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ORGANELLE INHERITANCE AND VARIATION IN TOTAL PROTEIN, RIBULOSE-1,5-BIPHOSPHATE CARBOXYLASE, AND PLASTOME COPY NUMBER IN ALFALFA, MEDICAGO SATIVA

presented by

CAROL M. SCHUMANN

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James F Hancock
Major professor

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By

Carol M. Schumann

A DISSERTATION

Submitted to
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1990

ABSTRACT

ORGANELLE INHERITANCE AND VARIATION IN TOTAL PROTEIN, RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE, AND PLASTOME COPY NUMBER IN ALFALFA, *MEDICAGO SATIVA*

By

Carol M. Schumann

Mitochondria and plastids are organelles that contain DNA and are generally inherited from the maternal parent in angiosperms. Even in species which exhibit biparental plastid inheritance, generally low levels of plastids are transmitted from the paternal parent. In contrast to the usual patterns of maternal or biparental inheritance of plastids, in the angiosperm alfalfa (*Medicago sativa*) plastids are transmitted almost exclusively from the paternal parent. Plastid and mitochondrial DNA was isolated from progeny of a reciprocal cross between genotypes of *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata*. RFLP analysis indicated that 28 out of 30 had only paternal plastid DNA, 1 out of 30 had only maternal plastid DNA, and 1 out of 30 had both maternal and paternal plastid DNA. All progeny had only maternal mitochondrial DNA, as is the case with most species.

Parental plants differed significantly for total soluble protein content, ribulose-1,5-bisphosphate carboxylase (Rubisco) content, and plastid DNA (plastome) copy number per cell. Progeny from self pollinations and from reciprocal crosses were analyzed to determine the

genetic components of the differences between the parents. Levels of total protein and Rubisco were similar between reciprocal populations, indicating regulation by nuclear genes. Significant differences between progeny of reciprocal crosses were observed in plastome copy number per cell, indicating an influence by cytoplasmic genes. However, in one case the cytoplasmic effect could be overridden by a particular nuclear background. Populations from self pollinations had lower plastome copy number than parents, which may have been caused by inbreeding effects.

DEDICATION

In memory of Marguerite Schumann and Joseph Scaletta, who both believed that I could and would complete this degree, even when I wasn't so sure.

ACKNOWLEDGMENTS

The evolution and successful completion of this thesis project can be largely credited to Dr. Jim Hancock, who provided a valuable combination of intellectual stimulation, freedom, and guidance. Additionally, he posesses the unique ability to come into the lab in late afternoon and interpret the day's molecular genetic data while mud from the field still clings to his clothes.

Thanks are also due to lab mates, past and present, who tolerated alfalfa in the greenhouse and lab, in an otherwise small fruit oriented program.

Special thanks are due to Coleen Mulinix, who produced the alfalfa populations that were so useful to me (and who was restrained from throwing them out when she very much wanted to). Thanks also to Pete Callow, whose painfully collected data on plastid numbers provided excellent background information. And thanks to Kobra Haghighi, who was vigilant in holding off other users to assure that all possible pieces of lab equipment and supplies were available for my use alone in the final push to finish my experiments.

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General Introduction

The original goal of this thesis was to determine the extent of coordination between nuclear and plastid genomes in alfalfa, Medicago sativa. Specifically, we wanted to examine genetic influences on chloroplast number in mature mesophyll cells, determine plastid genome (plastome) copy number in plastids and cells, and examine how these parameters might be influencing levels of a protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), whose production requires expression of genes from the separate genomes. Additionally, we wanted to determine whether level of expression was most influenced by nuclear or cytoplasmic factors. Since there had been several reports of biparental inheritance of plastids in Medicago sativa (Smith et al, 1986; Lee et al, 1988), we needed to establish the extent to which this had occurred in our populations. Surprisingly, RFLP analysis of organelle DNA revealed that although mitochondrial DNA was inherited strictly from the maternal parent in reciprocal crosses, plastid DNA was inherited almost entirely from the paternal parent. This was the first report of this type of organelle inheritance in an angiosperm, and is described in the first section of the thesis. Since publication of the study (Schumann and Hancock, 1989), a report by Masoud et al (1990)

has shown a similar bias toward paternal inheritance of plastid DNA in alfalfa crosses at both the diploid and tetraploid levels. Additionally, plastid DNA has been shown to be inherited paternally in one cross of Daucus species (Boblenz et al, 1990).

The original question, that of interaction between nuclear and plastid genomes, is addressed in the second section of the thesis.

CHAPTER 1

INTRODUCTION

Plastids are one of two plant cellular organelles which contain extra-nuclear DNA. Previous reports have indicated that they are inherited primarily from the maternal parent in the angiosperms, although a low level of biparental inheritance has been documented in many species (reviewed in Kirk and Tilney-Bassett, 1978; Hagemann, 1979; Sears, 1980).

Biparental plastid inheritance has commonly been noted when green and white sectored tissue has appeared in progeny following crosses between a normal green plant and one having a plastome-encoded chlorophyll deficiency. Oenothera (Kirk and Tilney-Bassett, 1978; Sears, 1980; Chiu et al, 1988) and Pelargonium (Kirk and Tilney-Bassett, 1978; Sears, 1980; Tilney-Bassett, 1973) are two well known examples of genera exibiting biparental plastid inheritance that have been documented in this way. A drug resistance marker has also been used to show that rare biparental plastid inheritance occurs in Nicotiana (Medgyesy et al, 1986), a taxa which was previously considered to have exclusively maternal inheritance of plastids (Kirk and Tilney-Bassett, 1978; Corriveau and Coleman, 1988).

In contrast to the angiosperms, plastid transmission in the gymnosperms is mostly paternal. Utilization of a plastid mutant has indicated greater than 95% paternal transmission of plastids in Cryptomeria (Ohba et al, 1971). Similarly,

restriction fragment length polymorphism (RFLP) analyses have documented high levels of paternal inheritance in Pinus (Wagner et al, 1982), Larix (Szmidt et al, 1987), Pseudotsuga (Neale et al, 1986), and Picea (Stine, 1988).

In this report, we describe the first evidence of predominantly paternal transmission of normal green plastids in an angiosperm, Medicago sativa, based on data from RFLPs. This technique allows one to follow the transmission of normal green plastids through sexual crosses, thus eliminating any differential survival bias which might exist against a mutant type.

Inheritance of mitochondria, the other DNA containing plant organelle, was also determined.

MATERIALS AND METHODS

Plant Material

Reciprocal crosses were performed between two individuals from different subspecies of alfalfa, Medicago sativa. Subspecies freely intercross but are morphologically distinct. M. sativa ssp sativa has an upright growth habit and purple flowers, while ssp falcata has a more spreading growth habit and yellow flowers. Based on subspecies designation, the organelles of the sativa parent (an individual from population PI 299049, obtained from the USDA North Central Regional Plant Introduction Station at Ames,

Iowa) will be referred to as "S", and those of the falcata parent (an individual from population W71-42, described by Bingham, 1975) as "F".

Organelle DNAs of both parents and a total of 30 progeny were characterized.

DNA Isolation

Total cell DNA was isolated from leaf tissue by phenol/ chloroform extraction and CsCl ultracentrifugation (Maniatis et al, 1982). Plastid DNA was isolated by sucrose step gradients and CsCl ultracentrifugation (Palmer, 1986).

RFLP Detection

DNA restriction reactions were according to manufacturers instruction (Boehringer Mannheim). Restriction fragments from 3 µg plastid DNA or 4 µg total cell DNA were separated on 1 or 1.25% agarose gels at 20 or 25 V, blotted to nitrocellulose, and probed with pLecP4, which contains a 19.4 kb fragment of tomato plastid DNA (Phillips, 1985), or pmtSylSa8, which contains a 22.1 kb fragment of Nicotiana sylvestris mitochondrial DNA (Aviv et al, 1984). Probes were labeled through random priming (Boehringer Mannheim kit), and hybridization conditions were as described by Thomashow et al (1981).

RESULTS

A plastid DNA polymorphism was present in the parents that allowed us to follow plastid inheritance in the progeny. As seen in Figure 1, B, Hind II digests produced a 2.3 kb band unique to the F genome, and a 4.6 kb band unique to the S genome. The 4.6 kb fragment is visible in the gel as a double molar band (A, lane 2). In Msp I digests, a 2.1 kb band is unique to the F genome while a 3.5 kb band is unique to the S genome.

Thirty progeny were analyzed by restriction with both diagnostic enzymes. There was 100 % agreement in inheritance data between enzymes; only Msp I digests are shown. Eighteen progeny are shown in Figure 2. Plastid inheritance was strongly paternal in both directions of this cross. When the sativa subspecies was the paternal parent, all progeny had exclusively S plastid DNA (fig. 2, lanes 3- 12; 6 progeny not shown). When the falcata subspecies was the paternal parent, inheritance was also strongly paternal, but some maternal plastids were transmitted (fig. 2, lanes 13-20, and 6 progeny not shown). Of the fourteen M. s. ssp sativa x M. s. ssp falcata progeny analyzed, 12 had exclusively F plastids, 1 had exclusively S plastids, and one plant had both types of plastid DNA (lane 18 in fig. 2).

Even though plastid inheritance was largely paternal in our population, mitochondrial inheritance was entirely maternal. Differences in mitochondrial genomes between the falcata and

Figure 1. Ethidium bromide stained gel (A) and Southern blot (B) of purified chloroplast DNA (lanes 1-4) and total cell DNA (lanes 5-8) of parents. Unique fragments are indicated by fine arrows (A) and bold arrows (B). Lanes 1, 3, 5, and 7, F; lanes 2, 4, 6, and 8, S. Lanes 1-2, 5-6, Hind II (Hinc II) digest; lanes 3-4, 7-8, Msp I digest. Size markers from Hind III digests of lambda DNA are indicated. DNA fragments were separated on a 1.25% agarose gel at 20 V. The probe used in B was pLecP4 (Phillips, 1985).

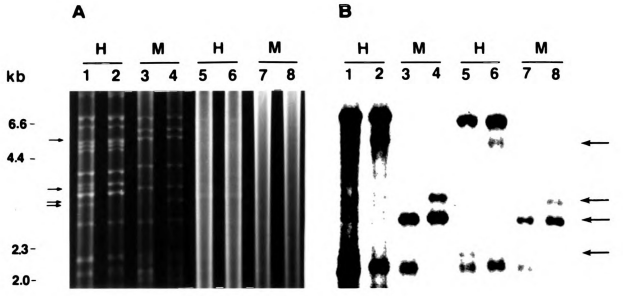

Figure 1

sativa parents allowed RFLP analysis of progeny, and can be seen in Fig. 3. Inheritance was strictly maternal in both directions of this cross, including the one progeny that was biparental for plastid type (lane 18).

Single shoot analysis of the plant carrying both parental types of plastid DNA (lane 18, figs. 2 and 3) can be seen in Fig. 4. Panel A indicates that individual shoots have sorted into pure tissue of one plastid type or the other, producing a fixed chimera. Vegetative sorting out has classically been observed via visible green and white sectors in leaves, and here is documented at the DNA level as well. Panel B indicates that mitochondrial type is strictly maternal in these shoots, with no trace of paternal input.

Figure 2. Southern blot of Msp I digested DNA of parents and progeny from a reciprocal cross, showing plastid DNA polymorphism. Lane 1, F; lane 2, S; lanes 3-12, progeny from the cross FxS; lanes 13-20, progeny from the cross SxF (maternal parent is listed first in crosses). Four µg total cell DNA was separated on a 1% agarose gel at 25 V.

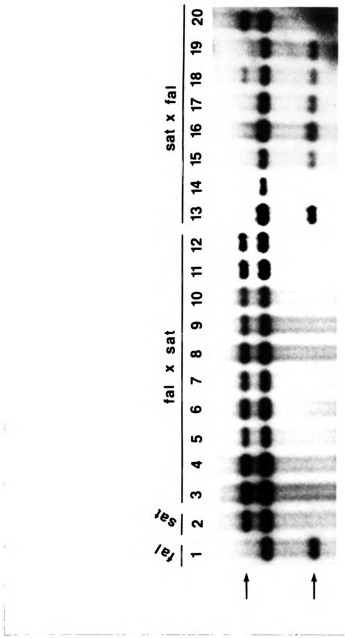
fal
sat
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
fal x sat
sat x fal

Figure 2

Figure 3. Southern blot of Msp I digested DNA of parents and progeny from a reciprocal cross, showing mitochondrial DNA polymorphism. Lane 1, F; lane 2, S; lanes 3-12, progeny from the cross FxS; lanes 13-20, progeny from the cross SxF. Blot is from the same filter as Fig. 2, which has been stripped and re-probed with pmtSylSa8 (Aviv et al, 1984).

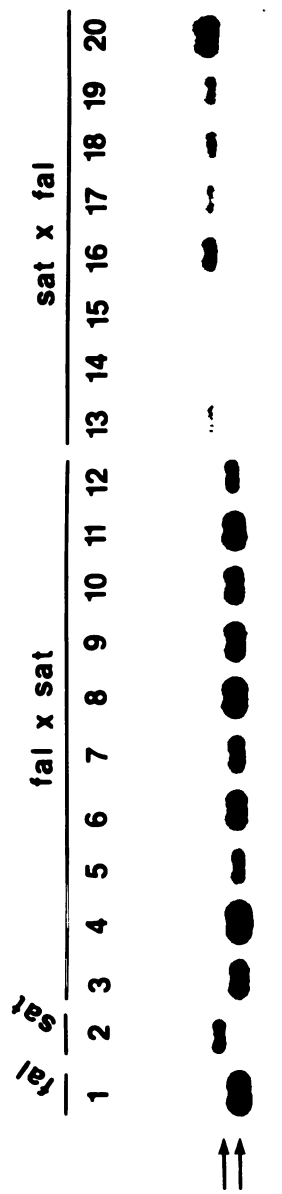
fal
sat
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
fal x sat
sat x fal

Figure 3

Figure 4. Southern blot of Msp I digested DNA extracted from single shoots of one plant (lane 18 in Figs. 2 and 3) from the cross SxF. Lane 1, F; lane 2, S; lanes 3-8, individual shoots. A, probed with labeled plastid DNA as in Fig. 2. B, probed with labeled mitochondrial DNA as in Fig. 3.

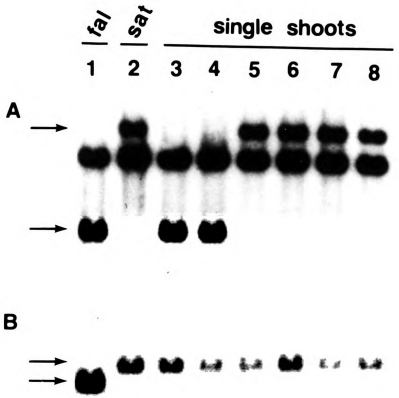

Figure 4

DISCUSSION

Although there have been reports of uniparental paternal inheritance of plastids in the angiosperm Pelargonium (Tilney-Bassett and Birkey, 1981), entirely paternal progeny have predominated only in a cross when the maternal plastids were white. It has been documented in Pelargonium that white plastids are less successfully transmitted than are green plastids (Kirk and Tilney-Bassett, 1978; Hagemann, 1979; Tilney-Bassett and Birky, 1981), and paternal transmission of green plastids over green maternal plastids has not been reported. Thus ours is the first report of paternal plastid inheritance in an angiosperm when both plastid types were phenotypically normal.

Our results of maternal mitochondrial inheritance are in contrast to a report by others of biparental inheritance of mitochondria in this species (Fairbanks et al, 1988). However, our results are similar to those observed in conifers and other plant species. Although paternal inheritance of plastids is common among gymnosperms, less data exists regarding the mode of mitochondrial inheritance. However, results of one study indicate that mitochondrial inheritance is maternal in Pinus taeda, loblolly pine (Neale and Sederoff, 1989). Maternal inheritance of mitochondria has been extensively documented in angiosperms, mammals, and insects (Connett, 1987; Lansman et al, 1981). Thus, there is little

precedent for other than strict maternal inheritance of mitochondria, even when plastid inheritance is strongly paternal.

The independent inheritance of chloroplasts and mitochondria in this population may suggest that two separate organelle exclusion mechanisms are operating. Since progeny from one cross (M. s. ssp sativa x M. s. ssp falcata) show three distinct types of plastid inheritance (uniparental paternal, biparental, uniparental maternal), it is likely that plastids from both parents were transmitted to the zygote followed by vegetative sorting out. The bias in favor of the paternal plastids may have occurred because there was an unequal input of plastids from the gametes of each parent. The zygotes may have initially contained more paternal plastids than maternal ones, and the maternal plastids may have been diluted out in subsequent cell divisions. It is also possible that paternal plastids were preferentially replicated, and ultimately outcompeted maternal ones. No evidence exists for paternal input of mitochondria.

Examples of nuclear control of plastid transmission have been described in Pelargonium (Kirk and Tilney-Bassett, 1978; Tilney-Bassett and Birky, 1981) and Petunia (Cornu and Dulieu, 1988), and variation for this trait may exist among Medicago genotypes as well. Such variation would reconcile our observations with those of others (Smith et al, 1986; Lee et al, 1988) who report biparental plastid inheritance in this species. These studies utilized mutant plastids, however,

which may have introduced a bias similar to that observed in Pelargonium.

Our sample size was too small to draw conclusions about differential strengths of the two plastid types, but since the S plastid was transmitted both maternally and paternally, while the F plastid was only transmitted paternally, it is possible that S may have a competitive advantage over F. Such a system operates in Oenothera (Kirk and Tilney-Bassett, 1978; Chiu et al, 1988).

The question of plastid strength in Medicago can be addressed by utilizing vegetative clones of the chimeric plant. This provides a system for comparing the transmission of the two plastid types in exactly the same nuclear background. Reciprocal crosses of these plastid types to a third plastid type in a different nuclear background will allow a direct comparison of plastid strength between the S and F types.

CHAPTER 2

INTRODUCTION

Eukaryotic cells are fundamentally distinct from prokaryotes by having a membrane-bound nucleus, which contains the majority of the cell's genes. The remainder of eukaryotic genes are located in specialized cytoplasmic organelles. Mitochondria, the eukaryotic organelle responsible for respiration, contains DNA that codes for components of respiratory complexes and its own transcription/translation system (reviewed by Tzagoloff et al, 1979). In addition to mitochondria, plants have a second type of DNA containing organelle -- the plastid. Plastids differentiate into specialized types, depending on function. Perhaps the most important plastid function is photosynthesis, which occurs in chloroplasts. Other important functions occur in chromoplasts, amyloplasts, and leucoplasts. Plastid DNA codes for components of photosynthetic complexes and its own transcription/translation system (reviewed by Whitfield and Bottomley, 1983). Much information has accumulated in recent years regarding regulation of chloroplast proteins. The multimeric protein ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the first step in the Calvin cycle and is the most abundant chloroplast protein, has been the focus of much of this research. It provides an interesting model for plant gene regulation,

because it is composed of both plastid-encoded and nucleus-encoded subunits, which assemble in equimolar amounts in the chloroplast to form the holoenzyme. The small subunit (SSU) is coded by a family of nuclear genes (rbcS) and is present in two to eight copies per haploid genome (Dean et al, 1989). Its mRNA is translated on cytosolic ribosomes as a precursor protein which contains an amino-terminal transit peptide. This transit peptide is a general characteristic of nucleus encoded proteins which function in the chloroplast, and is cleaved off during or shortly after import (Chua and Schmidt, 1979; Pfisterer et al, 1982; Robison and Ellis, 1984). The large subunit (LSU) is coded by a single gene on the plastid genome (rbcL), and is transcribed and translated in the chloroplast. The two types of subunits associate into a hexadecamer consisting of eight large and eight small subunits, which is located in the chloroplast stroma (reviewed in Ellis, 1981). Rubisco comprises up to 50% of soluble plant proteins, and is said to be the most abundant protein on earth.

The production of Rubisco requires coordinate expression of genes from two distinct genetic systems, the nuclear and plastid genomes. Although the protein products of the genes, SSU and LSU, combine in equimolar amounts, the cellular copy numbers of the genes themselves, rbcS and rbcL, are very different. As stated above, SSU genes are present in 2-8 copies per haploid genome. Thus diploid plants have a maximum of 16 copies per cell, and tetraploid plants have 32 or less. Cellular copies of LSU, however, are much higher than copies

of SSU. Although rbcL is generally present as a single copy on the circular plastid genome (reviewed in Whitfeld and Bottomley, 1983), there may be up to 900 copies of the plastome per chloroplast (Boffey and Leech, 1982). Additionally, there may be as many as 300 chloroplasts per cell (Possingham and Saurer, 1969). Thus rbcL may be present from 500 to as many as several thousand copies per cell (Bendich, 1987; O'Neal et al, 1987). The genes must be regulated quite differently to compensate for such dramatic differences in copy number.

Despite the separate location of genes coding for the subunits of Rubisco, little evidence exists for the overproduction of one or the other. Niveson and Stocking (1982) examined subunit accumulation along the length of barley leaves, where developmental differences in the amount of holoenzyme occur in the different aged cells. They found that although the rate of synthesis of LSU and SSU varied along the leaf, the ratio between the subunits was constant. Additionally, blocking synthesis of one subunit with a translation inhibitor in vivo resulted in a decrease in the synthesis of the other. They concluded that production of the two subunits was tightly coupled.

Regulation of SSU appears to occur at several levels. The amount of SSU mRNA present in leaves of dark grown plants varies tremendously between species, ranging from non-detectable levels in pea (Smith and Ellis, 1981; Bennett et al, 1984) to 50% of light levels in cucumber (Walden and

Leaver, 1981) and maize (Nelson et al, 1984). However, in all species examined, mRNA levels increase in response to both red and blue light, indicating transcriptional regulation by phytochrome and a blue light receptor (reviewed in Watson, 1989). Different members of the gene family may vary in light responsiveness, which could account for species variation in dark expression (Harpster and Apel, 1985). Polans et al (1985) suggest that clustering of rbcS and cab genes to one locus in pea may facilitate coordinate regulation. This hypothesis is supported by the observations that SSU mRNA is undetectable in dark-grown pea leaves (Bennett et al, 1984; Smith and Ellis, 1981). Perhaps in species that show significant levels of SSU expression in the dark, such as cucumber (Walden and Leaver, 1981) and maize (Nelson et al, 1984), the genes are scattered among several loci which are differentially responsive to light. This remains speculative, however, because rbcS genes have not yet been mapped in these species.

Recent studies from several groups indicate that a signal of chloroplast origin is required for transcription of rbcS, as well as other nucleus encoded chloroplast proteins. Photo-oxidation of plastids, achieved either through use of carotenoid mutants or through treatment with an inhibitor of carotenoid synthesis, resulted in a dramatic decrease in the levels of rbcS and cab mRNAs in maize (Mayfield and Taylor, 1987) and mustard (Oelmuller and Mohr, 1986), while levels of other nuclear mRNAs were unaffected. Post-translational

regulation may occur through instability and rapid turnover of free, unassociated subunits (Schmidt and Mishkind, 1983). However, it is thought that SSU levels are the limiting factor in assembly of the holoenzyme, so unassociated SSU may not occur at any appreciable level (Rodermel et al, 1988).

In contrast to the major role of transcriptional control in regulating levels of SSU, transcription of LSU and other plastid-encoded proteins may be template limited. Important post-transcriptional factors regulate plastid gene expression, including RNA splicing, message stability, and translational efficiency (Deng and Gruissum, 1987). Rodermel et al (1988) produced transgenic tobacco plants expressing an antisense SSU RNA. They observed greatly reduced levels of sense mRNA, a similar decrease in levels of small and large subunits, but near control levels of LSU mRNA. They concluded that LSU levels were being regulated translationally or posttranslationally, with little or no transcriptional regulation. Bendich (1987) suggests that the need for high plastid translation rates may demand high levels of rRNA, and this need can most easily be met by an increase in plastome copy number. Since transcriptional control plays a comparatively minor role in the regulation of organelle genes (Mullet, 1988), it is therefore possible that gene copy number of LSU could influence final levels of protein product.

Many studies have focused on the developmental regulation of plastome copy number. In young dividing cells of spinach, chloroplasts both divide and synthesize DNA; thus a low amount

of DNA per plastid is maintained. When cells stop dividing and begin to expand, plastid DNA synthesis outpaces chloroplast division, resulting in high levels of DNA per plastid. As cells approach a mature, fully expanded state, plastid DNA synthesis stops while the plastids continue to divide (Lawrence and Possingham, 1986). Thus plastids in fully expanded cells have somewhat lower amounts of DNA than those found in younger cells, although the amounts per cell may be similar. Similar developmental patterns of change have been observed in oats (Possingham et al, 1988), wheat (Boffey and Leach, 1982), and barley (Baumgertner et al, 1989).

Investigations into genetic variation in Rubisco level and plastome copy number have generally been done across nuclear ploidy levels of the same or related species. Rubisco content per cell has been found to be positively correlated with nuclear ploidy level in wheat (Dean and Leech, 1982; Leech et al, 1985) and alfalfa (Molin et al, 1982). Plastid DNA per cell has also been positively correlated with nuclear ploidy in wheat (Dean and Leech, 1982; Bowman, 1986) and alfalfa (Meyers et al, 1982).

Genetic variation for Rubisco content within a ploidy level has been observed in pea (Hobbs et al, 1990), and suggested to be due to differences in the rate of light-induced accumulation of rbcS transcripts. Variation in Rubisco content within a ploidy has also been observed in alfalfa, and shown to be regulated by nuclear genes (Daday et al, 1987). Based on information from other systems, Daday et

al postulate that variation in SSU gene expression is causing the Rubisco variation that they observed.

The purpose of this study was to examine genetic variation in Rubisco content and plastome copy number within a segregating population of tetraploid alfalfa that was known to exhibit variation in the number of chloroplasts present in mesophyll cells of similar size. Previous work has described high heritability of Rubisco levels in tetraploid alfalfa (Daday et al,1987) and correlation of Rubisco level with chloroplast number across ploidy (Molin et al, 1982). It was our goal to: 1) examine both of these parameters in the same genetic background, 2) determine whether differences in chloroplast number were reflected in different cellular plastome copy numbers, and 3) determine whether cellular plastome copy number influenced levels of Rubisco. Because Rubisco comprises such a significant proportion of the soluble protein in plants, total protein was also determined.

MATERIALS AND METHODS

Plant Material

Populations were produced from reciprocal crosses and from self-pollinations of genotypes of two subspecies of tetraploid alfalfa, Medicago sativa (described in Chapter 1).

Populations will be referred to by subspecies designation (ie F, ssp falcata; S, ssp sativa). Chloroplast number per mesophyll cell differed between the parents (24 in falcata vs 20 in sativa), and segregated in progeny populations (P. W. Callow, unpublished data; see Appendix 1). Plastids were shown to be inherited almost exclusively from the paternal parent in progeny from reciprocal crosses (Schumann and Hancock, 1989), so cytoplasmic effects could be examined by comparing reciprocal populations. One exceptional plant, from the cross falcata x sativa, was chimeric for plastid type. Shoots of this plant, which had segregated into pure tissue of one type or the other, were vegetatively propagated and analyzed separately.

Protein Purification

Rubisco was purified from leaf tissue of the falcata parent following the procedures of McCurry et al, 1982. Two hundred grams of leaves were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. All subsequent steps were at 4°C. Six hundred ml Bicine buffer (25 mM Bicine, 1mM EDTA, 10 mM EtSH, pH 8.0) was added and stirred into the frozen leaf powder to form a semi-frozen slurry. When the slurry thawed, it was filtered through 5 layers of cheesecloth and 2 layers of Miracloth (Calbiochem). The filtrate was then centrifuged for 15 min. at 9000 x g to remove any remaining cellular debris. The supernatant was decanted and made 37% saturated with ammonium sulfate, and

centrifuged for 45 min at 9000 x g. The supernatant was again decanted, ammonium sulfate increased to 50% saturation, and the precipitate collected by centrifugation. The precipitate was resuspended in 10 ml Bicine buffer and applied to a 2.5 x 75 cm Sepharose 4B (Sigma) gel filtration column which had been equilibrated in Bicine buffer. Ten ml fractions were collected and monitored by absorbance at 280 nm on a Gilford Response II spectrophotometer (Ciba-Corning, Oberlin, OH). Fractions representing the absorbance peak were pooled, concentrated by re-precipitation with ammonium sulfate, resuspended in a small volume of Bicine buffer and quantified by a modification of the Lowry procedure (Bensadoun and Weinstein, 1976).

Antibody Production

A polyclonal antibody was raised against Rubisco from alfalfa, and was used for quantifying the enzyme in crude extracts.

Injection Regime

Purified alfalfa Rubisco was dialyzed against Bicine buffer to remove residual ammonium sulfate and emulsified in Freund's adjuvent (Sigma) at 1 mg/ml. A New Zealand White rabbit was purchased from and housed at the Laboratory Animal Care facility on the Michigan State University campus. Workers at the facility performed the injections and bleedings. The initial injection was 1 mg antigen in complete adjuvent injected sub-cutaneously. Booster injections of 0.5

mg in incomplete adjuvent were at 14 and 28 days. Titer was tested just prior to booster injections, and blood was collected after 42 days. After clotting, crude sera was decanted and stored at -70°C for later use.

Protein Extractions

Greenhouse grown leaf material (0.5 g) was ground to a fine powder with liquid nitrogen in a mortar and pestle, and then blended with 10 ml buffer (50 mM Tris, 10 mM EDTA, pH 8.0). Cell debris were removed by low speed centrifugation (5000 x g, 10 min) and frozen at -20°C for later quantification of soluble protein and Rubisco.

Soluble Protein Quantification

Protein was quantified by a modification of the Lowry et al (1951) procedure that eliminates interference by Tris buffers (Bensadoun and Weinstein, 1976). Fifty µl aliquots of protein extracts were mixed with 1.5 ml dH_2O and 25 µl 1% DOC, and incubated at room temperature for 15 min. Five hundred µl 24% TCA was added, vortexed, and the protein precipitate collected by centrifugation (300 x g, 15 min). The precipitate was then disolved in 1.5 ml Lowry reagent C (2% Na_2CO_3, 0.02% Na-tartrate, 0.4% NaOH, 0.05% $CuSO_4.H_2O$), and incubated at room temperature for 10 min. Three hundred µl of 0.5 N Folin and Ciocalteu's phenol reagent (Sigma) was added and vortexed, and the absorbance at 660 nm determined exactly 30 min later on a spectrophotometer. All samples were done in

triplicate. A standard curve was generated using fraction V bovine albumin (Sigma) which was linear in the range of the unknown samples.

Rubisco Quantification

Rubisco quantity in crude extracts was determined by the enzyme-linked immunosorbant assay (ELISA) procedure (Clark and Adams, 1977). Wells of microtiter plates (Corning) were filled with 200 µl Rubisco antisera diluted 1000x in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$), incubated at 37°C for 4 hours, and washed 3x with PBS-Tween (140mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2.7mM KCl, 0.05% Tween-20, pH 7.4), with 3 min between washes. Crude protein extracts were diluted with blocking buffer (2% PVP, 2% BSA in PBS-Tween) to contain approximately 500 ng total protein (determined by Lowry as above) in 200 µl, and loaded into wells. Purified Rubisco standards of 10 ng to 1 µg were included on each plate, and five replicate plates were prepared. Plates were incubated at 37°C for 4 hours, then held at 4°C overnight. Wells were washed 3x with PBS-Tween as above, followed by a 4 hour, 37°C incubation with 200 ul Rubisco antisera (1000x dilution in blocking buffer). After washing as above with PBS-Tween, 200 µl goat anti-rabbit antisera conjugated to alkaline phosphatase (Sigma) was added to the wells (1000x dilution in blocking buffer). Plates were incubated at 37°C for 4 hours and washed 3x with PBS-Tween. Two hundred µl freshly prepared alkaline phosphatase substrate (1 mg/ml p-nitrophenol

phosphate in 10% diethanoleamine) was added to each well. The colorimetric reaction was slowed after 4 min by addition of 50 µl 3M NaOH to each well. Results were quantified by determining spectrophotometric absorbance at 405 nm with an EIA reader (Bio-Tek model EL-307, Bio-Tek Instr., Burlington, VT).

DNA Extraction

Two grams of greenhouse grown leaf material were ground to a fine powder with liquid nitrogen in a mortar and pestle, then allowed to thaw in 10 ml DNA extraction buffer (50 mM Tris, 10 mM EDTA, 1M NaCl, 0.1% Sarkosyl, pH 8.0). Cell debris was removed by low speed centrifugation (5000 x g, 10 min). Samples were extracted once with an equal volume of Tris-equilibrated phenol (pH 8.0) and once with an equal volume of chloroform/isoamyl alcohol (24:1). Nucleic acids were then precipitated by adding one volume of isopropanol, and scooped out with a glass hook. The samples were dissolved in TE, digested with RNase A (40 µg/ml) at 30°C for 1 hour, and the DNA precipitated by addition of NaCl (to 0.4 M) and 100% EtOH (2 volumes). DNA was collected by centrifugation, rinsed in 70% EtOH, air dried, and dissolved in TE. It was then quantified via its absorbance at 260 nm on a spectrophotometer.

Plastome Copy Determination

Plastome copy number was determined by the slot blotting

procedure (Riven, 1986), with three replicates. Known amounts of genomic DNA were denatured by heating to 100°C for 5 min in the presence of 0.4N NaOH, neutralized by the addition of NH_4-acetate (ammonium acetate, CH_3COONH_4) to 1M concentration, then applied to a nitrocellulose membrane (Biorad) through a slotted template. The template contained wells which were bevelled down to 6x1 mm openings, and was made of 1/4" plexiglass at the Engineering Research machine shop on the MSU campus. The apparatus was assembled into a sandwich consisting of 1/4" plexiglass backing, a 1 cm stack of paper towels (trimmed to exact size of template), 4 layers of dry filter paper (Whatman 3MM), one layer of filter paper wet in 1M NH_4-acetate, the nitrocellulose filter which had been wetted in 1M NH_4-acetate, and the slotted template which had been soaked in 1M NH_4-acetate and 100µg/ml sheared salmon sperm DNA (Sigma). The entire sandwich was clamped tightly together. DNA samples were applied in 56 µl volume, which wicked through the slot in 1-2 min. Wells were rinsed with 56 µl 1M NH_4-acetate. After the rinse had also wicked through, the sandwich was disassembled, the nitrocellulose air dried, then baked at 80°C for 2 hours under vacuum. Dilutions of linearized, denatured plasmid DNA containing the cloned DNA to be used as a probe were included on the filters as standards. The plasmid, pIC20R, contained a 1.2 kb fragment of the rbcL gene from pea (gift of J.Palmer).

For labeling, the cloned fragment was purified by agarose gel electrophoresis following digestion with the restriction

enzymes HindIII and PstI. The fragment was cut out of the gel, electro-eluted, precipitated with EtOH, and resuspended in an appropriate volume of TE. Twenty-five nanograms were radiolabeled by random priming (Boeringer-Mannheim kit) using α-^{32}PdCTP (NEN DuPont). Non-incorporated nucleotides were removed by spin-column centrifugation (Maniatis et al, 1982).

Southern hybridizations followed the protocol of Thomashow et al, 1981. Prehybridization was done for 2-4 hours at 68°C, in 6x SSC, 10x Denhardt's. Filters were placed in hybridization fluid (6x SSC, 5x Denhardt's, 20mM Tris, pH 7.6, 0.1% SDS, 2mM EDTA, 100ug/ml sheared salmon sperm DNA) at 68°C for 2 hours without probe, then the radiolabeled probe was added to 10^6 dpm/ml fluid. Hybridizations occurred overnight (approximately 18 hours). Filters were washed (6x SSC, 0.2% SDS, 5mM EDTA) for 2 hours with 4 changes of solution, and allowed to air dry. Bound radioactivity was quantified using a radioanalytic imaging system (Ambis Systems, San Diego, CA) located in the Department of Horticulture, Michigan State University. Plastome copy number was determined by comparison to known copies of probe in the plasmid standards, and converted to a cell basis by estimating nuclear DNA amount to be 4.4 pg. This estimate is from Winicov et al (1988), who determined the 1X amount in diploid alfalfa to be 1.1 pg by reassociation kinetics.

Abreviations

Bicine -- N,N-bis(2-hydroxyethyl)glycine

BSA -- bovine albumin, fraction V

DOC -- deoxycholate

EDTA -- ethylenediamine tetraacetic acid, disodium salt

EtOH -- ethanol

EtSH -- 2-mercoptoethanol

PVP -- polyvinylpyrolidone, mw 40,000

SDS -- sodium dodecyl sulfate

TCA -- trichloroacetic acid

TE -- 10mM tris, pH 8.0, 1mM EDTA

Tris -- Tris(hydroxymethyl)aminomethane

Tween 20 -- Polyoxyethylenesorbitan monolaurate

1x Denhardt's = 0.02% Ficoll (Sigma), 0.02% PVP, 0.02% BSA

1x SSC = 150mM NaCl, 15mM Na_3 citrate dihydrate, pH 7.0

RESULTS

The amount of Rubisco differed between the parents, with sativa (S) being significantly ($P<0.05$) higher than falcata (F) (Table 1). Progeny from the reciprocal crosses were not significantly different and population means of both families were skewed toward falcata, the low parent. Progeny from self pollinations of falcata did not differ significantly from the falcata parent, although progeny from self pollinations of sativa were lower than the sativa parent.

Parents and reciprocal populations showed similar relationships for total soluble protein content (Table 2). Between parents, sativa was significantly higher than falcata, and populations from reciprocal crosses did not differ significantly from each other. However, in contrast to being skewed toward the low parent for Rubisco content, they were skewed toward the high parent, sativa, for total protein. Falcata and sativa again responded differentially to selfing. The mean of self pollinated progeny from falcata was dramatically higher than that of falcata, while the mean of self pollinated progeny from sativa was much lower than that of sativa. This resulted in the falcata self pollinated population being higher than the sativa self pollinated population, a reversal of the relationship between parental falcata and sativa plants.

Figures 5 and 6 show the relationship between Rubisco and total protein content among progeny from reciprocal crosses

Table 1. Mean Rubisco level (µg/mg fresh wt) in parental genotypes and progeny populations, standard deviation (s), t test results, and level of significance (P) in pair wise comparisons (ns, not significantly different).

Comparison	N	Rubisco	s	t statistic
F	5	9.7	1.09	t=2.05
S	5	11.4	1.54	P<0.10
FxS	15	9.6	1.15	t=0.11
SxF	13	9.7	1.66	ns
F selfed	12	8.9	2.42	t=1.83
S selfed	8	7.6	0.48	P<0.10
F	5	9.7	1.09	t=0.92
F selfed	12	8.9	2.42	ns
S	5	11.4	1.54	t=5.35
S selfed	8	7.6	0.48	P<0.01

Table 2. Mean total soluble protein (µg/mg fresh wt) in parental genotypes and progeny populations, standard deviation (s), t test results, and level of significance (P) in pair wise comparisons (ns, not significantly different).

Comparison	N	Protein	s	statistics
F	5	15.3	2.0	t=7.33
S	5	24.1	1.8	P<0.001
FxS	15	22.6	5.9	t=.074
SxF	13	24.7	8.6	ns
F selfed	12	28.3	11.8	t=3.16
S selfed	8	16.7	3.9	P<0.01
F	5	15.3	2.0	t=3.70
F selfed	12	28.3	11.8	P<0.01
S	5	24.1	1.8	t=4.63
S selfed	8	16.7	3.9	P<0.01

between falcata and sativa (Figure 5) and among the progeny resulting from self pollinations (Figure 6). Although the two parameters are significantly correlated among progeny from reciprocal crosses, ($R=0.584$, $df=28$, $P<0.01$), there was substantial variation in the amount of Rubisco as a proportion of total protein (tabular values can be found in Appendix 2). Rubisco and total protein were not significantly correlated among progeny from self pollinations ($R=0.446$, $df=16$).

Plastome copy number per cell differed significantly between parents, with falcata being higher than sativa (Table 3). Both populations of self pollinated progeny were lower than the respective parents, although the relationship between them paralleled the differences between falcata and sativa. The populations from reciprocal crosses between falcata and sativa differed significantly, with the mean of progeny from the cross sativa x falcata being higher than that of falcata x sativa. Differences between reciprocal crosses indicates a cytoplasmic effect, usually maternal. However, in these populations plastids were inherited from the paternal parent. Thus the differences between reciprocals parallel the differences observed between parents when plastid source is considered. Plastome copy number was not significantly associated with Rubisco level among progeny from reciprocal crosses (Figure 7, $R=-0.200$, $df=23$) or among progeny from self pollinations (Figure 8, $R=0.447$, $df=13$).

Shoots of the chimeric plant which had different plastid

Figure 5. Correlation between Rubisco level and soluble protein content among progeny from reciprocal crosses between genotypes of falcata and sativa. Parents are indicated on the figure for comparison, but are not included in the correlation.

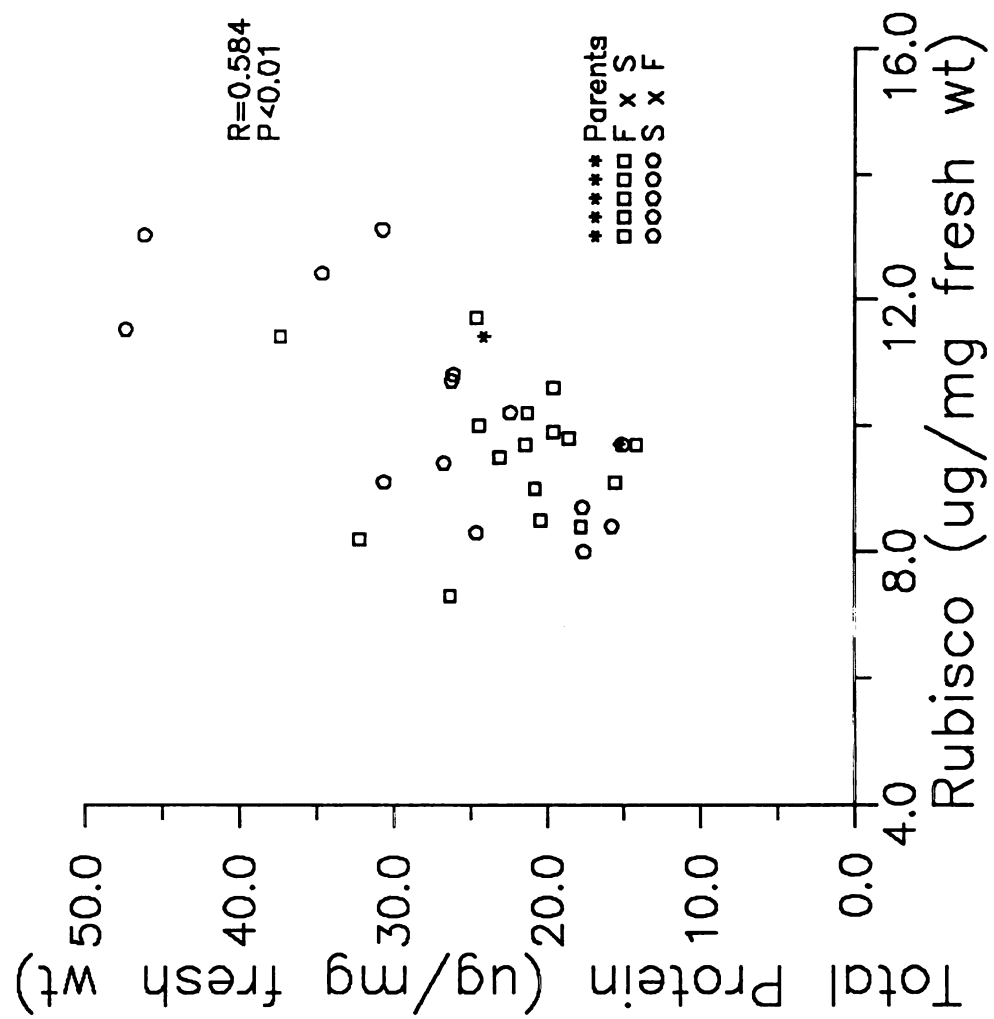
R=0.584
P<0.01
***** Parents
□□□□□ F x S
ooooo S x F
Total Protein (ug/mg fresh wt)
50.0
40.0
30.0
20.0
10.0
0.0
4.0
8.0
12.0
16.0
Rubisco (ug/mg fresh wt)

Figure 5

Figure 6. Correlation between Rubisco level and soluble protein content among progeny from self pollinations of genotypes of falcata and sativa. Parents are indicated on the figure for comparison, but are not included in the correlation.

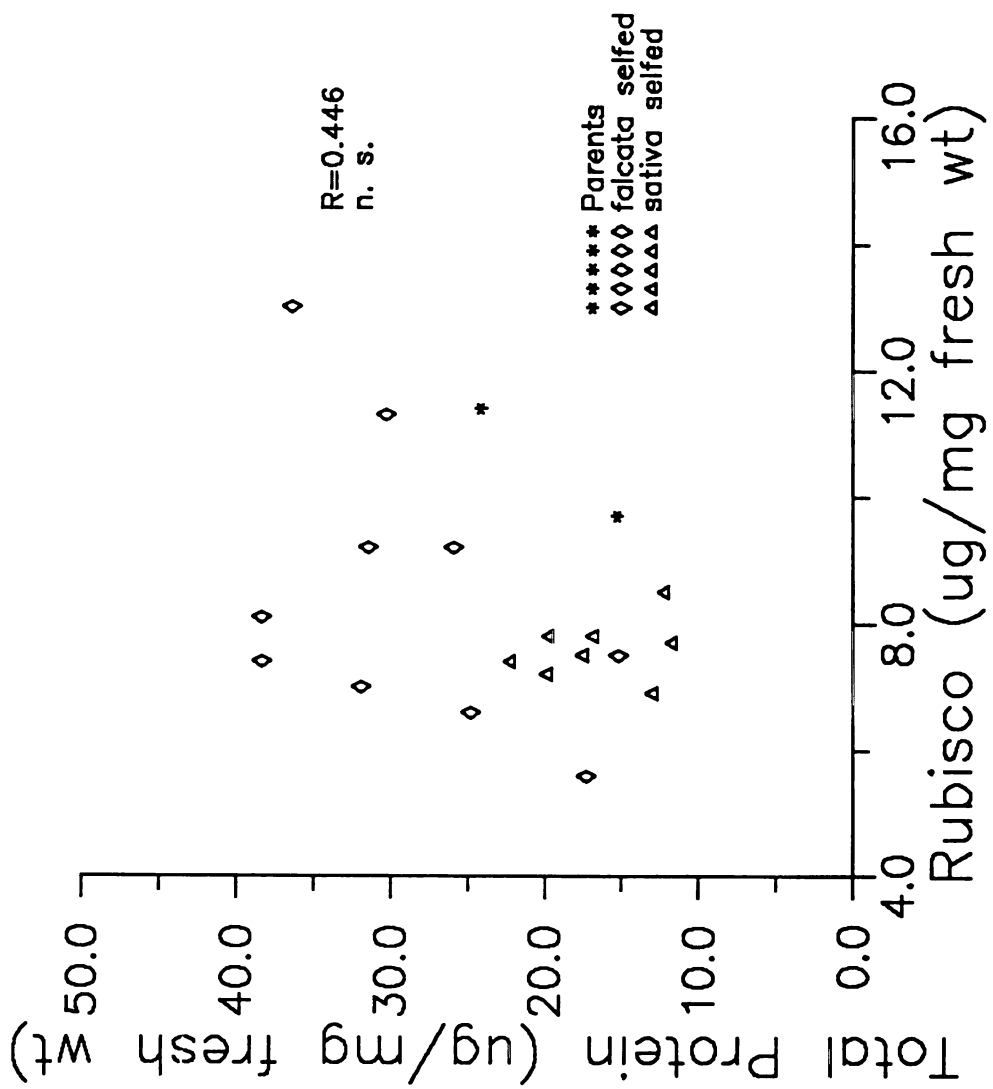
Total Protein (ug/mg fresh wt)
50.0
40.0
30.0
20.0
10.0
0.0
4.0
8.0
12.0
16.0
Rubisco (ug/mg fresh wt)
R=0.446
n. s.
***** Parents
◊◊◊◊◊ falcata selfed
ΔΔΔΔΔ sativa selfed

Figure 6

Table 3. Mean plastome number per cell in parental genotypes and progeny populations, standard deviation (s), t test results, and level of significance (P) in pair wise comparisons (ns, not significantly different).

Comparison	N	Plastomes	s	statistics
F	7	2155	284	t=3.93
S	7	1691	130	P<0.01
FxS	14	1845	895	t=2.21
SxF	11	2374	1093	P<0.05
F selfed	10	1810	384	t=3.28
S selfed	5	1098	402	P<0.05
F	7	2155	284	t=2.13
F selfed	10	1810	384	P<0.10
S	7	1691	130	t=3.19
S selfed	5	1098	402	P<0.05

Figure 7. Association between plastome copy number and Rubisco level in progeny from reciprocal crosses between genotypes of falcata and sativa. (R=-0.200, not significant)

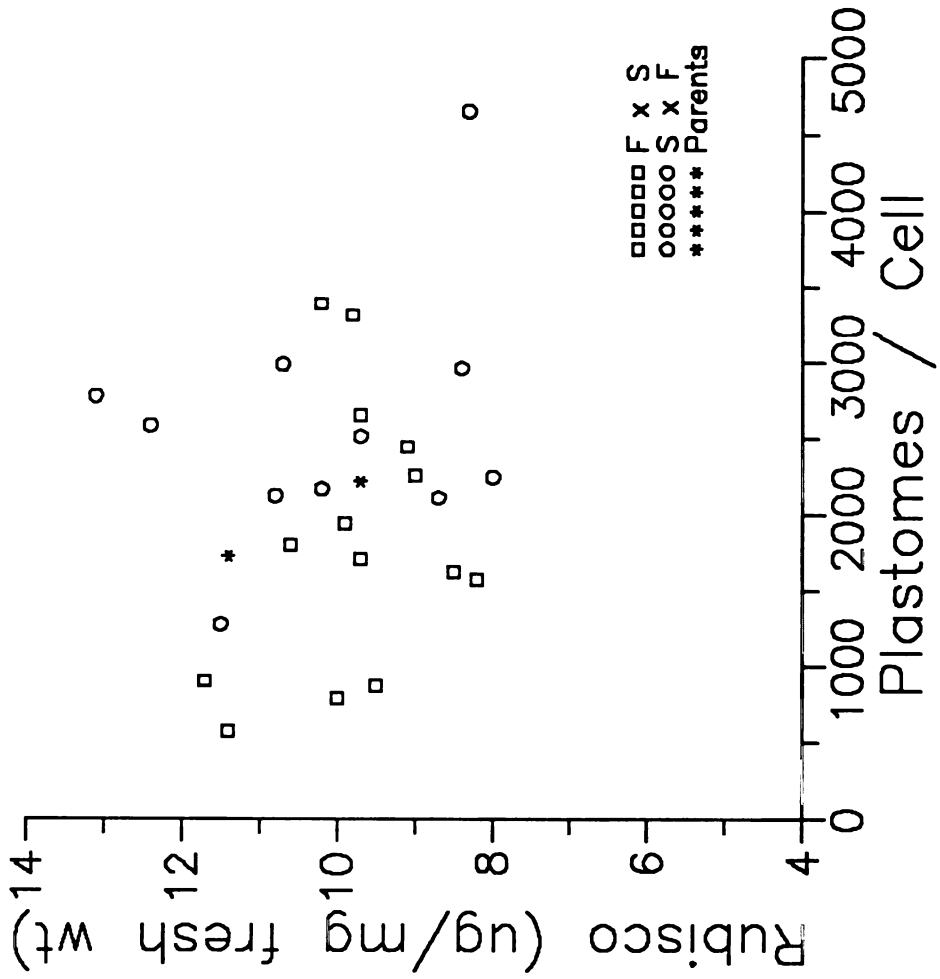
Rubisco (ug/mg fresh wt)
14
12
10
8
6
4
0
1000
2000
3000
4000
5000
Plastomes / Cell
□□□□□ F x S
ooooo S x F
***** Parents

Figure 7

Figure 8. Association between plastome copy number and Rubisco level in progeny from self pollinations of genotypes of falcata and sativa. (R=0.447, not significant)

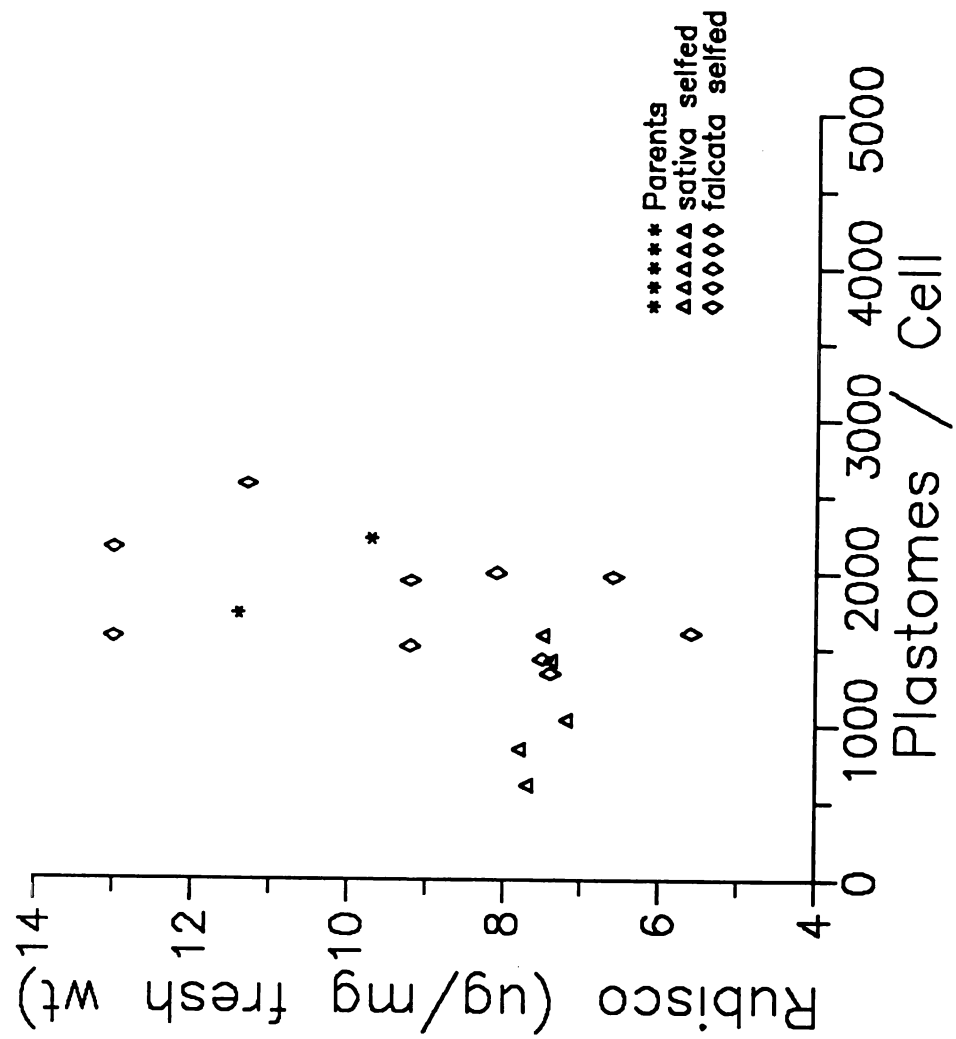
Rubisco (ug/mg fresh wt)
14
12
10
8
6
4
0
1000
2000
3000
4000
5000
Plastomes / Cell
***** Parents
ΔΔΔΔΔ sativa selfed
◊◊◊◊◊ falcata selfed

Figure 8

types in the same nuclear background showed no significant differences in Rubisco, total protein, or plastome copy number (Table 4). Values were lower than, or not significantly different than, the low parent, but were within the ranges of the progeny populations (Rubisco range: 8.0-13.1; Total protein range: 14.2-45.2; Plastome range: 574-3387).

Table 4. Rubisco content, total protein, and plastome copy number in parental genotypes and shoots of chimeric progeny plant.

Parent or cross	Rubisco (µg/mg f wt)	Protein (µg/mg f wt)	Plastomes per Cell
F	9.7 t=2.05	15.3 t=7.33	2155 t=3.93
S	11.4 P<0.10	24.1 P<0.001	1691 P<0.01
SxF-11(F)[1]	9.7 t=0.32	15.5 t=0.20	1368 t=0.21
SxF-11(S)[1]	9.4 n.s.	15.1 n.s.	1388 n.s.

[1] Plastome type is in parenthesis.

DISCUSSION

Data in Table 1 indicate that Rubisco amounts are more strongly controlled by nuclear genes than plastid ones, because there was no significant difference between reciprocal crosses. This result agrees with those of Daday et al (1987), who recovered high and low Rubisco lines of alfalfa after three generations of intercrossing and selection for nitrogen content. They also observed considerable variation in the proportion of cellular protein that was accounted for by Rubisco, which also agrees with our findings.

The differential response to selfing in our populations may be due to varying levels of sensitivity to inbreeding depression. Alfalfa, being an autotetraploid, is a classic example of a plant that does poorly after even moderate amounts of inbreeding (Busbice and Wilsie, 1966). Progeny from self pollinations of falcata were not significantly different than the parent for Rubisco amount, although progeny of sativa self pollinations were markedly lower. It may be that the falcata parent is more polymorphic than the sativa parent, and therefore tolerates the inbreeding effect of selfing better. This theory is supported by the observation of a low standard deviation (s) in the Rubisco population mean of progeny from sativa self pollinations relative to other populations in Table 1. This low standard deviation may have resulted from lack of segregation at rbcS loci, evidence of reduced heterozygosity in the sativa parent. This differential response to self pollination might account for

the reversal of parental rankings when comparing populations from self pollinations to each other.

Similar relationships between the populations were observed for total soluble protein amount. The sativa parent was higher than falcata, but progeny from self pollinations of sativa were quite low. However, progeny from self pollinations of falcata were much higher than falcata. The absence of inbreeding depression in progeny from self pollinations of falcata may partially explain this result, but does not explain why the mean of the population from self pollinations is higher than the parental plant. It is possible that differences between populations from self pollinations and parent plants were due to sampling errors that occurred from differential survival of inbred individuals containing various combinations of deleterious genes. However, since Rubisco levels were similar in falcata and progeny from self pollinations of falcata, the high protein level observed in the population resulting from self pollinations of falcata was not due to an increase in Rubisco level.

Plastome copies per cell differed between falcata and sativa. Falcata, the plant that had lower levels of protein and Rubisco, had a higher number of plastomes per cell than sativa. Interestingly, this plant also had a higher number of plastids per mesophyll cell than did sativa (24 vs 20; see Appendix 1). Thus the plastome copy numbers per plastid were similar (92 in sativa vs 86 in falcata). However, plastomes

per cell and plastids per cell were not significantly correlated in the progeny (Appendix 3). Since falcata and sativa have different nuclear backgrounds as well as different plastid types, it is impossible to distinguish between the effects of nuclear and cytoplasmic genes on plastid and plastome number in the parents. However, our populations from reciprocal crosses and self pollinations allow us to separate these effects.

Because of Mendelian inheritance, populations resulting from reciprocal crosses should have similar distributions of nuclear genes. However, we observed significant differences in plastome copy number between reciprocal populations, which implies a cytoplasmic effect. Since plastids were inherited from the paternal parent in these populations, the population that had a high plastome copy number (sativa x falcata) carried the plastid of the parent that had a high plastome copy number (falcata). Thus the cytoplasmic effect on plastome copy number may be due to plastid type. Plastome influences on the extent of biparental inheritance in Oenothera have been suggested to partially be due to differences in replication rates (Chiu et al, 1988). Similar differences in F and S plastomes may result in different final numbers per cell.

The plastid genomes did not have complete dominance over nuclear genes, as the chimeric plant did not have sectors showing significant differences in Rubisco amount, protein content, or plastome copy number. This indicates domination

by nuclear genes of all three parameters, and contrasts with our results from the reciprocal populations. Since Rubisco, protein, and plastome copy numbers in these shoots were near the low end of the population ranges, it is possible that this specific nuclear background contains a combination of genes which strongly regulate plastome copy number, and override plastid influences. A scenario of primary determination by nuclear genes with secondary influences by plastid type would also explain the lower plastome copy numbers observed in progeny from self pollinations relative to parents. These lower plastome numbers could be due to inbreeding depression resulting from loss of polymorphism of nuclear genes; it may be more pronounced in self pollinations of sativa than in self pollinations of falcata, either because of more extreme inbreeding depression, or because of differences in plastid type.

In summary, it appears from our study that nuclear genes are important in determining cellular levels of Rubisco, total soluble protein, and plastome copy number in alfalfa. However, the influence of the genes regulating plastome copy number may be modified by the plastome. Additionally, levels of all three parameters may be modified to varying degrees by the inbreeding effects of self pollination.

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APPENDICES

Appendix 1. Mean plastid number in isolated mesophyll cells of alfalfa populations (P. W. Callow, personal communication). Asterisks refer to significant differences in pairs of means.

Population	Plastid Number
ssp *sativa*	20.9
ssp *falcata*	24.1*
sativa selfed	20.9
falcata selfed	24.2**
sativa x *falcata*	23.7
falcata x *sativa*	24.2

* Significantly different at $P<0.05$

** Significantly different at $P<0.01$

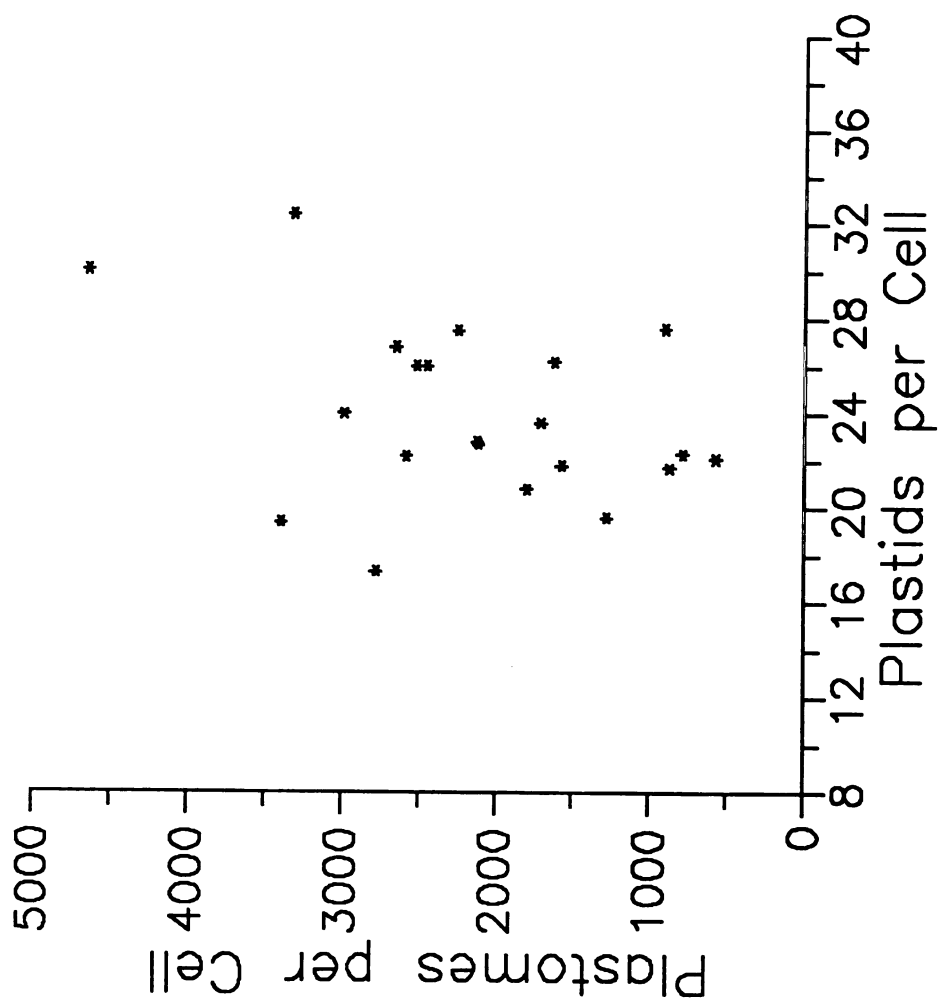
Appendix 2. Rubisco and total soluble protein in genotypes of alfalfa (µg/mg fresh wt). Fraction is the ratio of Rubisco to total protein.

Genotype	Rubisco	Protein	Fraction
F (M. fal)	9.7	15.3	.63
S (M. sat)	11.4	24.1	.47
FxS-1	9.5	23.1	.41
FxS-2	10.6	19.6	.54
FxS-3	9.1	15.6	.58
FxS-4	10.2	21.3	.47
FxS-5	9.9	19.6	.51
FxS-6	9.7	14.2	.68
FxS-7	9.8	18.6	.53
FxS-8	11.7	24.6	.48
FxS-9	11.4	37.3	.31
FxS-10	10.0	24.4	.41
FxS-11	8.2	32.2	.25
FxS-12	7.3	26.3	.36
FxS-13	8.5	20.4	.42
FxS-14	9.7	21.4	.45
FxS-15	9.0	20.8	.43
SxF-2	8.4	17.8	.47
SxF-3	8.7	17.7	.49
SxF-4	13.1	30.7	.43
SxF-5	8.0	17.6	.45
SxF-6	9.7	15.1	.64
SxF-7	10.7	26.2	.41
SxF-8	8.4	15.8	.53
SxF-9	8.3	24.6	.34
SxF-10	10.2	22.4	.46
SxF-12	11.5	45.3	.25
SxF-13	10.8	26.1	.41
SxF-14	12.4	34.6	.36
SxF-15	9.4	26.7	.35

Appendix 2 (continued)

Genotype	Rubisco	Protein	Fraction
F self 1	9.1	30.6	.30
F self 2	13.0	46.1	.28
F self 3	13.0	36.3	.36
F self 4	11.3	30.2	.37
F self 5	8.1	38.8	.21
F self 7	7.5	15.2	.49
F self 9	7.0	31.9	.22
F self 10	6.6	24.8	.27
F self 11	9.2	31.4	.29
F self 12	5.6	17.3	.32
F self 13	7.4	38.3	.19
F self 14	9.2	25.9	.35
S self 1	7.2	19.9	.36
S self 6	7.8	16.9	.46
S self 7	8.5	12.3	.69
S self 8	7.7	11.8	.65
S self 9	6.9	13.1	.53
S self 12	7.5	17.6	.43
S self 13	7.8	19.8	.39
S self 14	7.4	22.3	.33

Appendix 3. Association between plastids per cell and plastomes per cell in progeny from reciprocal crosses and self pollinations of genotypes of *falcata* and *sativa*. (R=0.355, not significant)


Plastomes per Cell
5000
4000
3000
2000
1000
0
8
12
16
20
24
28
32
36
40
Plastids per Cell

Appendix 3