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dissertation entitled
The Genetics of Plastid Transmission after Somatic Hybridization in Higher Plats: Plastid Transmission in Somatic Hybrids of Solanum lycopersicoides and Lycopersicon esculentum

presented by

Brent Lee Ridley

has been accepted towards fulfillment
of the requirements for

~~Master of Science~~ degree in ~~Plant Breeding~~ and Genetics

John F. Kelly
Major professor

Date October 22, 1990

THE GENETICS OF PLASTID TRANSMISSION AFTER SOMATIC HYBRIDIZATION IN HIGHER PLANTS: PLASTID TRANSMISSION IN SOMATIC HYBRIDS OF *Solanum lycopersicoides* AND *Lycopersicon esculentum*

By

Brent Lee Ridley

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1990

ABSTRACT

THE GENETICS OF PLASTID TRANSMISSION AFTER SOMATIC HYBRIDIZATION IN HIGHER PLANTS: PLASTID TRANSMISSION IN SOMATIC HYBRIDS OF *Solanum lycopersicoides* AND *Lycopersicon esculentum*

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Restriction fragment length polymorphisms were used to examine the plastid transmission of somatic hybrids of tomato with *Solanum lycopersicoides*. The plastids in 68 of 70 hybrids were from tomato. One hybrid, plant 240 had *S. lycopersicoides* plastids and another, plant 63 had a mixture of parental plastids. Plastid DNA from several branches was analyzed to follow plastid segregation in hybrid 63. This hybrid had sorted into branches with pure plastids, although one branch may have had a plastid mixture.

Plastid transmission after somatic hybridization is reviewed in consideration of plastid evolution and genetics. Plastid genomes are highly conserved during evolution, and highly resistant to genetic changes during somatic hybridization. Plastid fusion and plastid DNA recombination are very rare after either somatic or sexual hybridization. Plastids segregate rapidly, and often stochastically in both sexual and somatic hybrids. When plastid transmission deviates from random behavior it is probably for similar reasons in both somatic and sexual hybrids.

I would like to dedicate this thesis to my parents whose love and support has provided me with so many possibilities.

ACKNOWLEDGMENTS

I am very grateful to Dr. David Dilley and Dr. James Hancock for their guidance. I would like to thank Dr. Kenneth Sink for making this research possible. I would especially like to thank my major professor Dr. John Kelly for his support. I am grateful to Dr. Barbara Sears for her expert advice and critical review of my manuscripts. I also appreciate the efforts of Dr. Thomas Friedman as a member of my guidance committee. I appreciate the technical advice I received from Dr. Jeff Palmer. I am grateful to Dr. A. L. Phillips for the tomato cpDNA library, Dr. C. M. Rick for seed of LA 1990, Dr. Patrick Moore for parental nuclear DNA samples, and Mr. Amnom Levi for parental mitochondrial DNA samples. I am also grateful to Mrs. Marlene Camron and Mr. Amnom Levi for assistance with the plant propagation

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PAGE

CHAPTER 1

Literature Review

Introduction

About two-thirds of the angiosperms investigated have maternal inheritance of plastids during sexual reproduction, and most of the remaining species have bi-parental inheritance (Kirk and Tilney-Bassett, 1978; Sears, 1980b). Maternal inheritance precludes the independent assortment of agronomically important cytoplasmic traits in almost all crop plants. Independent assortment of cytoplasmic genomes would facilitate the breeding of improved crop plants. Agronomically important mitochondrial traits include cytoplasmic male sterility and resistance to some diseases (Pring and Lonsdale, 1985). Plastomes (plastid chromosomes) encode atrazine resistance (Robertson, 1985), resistance to certain diseases (Vaughn and Duke, 1984), and control of the rate of photorespiration (Ogren, 1984).

Artificially induced fusion of protoplasts from plants with different cytoplasmic genomes produces heteroplasmic cells. Subsequent sorting of the organelles during cell division often results in novel combinations of cytoplasmic genomes. The plastomes in somatic hybrid plants are almost always transmitted without recombination and often are inherited randomly from either parental protoplast (Fluhr 1983; Kumar and Cocking, 1987). Shoots with mixed plastids are rare because sorting out usually is completed before shoot regeneration can occur. This is a consequence of the rarity of plastid fusion, which precludes cpDNA (chloroplast DNA) recombination (Possingham and Lawrence, 1983), and rapid sorting out of plastids during cell division (Akada and Hirai, 1986). In contrast, mitochondrial genomes almost always undergo recombination or rearrangement in somatic hybridization experiments (Hanson, 1984). Mitochondria probably sort more slowly than plastids in somatic hybrids, and this may stem from the prevalence of mitochondrial fusion (Bendich and Gauriloff, 1984) and mtDNA (mitochondrial DNA) recombination (Izhar *et al.*, 1983; Aviv and Galun, 1986; Aviv and Galun, 1987).

Plastid Biochemistry, Evolution, and Genetics

Photosynthesis is the primary function of plastids, but they have other important metabolic duties as well. Plastids participate in chlorophyll, amino acid and fatty acid biosynthesis; nitrate and sulfate reduction; and starch metabolism (Kirk and Tilney-Bassett, 1978). About eighty to ninety percent of the proteins found in plastids are encoded in the nucleus, translated on cytosolic ribosomes, and transported into the organelles. The remaining proteins are plastome encoded, and most function in the plastid transcription and translation systems, photosynthetic complexes, and chlororespiration system (Ohyama *et al.*, 1988; Wu and Yang, 1989). Plastome genes are transcribed and translated in the plastid by enzymes which are distinct from those found in the nucleus and cytosol.

The evolutionary origin of plastids and the physiological processes involved in the transmission of their genetic information have impacted the structure of modern plastomes. Careful examination of the plastomes in this context provides information regarding the mechanisms controlling their inheritance. Consequently, review of the relevant issues of plastome evolution, sexual inheritance and somatic transmission is useful in understanding plastid transmission in cell fusion hybrids.

From an overall perspective the plastid genome is more evolutionarily conserved than other organelle genome (Palmer, 1985d). Plastids probably originated as photosynthetic procaryotes which became endosymbiotic after they were engulfed by the predecessors of modern eucaryotes (Wallace, 1982; Gray, 1983). These organelles have evolved extensively from their procaryotic ancestors, mainly through the transfer of most of their genetic functions to the nucleus (Palmer, 1985d; Brinkmann *et al.*, 1987). Plastids and cyanobacteria, their putative ancestors, have a very similar arrangement of some their genes (Palmer, 1985a). Furthermore, the sequences of plastid genes have considerable homology to cyanobacterial genes having similar functions. However, almost all cpDNAs examined from diverse groups of plants and algae are consistently 20-30 times smaller than their presumed

ancestors. Nearly all cpDNAs analyzed are circular molecules between 120 and 217 kilobase pairs (kb) in size (Palmer, 1985b).

This rather tight conservation of size is observed in all of the over 200 angiosperms examined, in representatives of several groups of land plants outside the angiosperms, and in representatives of several groups of algae. In contrast, plant mitochondrial genomes vary greatly in size, ranging in kinetic complexity from 200 to 2000 kb in the angiosperms (Palmer, 1987). The difference in the conservation of size between the genomes of different organelles suggests that selection may restrict plastid genome size. The uniformity of plastid genome size suggests that the reduction occurred soon after the beginning of endosymbiosis, and the genome remained relatively stable thereafter (Palmer, 1985a).

The angiosperm plastome architecture and genic content has been studied intensively and such analyses provide interesting evolutionary insights (Palmer, 1985c). Plastomes are amenable to molecular analysis of this sort because they are relatively small. Unfortunately, cpDNA analysis of land plants outside the angiosperms has been limited to a few observations in the gymnosperms, ferns and liverworts. Tight conservation of architecture and coding complement are the most striking features of plastomes. The cpDNA of spinach was the first to be investigated thoroughly and it has the putative angiosperm ancestral gene arrangement (Palmer, 1987; Figure 1).

The most distinctive features of the plastome map are the large inverted repeats (Figure 1). In land plants these repeats always contain the rRNA (ribosomal RNA) genes and are always located asymmetrically, dividing the genome into small and large single-copy regions. Plastid genomes with large inverted repeats are often devoid of any other repeated sequences (Palmer, 1985b). However, direct repeats are found in the green algae *Chlamydomonas* (Gelvin and Howell, 1979), and tandom repeats are found in the angiosperm *Oenothera* (Blasko *et al.*, 1988) in addition to the large inverted repeats. Plastid DNAs rapidly undergo homologous recombination across the large inverted repeats (Palmer, 1983). Hence cpDNAs exist as an equal proportion of two isomers (flip-flop isomers), each having the small single-copy region in an opposite orientation relative to the large single-copy region.

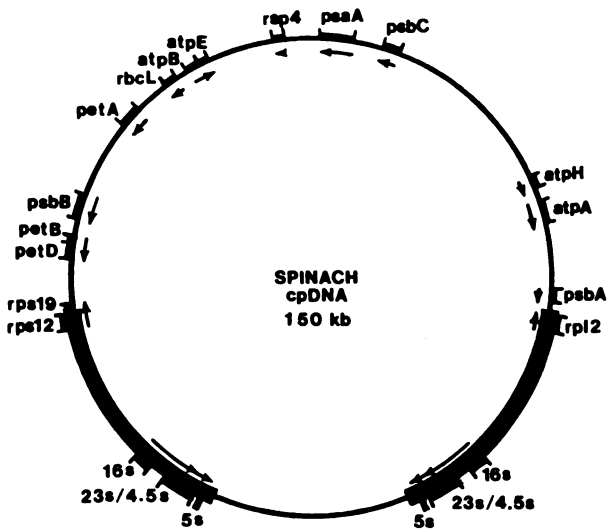

Figure 1. The gene map of spinach plastid DNA. The abbreviations for the genes are standard (Hallick and Bottomley, 1983). Redrawn from Palmer, (1985a).

The size of the inverted repeat varies from 10 kb to 76 kb in different angiosperms; most differences in overall plastome size result from differences in the size of the inverted repeat (Palmer, 1985c). In fact, the range of plastome size for all land plants examined is only 110-150 kb when variation due to the inverted repeat is removed (Palmer, 1987). The inverted repeats are a general feature of angiosperm cpDNAs, and probably the cpDNAs of all land plants. However, this conclusion is based on limited observations in a small number of groups outside the angiosperms.

The arrangement of genes encoded by the cpDNA is highly conserved among the land plants which have been examined (Palmer, 1985d). The order of cpDNA sequences is essentially the same between the angiosperm spinach, the gymnosperm *Ginkgo biloba*, and the fern *Osmunda cinnamomea* (Palmer and Stein, 1986). The entire plastome of the angiosperm *Nicotiana tabacum* (Shinozaki *et al.*, 1986b) and the liverwort *Marchantia polymorpha* (Ohyama *et al.*, 1986) has been sequenced, and the genes and open reading frames are arranged in nearly the same order, except for a large inversion in the large single-copy region. When the orders of all available cpDNA sequences from land plants are compared, many of the differences between them can be attributed to single inversions within the single-copy regions (Palmer and Stein, 1986). This contrasts with those plants

lacking the large inverted repeat, which have incurred many cpDNA rearrangements (Palmer *et al.*, 1987; Strauss *et al.*, 1988).

With just a few exceptions the same set of genes is encoded by the plastid genomes of all land plants and two algae, *Chlamydomonas reinhardtii* and *Euglena gracillis* (Palmer, 1985a). Consequently, the nuclear genomes of diverse organisms probably encode a similar set of plastid proteins. The plastome is large enough to encode more than 100 proteins and stable RNAs. Measurements of the proportion of the plastome transcribed into RNA suggest that most of this coding potential is utilized in diverse organisms, including *E. gracillis* (Koller and Delius, 1984), *C. reinhardtii* (Howell and Walker, 1977), and angiosperms (Oishi *et al.*, 1981; Poulsen, 1983). Nucleic acids have not been shown to cross the plastid membrane so cpDNAs probably encode all the stable RNAs of the organelle and any proteins synthesized on plastid ribosomes (Ellis, 1981). Isolated pea chloroplasts synthesize up to 80 soluble peptides *in vitro* (Ellis *et al.*, 1980). The gene content encoded by the cpDNA sequences of *N. tabacum* and *M. polymorpha* is strikingly similar (Shinozaki *et al.*, 1986b; Ohyama *et al.*, 1986). Tobacco has 122 and *M. polymorpha* has 121 different genes and unidentified opened reading frames. About 100 of these have been identified either functionally or based on sequence homology with genes from other organisms. The coding complement is essentially conserved between these distantly related plants (Weil, 1987).

Convincing evidence for the endosymbiotic origin of plastids is based on comparison of the sequence and organization of plastid and bacterial rRNA genes. The rRNA genes are always plastid-encoded, without exception, in numerous plants examined thus far (Palmer, 1985a). The 16S rRNA, 23S rRNA and the intergenic spacer are oriented the same way and cotranscribed similarly in eubacteria and plastids (Figure 2). This large transcript also includes tRNA genes for isoleucine and alanine within the intergenic spacer, and these are arranged in the same orientation relative to the rRNA genes in eubacteria and plastids. This precursor is cleaved to yield the individual RNA products in both cases (Crouse *et al.*, 1984). The 5S rRNA gene is part of this transcript in eubacteria, but probably is transcribed independently in angiosperm plastids (Bohnert *et al.*, 1982).

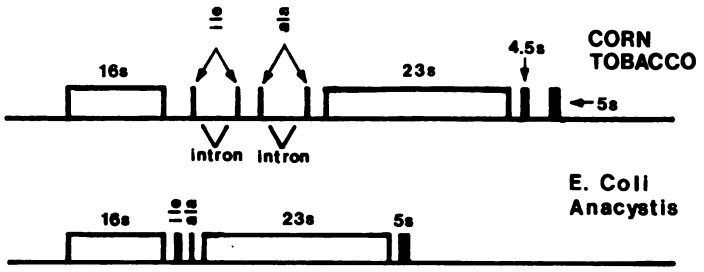

Figure 2. Ribosomal RNA operons of plastids and eubacteria. Abbreviations for the gene products are standard. Redrawn from Palmer (1985a).

Comparison of the 16S and 23S rRNA gene sequences from the cyanobacteria *Anacystis nidulans*, to the sequences from angiosperm plastids reveals remarkable conservation (Gillham *et al.*, 1985). There is 83% homology between the 16S rDNAs and 78% homology between the 23S rDNAs of *N. tabacum* and *A. nidulans* (Palmer, 1985a). To add perspective to this, the 16S and 23S rDNAs of the monocot *Zea maize*, and the dicot *N. tabacum* are 96% and 92% homologous, respectively (Tomioka and Sugiura, 1983; Kumano *et al.*, 1983; Douglas and Doolittle, 1984; Palmer 1985a). The secondary structure of rRNAs are also highly conserved between eubacteria and plastids. Glotz *et al.* (1981) observed over 450 compensating base changes between *Zea maize* and *Escherichia coli* in rRNA regions that can form stem-loop structures. Many of the regions that are highly conserved in plastids are those that are functionally important in eubacteria, such as the region in the 3' end of the 16S rRNA, which is complementary to the Shine-Delgarno sequence (involved in ribosome binding) found on all procaryotic mRNAs. Most chloroplast mRNAs can be translated efficiently on *E. coli* ribosomes, but not on plant cytoplasmic ribosomes (Bottomley and Whitfield, 1979). This demonstrated the functional similarity of the plastid and eubacterial translational apparatus.

Studies of several groups of land plants and algae indicate that all plastomes probably encode a complete set of tRNA genes (Crouse *et al.*, 1984). The cpDNA of *M. polymorpha* encodes 32 species of tRNA (Ohyama *et al.*, 1986), and that of *N. tabacum* encodes 30 (Shinozaki *et al.*, 1986b).

These tRNAs are sufficient to read all 61 codons which signify amino acids, when the wobble rules and anticodon base modifications are considered (Weil, 1987). The *Euglena* plastome tRNAs are encoded polycistronically, in clusters of two to six genes. In contrast, the plastid tRNA genes of land plants are not clustered significantly and are transcribed independently, with the exception of the two tRNAs in the rRNA intergenic spacer (Palmer, 1985a). The sequence of plastid tRNAs is highly conserved between plants and algae (Gillham *et al.*, 1985). In fact, the tRNAs of plastids have much more sequence homology to their eubacterial tRNA analogues than to cytoplasmic analogues from the same plant. The secondary structure of most domains of the tRNAs are also highly conserved between plastids and eubacteria.

About 20 of the 60-65 plastid ribosomal proteins are encoded by cpDNAs, and the remainder are nuclear-encoded (Palmer, 1985a). Nineteen putative plastid ribosomal protein genes have been identified in both *N. tabacum* and *M. polymorpha* by comparison of the amino acid sequence (deduced from the DNA sequence) with that of the homologous genes from *E. coli* (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986b). About half of these are organized in clusters reminiscent of those in *E. coli*, and the others are scattered throughout the genome. Furthermore, some plastid ribosomal proteins have considerable immunological homology with their eubacterial homologues (Dorne *et al.*, 1984). Plastome-encoded plastid ribosomal proteins from *C. reinhardtii* have considerably more antigenic, size and charge similarities to eubacterial ribosomal proteins than do those that are nuclear-encoded and imported from the cytoplasm (Schmidt *et al.*, 1984). This may indicate slower evolution of plastid-encoded genes relative to nuclear ones and/or selective transfer of genes which are less functionally constrained to the nucleus (Palmer, 1985a).

Plastid genomes encode about 16 of the polypeptides which form the four supramolecular complexes (the ATPase complex, the cytochrome b6-f complex, and the photosystem I and II complexes) essential for the light reactions of photosynthesis (Weil, 1987). The arrangements and transcription patterns of several of these genes also resemble their procaryotic equivalents (Palmer, 1985a).

Table 1. Comparison of the deduced amino acid sequence homologies of some plastid and *E. coli* genes (Palmer, 1985a). The abbreviations for the genes are standard (Hallick and Bottomley, 1985).

	rbcL	atpB	atpE	atpH	atpA
Dicot-dicot					
Pea-spinach	94	92	82	--	--
Tobacco-spinach	93	96	87	--	--
Monocot-dicot					
Maize-tobacco	90	91	70	--	--
Wheat-spinach	--	--	--	100	--
Monocot-monocot					
Maize-barley	95	97	98	--	--
Angiosperm-*E. coli*					
Tobacco-*E. coli*	--	62	27	--	54
Spinach-*E. coli*	--	67	26	30	--
Maize-*E. coli*	--	58	23	--	--

Six of the eight subunits of the chloroplast ATPase complex are encoded by cpDNAs (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986b), and the sequences of four of these (atpA, atpB, atpE, and atpH) have been compared (Table 1). These genes have 82-100% amino acid sequence homology among diverse angiosperms and 30-67% homology between the angiosperms and *E. coli* (Palmer, 1985a). Five genes from photosystem II are encoded by angiosperm cpDNAs. The gene for the 32 kilodalton (kd) photosystem II thylakoid membrane protein which binds the herbicide atrazine, has been sequenced in several organisms. This gene is extremely conserved among diverse groups of organisms. The conservation of amino acid sequence of the 32 kd protein among five distantly related angiosperms approaches 100%, and is about 87% between *Euglena* and the angiosperms (Palmer, 1985a).

Plastid genomes also encode a component of one enzyme of the Calvin cycle, the large subunit of ribulose-1-5-bisphosphate carboxylase-oxygenase (RuBisCO). The sequence of the gene for the large subunit of RuBisCO (rbcL) has been compared among several angiosperms, the green algae *Euglena gracilis*, two species of cyanobacteria, and a nonsulfur purple bacterium (Palmer, 1985a). The amino acid sequence of this gene is highly conserved in every case except between the

cyanobacteria-plastid group and the nonsulfur purple bacteria (Table 1), attesting the close evolutionary relationship of plastids and cyanobacteria.

The regulatory sequences of plastid genes parallel those of procaryotes. Plastid and *E. coli* promoter consensus sequences have nearly perfect homology in both the -10 and -35 regions (Briat *et al.*, 1986; Kung *et al.*, 1986; Zurawski and Clegg, 1987). Furthermore, at least some plastid promoters function with the *E. coli* transcriptional machinery in cell-free systems (Bottomley and Whitfield, 1979) or when cpDNA is cloned randomly into a plasmid vector designed to select promoter sequences (Kong *et al.*, 1984; Kung and Lin, 1985). Plastid termination sequences also resemble procaryotic sequences (Palmer, 1985a). Short inverted repeats which can form hairpin loops are characteristic of *E. coli* rho-independent terminators and are found downstream from the coding regions of many plastid genes (Crouse *et al.*, 1984). However, evidence for rho-dependent termination in plastids is lacking (Zurawski and Clegg, 1987). Most plastid mRNAs have a highly conserved ribosome-binding site (Shine-Delgarno sequence) which is complementary to the 3' end of the plastid 16S rRNA (Kozak, 1983). It is clear from this and other evidence that plastid transcription and translation strikingly resemble that of procaryotic organisms.

Nevertheless, in plastid genomes the polycistronic expression of related genes found in procaryotes has often been dismantled (Palmer, 1985a). This is probably the result of the transfer of many genes to the nucleus (Brinkmann *et al.*, 1987). In addition, rearrangements have dispersed genes more randomly throughout the plastome (Palmer, 1985b). Many genes belong to smaller transcriptional units or are transcribed independently, although some of the ancient operons, such as the rDNA operon, remain virtually intact (Whitfield and Bottomley, 1983). Most of these changes probably occurred very early in the evolution of land plants since most plastid genomes have nearly the same complement of genes in nearly the same arrangement.

The plastome of the alga, *Euglena gracilis* has at least 50 introns (Koller and Deluis, 1984). In contrast, the plastid genomes of land plants have few introns. The tobacco plastome has 15 introns and the *M. polymorpha* plastome has 18 introns, in both protein-coding and tRNA genes (Shinozaki *et al.*, 1986b; Ohyama *et al.*, 1986). In addition, the mRNA from the *N. tabacum* plastid gene encoding

the ribosomal protein S12 is trans-spliced from two independent transcriptional units (Hildebrand *et al.*, 1988; Plant and Gray, 1988). Two groups of introns with different excision mechanisms have been detected in plastomes (Shinozaki *et al.*, 1986a; Weil, 1987; Plant and Gray, 1988) using sequence or structural similarities to well characterized examples from other genetic systems (Sharp, 1985). Group I introns, which splice autocatalytically *in vitro*, are structurally homologous to introns in the rRNA gene *Tetrahymena* and some mitochondrial introns of fungi (Shinozaki *et al.*, 1986a; Plant and Gray, 1988). Most of the introns of land plant plastid genomes belong to group II (Plant and Gray, 1988). Group II introns sometimes splice autocatalytically *in vitro* and can be folded into secondary structures similar to those postulated for the introns in maize mitochondrial cytochrome oxidase, yeast cytochrome oxidase and yeast cytochrome b (Michael and Dujon, 1983).

The only angiosperms lacking the large inverted repeat, of 200 or more species examined, belong to eight related genera of the legume family; *Pisum*, *Vicia*, *Lathyrus*, *Cicer*, *Medicago*, *Trifolium*, *Melilotus*, and *Wisteria* (Palmer, 1985a). Interestingly, the cpDNA sequences of most members of this group are highly rearranged relative to the ancestral order and these changes have occurred after the loss of the inverted repeat (Palmer *et al.*, 1987). Two gymnosperms; the Pinaceae, *Pseutosuga mensiesii* and *Pinus radiata*, also are missing the inverted repeat and have incurred many cpDNA rearrangements (Strauss *et al.*, 1988). The extensive rearrangements occurring in the cpDNAs of those species having lost the large inverted repeat suggest the repeat has a stabilizing influence (Palmer *et al.*, 1985; Strauss *et al.*, 1988).

It is readily apparent from the above discussion that plastid genomes have evolved slowly. However, the physiological and/or genetic causes of this are more difficult to ascertain. Perhaps the dense arrangement of genes in cpDNAs inhibits rearrangement since most rearrangements would disrupt functional sequences (Palmer, 1985b). However, rearrangement is common in the smaller plastid genomes of species having lost a large inverted repeat suggesting that other mechanisms are involved (Palmer *et al.*, 1987; Strauss *et al.*, 1988).

It has been difficult to demonstrate intermolecular recombination of cpDNAs in higher plants (Chiu and Sears, 1985), and genetic or molecular evidence of it is available only from two experiments

with higher plants (Medgyesy *et al.*, 1986; Thanh and Medgyesy, 1989), and from species of *Chlamydomonas* (Sears, 1980a; Mets and Geist, 1983; Lemieux *et al.*, 1984). In both cases, the recombinant molecules have crossover points at frequent intervals, as determined by genetic and restriction enzyme analysis. The presence of many crossover points is evidence for a mechanism involving gene conversion (non- reciprocal recombination).

If gene conversion is the major mode of intermolecular recombination in plastids, it probably occurs intramolecularly as well. Accordingly, both large inverted repeats from several organisms have been sequenced, and they are identical (Kato *et al.*, 1981; Speilmann and Stutz, 1983; Sugita *et al.*, 1984; Zurawski *et al.*, 1984; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986b). Furthermore, mutations have been recovered in the large inverted repeat of *Chlamydomonas*, and both copies are identically altered (Dron *et al.*, 1983; Erickson *et al.*, 1985). This implies that there is a mechanism for copy correction (gene conversion) between the repeats. It seems plausible that copy correction would also occur between the multiple copies of the cpDNA within each plastid. With such a mechanism in operation, a mutant molecule within the population of cpDNAs in a plastid could be either eliminated or fixed through successive rounds of gene conversion (Birky, 1978). A mutation would occur first in one molecule in a population of 20-200 wild type molecules per plastid (Possingham and Lawrence, 1983; Steel-Scott *et al.*, 1984; Bendich, 1987), so there would be a greater chance that the mutation would be eliminated rather than fixed (Sears, 1983). Once established within a single plastid the mutation must also compete in a population of perhaps 10-200 plastids per cell (Possingham and Lawrence, 1983). Cells with the mutant plastids must also segregate into the germ line if they are to be carried to subsequent generations (Kirk and Tilney-Bassett, 1978). It is likely that these are stochastic processes, so the chances for successful establishment of a mutation are low, because of its initially low abundance (Birky, 1983). Thus the high ploidy of the plastome and the large number of plastids per cell may contribute significantly to the conservation of cpDNA.

There is abundant evolutionary evidence pertaining to mutations in the cpDNAs of angiosperms (Palmer, 1987). Sequence inversions in plastid genomes having the large inverted repeats are quite rare evolutionarily. All of the inversions discovered upon examination of about 160

species of flowering plants having large inverted repeats are contained within the single-copy regions (Palmer, 1985a). Insertion and deletion mutations are more common. The great majority of these mutations are 1-10 base pairs (bp), and those of about 50-1000 bp are somewhat infrequent (palmer, 1985a). Large length mutations (greater than 1-2 kb) are extremely rare in cpDNA of land plants (Palmer, 1987). Gain or loss of restriction sites due to small length mutations and base substitutions is responsible for most of the interspecific changes in cpDNA restriction pattern that are observed (Palmer, 1985b).

The rate of silent substitutions in cpDNAs is comparatively low; about 1.0 - 3.0 x 10^{-9} per year for several genes in the single-copy regions, and at least three times lower for sequences in the inverted repeats (Zurawski *et al.*, 1984; Wolfe *et al.*, 1987). In comparison, the silent substitution rate is estimated at 0.1 - 11 x 10^{-9} per year for mammalian nuclear genes, 5 - 30 x 10^{-9} per year for plant nuclear genes, 18 - 54 x 10^{-9} per year for mammalian mitochondrial genes and 0.2 - 1.0 x 10^{-9} per year for plant mitochondrial genes (Wolfe *et al.*, 1987). The estimate for the plant nuclear genome is tentative since few sequences for the same gene are available from different species. Additionally, many of the dates of lineage divergence used to calculate substitution rates in plant genomes are unreliable because fossil evidence of plant evolution is often scarce.

Although intraspecific variation in cpDNA restriction pattern is somewhat rare among angiosperms it has been detected, particularly in larger samples. Such variation has been observed between populations (Palmer *et al.*,1985; Teeri *et al.*, 1985; Banks and Birky, 1985), among individuals within the same population (Robertson, 1985; Wagner *et al.*, 1987), and even within individual plants. Moon *et al.* (1987) sequenced two cpDNA fragments derived from an individual rice plant, which encode the same region of the genome. One of the fragments had a truncated rbcL gene and a frame-shifted atpB gene and the other had functional genes. The defective fragment was always 10 times less abundant than the normal one in bulk DNA isolations from many plants or isolations from individual plants. The authors suggested that the polymorphic cpDNAs are maintained stably by genetic complementation of different defects in the different types of molecules. Rose *et al.* (1986) examined the restriction pattern of cpDNA from 23 *Medicago sativa* protoclones derived from two

clonally propagated plants. A novel restriction pattern was detected in several of the protoclones, but the novel pattern was exactly the same in each protoclone. The cpDNA of selfed progeny of one of the plants used to derive the protoclones segregated, giving both the original pattern and the novel one. These results indicate cpDNA variation can occasionally be maintained within a population or by an individual plant, and this variation may be difficult to detect.

The inheritance of plastids in the plant kingdom is usually maternal, but many taxa inherit plastids biparentally or paternally (Kirk and Tilney-Bassett, 1978; Tilney-Bassett and Abdel-Wahab, 1979; Sears, 1980b; Whatley, 1982). Cytoplasmically inherited traits, such as restriction fragment length polymorphisms (RFLPs), pigment mutations, and antibiotic resistance must be used to follow organelle inheritance plants. Three genetic criteria that may be used to establish the cytoplasmic inheritance of a trait are: 1) non-Mendelian inheritance such an reciprocal differences after reciprocal crosses, and 2) the sorting out of genetic entities during development 3) molecular genetic evidence that the trait is cytoplasmically encoded (Kirk and Tilney-Bassett, 1978). Genetic analysis is necessary to establish the mode of organelle inheritance definitively, since cytological observation of the organelles during the reproductive process is sometimes misleading (Sears, 1980b). In higher plants, cytoplasmically inherited traits must be assigned to either plastids or mitochondria by morphological, cytological or biochemical analysis (Michaelis, 1966; Kutzelnigg and Stubbe, 1974; Fluhr *et al.*, 1985; Börner and Sears, 1986).

By far the largest body of evidence concerning the mode of plastid inheritance is available for the angiosperms. Both maternal and biparental inheritance are found in both dicot and monocot species, and strictly paternal inheritance is very exceptional (Tilney-Bassett and Birky, 1981). It is interesting that some genera, such as *Epilobium* (Schmitz and Kowallik, 1986) and *Solanum* (Simmonds, 1969), initially thought to have strictly maternal plastid inheritance transmit paternal plastids very infrequently. Similarly, Medgyesy *et al.* (1986) discovered very minimal paternal cpDNA inheritance in *Nicotiana*, which was previously considered to have strictly maternal inheritance. They accomplished this by fertilizing *N. plumbaginifolia* plants using pollen from a line with plastome-encoded streptomycin resistance. The seedlings from these crosses were induced to form callus on

streptomycin-containing media. Resistant sectors were green on a background of white cells, since streptomycin bleaches sensitive cells, but does not kill them. Thus, tiny sectors of paternal plastids were found in 0.07% to 2.5% of these calli. Earlier studies would not have discovered paternal plastids in *Nicotiana* because the plants having them had so few that they were undetectable by conventional methods. This suggests that other species thought to have strictly maternal plastid inheritance might likewise inherit some paternal plastids.

Largely paternal plastid inheritance seems to be the general rule in the gymnosperms although data is available for only a few groups. Genetic evidence for paternal inheritance is available for species of *Picea*, *Pinus*, *Pseudotsuga* and *Cryptomeria* (Stein and Keathley, personal communication; Neale *et al.*, 1986; Wagner *et al.*, 1987). However, Ohba *et al.* (1971) observed 0% to 3% biparental plastid transmission in crosses of *Cryptomeria japonica*. This points to the possibility of traces of maternal plastid inheritance in the other gymnosperms since this study examined a much larger population than the others. Cytological examination of spermatogenesis and fertilization suggests that plastids are inherited through a variety of mechanisms in the gymnosperms but genetic and biochemical evidence is needed to confirm this (Sears, 1980b).

Different methods of plastid inheritance have arisen on several independent occasions in the angiosperms, since more than one method of plastid transmission exists within several orders and even within some families (Tilney-Bassett and Abdel-Wahab, 1979; Sears, 1980b). Most higher plants transmit plastids uniparentally and this could contribute to the conservation of the plastome. However, the plastome is also conserved in biparental species such as the *Euoenothera* (Gordon *et al.*, 1982) suggesting that other factors are involved. It is also possible that the high ploidy and frequent gene conversion of plastid genomes have restrained them from genetic alteration by eliminating most recombinant molecules (Birky, 1978; Birky, 1983; Sears, 1983). Moreover, the genomes from different plastids probably seldom interact since plastids rarely fuse (Esau, 1972, Vaughn, 1981, Sears, 1983). Thus plants would be released from selection for biparental exchange of plastomes since advantageous recombinant genotypes would be produced extremely rarely (Medgyesy, *et al.*, 1985; Chiu and Sears, 1985; Thanh and Medgyesy, 1989). Hence the mechanisms of plastid inheritance would be free to

evolve in response to other pressures (Sears, 1983; Birky, 1983). This may explain the heterogeneity of plastid transmission since different mechanisms may have arisen in response to a variety of selection pressures on a variety of occasions.

Sears (1980b) and Whatley (1982) reviewed the voluminous cytological literature on sexual reproduction in plants and noted that the male or female plastids are eliminated during a variety of reproductive phases. In angiosperms the plastids can be eliminated from the male gametes by exclusion from the generative cell. Those which are not excluded may disintegrate or be genetically incapacitated as the pollen grain matures (Whatley, 1982; Day and Ellis, 1984). In addition the male plastids of pollen-bearing plants can be excluded from the egg during the process of fertilization. In several species of gymnosperms the female plastids appear to degenerate within the egg cell following fertilization. In the lower land plants, which have motile sperm, the plastids are often collected into a cytoplasmic vesicle and ejected from the male gamete prior to fertilization (Sears, 1980b; Whatley, 1982).

More cytological evidence is available regarding the mechanisms of cytoplasmic inheritance in angiosperms than for any other group. During angiosperm pollen production haploid microspores divide asymmetrically to produce a smaller generative cell and a larger vegetative cell. All or most of the plastids partition into the vegetative cell during this division in most species (Reviewed by: Sears, 1980b; Whatley, 1982; Connett, 1987; e.g. Russell, 1983; Russell, 1984; Schroder, 1985). The plastids within the generative cell are probably the only pollen plastids which can enter the egg (Whatley, 1982). Complete elimination of plastids from generative cells is difficult to prove using light or electron microscopy, and some species in which no generative cell plastids were detected inherit paternal plastids (Connett, 1987). This may stem from the difficulty in distinguishing small proplastids from mitochondria in the pollen of many species (Connett, 1987). Additionally, complete serial sectioning of a number of cells is needed to be sure that a few plastids do not remain undetected (Sears, 1980b). This requires a large amount of work and has seldom been done.

Plastomes probably are altered during pollen production in some angiosperms. Ultrastructural evidence indicates that plastids disintegrate in the generative cell before or after it

divides mitotically to form the sperm cells in species of *Nicotiana* (Clauhs and Grun, 1977) and *Epilobium* (Schmitz and Kowallik, 1987). The haploid plants regenerated from anther culture of certain species are frequently albino. Albino rice plants derived from anther culture have plastids with highly abnormal ultrastructure (Vaughn *et al.*, 1980). Day and Ellis (1985) examined the cpDNA from anther-culture-derived albino wheat and barley plants using Southern blotting. In some of the plants up to 70% of the genome had been deleted. They propose that a mechanism involved in maternal plastid inheritance is responsible for these deletions (Day and Ellis, 1984). Plastid DNA degradation probably begins prior to the microspore mitosis since the haploid plants studied only regenerated from immature pollen, prior to formation of the sperm cells (Vaughn *et al.*, 1980), and cultured plant cells probably are inviable without plastids. In support of this, Mogensen and Rusche (1985) did not find any recognizable plastids in immature sperm cells of barley after examining electron micrographs of complete serial sections from several pollen grains.

Fertilization also provides an opportunity for the elimination of paternal plastids in angiosperms (Sears, 1980b; Whatley, 1982; Connett, 1987). In spinach the cytoplasms of the pollen tube (derived from the vegetative cell) and the sperm cells (derived from the generative cell) have plastids when the pollen tube grows through the filiform apparatus into the degenerating synergid (Wilms, 1981a; Wilms, 1981b). After the pollen tube discharges, the tube plastids remain in the degenerating synergid where they disintegrate. The nucleus of one of the sperm cells enters the egg for fertilization, leaving its organelle-containing cytoplasm in the degenerating synergid, thus excluding the male plastids. In contrast, *Plumbago zeylanica* lacks synergids and the pollen tube discharges into the intracellular space between the egg and central cells (Russell, 1983). When the sperm cell and egg cell membranes fuse during fertilization the sperm cytoplasm, with its organelles, enters the egg along with the nucleus.

Plants with biparental plastid inheritance allow the study of some unique features of cytoplasmic inheritance. Such plants produce embryos with either maternal, paternal, or mixed plastid complements (Kirk and Tilney-Bassett, 1978). In plants derived from mixed embryos the maternal and paternal plastids segregate during vegetative development, ultimately producing pure or chimeric

shoots. Chimeric shoots have different plastid types in the different concentric tissue layers of their apical meristems (Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1986). Plastid chimeras can be relatively stable during plant growth. Plastome-encoded, pigment-deficient mutants can be used to follow the progress of this segregation during plant growth (Kirk and Tilney-Bassett, 1978). There are four predominant phases in the process: 1) the initial phase with mixed cells only and no visible manifestation, 2) a fine mosaic phase during which the mixed cells sort-out as they divide producing pure white and pure green cells, 3) a coarse mosaic phase with few remaining mixed cells during which pure tissues sort-out from a mosaic of pure cells, and 4) a final phase during which pure white, pure green, or chimeric shoots segregate from pure tissues.

Plastid segregation is a stochastic process if the different plastids replicate with equal efficiency, partition randomly between progeny cells, and do not have a differential effect on cell division or tissue development (Michaelis, 1967a). If plastid segregation is a stochastic process, statistical models can be created to predict its features. Michaelis (1967a) proposed such a model, and described the mathematical details. This model requires the following additional assumptions: 1) Prior to division the number of plastids per cell is a constant (2N). 2) Plastids always partition equally between the two daughter cells (1N per cell). 3) Each plastid replicates once between each cell division.

The model predicts several general features of plastid somatic segregation (Kirk and Tilney-Bassett, 1978). A cell must divide approximately 10 x N times to achieve almost complete (>99%) sorting-out. Furthermore, the most abundant class of mixed cells always has the same proportion of plastid types as the initial cell had. After sorting out is complete, the proportion of cells with each plastid type is the same as the proportion of each plastid type in the initial cell. This model provides an excellent basis for an understanding of plastid somatic segregation; most plastid traits sort out as predicted, although deviations are fairly common (Michaelis, 1967b; Kirk and Tilney-Bassett, 1978; Birky, 1978; Kumar and Cocking, 1987).

I have, until this point, assumed that individual plastids are the units of plastid segregation. However, it is logical that cpDNA molecules should be the segregation units, since they encode the plastid genetic information. Higher plants have about 500-50,000 copies of the plastome per cell

(Possingham and Lawrence, 1983; Bendich, 1987), so it would take at least 2500 (10 x N) cell divisions for complete sorting-out if cpDNA molecules were the segregation units. An annual plant would need several reproductive generations to complete 2500 cell divisions. However, annuals usually sort out completely in only one or two generations (Michaelis, 1967a). This suggests that the predominant segregation units must be plastids. There are approximately 10-30 plastids in the meristematic cells of higher plants (Possingham and Lawrence, 1983; Steele-Scott *et al.*, 1983) so complete sorting-out should take about 50-150 cell divisions. This number corresponds well with the cell division requirements for one to two generations in annual species (Michaelis, 1967b).

Unequal plastid partitioning into daughter cells, incomplete plastid mixing between divisions and plastid selection will always reduce the *effective* number of segregation units, so calculations using the number of plastids per cell give the maximum number of divisions needed for segregation (Michaelis, 1967a). For example, the first division of angiosperm zygotes is unequal and only the smaller of the cells contributes to the adult plant, thus reducing the effective number of plastids per cell (Whatley, 1982). Plastids have been observed to mix more slowly than most other cytoplasmic particles (Honda *et al.*, 1964), so sister plastids may partition into the same daughter cell more frequently than expected (Michaelis, 1967b; Kirk and Tilney- Bassett, 1978). Electron microscopic observations in *Oenothera* suggest that plastid mixing in the zygote is particularly slow (Meyer and Stubbe, 1974).

It is implicit that plastids do not exchange genetic information regularly since they, not cpDNA molecules, are the predominant units of vegetative segregation. Cytological observations confirm that plastid fusion occurs very rarely (Esau, 1972; Vaughn, 1981), if at all (Sears, 1983) in angiosperms. Chiu and Sears (1985) exploited the biparental plastid inheritance and available pigment mutations of *Oenothera* to look for plastid recombination. No green recombinant sectors were detected in an estimated 1,000 to 3,700 seedlings with mixed plastids. They suggested that rare recombinant molecules may have remained undetected if there had been insufficient cell divisions for complete sorting-out in these seedlings. Medgyesy *et al.* (1985) successfully selected a recombinant *Nicotiana* somatic hybrid in callus culture using a clever scheme. One cell line was green, streptomycin-sensitive

and lincomycin-resistant. The other line had two cpDNA mutations; one conferring pigment deficiency and the other conferring streptomycin-resistance. Streptomycin-sensitive cells bleach, but continue to grow slowly on streptomycin-containing media. Since the plastids of the other cell line were also white due to the cpDNA mutation, only the cells with fused and/or recombinant plastids could turn green on streptomycin-containing media. The scheme maintained heteroplasmic cells, but selected green recombinant cells which grew faster than the white cells. Later, growth on lincomycin media was used to distinguish the rare recombinants from pigment deficiency revertants. The authors tentatively estimate that 2000 to 20,000 heteroplasmic cells were screened to obtain one recombinant. The extreme rarity of cpDNA recombination in angiosperms (Kutzelnigg and Stubbe, 1974; Medgyesy, *et al.*, 1985; Chiu and Sears, 1985) is in accord with the rarity of plastid fusion and the rapid pace of plastid vegetative segregation.

Carefully designed experiments attempting to correlate cytological observation of the plastid input with genetic analysis of the output, using either sexual or somatic hybridization have not been reported. There is, however, some evidence suggesting that plastid input and output can be correlated as predicted by the Michaelis model if neither plastid genotype has a selective advantage. Electron microscopic observations reveal that *Oenothera* egg cells contribute about 3-4 times more plastids to the zygote than sperm cells do, and this roughly correlates with the general maternal bias of plastid inheritance in the genus (Meyer and Stubbe, 1974; Kirk and Tilney Bassett, 1978). Akada and Hirai (1986) conducted more convincing experiments using somatic hybridization of wild *Nicotiana* species. They used isoelectric-focusing of the large subunit of RuBisCO to analyze the plastids of 23 calli presumably derived from single fusions. They assumed each of the mesophyll-derived fusion partners contributed the same number of plastids and neither plastid type had a selective advantage. They found balanced plastid transmission as predicted by the Michaelis model. Furthermore, the degree of sorting-out corresponded to that expected after 40 divisions of cells having 20 plastids. Plastid counts of the material they used are lacking. However, roughly 20 plastids per cell have been observed after three or more divisions of mesophyll-derived protoplasts of *N. tabacum* (Thomas and Rose, 1983).

More rapid division of the plastids from one parent can increase their frequency in subsequent cell generations. Contrary to an assumption of the Michaelis model, each plastid probably does not replicate exactly once per cell cycle. Plastid division can occur continuously during the cell cycle but the maximum number of plastids per cell is limited, and this limit varies with the developmental regime of the tissue (Possingham and Lawrence, 1983; Thomas and Rose, 1983). When the number of plastids per cell is below the limit, those that divide more rapidly can reproduce more frequently and predominate in subsequent generations.

Oenothera has yielded genetic evidence supporting this concept. Schötz (1954) discovered differences in competition among the five different plastomes of *Oenothera* subsection *Euoenothera* when they were brought together by sexual matings. Each of the five plastome types was assayed using reciprocal crosses in which one parent carried a plastome-encoded albino mutant, and the other carried the wild-type plastome to be tested (Cleland, 1972, Hagemann, 1976; Kirk and Tilney-Bassett, 1978). The germinating seeds from these crosses were scored by measuring the relative size of the green sectors on their cotyledons, giving a quantitative estimate of the relative competitiveness of each plastome. This "variegation rating" ranked the five plastomes in nearly the same order regardless of the direction of the cross or the nuclear background (Kirk and Tilney-Bassett, 1978; Chiu *et al.*, 1988). Schötz (1968) attributed this phenomenon to differences in the rate or onset of plastid division. The nuclear genome also influences the variegation rating, but not to the same extent as the plastome.

After certain interspecific hybridizations of *Oenothera* the plastome cannot function properly with the hybrid nuclear genome, causing several types of physiological disturbances (Cleland, 1972; Kutzelnigg and Stubbe, 1974; Kirk and Tilney-Bassett, 1978). Some manifestations of this phenomenon are: periodic chlorophyll deficiency and re-greening, complete chlorophyll deficiency, impaired growth, deformed leaves, inhibition of embryo development, and lethality (Kutzelnigg and Stubbe, 1974; Kirk and Tilney-Bassett, 1978). Nuclear control of these "plastome- genome" incompatibilities is usually polygenic (Kirk and Tilney-Bassett, 1978). The phenomenon is called hybrid variegation when the affected plastids are deficient in chlorophyll development.

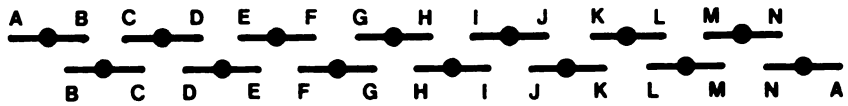

Figure 3. Diagram of the chromosomal pairing of an *Oenothera* ring chromosome. The chromosomal arms marked "A" pair to form a continuous chain or "ring chromosome". Redrawn from Kirk and Tilney-Bassett (1978).

Such plastids behave similarly to plastids with pigment mutations except they green when reunited with a compatible nuclear genome since the plastome is not altered (Schötz, 1962).

Hybrid variegation has been demonstrated in *Pelargonium* (Metzlaff *et al.*, 1982; Pohlheim, 1986) and other genera (Menczel *et al.*,1987), but has been studied most extensively in *Oenothera* (Kirk and Tilney-Bassett, 1978). *Oenothera* subsection *Euoenothera* is well suited for such experiments since evolutionarily distinct haploid nuclear complements occupying the same nucleus can form ring chromosomes,suppressing genetic reassortment (Cleland, 1972). Ring chromosomes result from multiple reciprocal translocations of chromosomal arms within the haploid complements. During meiosis chromosomal complements with different translocations pair in a zig-zag arrangement (called a ring chromosome) since homologous arms are displaced to different chromosomes (Figure 3). When the entire genome forms one ring each haploid complement segregates as a unit and recombination is greatly suppressed.

Because of the prevalence of ring chromosomes in *Euoenothera* cytoplasmic substitution is possible without repeated back-crossing. Stubbe (1960) studied the reaction of the five different plastid types of subsection *Euoenothera* and found profound differences in their ability to green with different combinations of the three basic haploid nuclear complements (Figure 4). The manifestation of this plastome-genome incompatibility ranged from small changes in chloroplast pigmentation to complete lethality.

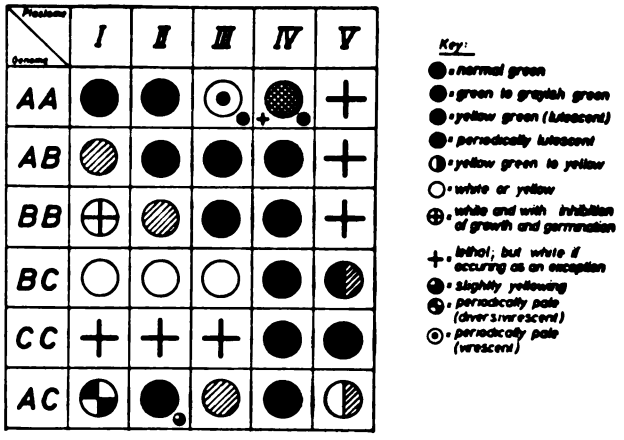

Figure 4. Compatibility relations between different nuclear genome complements and plastomes of *Euoenothera* (from Stubbe, 1959). The use of more than one sign in some squares depends on slight differences between different A-complexes.

Not surprisingly, each plastome type is more compatible when associated with genomes closely related to the one with which it co- evolved (Cleland, 1972; Kirk and Tilney-Bassett, 1978).

Replication speed might also be important in the evolution of the subsection, as depicted in Figure 5 (Stubbe, 1964). The heavy line in the figure encloses all the nuclear genome- plastome combinations which green normally, and the species are written in the boxes corresponding to their genetic complements. Whenever a functional complement is not represented by a species there is a modern species with same nuclear complement and a more rapidly replicating plastid. On this basis Stubbe (1964) has suggested that more rapidly replicating plastids have replaced slower ones during the evolution of some species. Restriction analysis of the five plastomes is consistent with this hypothesis; the slowest plastid might be the most evolutionarily primitive, and the most rapid plastid types might be the most modern (Gordon *et al.*, 1982). However, cladistic of analysis a larger number of evolutionarily informative restriction site differences is needed to verify this conclusion.

plastome / genome	I	>	III	>	II	>	IV	<	V
AA	*elata* *hookeri* *strigosa*								
AB			*biennis-1*		*biennis-2*				
BB			*biennis-3* *grandifolia*						
BC							*parviflora-1*		
CC									*argillicola*
AC							*parviflora-2*		

Figure 5. Distribution of the natural species among the compatible combinations (framed squares) of *Euoenothera*. The signs between the plastomes I-V refer to the relative strength (multiplication rate) of the plastids. Adapted from Stubbe (1964) as modified by Chiu *et al.*, (1988).

The genetics of biparental plastid inheritance in the cultivated geranium, *Pelargonium* x *Hortorum* Bailey has been studied extensively (Kirk and Tilney-Bassett, 1978; Kirk and Tilney-Bassett 1978). A single nuclear locus in conjunction with some modifying genes controls the mode of plastid inheritance in *Pelargonium* (Tilney-Bassett, 1976). The alleles at this locus switch the plastid inheritance pattern between type I; maternal zygotes (MZ) > biparental zygotes (BPZ) > paternal zygotes (PZ), and type II; MZ > BPZ < PZ. Type I plants (Pr_1Pr_1) breed true, and Type II plants (Pr_1Pr_2) always segregate, usually with an "unexpected" 1:1 ratio of type I to type II. Tilney-Bassett and Abdel-Wahab (1982) found no homozygous (Pr_2Pr_2) plants after extensive analysis of many crosses. To explain their perplexing data they propose that this locus encodes a rather complex self-incompatibility system and that the same or a tightly linked locus also controls the patterns of plastid inheritance.

Neither type of plastid segregation pattern in *Pelargonium* is consistent with the Michaelis model, because the distribution of maternal and paternal plastids in the biparental plants from

particular crosses has no mode corresponding to the average plastid composition of the zygotes (Tilney-Bassett and Birky, 1981). Furthermore, the degree of sorting-out is extremely variable in both type I and type II crosses. These inconsistencies can be explained in terms of another model which was originally proposed to describe *Chlamydomonas* plastid and yeast mitochondrial inheritance (Birky, 1975; Birky, 1978; Birky; 1983; Sears, 1980; Thraikill *et al.*, 1980; Birky *et al.*, 1981; Galloway and Holden, 1985).

According to this model the organelles within a cell constitute a population which follows the rules of population genetics. In contrast to the Michaelis model wherein each organelle replicates exactly once, a smaller subsample of the intracellular population is selected at random to form the next generation. The model does not stipulate how the subsample is selected; it could involve differential organelle replication, differential organelle degradation, or other mechanisms. The subsample is small so there is random drift in the frequency of organelle types. Tilney-Bassett and Birky (1981) suggest that genes at the Pr locus act in the zygote to control the selection of a subpopulation of plastids to be transmitted. Type I (MZ > BPZ > PZ) plants preferentially select maternal plastids, but often include a few paternal ones. Type II (MZ > BPZ < PZ) plants may select either maternal of paternal plastids, but often completely exclude all the plastids from the other parent. Careful cytological observation with precise plastid counts during the *Pelargonium* fertilization, embryo development and germination processes would enhance our understanding of its organelle inheritance.

Plastid Transmission in Somatic Hybrids

Plastid evolution and sexual reproduction has interesting parallels with plastid behavior during somatic hybridization experiments, despite the artificial conditions created through somatic hybridization (for reviews of plastid transmission following somatic hybridization see: Fluhr, 1983; Harms, 1983: Gleba and Syntnik, 1984; Pelletier and Chupeau, 1984; Pelletier, 1986; Maliga and Menczel, 1986; Grun, 1986; Kumar and Cooper-Bland, 1986; Kumar and Cocking, 1987). The

striking evolutionary conservation of the plastome gene complement and arrangement implies that plastomes are highly resistant to genetic changes. Indeed, the cpDNA-coding complement and arrangement is stable during somatic hybridization (Medgyesy *et al.*, 1985; Thanh and Medgyesy, 1989). Plastome recombination is extremely rare in both sexual and somatic crosses (Kutzelnigg and Stubbe, 1974; Chiu and Sears, 1985; Medgyesy *et al.*, 1985) probably due to the rarity of plastid fusion. Plastid fusion has not been observed in viable angiosperm cells after fusion (Fowke and Rennie, 1976; Fowke *et al.*, 1977; Rennie *et al.*, 1980; Hodgson and Rose, 1984). However, plastid fusion may be required for plastome recombination and recombination does occur rarely after somatic hybridization (Medgyesy *et al.*, 1985; Thanh and Medgyesy, 1989).

The rapid pace and stochastic characteristics of plastid segregation in somatic hybrid cells (Akada and Hirai, 1986) are consistent with observations of the process in sexual hybrids (Michaelis, 1967a; Birky, 1978). Michaelis' (1967a) mathematical model of plastid segregation predicts that when plastid selection causes unbalanced transmission, mixed cells will be maintained for fewer cell divisions than if there were no selection. Mixed shoots may be more unusual in hybridization experiments with unbalanced plastid transmission than in experiments with balanced transmission. There have been at least twenty reports of somatic hybridization experiments where at least 10 hybrids were analyzed and there was non-random plastid transmission. Only five of these report the recovery of shoots with mixed plastids. On the other hand, twelve hybridizations where more than 10 plants were analyzed seem to have had balanced plastid transmission and five of these had shoots with mixed plastids (Table 3).

Furthermore, plastid transmission in somatic hybridization experiments frequently deviates from the expectations of purely random behavior (Table 3) and these deviations can usually be explained using concepts developed to explain similar phenomena in sexual crosses. The possible causes of unequal transmission of organelles in somatic hybrids are: 1) an initially larger contribution of organelles from one fusion partner, 2) more efficient replication of the organelles from one partner, 3) genetic incompatibility of the nuclear genome with the organelles from one partner, or 4)

selection of the organelles from one partner *in vitro* using antibiotics, herbicides or culture conditions (Kumar and Cocking, 1987).

The relative number of plastids contributed by the donor cells could influence plastid transmission in sexual and somatic hybrids similarly (Whatley, 1982; Flick and Evans, 1982; Fluhr, 1983; Connett, 1987). Differential transmission of plastids due to unequal input from the gametes is a well-documented aspect of sexual reproduction in higher plants (Sears, 1980b; Whatley, 1982; Connett, 1987). If segregation in somatic hybrids were purely stochastic, the proportion of pure cell lines after complete sorting-out should reflect the original plastid ratio in the heterokaryon (Michaelis, 1967a; Birky, 1978; Akada and Hiria, 1986). Mesophyll and suspension cells probably contribute a significantly different number of plastids when they are fused. Mesophyll cells have more plastids than any other tissue examined (Steele-Scott, 1984), about 10-fold more than meristematic cells (Possingham and Lawerence, 1983). No information about the number of plastids found in long term suspension cells is available, but these rapidly dividing cells are similar to the meristematic cells of whole plants and probably have relatively few proplastids. Thus, when mesophyll and suspension cells are fused the plastids from the mesophyll partner should predominate in the heterokaryon.

Akada *et al.* (1983) examined plastid segregation in *Nicotiana glauca* + *N. Langsdorfii*[*] hybrid callus after reciprocal fusions of suspension and mesophyll cells, or fusion of suspension cells from both species. Samples of 10-13 calli from each of the fusions seem to have had balanced transmission, suggesting that the tissue source was unimportant. This fusion pair was well chosen since it has had primarily balanced plastid transmission in other experiments (Table 3), reducing the possibility of factors other than tissue source confounding the result. However, the sample size they analyzed was small, particularly since samples with mixed plastids are uninformative without analysis of their plastid ratio, and the authors list over half the calli they analyzed as mixed without giving the ratio.

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[*] hereafter "+" denotes somatic hybridization and an "x" denotes sexual hybridization

Table 2. Plastid competitive ability in *Nicotiana* somatic hybrids. This table only includes data from somatic hybridization experiments which did not involve plastid selection using antibiotics herbicides or other chemicals. The data from all the hybridizations where the parental species pair has the same isoelectric-focusing pattern for the large subunit of RuBisCO have been combined, and cumulative ratio is given in the same order as the parental isoelectric-focusing patterns. Statistical significance is based on the confidence limits of the binomial distribution with the null hypothesis of an equal proportion of each plastid type, and all the categories listed as not significant (N.S.) are not significant at the 10% confidence level.

Parental Plastid RuBisCO Types	Number of Exps.	Mode of Transmission	Cumulative Ratio	Statistical Significance
Type II + Type III	10	Unbalanced	28:110	<1%
Type II + Type III	3	Unbalanced	26:4	<1%
Type II + Type A	5	Unbalanced	33:79	<1%
Type II + Type I	6	Balanced	64:55	N.S.
Type II + Type II	4	Balanced	92:102	N.S.
Type III + Type III	1	Balanced	10:12	N.S.

There are eight reports of mesophyll-suspension cell fusions of various species where mesophyll-derived plastids clearly predominated, none where the plastids from the suspension partner predominated and two reports of relatively large populations having balanced transmission (Table 3). The cumulative ratio of plastids from mesophyll fusion partners to suspension fusion partners from all these experiments is 185 to 97 (Table 3). However, the implications of this are uncertain because the number of comparisons is small and many uncontrolled factors could affect the result.

Examination of the process of plastid segregation as heterokaryons divide to produce callus exposes some pitfalls inherent in investigations of plastid transmission in somatic hybrids. Akada and Hirai (1983, 1986) demonstrated that different sectors of hybrid calli have very different plastid ratios. The largest difference between sectors of the same callus was about 80%. This illustrates the possibility of inadvertently selecting one type of plastid during routine culturing of small numbers of hybrid calli, if portions of the calli are discarded.

The relative replicative competitiveness of different plastids is important in determining plastid sexual transmission in *Euoenothera* (Schötz, 1954; Kirk and Tilney- Bassett, 1978), and may have had

a role in the evolution of this subsection (Stubbe, 1964). Perhaps replicative competitiveness is a determinant of plastid transmission of somatic hybrids. In fact, there is a relationship between the behavior of plastids in *Nicotiana* somatic hybrids and a plastome encoded biochemical marker (Table 2; Table 3). This marker is the isoelectric-focusing pattern of the large subunit of the Calvin cycle enzyme, ribulose bisphosphate carboxylase-oxygenase (RuBisCO). The enzyme probably is not functionally related to plastome replicative competitiveness. However, the isoelectric-focusing pattern of the large subunit of RuBisCo has been highly conserved during evolution, and has been used to follow evolution within several genera including *Nicotiana* (Chen and Wildman, 1981). Sixty-three species of *Nicotiana* have only four RubisCO large subunit isoelectric-focusing patterns and the patterns of the remaining two species are unknown. Twenty-eight species have pattern III, 23 have pattern A, 7 have pattern II, and 5 have pattern I (Chen and Wildman, 1981).

All five somatic hybridizations between different pairs of *Nicotiana* species having the same large subunit pattern appear to have balanced plastid transmission (Table 2; Table 3). The three hybridizations between different species pairs with patterns II and I also appear to have balanced transmission. The two hybridizations between different pairs of species with patterns II and A both have unbalanced transmission, and the type A plastids are always favored. The suggested order of replicative efficiency for these plastid types is type I = type II < type A. This conclusion is tentative since the number of different species pairs hybridized is small and many uncontrolled factors could affect the result. The type II plastome may be the evolutionary progenitor of the type A plastome (Kung *et al.*, 1982). This suggests that relative replicative competitiveness may have a role in the evolution of *Nicotiana* species that is similar to its putative role in *Euoenothera* evolution (Stubbe, 1964; Chiu *et al.*, 1988).

The results of fusions between type II and III species are more complex. There are seven groups of hybridization experiments having different parental species pairs with these RuBisCO patterns excluding experiments without enough observations for statistical significance (Table 3). In four of these type II + type III fusions, type III plastids prevail, and in three fusions type II plastids prevail (Table 2; Table 3, pp. 43-45). *Nicotiana tabacum* and *N. sylvestris* are the only type II species

used for all the type II + type III fusions. Differences between these species may not be responsible for the differences between transmission patterns type II + type III fusions, since a fusion between them has balanced transmission. Furthermore, *N. tabacum* is thought to have arisen from an interspecific hybridization with *N. sylvestris* as the cytoplasmic parent (Gray *et al.*, 1974). The differences in transmission are probably due to differences between the five type III species used for the fusions. More species have type III plastids than any of the other types and most of these are native to South America, the center of origin of the genus (Chen and Wildman, 1981; Goodspeed, 1954). Therefore, it would not be surprising to find greater plastid diversity within this group.

Examination of the reaction of these species to the fungal toxin, tentoxin is also relevant. Tentoxin is another plastome encoded trait which probably has no functional relationship to plastid replicative competitiveness (Durbin and Uchytil, 1977b). All three type III species used in the fusions where type II plastids prevailed are sensitive to tentoxin (Table 3). Conversely, three of the four type II + type III fusions where type III species prevailed utilized tentoxin insensitive type III species.

Fusions between *N. tabacum*, *N. sylvestris*, and *N. undulata*, in all three combinations may demonstrate that plastome-controlled differences in replicative competitiveness are not the only influence of plastid transmission in somatic hybridizations. The *N. tabacum* + *N. sylvestris* fusion has balanced transmission (Table 3), yet *N. undulata* plastids prevail in the *N. tabacum* + *N. undulata* fusion (Table 3) and *N. sylvestris* plastids prevail in the *N. sylvestris* + *N. undulata* fusion (Table 3). However, the origin of the cytoplasm of the male sterile line of *N. tabacum* used in the *N. tabacum* + *N. undulata* fusion was questionable, and may not have been *N. undulata*. The identity of this unknown cytoplasm was surmised using cpDNA restriction, RuBisCO large subunit isoelectric-focusing, and the male sterile flower morphology, but the data was not published (Bonnett and Glimelius, 1983). Of the three methods used only the cpDNA restriction analysis was potentially definitive. However, their identification is very questionable since cpDNA restriction was used only used to determine that the plastids were not from *N. plumbaginifolia* as thought originally. Plastid DNA restriction data distinguishing only four of the twenty- three *Nicotiana* species, not including *N. undulata*, was available when they published (Kung et al., 1982).

The influence of the nuclear genome on plastid transmission in somatic hybrids is almost certainly significant. By far the largest portion of proteins needed for normal plastid function are nuclear-encoded (Parthier, 1982; Palmer, 1987). However, evidence for a nuclear influence on somatic hybrid plastid transmission is lacking, primarily because of the lack of relevant nuclear-encoded genetic markers. The nuclear genome is much larger and more recombinationally fluid than the plastid genome, so the vast majority of nuclear-encoded traits would not be genetically linked to genes that influence plastid transmission. Only those genes which directly influence plastid transmission or closely linked genes could co-segregate with changes in the plastid transmission of somatic hybrids.

There is no obvious pattern when the of mode plastid transmission in *Nicotiana* somatic hybrids and various nuclear-encoded traits of their parents are compared (for some examples see Table 3). The parental traits used for these comparisons are the phylogenetic relationship, which was determined by extensive morphological and cytogenetic analysis (Goodspeed, 1954; Burbidge, 1960), the sexual cross compatibility (Pandy, 1979), and various properties of the small subunit of RuBisCO (Kawashima *et al.*, 1974; Gray, 1977; Chen and Wildman, 1981). In addition, plastid transmission is not noticeably different when intergeneric and intrageneric fusions, or fusions of sexually compatible and incompatible species are compared (Table 3).

Hybrid bleaching, which is fairly common following interspecific sexual hybridization, involves a chlorophyll deficiency resulting from genetic incompatibility between the plastome and the nucleus (Kirk and Tilney-Bassett, 1978). The only reports of hybrid bleaching after somatic hybridization involve *Brassica napus* cybrids with *Raphanus sativus* plastids (Pelletier *et al.*, 1983; Menczel *et al.*, 1987). However, hybrid bleaching was initially discovered following sexual hybridization of these species. It is curious that no other instances of hybrid bleaching have been reported after somatic hybridization. Perhaps the strong plastome- nuclear genome incompatibility which results in hybrid bleaching reduces the likelihood of obtaining somatic hybrid plants.

Certain aspects of the somatic hybridization process can influence plastid transmission, but the role of such influences is not always completely clear. Clearly, *in vitro* selection of plastome-encoded

traits, such as antibiotic resistance (Medgyesy *et al.*, 1980; Menczel *et al.*, 1981; Fluhr *et al.*, 1983; Pental *et al.*, 1984; Bourgin *et al.*, 1986), herbicide resistance (Robertson *et al.*, 1987; Guri *et al.*, 1988) or pigment deficiency (Nakata and Oshkima, 1982; Aviv *et al.*, 1984; Barsby *et al.*, 1984; Fluhr *et al.*, 1984; Menczel *et al.*, 1986; Glimelius and Bonnett, 1986) almost always results in exclusive transmission of the selected plastid (Table 3).

Iodoacetate is a nonspecific metabolic inhibitor which acts on the sulfhydryl groups of proteins and other biomolecules. Iodoacetate treatment has been used to inhibit cell division of the protoplasts from one fusion partner, and the heterokaryons formed after fusions with such protoplasts can divide due to metabolic complementation with the biochemical constituents from the untreated fusion partner (Wright, 1978). Iodoacetate treatment of *N. plumbaginifolia* protoplasts may have selected against plastids from the treated fusion partner after a fusion with *N. tabacum*, since this fusion pair has balanced transmission when iodoacetate is not used (Sidorov *et al.*, 1981; Table 3). However, the drug does not seem to affect the plastid transmission of some other fusions (Table 3), perhaps because it was used at a lower concentration (Fluhr *et al.*, 1983; Aviv and Galun, 1986, Morgan and Maliga, 1987). It is interesting that both somatic hybridizations of *N. langsdorfii* and *N. glauca* utilizing electrically induced protoplast fusion only generated hybrids with *N. langsdorfii* plastids (Chapel *et al.*, 1986; Morikawa *et al.*,1987) In contrast, both fusions with the same partners utilizing the conventional polyethylene glycol method had balanced plastid plastid transmission (Akada and Hirai, 1983; Chen *et al.*, 1977). Perhaps the fusion or the subsequent culture conditions used in the electrofusion experiments influenced plastid transmission in the hybrids. Changes in the culture conditions are postulated to have affected the plastid transmission of *N. tabacum* + *N. knightiana* hybrids, but this conclusion is quite tentative (Maliga *et al.*, 1978; Maliga *et al.*, 1980; Menczel *et al.*, 1981; Table 3).

Somatic fusion can be used to study plastid genetics in plant species lacking biparental inheritance. As discussed above, plastids have behaved similarly after sexual and somatic hybridization. However, somatic hybridization procedures are both difficult and laborious so the number of experimental observations is restricted. Perhaps as a consequence of this, somatic

hybridization experiments designed specifically to study plastid genetics have been rare. Therefore, most conclusions about plastid behavior after somatic hybridization are based on comparisons of several experiments from different laboratories. Such conclusions are subject to error due to the influence of many uncontrolled factors. In addition, the *in vitro* culture conditions used during somatic hybridization can influence plastid transmission in ways that are not always readily apparent.

Table 3. **Chloroplast transmission after somatic fusion.** All fusion experiments involving the same plastid donors are grouped together. "NO SELECTION" indicates that no *in vitro* selection of plastids was employed. "SELECTION" indicates that *in vitro* selection of plastids was employed, and the plastome encoded trait used for selection follows. "SELECTION?" indicates that *in vitro* selection of plastids is suspected, and the putative selective agent follows. **Species:** The species which originally donated the plastids. The chromosome number of the species is in brackets. **Species Origin:** The continent where the species originated. North America (N.A.), South America (S.A.), Australia (Aus.). **Tentoxin:** The reaction of the species to the fungal toxin tentoxin. Sensitive (S), Insensitive (I). **RuBisCO:** Isoelectric focusing data for the enzyme ribulose bisphosphate carboxylase. Small subunit (SSU), Large Subunit (LSU). The nomenclature is from Chen and Wildman (1981). Each of isoelectric points of the SSU peptides from the entire genus is given a consecutive number starting with the most alkaline. Species with numbers in common have SSU peptides with the same isoelectric point. **Nuclear Genome:** "Hybrid" indicates that the plants analyzed contain nuclear genomes from both donors. The first three letters of the species donating nuclear genomes appear in parentheses when these species are not the same as the species donating the plastids. "Cybrid" indicates that the plants analyzed contain nuclear genomes from only one donor and the first three letters of that species appear in parentheses. **Verification:** The method used to confirm that the plants analyzed were hybrids or cybrids. Chromosome number (C#), Chloroplast transfer (cp-T), Isozyme analysis (IZ), Microscopic isolation of heterokaryons (Micro-iso), Interspecific mitochondrial DNA recombination (mt-R), Induction of male sterility (MS), Complementation of different parental nuclear encoded genetic markers (NC), Hybrid pattern of the small subunit of RuBisCO (SSU). **Tissue Source:** The tissue used for protoplast isolation is listed in the same order as the plastid donor species. Callus culture (C), Etiolated shoots (ES), Hypocotyl (HC), Leaf mesophyll (M), Suspension culture (S). **Plastid Ratio:** The number of plants or calli with plastids of each type is listed in the same order as the plastid donor species. eg. 1st parent/mixed/2nd parent. **Statistical Inference:** "Unbalanced" indicates that the null hypothesis of an equal proportion of plastid types is rejected based on the 95% confidence limits of the binomial distribution (Steel and Torrie, 1980). Confidence limits other than 95% are given in parentheses.

Table 3

PARENTAL SPECIES						SOMATIC HYBRIDIZATIONS				
SPECIES (2a)	[1]SUBGENUS & SECTION	[1]SPECIES ORIGIN	[2]TEN-TOXIN	[3]RuBisCo SSU	[3]RuBisCo LSU	NUCLEAR GENOME	VERIFI-CATION	TISSUE SOURCE	PLASTID RATIO	STATISTICAL INFERENCE
Nicotiana										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		SELECTION: pigment deficiency			
N. undulata (20)	*Petunioides* Undulatae	S.A.	S	6,9	III	[5]cybrid (tab)	mt-R	M/M	0/47	unbalanced
(sexual compatibility not available)							SELECTION: antibiotic resistance			
						[6]cybrid (tab)	cp-T	M/M	8/0	unbalanced
									NO SELECTION:	
						[7]cybrid (tab)	C#	S/M	0/22	unbalanced
						[8]cybrid & hybrid	Micro-Iso	C/M	0/4/1*	—
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		NO SELECTION:			
N. otophora (24)	*Tabacum* Tomentosae	S.A.	-	7	III	[9]hybrid	N.A.	S/M	0/2	N.S.
(sexually compatible; unilaterally?)[4]										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		NO SELECTION:			
N. glutinosa (24)	*Tabacum* Tomentosae	S.A.	-	7	III	[10]hybrid	C#	S/M	1/4	N.S.
(sexually compatible)[4]										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		NO SELECTION:			
N. knightiana (24)	*Rustica* Paniculatae	S.A.	I	9	III	[16]hybrid	C#	C/M	18/21	N.S.
						[17]hybrid	IZ	S/M	0/28	unbalanced
(unilaterally sexually compatible; tab x kni)[4]								TOTAL:	18/49	unbalanced
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		SELECTION: antibiotic resistance			
N. rustica (48)	*Rustica* Rusticae	S.A.	I	6,9	III	[11]hybrid	SSU	M/S	11/0	unbalanced
(sexually compatible)[4]						[12]hybrid	cp-T	S/M	2000/62/0	unbalanced
							NO SELECTION:			
						[13]hybrid	SSU	M/M	2/2	N.S.
						[14]hybrid	SSU	M/S	1/5	N.S.
						[15]hybrid	SSU	S/S	3/11	unbalanced (90% level)
								TOTAL:	6/18	unbalanced
N. sylvestris (24)	*petunioides* alatae	S.A.	I	7	II		NO SELECTION:			
N. rustica (48)	*Rustica* Rusticae	S.A.	I	6,9	III	[18]cybrid (syl)	mt-R	M/M	3/7	N.S.
(sexually compatible)[4]						[19]hybrid	SSU	S/S	0/8	unbalanced
								TOTAL:	3/15	unbalanced
N. sylvestris (24)	*Petunioides* alatae	S.A.	I	7	II		NO SELECTION:			
N. biglovii (48)	*Petunioides* biglovianae	N.A.	S	6,9	III	[5]cybrid (syl)	MS	M/M	9/2/4	unbalanced (90% level)
(sexual compatibility not available)										

Table 3 (Cont'd.)

PARENTAL SPECIES						SOMATIC HYBRIDIZATIONS				
SPECIES (2n)	[1]SUBGENUS & SECTION	[1]SPECIES ORIGIN	[2]TEN-TOXIN	[3]RuBisCo SSU	[3]RuBisCo LSU	NUCLEAR GENOME	VERIFICATION	TISSUE SOURCE	PLASTID RATIO	STATISTICAL INFERENCE
Nicotiana										
N. sylvestris (24)	*Petunioides* alatae	S.A.	I	7	II	[5]cybrid (syl)	NO SELECTION: MS	M/M	10/0	unbalanced
N. undulata (24)	*Petunioides* undulatae	S.A.	S	6,9	III					
(sexually compatible; unilaterally?)[4]										
N. sylvestris (24)	*Petunioides* alatae	S.A.	I	7	II	[5]cybrid (syl)	NO SELECTION: MS	M/M	7/0	unbalanced
N. alata (18)	*Petunioides* alatae	S.A.	S	3,5	III					
(unilaterally sexually compatible; ala x syl)[4]										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II	[20]cybrid (tab)	NO SELECTION: MS	M/M	11/15	N.S.
N. debneyi (48)	*Petunioides* Saveolentes	Aus.	I	1,3, 5	A	[21]hybrid (tab)	mt-R	S/M	2/7	unbalanced
(sexually compatible)[4]								TOTAL:	13/22	unbalanced (90% level)
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II	[22]cybrid (tab)	SELECTION: pigment deficiency cp-T	M/M	0/12	unbalanced
N. suaveolens (23)	*Petunioides* Saveolentes	Aus.	I	2,5, 6,8	A	[23]cybrid & [24]hybrid	NO SELECTION: Micro-Iso	C/M	0/7/1*	—
(sexually incompatible)[4]						[24]hybrid	C#	S/M	6/19	unbalanced
						[25]cybrid (tab)	Micro-Iso	S/M	9/1/23	unbalanced
						[7]hybrid	C#	S/M	5/15	unbalanced
								TOTAL:	20/57	unbalanced
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II	[9]hybrid	NO SELECTION: N.A.	S/M	0/5	N.S.
N. nesophila (48)	*Petunioides* repandae	N.A.	S	9	I					
(sexually incompatible)[4]										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II	[25]cybrid (tab)	NO SELECTION: Micro-Iso	S/M	41/2/32	N.S.
N. repanda (48)	*Petunioides* repandae	N.A.	S	9	I					
(unilaterally sexually compatible; rep x tab)[4]										
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II	[23]cybrid & [26]hybrid	NO SELECTION: Micro-Iso	C/M	0/6/3*	—
N. glauca (24)	*Rustica* panicuulatae	S.A.	S	9	I		C#	S/M	3/0	N.S.
(unilaterally sexually compatible; gla x tab)[4]						[7]hybrid (tab)	C#	S/M	11/10	N.S.
								TOTAL:	14/10	N.S.

Table 3 (cont'd)

PARENTAL SPECIES						SOMATIC HYBRIDIZATIONS				
SPECIES (2n)	[1]SUBGENUS & SECTION	[1]SPECIES ORIGIN	[2]TEN-TOXIN	[3]RuBisCo SSU	[3]RuBisCo LSU	NUCLEAR GENOME	VERFI-CATION	TISSUE SOURCE	PLASTID RATIO	STATISTICAL INFERENCE
Nicotiana										
N. langsdorfii (36)	*Petunioides* Alatae	S.A.	S	3,5	II		SELECTION? electrofusion			
N. glauca (48)	*Rustica* Paniculatae	S.A.	S	9	I	[27]hybrid	SSU	M/M	7/0	unbalanced (90% level)
						[28]hybrid	SSU	M/M	All/0**	—
(unilaterally sexually compatible; lan x gla)[4]							NO SELECTION:			
						[29]hybrid	SSU	S/S	0/All/0**	—
						[30]hybrid	SSU	M/M	0/1	N.S.
						[31]hybrid	SSU	M/M	9/1/7	N.S.
								TOTAL:	9/8	N.S.
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		SELECTION: antibiotic resistance			
N. sylvestris (24)	*Petunioides* Alatae	S.A.	I	3	II	[32]cybrid (syl)	cp-T	M/M	117/0	unbalanced
							NO SELECTION:			
(sexually compatible)[4]						[6]cybrid	NC	M/M	8/18/9	N.S.
						[9](tab) hybrid	N.A.	S/M	1/0	N.S.
								TOTAL:	9/9	N.S.
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		SELECTION: antibiotic resistance			
N. plumbaginifolia (20)	*Petunioides* Alatae	S.A.	S	3	II	[33]cybrid (plu)	cp-T	M/M	All/0	—
						[34]cybrid (tab)	C#, cp-T	S/M	0/12	unbalanced
(sexually compatible)[4]							SELECTION? iodoacetate			
						[35]cybrid (plu)	IZ	M/C	31/5/3	unbalanced
							NO SELECTION:			
						[23]hybrid	C#	C/M	0/1/0*	—
						[35]hybrid	IZ	M/C	7/3/6	N.S.
N. tabacum (48)	*Tabacum* Genuianae	S.A.	I	7,9	II		SELECTION: antibiotic resistance			
N. tabacum (48)	*Tabacum* Genuiane	S.A.	I	7,3	II	[36]hybrid	cp-T	M/M	9/1	unbalanced
							NO SELECTION:			
(sexually compatible)[4]						[37]cybrid & hybrid	NC	M/M	76/87	N.S.
N. debneyi (48)	*Petunioides* Saveolentes	Aus.	I	1,3, 5	A		NO SELECTION:			
N. debneyi (48)	*Petunioides* Saveolentes	Aus.	I	1,3, 5	A	[38]hybrid	IZ	M/M	4/2	N.S.
(sexually compatible)[4]										
N. biglovii (24)	*Petunioides* bigelovianae	N.A.	S	4,6	III		NO SELECTION:			
N. undulata (20)	*Petunioides* Undulatae	S.A.	S	6,9	III	[39]cybrid (syl)	mt-R	M/M	10/12	N.S.
(sexual compatibility not available)										

Table 3 (cont'd)

PARENTAL SPECIES			SOMATIC HYBRIDIZATIONS				
SPECIES (2n)	[40]TAXONOMIC RELATIONSHIP	[41]SEXUAL CROSS	NUCLEAR GENOME	VERIFICATION	TISSUE SOURCE	PLASTID RATIO	STATISTICAL INFERENCE
Solanum							
S. tuberosum (48)	Subgenus; *Petotoe* section; Tuberosa	incompatible		NO SELECTION:			
S. nigrum (72)	Subgenus; *Solanum* section; Solanum		[42]hybrid	C#	M/M	3/1/8	N.S.
S. tuberosum (48)	Subgenus; *Petota* section; Tuberosa	incompatible		SELECTION: pigment deficiency			
S. brevidens (24)	Subgenus; *Petota* section; Eutuberosa		[43]hybrid	C#	M/M	0/29	unbalanced
S. pinatisectum (24)	Subgenus; *Petota* section; Pinnatisecta	compatible		NO SELECTION:			
S. phureja	Subgenus; *Petota* section; Tuberosa		[44]hybrid (pin + phu x tab)	IZ	M/M	0/6	N.S.
S. melongena (24)	Subgenus; *Leptostemonum* section; Melongena	incompatible		NO SELECTION:			
S. sisybriifolium (24)	Subgenus; *Leptostemonum* section; Olignethes		[45]hybrid	C#, IZ	S/S	0/8	unbalanced
S. melongena (24)	Subgenus; *Leptostemonum* section; Melongena	not available		NO SELECTION:			
S. torvum (24)	Subgenus; *Leptostemonum* section; Torva		[46]hybrid	C#, IZ	M/M	8/2/0	unbalanced
S. melongena (24)	Subgenus; *Leptostemonum* section; Melongena	not available		SELECTION: herbicide resistance			
S. nigrum (24)	Subgenus; *Solanum* section; Solanum		[47]hybrid	C#, IZ	M/M	0/3	N.S.
Lycopersicon							
L. esculentum (24)	"*esculentum* complex"	unilateral by ovule culture		NO SELECTION:			
L. peruvianum (24)	"*peruvianum* complex"	(esc x per)	[48]hybrid	C#	N.A.	0/5	N.S.
L. esculentum (24)	"*esculentum* complex"	unilateral (esc x pen)		NO SELECTION:			
L. pennellii (24)	"*esculentum* complex"		[49]hybrid	C#, IZ	C/M	1/1/2	N.S.
Petunia							
P. parodii (14)		unilateral (par x inf)		NO SELECTION:			
P. inflata (14)	Subgenus; *Eupetunia*		[50]hybrid	C#, IZ	M/S	10/0	unbalanced
Datura							
D. innoxia (24)		compatible by ovule culture		NO SELECTION:			
D. stramonium (24)			[51]hybrid	C#, IZ	M/M	6/1	N.S.
D. innoxia (24)		compatible by ovule culture		NO SELECTION:			
D. discolor (24)			[51]hybrid	C#, IZ	M/M	2/1/1	N.S.

Table 3 (Cont'd)

PARENTAL SPECIES			SOMATIC HYBRIDIZATIONS				
SPECIES (2n)	[40]TAXONOMIC RELATIONSHIP	[41]SEXUAL CROSS	NUCLEAR GENOME	VERIFICATION	TISSUE SOURCE	PLASTID RATIO	STATISTICAL INFERENCE
Brassica							
B. napus (38) *B. campestris* (20)		compatible	[52]hybrid (nap)	NO SELECTION: Micro-Iso	M/ES	15/4	unbalanced
Lycopersicon + Solanum							
L. esculentum (24)	Subfamily; Solanoideae Tribe; Solaneae	incompatible		NO SELECTION:			
S. nigrum (24)	Subfamily; Solanoideae Tribe; Solaneae		[53]hybrid	C♀, IZ	M/M	0/3	N.S.
L. esculentum (24)	Subfamily; Solanoideae Tribe; Solaneae	incompatible		NO SELECTION:			
S. tuberosum (24)	Subfamily; Solanoideae Tribe; Solaneae		[54]hybrid	C♀, SSU	S/M	11/7	N.S.
			[55]hybrid	C♀, SSU	M/M	0/4	N.S.
					TOTAL:	11/11	N.S.
L. esculentum (24)	Subfamily; Solanoideae Tribe; Solaneae	incompatible		NO SELECTION:			
S. rickii (24)	Subfamily; Solanoideae Tribe; Solaneae		[56]hybrid	C♀, IZ	M/S	0/4	N.S.
L. esculentum (24)	Subfamily; Solanoideae Tribe; Solaneae	unilateral by ovule culture (esc x tab)		NO SELECTION:			
S. lycopersicoides (24)	Subfamily; Solanoideae Tribe; Solaneae		[57]hybrid	C♀, IZ	M/S	68/1/1	unbalanced
Nicotiana + *Petunia*							
N. tabacum (48)	Subfamily; Cestroideae Tribe; Nicotineae	incompatible		SELECTION: pigment deficiency			
P. hybrida (28)	Subfamily; Solanoideae		[58]hybrid & cybrid (tab)	cp-T	M/M	0/83	unbalanced
				SELECTION: pigment deficiency			
			[59]hybrid & cybrid (pet)	cp-T	M/M	1/0	N.S.
Brassica + Raphanus							
B. napus (38)	Family; Cruciferae	compatible		NO SELECTION:			
R. sativus	Family; Cruciferae		[60]cybrid (nap)	Micro-Iso	M/HC	4/2	N.S.

Table 3 (Cont'd)

References: [1](Goodspeed, 1954; Burbidge, 1960; Smith, 1979;) [2](Durbin and Uchytil, 1977a; Durbin and Uchytil, 1977b; Burk and Durbin, 1978) [3](Chen and Wildman, 1981) [4](Goodspeed, 1954; Burbidge, 1960; Durbin and Uchytil, 1977; Pandy, 1979) [5](Aviv *et al.*, 1984a) [6](Fluhr *et al.*, 1983) [7](Bonnett and Glimelius, 1983) [8](Gleba *et al.*, 1985) [9](Flick and Evans, 1982) [10](Horn *et al.*, 1983) [11](Pental *et al.*, 1984) [12](Nakata and Oshkima, 1982) [13](Iwai *et al.*, 1980) [14](Hamill *et al.*, 1984) [15](Douglas *et al.*, 1981) [16](Menczel *et al.*, 1981) [17](Maliga *et al.*, 1978; Maliga *et al.*, 1980) [18](Aviv *et al.*, 1984b) [19](Gleddie *et al.*, 1983) [20](Kumashiro and Kubo 1986) [21](Belliard *et al.*, 1978; Belliard and Pelletier, 1980)[22](Fluhr *et al.*, 1984) [23](Gleba *et al.*, 1984) [24](Glimelius *et al.*, 1981) [25](Flick *et al.*, 1985) [26](Evans *et al.*, 1980) [27](Morikawa *et al.*, 1987) [28](Chapel *et al.*, 1986) [29](Akada and Hirai, 1983) [30](Kung *et al.*, 1975) [31](Chen *et al.*, 1977), [32](Medgyesy *et al.*, 1980) [33](Menczel *et al.*, 1982) [34](Menczel *et al.*, 1986) [35](Sidorov *et al.*, 1981) [36](Bourgin *et al.*, 1986) [37](Glimelius and Bonnett, 1981) [38](Scowcroft and Larkin, 1981) [39](Aviv and Galun, 1986) [40](Hawks *et al.*, 1978; Sink, 1984) [41](Hogenboom, 1979; Hermsen and Sawicka, 1979; Rao, 1979; Hawkes, 1979; Rick, 1979) [42](Gressel *et al.*, 1984) [43](Barsby *et al.*, 1984; Kemble *et al.*, 1986) [44](Sidorov *et al.*, 1987) [45](Gleddie *et al.*, 1986) [46](Guri and Sink, 1988a) [47](Guri and Sink, 1988b) [48](Piven *et al.*, 1986) [49](O'Connel *et al.*, 1987) [50](Schnabelrauch *et al.*, 1985; Clark *et al.*, 1986) [51](Schieder, 1978; Muller-Gensert and Schieder, 1985) [52](Yarrow *et al.*, 1986) [53](Guri *et al.*, 1988) [54](Melchers *et al.*, 1978; Schiller *et al.*, 1982) [55](Shepard *et al.*, 1983) [56](O'Connel and Hanson, 1986) [57](Handley *et al.*, 1986; Levi *et al.*, 1988) [58](Glimelius and Bonnert, 1986) [59](Pental *et al.*, 1986) [60](Morgan and Maliga, 1987)

*The numbers indicate the number of calli which regenerated shoots with plastids from only one parent or from both parents. eg. shoots of 1st parent/shoots of both parents/shoots of 2nd parent.

**The plastid type of hybrid calli is reported.

CHAPTER 2

Non-random plastid transmission in somatic hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*

Introduction

Maternal inheritance of plastids during sexual reproduction predominates among angiosperms, but a proportion of species have biparental inheritance (Sears 1980). Uniparental inheritance precludes the independent assortment of organelles genomes in most crop species examined so far. However, chemically or electrically mediated fusion of plant protoplasts can produce heteroplasmic cells. Subsequent sorting-out of the organelles in these cells during the cell divisions leading to regenerated plants often results in novel combinations of nuclear and cytoplasmic genomes (reviewed by Kumar and Cocking 1987).

The plastids in the somatic hybrids examined to date have sorted-out rapidly, without plastid DNA (cpDNA) recombination (Akada and Hirai, 1986; Chapter 1 of this thesis), excepting two demonstrations of cpDNA recombination using a very effective method of *in vitro* selection (Medgesey, *et al.*, 1985; Thanh and Medgyesy, 1989). Rapid sorting-out is a consequence of the rarity of plastid fusion, which precludes mixing of DNA between plastids and recombination of plastid genomes (Possingham and Lawerence, 1983). Sorting-out is usually complete before shoot regeneration, but occasionally chimeric shoots regenerate (Glimelius *et al.*, 1981; Nakata and Oshkima, 1982; Aviv *et al.*, 1984; Guri and Sink, 1988b). Shoots with mixed plastids are more common in fusions with balanced plastid transmission (Chapter 1) as predicted by a mathematical model of stochastic plastid assortment (Michaelis, 1967a). However, somatic hybrids can inherit plastids predominantly from one parent, contrary to the expectations of purely stochastic assortment (Chapter 1; Appendix I; Levi *et al.*, 1988; Clark *et al.*, 1986).

In comparison, mitochondrial genomes almost always undergo recombination or rearrangement in somatic hybridization experiments (Belliard *et al.*, 1979; Galun *et al.*, 1982; Boeshore *et al.*, 1983; Rothenberg *et al.*, 1985; Robertson *et al.*, 1987; reviewed by Hanson 1984). The

mitochondria in somatic hybrids probably sort-out more slowly than the plastids, and this may stem from the prevalence of mitochondrial fusion (Bendich and Gauriloff, 1984) and mitochondrial DNA (mtDNA) recombination (Aviv and Galun, 1986; Aviv and Galun, 1987). For example, mtDNA-encoded cytoplasmic male sterility in *Petunia* somatic hybrids continued to segregate for more than three meiotic cycles (Izhar *et al.*, 1983). A few investigations imply that non-random transmission of mitochondria also can occur in somatic hybrids, but interpretation of the data is ambiguous due to the complexity of the mitochondrial genome and the prevalence of mtDNA recombination and rearrangement (O'Connell and Hanson, 1985; O'Connell and Hanson, 1986; Levi *et al.*, 1988).

The possible causes of unequal transmission of organelles in somatic hybrids are; (1) an initially larger contribution of organelles from one fusion partner, (2) more efficient replication the organelles from one partner, (3) genetic incompatibility of the nuclear genome with the organelles from one partner, or (4) artificial selection of the organelles from one partner *in vitro* (Kumar and Cocking, 1987). Understanding the factors determining organelle transmission in somatic hybrids will lead to increased understanding of the interactions among the three different genomes within plant cells.

This chapter describes biased transmission of plastids in a large population of somatic hybrid plants in which the mitochondrial transmission probably also was biased (Appendix I; Levi *et al.*, 1988). Fusion of leaf mesophyll-derived protoplasts from *Lycopersicon esculentum* with suspension-derived protoplasts of *Solanum lycopersicoides* produced intergeneric somatic hybrid plants (Handley *et al.*, 1986). Stringent selective conditions were not used to recover the hybrid calli or plants (Sink *et al.*, 1986). The *S. lycopersicoides* suspension-derived protoplasts did not divide and calluses of the tomato cultivar rarely regenerated shoots. Putative hybrid calli were selected by their vigorous growth, and their distinct morphology and pigmentation. All of the plants studied herein have been verified as nuclear hybrids using *Got*-2 and *Got*-3 isozyme markers (Moore and Sink, 1987a).

Materials and Methods

All DNA analyzed was isolated from fully expanded leaves of plants grown in the greenhouse. *Lycopersicon esculentum* Mill. 'Sub Arctic-Maxi', *Solanum lycopersicoides* Dun. (LA 1990), their sexual hybrid, and 70 somatic hybrids were asexually propagated for the analysis. Whole cell DNA was isolated from bulk leaf samples collected from several areas of each somatic hybrid. Hybrids 63 and 240 were selected for more detailed study. Three clones from shoot culture of regeneratant number 63 and one shoot from regeneratant number 240 were rooted *in vitro* and transferred to the greenhouse. Several cuttings of hybrid 240 were propagated after it was transferred to the greenhouse. Whole cell DNA was isolated from several small samples (app. 0.5 g) collected from the individual branches of each of the cloned plants.

Chloroplasts were isolated from freshly harvested tissue using 5-step 20-60% sucrose gradients as described by Palmer (1986) with some modifications. The extraction and wash buffers both contained 0.4 M sucrose, 50 mM Tris pH 8, 6 mM Na EDTA; but 0.1% BSA (w/v), 0.15 PVP-40 (w/v), and 30 mM 2-mercaptoethanol were also added to the extraction buffer. Chloroplast DNA (cpDNA) was prepared by suspending the organelle fraction in CTAB buffer (10 mM Tris pH 8, 1.4 M NaCl, 20 mM Na EDTA, 2% cetyltrimethylammonium bromide (w/v), 120 mM 2-mercaptoethanol), extracting with 24:1 chloroform/isoamyl alcohol, then precipitating the nucleic acids by adding 2-3 volumes of ethanol (Saghai-Maroof *et al.*, 1984). The cpDNA pellets were dissolved and further purified on CsCl/ethidium Bromide (EtBr) gradients.

Plant total cellular DNA was isolated from fresh tissue or from frozen tissue that had been stored at -70 ^{o}C. The DNA was isolated by grinding 0.5 g of leaves in a mortar with 10 ml of the same buffer used for chloroplast extraction and centrifuging the slurry at 4,500 x g; 15 min. The pellet was resuspended in CTAB buffer and extracted with 24:1 chloroform/isoamyl alcohol. The nucleic acids were precipitated and further purified on CsCl/EtBr gradients. Electrophoresis, Southern

blotting, nick translation, hybridization, autoradiography, and photography were performed using standard procedures (Maniatis *et al.*, 1982).

A 15 kilobase (kb) tomato chloroplast *Pst* 1 clone (P-6) was used for the cpDNA analysis (Phillips 1985). Purified nuclear and mitochondrial DNA from both *L. esculentum* and *S. lycopersicoides* were the kind gifts of Dr. Patrick Moore (Moore and Sink, 1988b) and Mr. Amnom Levi (Levi *et al.*, 1988) respectively.

Results

Whole cell DNA was isolated from bulk leaf samples collected from several branches of each somatic hybrid in order to screen more regions for rare plastid sectors. *S. lycopersicoides* has an additional *Pst* I site which splits the tomato 15 kb cpDNA fragment corresponding to clone P-6 into 9 kb and 6 kb fragments (J. Palmer personal communication). When *Pst* I digests from 70 different somatic hybrids were blotted onto nitrocellulose and probed with P-6, all but two had the tomato cpDNA restriction pattern (Figure 1). Hybrid 240 had the *S. lycopersicoides* cpDNA pattern and hybrid 63 had a pattern corresponding to a mixture of parental cpDNAs (Figure 1). Hybrid 240 had *S. lycopersicoides* mitochondria; the mtDNA of hybrid 63 was not analyzed (Levi *et al.*, 1988). The sexual hybrid had the *L. esculentum* plastid pattern as expected (data not shown). No nuclear or mitochondrial homologies were detected when purified nuclear, mitochondrial and chloroplast DNAs from these species were probed with P-6 (Figure 1; data not shown).

The mixed cpDNA pattern of hybrid 63 could have been due to the maintenance mixed cells, a stable periclinal chimera, or sorting-out between branches. The original analysis using bulk samples could not distinguish these possibilities. In addition, bulking leaves from many branches reduces the sensitivity of the analysis by diluting the cpDNA from rare sectors. For these reasons hybrids 64 and 240 were analyzed using separate samples from several branches of each plant.

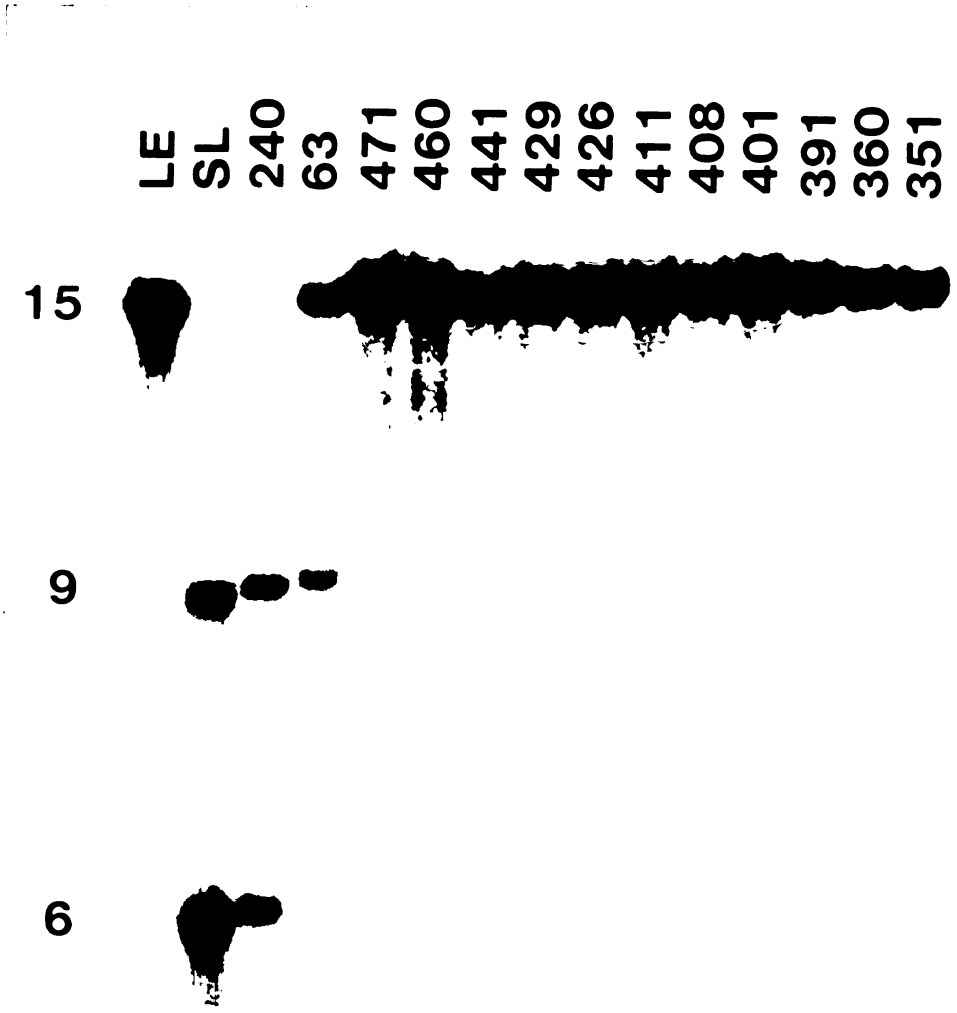

Figure 1. Southern analysis of plastid DNA from the parental lines and thirteen somatic hybrids. *Pst* I digests of whole cell DNA from the somatic hybrids or purified cpDNA from *L. esculentum* (LE) and *S. lycopersicoides* (SL) were electrophoresed on a 0.8% agarose gel, blotted to nitrocellulose, and probed with the ^{32}P labeled cpDNA clone P-6. The sizes of the fragments are given in kilobase pairs.

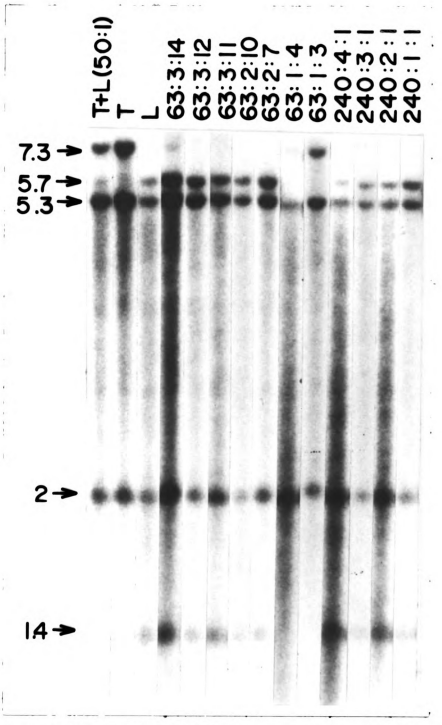

Figure 2. Southern analysis of the plastid DNA from the parental lines *S. lycopersicoides* (SL), and *L. esculentum* (LE), and eleven samples of somatic hybrids 240 and 63. *Eco* RV-*Pst* I double digests of whole cell DNA were electrophoresed on an 0.8% agarose gel, blotted to nitrocellulose, and probed with the ^{32}P labeled cpDNA clone P-6. The DNA samples are labeled first with the hybrid number, then with the plant clone number, and finally with the DNA sample number separated by colons. The lane marked LE + SL contains a 50:1 ratio of tomato to *S. lycopersicoides* whole cell DNA.

Seven samples from hybrid 240 and 13 samples from hybrid 63 were double-digested with *Sal* I and *Pst* I, or with *Eco* RV and *Pst* I. Southern blots of these double-digests were probed with the cloned cpDNA fragment P-6. In *Sal* I- *Pst* I digests *S. lycopersicoides* has an additional *Pst* I site which cleaves the tomato 8.6 kb fragment to yield 5.9 and 2.7 kb fragments (data not shown). The same *Pst* I site results in a missing 7.3 kb fragment and additional 5.7 and 1.4 kb fragments in *Eco* RV- *Pst* I digests of *S. lycopersicoides* (Figure 2). A faint 7.3 kb *L. esculentum* band could be detected in *Eco* RV- *Pst* I digests when a 50:1 mixture of *L. esculentum* and *S. lycopersicoides* DNAs was analyzed to test the sensitivity of the procedure (Figure 2). All seven samples from hybrid 240 had *S. lycopersicoides* plastids (Figure 2). Seven hybrid 63 samples from various clones had *S. lycopersicoides* plastids and five had *L. esculentum* plastids (Figure 2). The remaining hybrid 63 sample (63:3:14) from *in vitro* clone 63:3, had a faint 7.3 kb band which may have been due to a small amount of *L. esculentum* cpDNA or partial digestion (Figure 2). Similarly, a faint 8.6 kb band was observed in *Sal* I- *Pst* I digests of sample 63:3:14 (data not shown). All other DNA samples from clone 63:3 had only *S. lycopersicoides* fragments. Similarly, all samples from hybrid 63 *in vitro* clone 63:1 had the *L. esculentum* pattern, and all samples from hybrid 63 *in vitro* clone 63:2 had the *S. lycopersicoides* pattern.

Discussion

The two solanaceous species utilized in these experiments are unilaterally sexually compatible when tomato is the female parent, but the hybrids are highly sterile (Rick, 1979). Hence, these somatic hybrid plants afford the opportunity to study unique combinations of organelle genomes not normally possible following sexual mating. It is striking that the majority of these somatic hybrids seem to have chloroplasts and mitochondria from opposite fusion parents. They have predominantly (97%) tomato plastids and probably have exclusively *S. lycopersicoides* mitochondria (Appendix I; Levi *et al.*, 1988).

The plastid transmission in these somatic hybrids could have been determined by the relative number of plastids donated to the heterokaryon by the parental protoplasts. In these experiments,

the tomato protoplasts were isolated from leaf mesophyll cells and fused with suspension-derived *S. lycopersicoides* protoplasts. Mesophyll cells have about 10-fold more plastids than meristematic cells (Possingham and Lawrence, 1983), more than any other tissue that has been examined (Steele-Scott *et al.*, 1984). To my knowledge, the number of plastids found in long-term suspension cells has not been determined. However, these rapidly dividing cells are similar to meristematic cells of whole plants and probably have relatively few proplastids. For this reason tomato chloroplasts probably predominated in the heterokaryons. Following fusion, the partitioning of plastids during cell division would have fixed the predominant type (Akada and Hiria, 1986; Michaelis, 1967a). This process would have been quite rapid if the number of plastids in the hybrid cells decreased from around 100 to about 10 within 3-4 divisions as they did in cultured mesophyll protoplasts of *N. tabacum* (Thomas and Rose, 1983).

There are eight reports of mesophyll-suspension cell fusions of various species where mesophyll-derived plastids clearly predominated, none where the plastids from the suspension partner predominated and two reports of relatively large populations having balanced transmission (Chapter 1). The cumulative ratio from all the mesophyll-suspension fusions without plastid selection which are tabulated in Chapter 1, Table 3 is 185 with plastids from mesophyll partners to 97 with plastids from suspension partners. Clearly, our ratio of 68 hybrids with plastids from the mesophyll partner to 2 with plastids from the suspension partner much more skewed, suggesting other factors may be involved. However, the cumulative ratios are subject to uncertainty because the number of comparisons is small and many uncontrolled factors among the various experiments could have affected the result. Differential transmission of plastids due to unequal input from the gametes is a well documented aspect of sexual reproduction in higher plants (Whatley, 1982).

Another possibility is that more rapid proliferation of the plastids from *L. esculentum* resulted in biased transmission (Chapter 1). Plastids can divide continuously during the cell cycle but the maximum number per cell seems to be limited in a developmentally coordinated fashion (Possingham and Lawrence, 1983). Assuming that plastid multiplication is a stochastic process, plastids that can divide more rapidly when the number per cell is below the limit will predominate in subsequent cell

generations. *Oenothera*, with biparental plastid inheritance, has yielded genetic evidence supporting this concept. Schötz (1954) discovered differences in competition between five different plastomes (plastid chromosomes) of *Euoenothera* when they were brought together by sexual matings. Each of the five plastome types was assayed using reciprocal crosses in which one parent carried a plastome encoded albino mutant, and the other carried the wild-type plastome to be tested. The germinating seeds from these crosses were scored by measuring the relative size of the green sectors on their cotyledons, which gives a quantitative estimate of the relative competitiveness of each plastome. This "variegation rating" ranks the five plastomes in nearly the same order regardless of the direction of the cross in a constant nuclear background (Chiu *et al.*, 1988). Schötz (1968) attributed this to differences in the rate of plastid multiplication.

Perhaps the under-represented *S. lycopersicoides* plastids were less competitive in the hybrid cells due to incompatibility with the hybrid nuclear genome. Biased sorting out due to plastome-genome incompatibility may have occurred at any time between cell fusion and the time of analysis. Genetic evidence for the incompatibility of plastomes with certain nuclear genomes has been obtained for *Oenothera*. Stubbe (1960) studied the reaction of the five different plastomes of subsection *Euoenothera* and found profound differences in their ability to green when combined with different nuclear complements. The manifestation of this "plastome-genome" incompatibility ranged from small changes in chloroplast pigmentation to complete lethality. Each plastome type is more compatible when associated with a genome more closely related to the one with which it co- evolved (Kutzelnigg and Stubbe, 1974).

Incompatibility during plant regeneration, which is a complex process involving coordinate expression of many genes, may be particularly important in determining organelle transmission. Menczel *et al.*, (1981) reported two somatic fusion experiments using *Nicotiana tabacum* and *N. knightiana* with completely different results. In the first experiment they observed only *N. knightiana* chloroplasts in the hybrid plants, and in the second they observed balanced transmission of both types. They suggested the culture conditions used in the first experiment precluded regeneration from cells with *N. tabacum* plastids. Schnabelrauch and Sears (personal communication) studied regeneration

of isonuclear lines of *Oenothera* with five different plastomes, and observed an influence of plastome type on the efficacy of regeneration. Perhaps in our experiments, cells with different plastomes also responded to the culture medium differently.

The bulk DNA sample from hybrid 63 had cpDNA restriction fragments corresponding to both parents. Originally, the hybrid 63 shoot which regenerated had plastids from both species, but the plastids sectored as the plant was propagated via shoot culture. Segregation must have been nearly complete by the time the original shoot could be cloned in shoot culture since only one of the hybrid 63 *in vitro* clones may have had plastids from both species. This is in accord with observations of plastid segregation in other somatic hybrids. Mixed cells usually sort prior to shoot regeneration making hybrid plants with mixed plastids uncommon (Chapter 1; Kumar and Cocking, 1987). Stable plastid chimeras have not been reported after somatic hybridization, but are fairly common after sexual crosses. Furthermore, there is only one report of the stable maintenance of different cpDNAs within plant cells and it did not involve somatic hybridization (Moon *et al.*, 1987).

CHAPTER 3

Conclusion

The prevalence of uniparental inheritance of plastids and the scarcity of intergenomic recombination of plastomes can impede breeding efforts to improve crop plants. Some agronomically important traits encoded by plastomes are atrazine resistance (Robertson, 1985), resistance to certain diseases (Vaughn and Duke, 1984), and control of photorespiration (Ogren, 1984). The primary function of plastids is photosynthesis, but they also have other important metabolic functions. Plastids participate in chlorophyll, amino acid, and fatty acid biosynthesis; nitrate and sulfate reduction; and starch metabolism (Kirk and Tilney-Bassett, 1978). Therefore, it is implicit that plastomes encode other traits which could be manipulated for agronomic benefit. Somatic hybridization of plants by *in vitro* fusion of protoplasts produces heteroplasmic cells. Unique genetic complements of both cytoplasmic and nuclear genomes can result from sorting and recombination of genetic elements during the subsequent cell generations.

The evolutionary origin of plastids and the manner in which they transmit their genetic information has influenced the structure of modern plastomes. Examination of plastomes with this in mind provides information about the mechanisms governing plastome inheritance. Hence, consideration of certain aspects of plastid evolution and genetics is relevant in understanding plastid transmission in cell fusion hybrids.

Plastids are thought to have arisen through endosymbiosis of an organism related to blue green algae within a primitive eucaryotic cell. Plastomes from evolutionarily diverse groups such as algae and angiosperms encode nearly the same complement of genes. The plastomes of all the land plants examined to date encode about 120 genes, and those with known functions encode components of the plastid transcription and translation systems, photosynthetic complexes, and chlororespiration system. Plastid genes are transcribed and translated in the plastid by enzymes which are almost entirely distinct from those found in the nucleus and cytosol.

However, most proteins found in plastids (about eighty to ninety percent) are encoded by nuclear genes, translated on cytoplasmic ribosomes and transported across the plastid envelope (Kirk and Tilney-Bassett, 1978). Most of these genes were probably transferred from the plastome to the nucleus during the course of evolution. The fact that plastomes from diverse species encode nearly the same set of genes implies that most genes were transferred to the nucleus early in the evolution of the symbiosis, and that the plastome has been relatively stable ever since. In support of this, a bryophyte (*Marchantia polymorpha*) and an angiosperm (*Nicotiana tabacum*) have their plastid genes arranged in nearly the same order with the exception of a 30 kb inversion.

Plastids may be inherited maternally, paternally or biparentally in higher plants. Genetic analysis using appropriate plastid markers is needed to definitively establish the means of plastid inheritance, since cytological observation of organelles during reproduction can be unreliable (Sears, 1980b). Markers such as restriction fragment length polymorphisms, pigment mutations and antibiotic resistance have been used to follow organelle inheritance in plants. Some criteria that may be used to demonstrate that a trait is cytoplasmically encoded are: 1) non-Mendelian inheritance such as reciprocal differences after reciprocal crosses, 2) the sorting out of genetic entities during development, and 3) molecular genetic evidence that a trait is cytoplasmically encoded (Kirk and Tilney-Bassett, 1978; Medgyesy *et al.*, 1986). Cytoplasmic traits also must be assigned to either plastids of mitochondria by morphological, cytological or biochemical characterization (Michaelis, 1966; Kutzelnigg and Stubbe, 1974; Fluhr *et al.*, 1985; Börner and Sears, 1986).

Plastome encoded pigment-deficiency mutants in plants with biparental inheritance provide a convenient means to study the processes involved in sorting-out of plastids during development. In plants developing from biparental embryos the plastids from each parent segregate during vegetative growth until pure or chimeric shoots are formed (Kirk and Tilney-Bassett, 1978). Plastid chimeras may sort further, but can be relatively stable during continued plant growth (Tilney-Bassett, 1986). When a pigment-deficiency marker is used to follow the sorting process there are four visibly discernible stages: 1) the initial phase with only mixed cells and no visible manifestation, 2) a fine mosaic phase during which the mixed cells sort-out as they divide producing pure white and pure

green cells, 3) a coarse mosaic phase with few remaining mixed cells during which pure tissues sort-out from a mosaic of pure cells and 4) a final phase during which pure white, pure green, and chimeric shoots segregate from pure tissues (Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1986).

When the different plastids replicate at an equal rate, partition randomly into daughter cells, and do not differentially effect cell division or plant development, plastid segregation is a random process (Michaelis, 1967a). Michaelis (1967a) developed a mathematical model to predict the characteristics of plastid segregation when it is a stochastic process. The model is based on the additional assumptions that the number of plastids per cell is a constant (2N) prior to division, and each daughter cell receives half (1N) during cell division. Also, each plastid must replicate only once each cell cycle. It is clear from many investigations of plastid behavior that each of these assumptions is normally violated at different phases of the plant's life cycle (Sears, 1980b; Whatley, 1982; Possingham and Lawrence, 1983; Thomas and Rose, 1983; Bendich, 1987). However, Michaelis' model provides an excellent basis for understanding plastid behavior and plastids often sort-out as predicted, although exceptions are fairly common (Michaelis, 1967b; Kirk and Tilney-Bassett; 1978; Birky, 1978; Kumar and Cocking, 1987).

Several general features of plastid somatic segregation can be inferred from this model (Kirk and Tilney-Bassett, 1978). The summation of all the ratios of different plastid types in the progeny cells is always the same as the ratio the initial cell originally had. Hence, after sorting-out is complete the proportion of cells with each plastid type reflects the ratio in the initial cell. Furthermore, a cell must undergo about 10 x N cell divisions to attain nearly complete (>99%) sorting-out, where N is the number of plastids per cell immediately after division.

It is useful to at this point to digress and consider whether individual plastids or plastomes are appropriate as the units of segregation. Angiosperm cells each have about 500-50,000 copies of the plastome (Possingham and Lawrence, 1983; Bendich, 1987). Since meristematic tissues have the lowest cpDNA ploidy and are most important in the sorting-out process, at least 2500 (10 x N) cell divisions would be required for nearly complete sorting of cpDNA molecules. Most annual plants would require several reproductive cycles for this number of cell divisions. In comparison,

meristematic cells of angiosperms have about 10-30 plastids, corresponding to 50-150 divisions for nearly complete sorting-out of plastids (Possingham and Lawrence 1983; Steele-Scott *et al.*, 1983). Annual plants usually sort-out in one or two reproductive cycles and two cycles could usually be completed within 50-150 cell divisions (Michaelis, 1967b). It should be noted that the *effective* number of segregation units is always reduced when there is plastid selection, unequal efficiency of plastid replication, unequal partitioning into daughter cells or incomplete mixing of plastids between divisions (Michaelis, 1967a). Hence, calculations using the number of plastids per cell give the maximum number of divisions needed for sorting-out.

The high ploidy of the plastome and the large number of plastid per cell probably play a key role in the strong evolutionary conservation of cpDNAs. In the few instances where intermolecular cpDNA recombination has been demonstrated the recombinant molecules have many closely spaced crossover points (Sears, 1980a; Mets and Geist, 1983; Lemieux *et al.*,1984; Medgyesy *et al.*, 1986). This suggests that recombination occurred through gene conversion. There is also evidence that intermolecular copy correction through gene conversion occurs between the large inverted repeats (Chapter 1; Dron *et al.*, 1983; Erickson *et al.*, 1985). This implies that copy correction also occurs between multiple copies of cpDNA within each plastid. It follows that a mutant cpDNA molecule could either be eliminated or fixed through consecutive rounds of gene conversion (Birky, 1978). Since a mutant molecule must arise as one of a population of 20-200 wild type molecules in a plastid, it is much more likely to be eliminated than fixed (Possingham and Lawrence, 1983; Steel-Scott *et al.*, 1984; Bendich, 1987). If a mutation is to be passed on to subsequent generations it must be fixed in one plastid, compete successfully in a population of about 10-200 plastids per cell and be maintained in a cell lineage that gives rise to germ cells (Kirk and Tilney-Bassett, 1978; Possingham and Lawrence, 1983). Since this process is largely stochastic the chances for successful establishment of a mutation are low.

Cell fusion has been used to study the somatic genetics of plastids in plant species lacking biparental inheritance (for reviews of plastid transmission following somatic hybridization see: Fluhr, 1983; Harms, 1983; Gleba and Syntnik, 1984; Pelletier and Chupeau, 1984; Pelletier, 1986; Maliga and

Menczel, 1986; Grun, 1986; Kumar and Cooper-Bland, 1986; Kumar and Cocking, 1987). As discussed in Chapters 1 and 2 of this thesis, the behavior of plastids in somatic tissues is similar after sexual and somatic hybridizations. However, caution must be used since the *in vitro* culture conditions used during somatic hybridization could influence plastid transmission in ways that are not always readily apparent (Chapter 1). In addition, somatic hybridization procedures are both difficult and laborious so the number of experimental observations is restricted. Perhaps as a consequence, somatic hybridization experiments designed specifically to study plastid genetics have been rare (Akada and Hirai, 1983; Akada *et al.*, 1983; Akada and Hirai, 1986). Therefore, most conclusions about plastid inheritance in somatic hybrids are based in comparisons of several experiments from different laboratories (Chapter 1). Such conclusions are subject to error due to the influence of many uncontrolled factors, such as differences in the method of fusion, selection of hybrids, or plastid analysis.

The cpDNA coding complement and arrangement is stable during somatic hybridization, as expected given the striking evolutionary conservation of plastomes. In contrast, mitochondrial genomes regularly recombine or rearrange after somatic hybridization, as expected given their evolutionary fluidity (Palmer, 1985a; Hanson, 1984). Therefore in is not surprising that we observed cpDNA stability and mtDNA rearrangement in our studies of the *L. esculentum* + *S. lycopersicoides* somatic hybrids (Chapter 1; Appendix 1).

The contribution of plastids from each fusion partner can influence plastid transmission after hybridization. When sorting-out of plastids after somatic hybridization is completely random, the proportion of pure cell lines when sorting-out is complete should reflect the original plastid ratio in the heterokarion (Chapter 1; Michaelis, 1967a; Birky, 1978; Akada and Hiria, 1986). There are more plastids in mesophyll cells than any other tissue examined, and mesophyll derived plastids often predominate when mesophyll and suspension derived protoplasts are fused (Kumar and Cocking, 1987). I am aware of no reports of mesophyll- suspension fusions where the plastids from the suspension derived partner clearly predominated (Chapter 1). The cumulative ratio of mesophyll to suspension derived plastids from all the mesophyll- suspension fusions tabulated in Chapter 1 is 185

to 97. In our experiments the ratio of plastids from the mesophyll (*L. esculentum*) and suspension (*S. lycopersicoides*) partners is 68 to 2 (Chapter 2; Appendix 1). Differences in the plastid input probably contributed significantly to our results. However, ours is the most unbalanced ratio reported for a mesophyll- suspension fusion suggesting that other factors may have also been involved.

The relative replicative competitiveness of different plastids is important in determining plastid transmission in sexual crosses in *Euoenothera* (Chapter 1; Schötz, 1954; Kirk and Tilney-Bassett, 1978), and may have had a role in the evolution of this subsection (Stubbe, 1964). More rapid division of the plastids from one parent can increase their frequency in subsequent cell generations. Contrary to an assumption the Michaelis model, each plastid probably does not replicate exactly once each cell cycle. Plastid division can occur continuously during the cell cycle but the maximum number per cell is under stringent developmental regulation (Possingham and Lawrence, 1983); Thomas and Rose, 1983). When the number of plastids per cell is below the maximum, those that divide more rapidly can reproduce more frequently and predominate in subsequent cell generations. The relative replicative competitiveness of different plastids is very likely to influence plastid transmission in somatic hybrids as well (Appendix 1), and there is evidence for such an influence in hybrids from the genus *Nicotiana* (see Chapter 1). It is possible that the highly unbalance plastid transmission we observed in our *L. esculentum* + *S. lycopersicoides* somatic involved differential replicative competitiveness.

Plastid transmission in somatic hybrids is almost certainly influenced by compatibility with the nuclear genome. Eighty to ninety percent of proteins needed for normal plastid function are encoded in the nucleus (Parthier, 1982; Palmer, 1987). There is ample evidence of nucleo-cytoplasmic incompatibility from sexual hybridization in *Euoenothera* and *Pelargonium* (Chapter 1; Stubbe, 1960; Metzlaff *et al.*, 1982; Polheim, 1986). However, evidence for a nuclear influence of plastid transmission in somatic hybrids is scarce, primarily because of the lack of relevant nuclear-encoded genetic markers (Chapter 1; Pelletier *et al.*, 1983; Menczel *et al.*,1987; Kumar and Cocking, 1987). Our two somatic hybrids with *S. lycopersicoides* plastids grew vigorously and were visibly indistinguishable from the 68 with *L. esculentum* plastids (Appendix 1). Clearly, both plastid types

were compatible with the hybrid nuclear genome. However, the nuclear genome could have had a quantitative influence on plastid segregation in these hybrids.

The rapid pace and stochastic attributes of plastid segregation in somatic hybrids are congruent with observation of the segregation process after sexual crosses (Chapter 1; Michaelis, 1967a; Birky, 1978; Akada and Hirai, 1986). In summary, the possible causes of unequal transmission of organelles in somatic hybrids are: 1) a larger organelle contribution from one fusion partner, 2) more efficient replication of the organelles from one partner, 3) incompatibility of the nuclear genome with the organelles from one partner, or 4) *in vitro* selection of the organelles form one partner with antibiotics or culture conditions (Kumar and Cocking, 1987). My research determined the origin of the plastids in our somatic hybrids, but gave little information about the cause(s) of the unbalanced plastid transmission I observed.

Combinations of fusions between mesophyll and suspension derived protoplasts isolated from *L. esculentum*, *S. lycopersicoides*, and a third 'tester" species such as *L. pennellii* could be used to distinguish the influences of plastid replication efficiency and the initial input of plastids. Observation of the number and morphology of the plastids in each type of protoplast could help determine the role of plastid input. Combinations of hybridizations and cybridizations with {L. esculentum} and {S. lycopersicoides} could be used to examine the influence of the nuclear genome on plastid transmission. Understanding the role of these factors in determining organelle transmission in somatic hybrids will lead to better understanding of the interactions among the three genomes within plant cells.

APPENDIX

APPENDIX

Non-random organelle transmission in somatic hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*

A. Levi, B.L. Ridley and K.C. Sink

The following manuscript has been published in Current Genetics (14:177-182). The author of this thesis was responsible for the chloroplast analysis conveyed herein, and a large portion of the writing of the mannuscript.

Summary.

Restriction fragment length polymorphisms (RFLPs) were used to determine the transmission of organelle genomes in somatic hybrid plants of tomato and its wild relative *Solanum lycopersicoides*. Biased frequencies of organelle combinations were observed in a population of 70 somatic hybrid plants each derived from a separate callus. The plastids in 68 of 70 hybrids examined were from *L. esculentum*. One of the remaining hybrids, plant 240, had *S. lycopersicoides* plastids and the other, plant 63, had a mixture of parental plastids. Forty-six of the same 70 plants were analyzed for mtDNA and all had that of *S. lycopersicoides* including plant 240. One of these hybrids had novel mtDNA fragments which may have resulted from recombination or rearrangement. The non-random transmission may have resulted from an initial unequal input of organelles, differential replication of organelles, or nucleo-organelle incompatibility.

Key words: RFLP - tomato - plastid - mitochondria - protoplasts

Introduction

Maternal inheritance of organelles predominates among angiosperms during sexual reproduction with only a few species exhibiting biparental inheritance (Sears 1980; Smith et al. 1986). Such inheritance schemes preclude independent assortment of organelle genomes in almost all crop plants. Conversely, cell fusion of plant protoplasts induced by chemical or electrical methods produces heteroplasmic cells. Subsequent random sorting-out of the organelles during cell divisions and organogenesis often results in novel combinations of organelle genomes in the regenerated plants. The plastomes (plastid chromosomes) usually quickly sort-out in a random manner (Chen et al. 1977; Belliard et al. 1978; Scowcroft and Larkin 1981; review by Kumar and Cocking 1987) or unilaterally when a chloroplast marker is selected (Medgyesy et al. 1980; Fluhr 1983; Menczel et al. 1986). Each regenerated shoot usually has plastids from only one parent, but some experiments have produced chimeric shoots (Gleba et al. 1985; review by Kumar and Cocking 1987). These results stem from the lack of plastid fusion which precludes cpDNA recombination (Possingham and Lawrence 1983), and from rapid sorting-out of plastids during cell division (Akada and Hirai 1986). However, Medgyesy et al. (1985) demonstrated plastid recombination when the heteroplasmic state was prolonged. In contrast, mitochondrial genomes almost always undergo recombination or rearrangement in somatic hybridization experiments (Belliard et al. 1979; Galun et al. 1982; Boeshore et al. 1983; Rothenberg et al. 1985; Robertson et al. 1987; reviewed by Hanson 1984). Although the evidence is as yet meager, mitochondria may not sort-out as quickly as plastids (Aviv and Galun 1987). For example, cytoplasmic male sterility in *Petunia* somatic hybrids continued to segregate for more than three meiotic cycles (Izhar et al. 1983).

In this report we describe the biased transmission of both plastids and mitochondria in a large population of somatic hybrid plants. Fusion of leaf protoplasts from tomato with suspension- derived protoplasts of *Solanum lycopersicoides* produced intergeneric somatic hybrid plants (Handley et al.,

1986). Stringent selective conditions were not used to recover the hybrid calluses or plants (Sink et al. 1986). *S. lycopersicoides* suspension-derived protoplasts did not divide and calluses of the tomato cultivar line used rarely regenerated shoots. Putative hybrid calluses were selected by their distinct morphology, pigmentation and vigorous growth. All of the plants studied herein have been verified as nuclear hybrids using *Got*-2 and *Got*-3 isozyme markers (Moore and Sink, 1987).

These two solanaceous species are unilaterally sexually compatible when tomato is the female parent but the hybrids are highly sterile (Rick 1951). Hence, the somatic hybrid plants afford the opportunity to study unique combinations of organelle genomes not normally possible following sexual mating.

Materials and Methods

All DNA analyzed was isolated from fully expanded leaves of plants grown in the greenhouse. *Lycopersicon esculentum* Mill. 'Sub Arctic-Maxi', *Solanum lycopersicoides* Dun. (LA 1990), their sexual hybrid, and 70 somatic hybrids were asexually propagated for analysis. Chloroplasts were isolated using 5-step 20-60% sucrose gradients as described by Palmer (1986) with some modifications. The extraction and wash buffers both contained 0.4 M sucrose, 50 mM Tris pH 8, 6 mM Na EDTA; whereas, 0.1% BSA (w/v), 0.15% PVP-40 (w/v), and 30 mM 2-mercaptoethanol were added to the extraction buffer. Mitochondria were isolated according to Hanson et al. (1986) using DNAse treatment and omitting the sucrose gradient. Chloroplast DNA (cpDNA) or mitochondrial DNA (mtDNA) was prepared by suspending the organelle fraction in CTAB buffer (10 mM Tris pH 8, 1.4 M NaCl, 20 mM Na EDTA, 2% cetyltrimethylammonium bromide (w/v), 120 mM 2-mercaptoethanol), extracting with 24:1 chloroform/isoamyl alcohol, then precipitating the nucleic acids by adding 2-3 volumes of ethanol (Saghai-Maroof et al. 1984). The cpDNA pellets were dissolved and further purified on CsCl/ethidium Bromide (EtBr) gradients. The mtDNA pellets were used without further purification, and the yields were 3 to 15 ug of mtDNA/50 g of leaves, depending on genotype. Whole cell DNA was isolated by grinding 0.5 g of leaves in a mortar with 10 ml of the same buffer used for chloroplast extraction and centrifuging the slurry at 4,500 g; 15 min. The pellet

was resuspended in CTAB buffer and extracted with 24:1 chloroform/isoamyl alcohol. The nucleic acids were precipitated and further purified on CsCl/EtBr gradients. Electrophoresis, Southern blotting, nick translation, hybridization, autoradiography, and photography were performed using standard procedures (Maniatis et al. 1982).

A 15 kilobase (kb) tomato chloroplast Pst 1 clone (P-6) was used for the cpDNA analysis (Phillips 1985). The mtDNA was probed using pZmE1, a 2.4 kb Eco RI clone encoding corn cytochrome C-oxidase subunit II (Fox and Leaver 1981) and pmt SylSa8, a 22.1 kb clone containing 8.9, 8.4, and 4.8 kb Sal I mitochondrial fragments from *Nicotiana sylvestris* (Aviv et al. 1984).

Results

Total DNA was isolated from 70 hybrids, digested with Pst I, blotted onto nitrocellulose and probed with P-6. *S. lycopersicoides* has an additional Pst I site which splits the tomato 15 kb cpDNA fragment into 9 kb and 6 kb fragments (J. Palmer personal communication). Sixty-eight of the 70 hybrids examined had the *L. esculentum* 15 kb fragment (Fig. 1). Hybrid 240 had the *S. lycopersicoides* pattern and hybrid 63 had a pattern corresponding to a mixture of parental cpDNAs (Fig. 1). Hybrid 240 had *S. lycopersicoides* mitochondria; the mtDNA of hybrid 63 was not analyzed. The sexual hybrid had the *L. esculentum* plastid pattern as expected (data not shown). No nuclear or mitochondrial homologies were detected when purified nuclear, mitochondrial and chloroplast DNAs from these species were probed with P-6 (data not shown).

To detect RFLPs between tomato and *S. lycopersicoides* mtDNA, digestion patterns produced by 7 restriction enzymes were screened using the 2 mtDNA specific probes. No differences in restriction patterns of the parents were detected when the Pst I, Sst I and Xho I digests were probed with pZmE1; or when the Sst I digest was probed with pmt SylSa8 (Table 1). However, differences in the parental mtDNAs were detected using eight enzyme-probe combinations. To perform the mitochondrial analysis, Hind III or Eco RI digests of purified mtDNA from hybrid plants were probed with pZmE1 and pmt SylSa8, and Bam hi or Xho 1 digests were probed only with pmt SylSa8. The sexual hybrid had the maternal tomato mitochondrial genome as expected (data not shown).

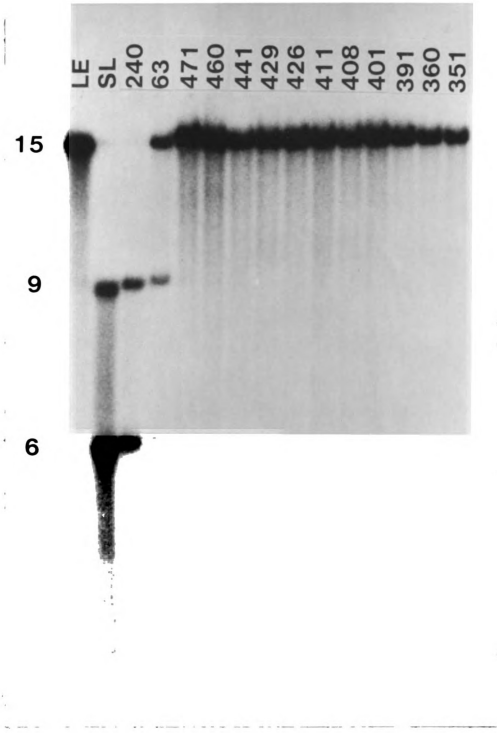

Figure 1. Southern analysis of plastid DNA from the parental lines and thirteen somatic hybrids. *Pst* I digests of whole cell DNA from the somatic hybrids or purified cpDNA from *L. esculentum* (LE) and *S. lycopersicoides* (SL) were electrophoresed on a 0.8% agarose gel, blotted to nitrocellulose, and probed with the ^{32}P labeled cpDNA clone P-6. The sizes of the fragments are given in kilobase pairs.

Table 1. Restriction fragment lengths of *L. esculentum* (LE) and *S. lycopersicoides* (SL) mitochondrial DNA in kilo-base pairs.

Enzyme	Probes					
	pmtSylSa8			pZmE1		
	Common Bands	Diagnostic Bands		Common Bands	Diagnostic Bands	
		SL	LE		SL	LE
Kpn I	–	–	–	None	23	24
Pst I	–	–	–	18, 15, 8.2	None	None
Sst I	24, 22.5, 12, 10, 8.5, 2.8	None	None	14, 8.2, 5.0, 0.8	None	None
Hind III	14.2, 12.5, 8.4, 5.9, 5.2, 2.5	6.8	None	9.4, 7.2, 3.4, 1.8	4.3	None
EcoRI	6.6, 6.5, 6.4, 3.5, 3.2, 3, 2.3, 1.8, 1.1	5.6, 5.1	6.3, 5.7	2.6	3.6, 1.2	4.6, 1.9
Bam HI	21, 20.5, 20, 13, 7.4, 5.4, 4.7, 2.2	22, 21.5, 2.8	21.7	10, 1.9	7.5	7.8
Xho I	17.5, 16, 13, 7.9, 4.6, 2.8, 1.3	40, 21, 8.3, 6.7	32, 30, 22	6.7, 2.8, 0.8	None	None

To determine the mt-DNA composition of the somatic hybrids, a random subset of 46 plants was analyzed. Forty-two hybrids were analyzed using both pmt SylSa8 and pZmE1, and 4 were analyzed using only one probe. Forty-five hybrid plants analyzed by Southern blotting had the same pattern as *S. lycopersicoides* (See hybrids 8 and 57, 38 and 51, and 69 in Figs. 2a, 2b and 3, respectively).

In contrast, when a Hind III digest of hybrid 23 was probed with pmt SylSa8 the pattern was the same as that of *S. lycopersiciodes* except a 14.2 kb fragment was missing and a novel 10.5 kb fragment appeared (Fig. 2a). In Bam HI digests of mtDNA from hybrid 23, the same probe hybridized to a novel 9.3 kb fragment and a 4.7 kb fragment with altered stoichiometry (Fig. 2b). This plant also had a more intense 8.0 kb band when a Hind III digest was probed with pZmE1 (data not shown). Hybrid 23 had no other changes from the *S. lycopersicoides* mitochondrial pattern when Southern blots of Eco RI digests were probed with pZmE1, or when the Hind III restriction pattern was examined on EtBr stained gels (data not shown). Interestingly hybrid 23 has tomato plastids, a highly abnormal leaf morphology, and is slow growing.

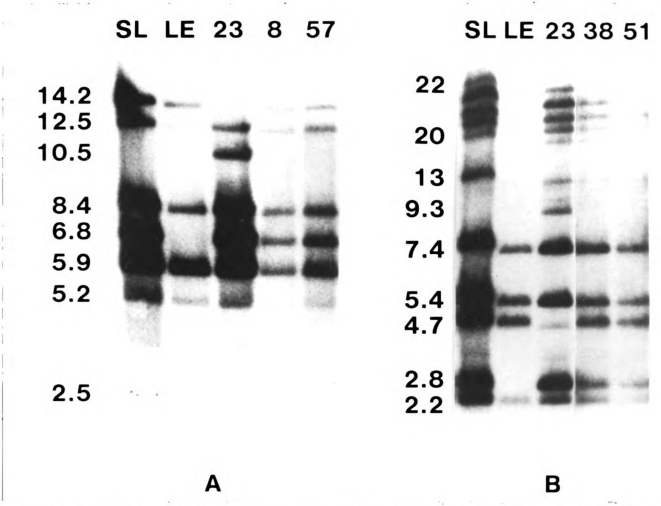

Fig. 2. Southern analysis of purified mtDNA from *L. esculentum* (LE), *S. lycopersicoides* (SL), and five somatic hybrids electrophoresed on 0.8% agarose gels. A) Hybridization of the pmt SylSa8 clone to Hind III digests; B) Hybridization of the pmt SylSa8 clone to Bam HI digests. Migration of the fragments in the SL lane was slightly retarded probably due to differential salt concentrations in the samples. The size of the fragments are given in kilobase pairs.

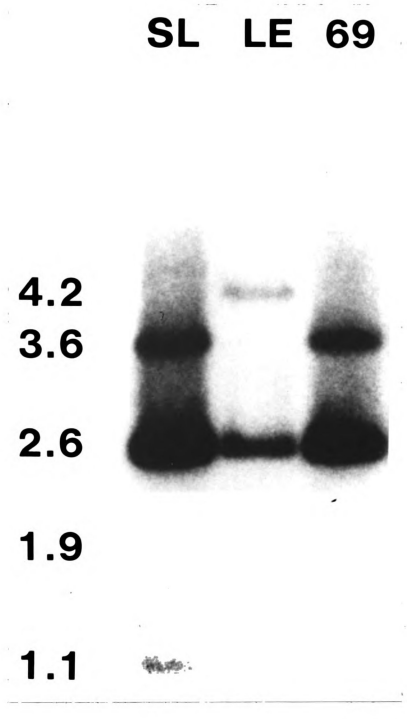

Fig. 3. Southern analysis of purified mtDNA from *L. esculentum* (LE), *S. lycopersicoides* (SL), and somatic hybrid 69 digested with Eco RI, electrophoresed on a 0.8% agarose gel and hybridized to pZmE1. Fragment sizes are given in kilobase pairs.

Discussion

It is striking that the majority of the somatic hybrids have chloroplasts and mitochondria from opposite fusion parents. They have predominantly (97%) tomato plastids and exclusively *S. lycopersicoides* mitochondria. Hybrid 63 had cpDNA restriction fragments corresponding to both parents. This could be due to chloroplast sorting-out between branches, a periclinal chimera, or maintenance of the mixed cell type.

Plastid transmission in these intergeneric somatic hybrid plants could have been determined by the relative number of plastids donated to the heterokaryon by the parental protoplasts. For example, mesophyll cells have about 10-fold more plastids than meristematic cells (Possingham and Lawrence 1983). In these experiments, the tomato protoplasts were isolated from leaf mesophyll cells and fused with suspension-derived *S. lycopersicoides* protoplasts. Mesophyll cells have more chloroplasts than those of any other tissue that has been examined (Steele-Scott et al. 1984). To our knowledge, the number of plastids found in long-term suspension cells has not been determined. However, these rapidly dividing cells are similar to meristematic cells of whole plants and probably have relatively few proplastids. For this reason tomato chloroplasts probably predominated in the heterokaryons. Following fusion, the partitioning of plastids during cell division would have fixed the predominant type (Akada and Hiria 1986). This process would have been quite rapid if the number of plastids in the hybrid cells decreased from around 100 to about 10 within 3-4 divisions as they did in cultured mesophyll protoplasts of *N. tabacum* (Thomas and Rose 1983). Differential transmission of chloroplasts due to unequal input from the gametes is a well documented aspect of sexual reproduction in higher plants (Whatley 1982).

Another possiblity is that more rapid division of the plastids from one fusion parent resulted in biased transmission. Plastid division can occur continuously during cell development and division but the maximum number of plastids seems to be limited in a developmentally coordinated fashion

(Possingham and Lawrence 1983). Assuming that plastid multiplication is a stochastic process after cell fusion, when the number of plastids per cell is below the limit, those that divide rapidly will predominate in subsequent generations. *Oenothera*, with biparental plastid inheritance, has yielded genetic evidence supporting this concept. Schotz (1954) discovered differences in competition between the five different plastomes of *Oenothera* when they were brought together by sexual matings. Each of the five plastome types was assayed using reciprocal crosses in which one parent carried a plastome encoded albino mutant, and the other carried the wild-type plastome to be tested. The germinating seeds from these crosses were scored by measuring the relative size of the green sectors on their cotyledons, which gives a quantitative estimate of the relative competitiveness of each plastome. This "variegation rating" ranks the five plastomes in nearly the same order regardless of the direction of the cross or the nuclear background (Chiu et al. 1987). Schotz (1968) attributed this to differences in the rate of plastid multiplication.

Our findings as well as the reports of others indicate that protoplast fusions involving species that are partially or entirely sexually incompatible are more likely to exhibit non-random plastid transmission (Menczel et al. 1981; Fluhr et al. 1983; Muller-Gensert and Schieder 1985; Clark et al. 1986; Gleddie et al. 1986; O'Connell and Hanson 1986). Conversely, fusions between highly sexually compatible species are likely to produce a random distribution of plastids (Glimelius and Bonnett 1981; Scowcroft and Larkin 1981; Sidorov et al. 1981; Flick et al. 1985; Kumashiro and Kubo 1986). This suggests some cytoplasmic genomes are less able to function physiologically with a foreign nucleus and this is more likely in hybrids between distantly related sexually incompatible species.

Genetic evidence for the incompatibility of plastomes with certain nuclear genomes has been obtained for *Oenothera*. Stubbe (1960) studied the reaction of the five different plastid types of the subsection *Euoenothera* and found profound differences in their ability to green when combined with different nuclear complements. The manifestation of this "plastome-genome" incompatibility ranged from small changes in chloroplast pigmentation to complete lethality. Each plastome type is more compatible when associated with a genome more closely related to the one with which it co-evolved (Kutzelnigg and Stubbe 1974). Perhaps the under-represented *S. lycopersicoides* plastids were less

competitive in the hybrid cells due to incompatibility with the hybrid nuclear genome. Biased sorting out due to plastome-genome incompatibility may have occurred at any time between cell fusion and the time of analysis.

Incompatibility during plant regeneration, a complex process involving coordinate expression of many genes, may be particularly important in determining organelle transmission. Menczel et al. (1981) reported two somatic fusion experiments using *Nicotiana tabacum* and *N. knightiana* with completely different results. In the first experiment they observed only *N. knightiana* chloroplasts in the hybrid plants, and in the second they observed balanced transmission of both types. They suggested the culture conditions used in the first experiment precluded regeneration from cells with *N. tabacum* plastids. Schnabelrauch and Sears (personal communication) studied regeneration of isonuclear lines of *Oenothera* with five different plastomes, and observed an influence of plastome type on the efficacy of regeneration. Perhaps in our experiments, cells with different plastome contents also responded differentially to the culture medium.

Our mitochondrial results parallel the findings of two other somatic hybridization experiments involving tomato with a related wild species. O'Connell and Hanson (1985) examined nine *L. esculentum* + *L. pennellii* somatic hybrid cell lines using one mitochondrial probe and found eight with the *L. pennellii* pattern and one which was a mixture. When the same workers used three probes to examine four *L. esculentum* + *S. rickii* somatic hybrid plants, all derived from one callus, they found only mitochondrial bands specific to *S. rickii* (O'Connell and Hanson 1986). They also noted changes in the relative intensities of some of the bands when these hybrids were probed with a mitochondrial ribosomal RNA clone, which suggests rearrangement or changes in the stoichiometry of sub-genomic circular molecules (Grayburn and Bendich 1987).

Of the forty-six hybrid plants examined in this study, only hybrid 23 had restriction pattern changes relative to *S. lycopersicoides* mtDNA, and no tomato specific fragments were detected in any hybrids. This suggests the tomato mitochondrial genome was rapidly eliminated and the changes in hybrid 23 represent rearrangement of the *S. lycopersicoides* genome rather than intergenomic recombination. It is, however, possible that tomato specific fragments in the hybrids were not

detected. When mtDNA digests of the hybrids were visualized on ethidium bromide stained agarose gels only *S. lycopersicoides* specific fragments were observed. Unfortunately, many regions of the gel were difficult to interpret due to dense spacing of the bands. Consequently, Southern analysis was used to obtain more definitive data. Since this procedure is more involved, we used two probes representing less than one tenth of the tomato mitochondrial genome (Palmer 1985) for the analysis.

The *S. lycopersicoides* mtDNA in these hybrids was derived from one year-old suspension cultures, and there are reports of mtDNA rearrangement in such cultures of tobacco (eg. Dale et al. 1981). Recent evidence indicates that the sequence arrangement of the master mtDNA chromosome is stable, but the stoichiometry of subgenomic circular molecules can change in suspension culture (Bailey-Serres et al. 1987; Grayburn and Bendich 1987). These changes in stoichiometry may have caused many of the changes in the mtDNA restriction patterns that have been reported (Negruk et al. 1986). Since no differences were observed between the restriction patterns of mtDNAs isolated from leaves of *S. lycopersicoides* and the somatic hybrids, except for hybrid 23, the mtDNA in the *S. lycopersicoides* cell suspension must have been fairly stable. The mtDNA changes in hybrid 23 cannot easily be explained by altered mtDNA stoichiometry since they involve novel fragments as well as changes in intensity of parental fragments. However, there is at least one report of a novel mtDNA fragment which was probably induced by tissue culture (Dewey et al. 1986).

The number of mitochondria may vary with the protoplast donor tissue (Bendich and Gauriloff 1984) in a manner analogous to that of chloroplasts. However, the mtDNA ploidy (the number of copies of mtDNA per cell) as well as the number of mitochondria per cell may influence mtDNA transmission. This is because mitochondria probably fuse frequently allowing the resident mtDNAs to interact (Bendich and Gauriloff 1984), whereas, plastids rarely fuse (Medgyesy et al. 1985). Mitochonrial fusion is probably a prerequisite of recombination and such recombination after somatic hybridization is common (Robertson et al. 1987; Rothenberg and Hanson 1987; Vedel et al. 1986; Rothenberg et al. 1985). Thus, depending on the rate of mitochondrial fusion, the mitochondrial genome from donors with higher mtDNA ploidy would be more likely to predominate in hybrid plants. However, the amount of mtDNA per cell remains relatively constant between developmentally distinct

tissues in pea, the only plant examined thus far (Lamppa and Bendich 1984). The role of these factors in mitochondrial transmission remains unknown since neither the mtDNA ploidy nor the number of mitochondria has been determined in plant suspension cells or compared between these two species.

It seems likely that the parental mitochondria were not maintained as a mixture since we did not observe extensive mtDNA recombination. This may suggest that nuclear-mitochondrial incompatibility was a contributing factor. However, we are not aware of any direct evidence for nuclear-mitochondrial incompatibility in plant somatic hybrids. Mitochondria and plastids have similar interactions with the nucleus (Douce 1985), suggesting mitochondria may be similarly capable of nucleo- cytoplasmic incompatibility relationships.

The authors gratefully acknowledge Drs. B.B. Sears and J.D. Palmer for technical assistance and advice during this research, Dr. A.L. Phillips for the tomato cpDNA clone bank, Dr. D. Aviv for mtDNA clones and Dr. C.M. Rick for seed of LA 1990.

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