varying levels of control on chloroplast numbers and therefore, some genes have a stronger effect on chloroplasts per cell than others.

In conclusion, there is often a tight correlation between chloroplast number per cell and cell face area among diploid and tetraploid genotypes of alfalfa, but significant differences in plastid density can be found. This indicates that while the size of the cell wields considerable control on plastid number, genes still exist which act independently of cell face area to regulate chloroplast number. Our controlled crosses demonstrated that chloroplast number per cell is largely controlled by the nucleus, but other non-chloroplastic factors appear to play a role.

BIBLIOGRAPHY

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Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson. 1983. Molecular Biology of the Cell. Garland Pub. Co. pp. 532.

Asahi,Y. and S. Toyama. 1982. Some factors affecting the chloroplast replication in the moss Plagiomnium trichomanes. Protoplasma 112:9-16.

Barlow, S.B. and R.A. Cattolico. 1981. Mitosis and cytokinesis in the Prasinophyceae. I. Mantoniella squamata (Manton and Parke) Desikachary. Am. J. Bot. 68:606-615.

Batschauer, A., E. Mosinger, K.Kreuz, I. Dorr and K. Apel. 1986. The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light-harvesting chlorophyll a/b protein. Eur. J. Biochem. 154:625-634.

Bingham, E.T. 1968. Stomatal chloroplasts in alfalfa at four ploidy levels. Crop Sci. 8:509-510.

Boffey, S.A., J.R. Ellis, G. Sellden and R.M. Leech. 1979. Chloroplast division and DNA synthesis in light-grown wheat leaves. Plant Physiol. 64:502-505.

Boffey, S.A. and R.M. Leech. 1982. Chloroplast DNA levels and the control of chloroplast division in light-grown wheat leaves. Plant Physiol. 69:1387-1391.

Bottomley, W. and H.J. Bohnert. 1982. The biosynthesis of chloroplast proteins. Encyclopedia of Plant Phys. 143:532-596.

Butterfass, T. 1973. Control of plastid division by means of nuclear DNA amount. Protoplasma 76:167-195.

_____ 1979. Patterns of chloroplast reproduction: A developmental approach to protoplasmic plant anatomy. Cell Biology monographs Vol. 6, Springer-Verlag, N.Y.

_____ 1980. The continuity of plastids and the differentiation of plastid populations. Chloroplasts, J. Reinert, ed. Springer-Verlag, N.Y.

_____ 1983. A nucleotypic control of chloroplast reproduction. Protoplasma 118:71-74.

_____ 1991. Cell sizes and chloroplast numbers per cell of hemiploid and polyploid plants. Cytologia 56:473-478.

Busbice, T.H. and C.P. Wilsie. 1966. Inbreeding depression and heterosis in autotetraploids with applications to Medicago sativa. Euphytica 15:52-67.

Cattolico, R.A., J.C. Boothroyd and S.P. Gibbs. 1976. Synchronous growth and plastid replication in the naturally wall-less alga Olisthodiscus luteus. Plant Physiol. 57:497-503.

Chaly, N. and J.V. Possingham. 1981. Structure of constricted proplastids in meristematic plant tissues. Biol. Cell 41:203-210.

Chaly, N., J.V. Possingham and W.W. Thomson. 1980. Chloroplast division in spinach leaves examined by scanning electron microscopy and freeze etching. J. Cell Sci. 46:87-96.

Deane-Drummond, C.E. and C.B. Johnson. 1980. Absence of nitrate reductase activity in San 9789 bleached barley seedlings (Hordeum vulgare cv. Midas). Plant Cell Environ. 3:303-307.

DeMaggio, A.E. and D.A. Stetler. 1971. Polyploidy and gene dosage effects on chloroplasts of fern gametophytes. Exp. Cell Res. 67:287-294.

Edwards, J.W. and G.M. Coruzzi. 1989. Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. The Plant Cell, Vol. 1:241-248.

Ellis, J.R. and R.M. Leech. 1985. Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves. Planta 165:120-125.

Ellis, J.R., A.J. Jellings and R.M. Leech. 1983. Nuclear DNA content and the control of chloroplast replication in wheat leaves. Planta 157:376-380.

Ellis, J.R., 1984. The nuclear domination of chloroplast development. Sci. Prog., Oxf. 69:129-142.

Feierabend, J. and B. Schubert. 1978. Comparative investigation of the action of several chlorosis inducing herbicides on the biogenesis of chloroplasts and leaf microbodies. Plant Physiol. 61:1017-1022.

Frandsen, N.O. 1968. Die Plastidenzahl als Merkmal bei der Kartoffel. Theor. Appl. Genetics 38:153-167.

Frearson, E.M., J.B. Power and E.C. Cocking. 1973. The isolation, culture and regeneration of Petunia leaf protoplasts. Dev. Biol. 33:130-137.

Gillham, N.W. 1978. Organelle Heredity, Raven Pr., N.Y. Giuliano, G. and P.A. Scolnick. 1988. Transcription of two photosynthesis associated nuclear gene families correlates with the presence of chloroplasts in leaves of the variegated tomato ghost mutant. Plant Physiol. 86:7-9.

Gruissem, W. 1989. Chloroplast gene expression: How plants turn their plastids on. Cell, Vol. 56:161-170.

Hancock, J.F. 1992. Plant evolution and the origin of crop species. Prentice Hall. N.J. pp. 100.

Harpster, M.H., S.P. Mayfield and W.C. Taylor. 1984. Effects of pigment deficient mutants on the accumulation of photosynthetic proteins in maize. Plant Mol. Biol. 3:59-71.

Hoober, J.K. 1984. Cholroplasts, Plenum Pr., N.Y. and London.

Karakashian, S.J., M.W. Karakashian and M. A. Rudzinska. 1968. Electron microscope observations on the symbiosis of Paramecium Bursaria and its intracellular algae. J. Protozool. 15:113-128.

Lamppa, G.K., L.V. Elliot and A.J. Bendach. 1980. Changes in chloroplast number during pea leaf development. An analysis of a protoplast population. Planta 148:437-443.

Lesney, M.S., P.W. Callow and K.S. Sink. 1986. A technique for bulk production of cytoplasts and miniprotoplasts from suspension culture-derived protoplasts. Plant Cell Rep. 5:115-118.

Lyndon, R.F. and E.S. Robertson. 1976. The quantitative ultrastructure of the pea shoot apex in relation to leaf initiation. Protoplasma 87:387-402.

Margulis, L. 1971. Symbiosis and evolution. Sci. Amer. 225(2):48-57.

Mayfield, S.P. and W.C. Taylor. 1984. Carotenoid-deficient maize seedlings fail to accumulate light-harvesting chlorophyll a/b bindingprotein (LHCP) mRNA. Eur. J. Biochem. 144:79-84.

McCourt, P., L. Kunst, J. Browse and C.R. Somerville. 1987. The effects of reduced amounts of lipid unsaturation on chloroplast ultrastructure and photosynthesis in a mutant Arabidopsis. Plant Phys. 84:353-360.

Molin, W.T., S.P. Meyers, G.R. Baer and L.E. Schrader. 1982. Ploidy effects in isogenic populations of alfalfa 2: Photosynthesis, chloroplast number, RUBP, chlorophyll and DNA in protoplasts. Plant Physiol. 70:1710-1714.

Oelmuller, R. and H. Mohr. 1986. Photo-oxidative destruction of chloroplasts and its consequences for expression of nuclear genes. Planta, 167:106-113.

Olszewska, M.I., B. Damsz and E. Rabeda. 1983. DNA endoreplication and increase in number of chloroplasts during leaf differentiation in five monocotyledonous species with different 2C DNA contents. Protoplasma 116:41-50.

Paolillo, D.J. and J. Kass. 1977. The relation between cell size and chloroplast number in the spores of a moss, Polytrichum. J. Exp. Bot. 28:457-467.

Possingham, J.V. 1980. Plastid replication and development in the life cycle of higher plants. Ann. Rev. Plant Physiol. 31:113-129.

Possingham, J.V., H. Hashimoto and J. Oross. 1988. Factors that influence plastid division in higher plants. Division and segregation of organelles. ed. S. Boffey and A. Lloyd. Society for experimental biology seminar, series 35. Cambridge pr.

Possingham, J.V. and M.E. Lawrence. 1983. Controls to plastid division. International review of cytology. Vol. 84:1-49. Academic pr.

Price, H.J., A.H. Sparrow and A.F. Nauman. 1973. Correlations between nuclear volume, cell volume and DNA content in meristematic cells of herbaceous angiosperms. Experientia 29:1028-1029.

Pyke, K.A. and R.M. Leech. 1987. The control of chloroplast number in wheat mesophyll cells. Planta, 170:416-420.

Ramachandran, D.D., and R.K.J. Narayan. 1985. Chromosomal variation in Cucumis. Theor. Appl. Genet. 69:497-502

Reiss, T. R. Bergfeld, G. Link, W. Thien and H. Mohr. 1983. Photo-oxidative destruction of chloroplasts and its consequences for cytosolic enzyme levels and plant development. Planta, 159:518-528.

Rose, R.J., D.G. Cran and J.V. Possingham. 1975. Changes in DNA synthesis during cell growth and chloroplast replication in greening spinach leaf disks. J. Cell Sci. 17:27-41.

Sagar, A.D., B.A. Horwitz, R.C. Elliott, W.F. Thompson and W.R. Briggs. 1988. Light effects on several chloroplast components in norflurazon-treated pea seedlings. Plant Physiol. 88:340-347.

Schumann, C.M. and J.F. Hancock. 1989. Paternal inheritance of plastids in Medicago sativa. Theor. Appl. Genet. 78:863-866.

Schumann, C.M. 1988. Master of Science thesis. Michigan State Univ. E. Lansing, Mi.

Scott, N.S. and J.V. Possingham. 1980. Chloroplast DNA in expanding spinach leaves. J. Exp. Bot. 31:1081-1092.

_____ 1982. Manipulation and expression of genes in eukaryotes. P. Nagey, A.W. Linnae, J.W. Peacock and J.A. Pateman, eds. Academic Pr., N.Y.

Scott, N.S. and J.N. Timmis, 1984. Homologies between nuclear and plastid DNA in spinach. Theor. app. gen. 67:279-288.

Sears, B.B., 1983. Genetics and evolution of the chloroplast. Stadler symp. Vol. 15 (1983), Univ. of Mo., Columbia. p.119-139.

Simpson, J., M. Van Montague and L. Herrera-Estrella. 1986. Photosynthesis associated gene families: differences in response to tissue specific and environmental factors. Sci. 233:34-38.

Slankis, T. and G.P. Gibbs. 1972. The fine structure of mitosis and cell division in the alga Ochromonas danica. J. Phycol. 8:243-256.

Standring, L.S., G.J. Pringle and B.G. Murray. 1990. The control of chloroplast number in Solanum muricatum Ait. and Cyphomandra betacea (Cav.) Sendt. and its value as an indicator of polyploidy. Euphytica 47:71-77.

Taylor, W.C., 1989. Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol. Biol., 40:211-233.

Von Wettstein, D. 1981. Chloroplast and nucleus: Concerted interplay between genomes of different cell organelles. In International Cell Biology, 1980-1981. H.G. Schweiger, ed. N.Y., Springer-Verlag.

Whatley, J.M. 1974. The behaviour of chloroplasts during cell division of Isoetes lacustris. New Phytol. 73:139-142.

_____ 1980. Plastid growth and division in Phaseolus vulgaris. New Phytol. 86:1-16.

Whatley, J.M. and B.E.S. Gunning. 1981. Chloroplast development in Azolla roots. New Phytol. 89:129-138.