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dissertation entitled

GENETICS OF NICOTIANA SOMATIC HYBRIDS

presented by

Jean Louise Roberts

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Crop and Soil Sciences and Genetics Program

Major professor

Date 8/12/82

THE GENETICS OF *NICOTIANA* SOMATIC HYBRIDS

By

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences
and
Genetics Program

1982

ABSTRACT

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Somatic hybridization provides one approach to a better understanding of the cytoplasmic genetics of higher plants. Protoplast fusion experiments combined homozygous nuclear albino, cytoplasm-substituted cells with nuclear wild-type, plastid albino cells. *Nicotiana tabacum* with "white seedling" or "Sulfur" nuclear albino markers, *N. megalosiphon* or *N. bigelovii* cytoplasms, and three different plastid albino genotypes were used. Complementation between different parental nuclear and cytoplasmic genomes permitted the selection of eight green fusion products during culture regeneration. The resulting somatic hybrid plants were tested for nuclear, mitochondrial and plastid markers.

Nuclear inheritance was examined by progeny tests for the nuclear albino marker and by ploidy determinations. The different mitochondrial genotypes were distinguished by a cytoplasmic male-sterile character. The albino/green visual marker, ribulose bisphosphate carboxylase/oxygenase isoelectric points, and tentoxin reactions marked the different plastid genomes. Results showed that these eight somatic hybrids include four different combinations of the marked nuclear, plastid and mitochondrial genomes.

The hybrid nuclear genotypes combined the parental nuclei in six plants; the albino parental nucleus was completely lost from two hybrid plants. The mitochondrial male-sterile marker was inherited independently from the plastid markers in these hybrids. Two plants had male-fertile anatomy, and six were male-sterile; no new floral phenotypes occurred. No recombination between the three plastid characters was detected in the eight green plants, but one variegated somatic hybrid plant was recovered.

Additional protoplast fusion experiments selected for recombination between different plastid albinos in somatic hybrids. No plastid recombinants were found.

The genotypes employed in these experiments permitted fusion product selection by complementation between nuclear and cytoplasmic albino markers and somatic hybrid analysis for three different cell genomes. Independent assortment of plastids and mitochondria in these hybrids resulted in new plant types with plastids and mitochondria derived from different Nicotiana species. These results contribute to our understanding of the genetic consequences of cytoplasmic hybridization in higher plants.

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INTRODUCTION

I.1. Prologue

This thesis discusses the theory and methods of cytoplasmic genetics and describes somatic hybridization experiments applying those methods to plant cells. These experiments were designed to yield both new techniques and information, not only to extend the formal knowledge of plant genetics, but also to make useful genetic variation accessible to plant breeders. Plastid genes influence economically important characters, such as chemical and disease resistance and photosynthetic efficiency; mitochondrial genes are implicated in cytoplasmic male sterility. Techniques to manipulate these genes could contribute to crop improvement.

Chapter I of this thesis examines the extent of our present-day understanding of organelle genetics. Chapter II details the methods used to obtain _Nicotiana_ somatic hybrids with marked nuclear, plastid and mitochondrial genomes. Chapter III presents the results of analysis of the hybrid plants.

I.2. Concepts of organelle genetics

I.2.1. Introduction

This survey of cytoplasmic genetics will place into perspective our current understanding of cytoplasmic genetics in plants. Several principles and techniques of cytoplasmic genetics differ from those of other branches of genetics. Genes outside the cell's nucleus have distinct non-Mendelian patterns of inheritance due to their independence from the cell processes and cytoskeletal machinery which govern the replication and distribution of chromosomal genes in meiosis and mitosis. Cytoplasmic genomes can vary greatly in copy number relative to nuclear genes, giving rise to quantitative genetic phenomena which require population analysis. This discussion will cover only mitochondria and chloroplasts, ignoring other heritable non-nuclear phenomena such as viruses, symbionts and maternal effects. The DNAs of mitochondria and chloroplasts behave genetically as distinct populations of prokaryotes, cooperating with their host cells in eukaryotic organisms.

I.2.2. Meiotic inheritance

The meiotic and mitotic genetics of cytoplasmic genomes follow the bulk distribution of cytoplasm in cell fusions and divisions. Thus, the number of genome copies each gamete effectively contributes to the zygote determines the inheritance of mitochondria and chloroplasts in the organism's sexual cycle. Differences in reciprocal crosses can result if gametes differ in size or if one parental organelle DNA is less competitive in replication. For these reasons, heterogametic species frequently exhibit maternal bias when organelle traits are followed through sexual crosses.

I.2.3. Mitotic inheritance

Mitosis distributes cytoplasm to cells irregularly. Random effects mediated by the symmetry of cell division, the vigor of cytoplasmic mixing, the number of genome copies, and any bias in replication cause genetic segregation of organelle DNAs in mitotic cell divisions. Thus, a cell containing a heterogeneous population of mitochondria or chloroplasts can beget different cell lineages which are uniform for organelle genotype. Somatic segregation is a phenomenon typical of cytoplasmic genetics.

I.2.4. Molecular aspects

Mitochondria and chloroplasts sequester special metabolic functions into membrane-delimited compartments.

The DNAs maintained within these organelles encode parts of enzyme complexes essential for respiration and photosynthesis, respectively, as well as parts of the translation machinery needed to express those structural genes *in situ*. Both mitochondria and chloroplasts also compartmentalize other biochemical functions, thereby contributing to the efficiency and regulation of cell metabolism. Physical fractionation *in vitro* separates both the biochemistry contained within the organelle and its genetic system from those of nucleus and cytoplasm.

The apparatus for replication and genetic expression encoded in organelle genomes shares several characteristics with prokaryotic systems, notably DNA circularity, polyteny and membrane attachment, and ribosome size, structure and sensitivity to antibacterial antibiotics. While suggestive of a disparate evolutionary origin for nuclear and organelle genomes, these features have also been widely exploited in the study of organelle genetics and biochemistry. Physical differences, antibiotic sensitivity, and the use of organisms which can tolerate organelle dysfunction have permitted *in vivo* dissection of nuclear and organellar contributions to metabolic traits. It is generally observed that the nuclear and organelle genomes cooperate to produce the organelle phenotype.

I.2.5. Differentiation

The physical separation of organelle DNAs into different membrane-bounded compartments can limit their genetic interaction. Eukaryotic differentiation involves organelle differentation, with accompanying changes in the DNA copy number within the organelle, the number of organelles per cell, physical characteristics of the membranes, and in the level of expression of organelle genes. This phenomenon of organelle differentiation affects the study of chloroplast and mitochondrial traits.

The biology of organelles dictates that inheritance studies of mitochondria and chloroplasts draw from population genetics to describe quantitative effects, while using molecular approaches to the specialized biochemistry of these subcellular compartments. Examples of the phenomena and techniques of organelle genetics in unicellular eukaryotic models and in higher animals and plants are detailed below.

I.3. Mitochondrial genetics

I.3.1. Yeast

The paradigm of mitochondrial genetics is the baker's yeast, *Saccharomyces cerevisiae*. The combination of historical precedence (1), facultative organelle function, and active genetic recombination have made yeast mitochondrial DNA (mtDNA) the best understood organelle genome. Though some features are not typical of all organelles, the study of yeast mitochondrial genetics contributes much to our understanding of organelle DNA inheritance and expression.

Yeast are homogametic. Each gamete of normal genotype and physiology contributes roughly the same population size of mitochondria to the zygote. When populations descended from a mass mating are examined for organelle markers, biparental inheritance and recombination of the markers are observed. This evidence implies that mitochondria must interact and their DNAs associate. Recombination is so frequent that an unambiguous genetic map cannot be constructed by this method. In contrast, pedigree studies of mitotic progeny from single zygotes show rapid segregation and fixation of markers (2). The most important form of exchange may be nonreciprocal gene conversion (3,4).

The mitochondrial DNA of yeast is circular, with a size of 50 megadaltons, corresponding to 76 kilobase pairs (2,5). Though a haploid cell contains about 50 copies of mtDNA within about 20 organelles (4), it behaves genetically as if about ten mtDNAs were replicating (2,3). Several factors may contribute to this apparent discrepancy in copy number. In yeast, mitosis by budding gives the daughter cells unequal amounts of cytoplasm. Genetic segregation in mitosis could be accelerated by incomplete cytoplasmic mixing, asymmetric cell division, segregation by organelle units, replicative competition, and by mitochondrial gene conversion. These would limit the duration of the heterozygous condition and thereby lower recombination and mutation frequencies in pedigree studies (2,4). A limited number of replication enzymes or DNA-membrane attachment sites may be available (4). It has also been suggested that recombination occurs only when mitochondria are dedifferentiated for a brief period in the zygote (2), and that the state of differentiation of the mitochondria, primarily due to demand for aerobic respiration, affects the number of mtDNA molecules (6).

Yeast are facultative anaerobes; their mitochondrial genomes are studied with respiratory defective mutants, antibiotic resistant mutants, and with petites. Petites are a class of respiratory defectives with a characteristic high frequency of occurrence spontaneously or following mutagenesis (2,3,5). They have been shown to contain deletions and amplifications of mtDNA. In some petites, a few sequences are retained and amplified to whole genome size (5); the amplified tandem repeat may be either nonsense or coding sequences. Other petites have no detectable mitochondrial DNA. Both types of petites develop no functional mitochondrial ribosomes and cannot express their mtDNA complement. The phenomenon generating and tolerating these deletions is not known to exist in any other organelle genetic system.

Deletion mutagenesis to the petite condition by treatment with intercalating acridine dyes permits mtDNA mapping by co-retention of sequences. This approach yielded a reliable circular map of mtDNA (5). Petites containing mtDNA must be crossed to a respiratory-sufficient genotype to express the retained sequences. This provides another example of organelle differentiation affecting the study of organelle genes. Complementation mapping of mitochondrial genes was possible in zygotes due to mitochondrial fusion (5). When progeny from petite crosses are examined, it is evident that some petites have a strong advantage in mtDNA replication suggesting intracellular selection (4).

Genes definitely located on mtDNA can be enumerated. Mitochondrial ribosomal RNAs are mtDNA-encoded. Yeast mitochondria lack the 5S rRNA (5). Variation in these loci confers useful resistance markers for the two antibiotics erythromycin and chloramphenicol (5). All 26 transfer RNAs found in mitochondria are mtDNA-encoded (5). Mutants in these display a lack of protein synthesis but have a complete ribosomal complement. One mtDNA-encoded polypeptide in yeast is a single ribosomal protein; its mutant phenotype is variable. The rest of the ribosomal proteins are synthesized outside the organelle and are encoded in the nucleus (5).

The yeast mitochondrial translation system is less prokaryotic in structure than in most organelles. Heterologous associations of ribosome subunits or messages plus ribosomes from *E. coli* and *S. cerevisiae* will not synthesize protein, though translation apparati of *E. coli* and *Chlamydomonas* chloroplast components will function together *in vitro* (5). Too, *S. cerevisiae* mitochondrial rRNA is processed from a transcript containing an intron (5). Different tRNA anticodons are used (5). These features distinguish yeast mitochondria from other organelle genetic systems and may reflect a more advanced, eukaryotic trend in evolutionary adaptation for this organelle.

Respiratory defective mutants with normal mitochondrial DNA and ribosomes map to seven mtDNA loci with known gene products comprising one out of the seven subunits of the cytochrome b complex, and three out of seven peptides of the cytochrome oxidase assembly (5). Mutants in these loci have atypical cytochrome absorbance spectra. The chemiosmotic transducer, Coupling Factor F1, includes three subunits encoded in mtDNA and seven encoded in the nucleus. Mutants in these genes are resistant to ionophores or to the antibiotic oligomycin (5). These hydrophobic proteins may require synthesis close to the site of assembly of the membrane-bound enzyme complexes; they would be unable to cross the mitochondrial membranes as do other nuclear-encoded, cytoplasmically translated components (7).

Expression of the mitochondrial genes is mediated by nuclear genes. In addition to the remaining components of the above-mentioned multimeric complexes of ribosomes and the electron transport system, mitochondrial DNA and RNA polymerases are nuclear-encoded (2). The mode of regulation of expression of cooperating nuclear and mitochondrial genes in producing organelle phenotype is as yet undiscovered.

MtDNA must contain a replication origin sequence (5). This origin is identifiable in petites with a strong mtDNA replication advantage. Physical techniques show that such successfully competitive petites have many copies of the replication sequence, whereas wild-type yeast have several such loci (8). One other known genetic locus, "omega", confers bias of mitochondrial gene transmission in crosses and is probably an initiation site for gene conversion (5). The generality of this gene conversion bias factor in organelle systems is unproven (9).

Four additional hydrophobic polypeptides with unknown function are encoded by mtDNA (10). There may be other coding sequences as yet undiscovered in yeast mtDNA.

The population genetics of yeast mtDNA transmission has drawn from phage genetics to describe a panmictic pool of replicating, recombining DNAs (4,9). Concepts of random drift (4,11) and competition (9) derive from evolutionary population genetics. The molecular aspects of copy number and recombination mechanism are obscured by the effects of organelle differentiation, asymmetric cell division, and prolific gene conversion during active recombination. A quantitative model to adequately describe all known features has not been completely worked out.

I.3.2. Mitochondrial genetics of animals

Animal cells cannot dispense with mitochondrial function. The unavailability of facultative fermenters limits genetic studies to antibiotic resistance markers or physical characterizations of the mitochondrial genome. Transmission genetics of animal mitochondria has been restricted to studies of maternal transmission and evolutionary mtDNA sequence divergence in nature (12-14), and to transfers of antibiotic resistances (15,16) and mtDNA restriction patterns by animal cell fusions (17). In contrast, the molecular study of animal mitochondria has proven richly productive.

Human mitochondrial DNA is among the smallest of organelle genomes, being a 16.5 kilobase pairs long circle (18), of 10 megadaltons molecular weight (2). A laborious collaboration recently produced the complete nucleotide sequence of human mtDNA from Hela and placental preparations (19). This remarkable piece of work depended on both molecular biological and information processing advances of the past decade.

Restriction fragments of human mtDNA, cloned in *E. coli*, provided templates for sequencing by the Sanger dideoxy chain termination method; a computer program correlated the results (19). The mtDNA sequence obtained was compared with the 5' and 3' terminal sequences of mitochondrial polyA RNAs produced in vivo, and with known amino acid sequences of mitochondrial proteins, to clarify transcription origins and termini (20,21). The results describe a highly evolved, compact genome with unusual features contributing to its economy of maintenance. New genes were discovered, and previously postulated functions were assigned loci (19).

The replication origin of this mtDNA and its transcription and translation origins are now known. Both strands of mtDNA are completely transcribed with a single promoter each, which have also been identified. Posttranscriptional processing to mRNA is apparently mediated by immediately adjacent tRNA sequences. The previously known mtDNA structural genes for three cytochrome c oxidase subunits, one cytochrome b subunit, and F1 ATPase subunits can now be precisely located. Eight additional previously unknown protein-encoding genes were discovered in the mtDNA sequence; transcripts of most of these could then be identified in mitochondrial polyA-mRNA preparations. Economy in transcription and translation also results from the use of only two undersized rRNAs (no 5S rRNA), a lack of mRNA leader sequences, the synthesis of translation termini by posttranscriptional polyadenylation of transcripts, and by the sufficiency of only 22 tRNAs. Remarkably, the 'universal' genetic code proves to not apply to human mitochondria. These organelles employ different translation initiation and termination codons.

Physical characterization of animal mtDNA suggests that base substitution plays a much greater role in its evolution than does rearrangement. Very little physical rearrangement of mtDNAs is found when distantly related species are compared (13).

Some features of animal mitochondria clearly distinguish them from prokaryotic systems. Mitochondrial ribosomes are structually different from, and share little sequence homology with, bacterial ribosomes (19). Translation depends upon some different codons. The tRNA-mediated posttranscriptional processing of mitochondrial mRNA has no precedent in prokaryotes. Unlike the situation in yeast, economy of code in animal mtDNA evidently permits no introns (19). Evolutionists might speculate profitably on the processes by which loss and modification of organelle genomes could yield the existing differences and similarities between prokaryoes and the various organellar systems.

I.3.3. Plant mitochondria

Plant mitochondria present a somewhat different experimental picture. Unlike yeast, plant cells appear to be obligate aerobes. There are presently no antibiotic resistance markers for use for *in vivo* meiotic or mitotic genetic analysis of the organelle. Additionally, because plant cells enclose two separate organelle systems, it becomes essential to separate the contributions of mitochondria from those of chloroplasts in determining plant characters. For these reasons, physical analysis of plant mtDNA and *in vitro* studies on mitochondrial protein synthesis have supplied almost all the genetic information currently available about this organelle.

In contrast to animal and fungal mitochondria, each plant mitochondrion can enclose different size classes of circular DNAs which seem to represent various rearrangements of a basic mitochondrial genome, with the possible addition of episomal or plasmid-like genetic elements (22,23). It is unclear whether all DNA size classes contain the same sequences; different mtDNA species could coexist and undergo mutual rearrangements. One piece of evidence suggestive of mtDNA rearrangement will be discussed below.

Evolutionary distance between plant species is reflected in mtDNA sequence rearrangements, rather than base substitutions as is the case in animals (24). The mtDNA of plants is much larger than the minimal animal mtDNA, and its isolation in different size classes makes it difficult to assign a molecular weight to the basic mitochondrial genome by analysis of restriction digests (22). Estimates of mtDNA size by these methods range from 70 megadaltons (25) to 320 megadaltons (24). Measurements of reassociation kinetics showed that in different cucurbit species, mitochondrial DNA ranges from 220 to 1600 megadaltons in sequence complexity, with only a small repetitive component (26). The origin and function of this mtDNA sequence abundance in plants, and its arrangement on variable plant mtDNA molecular sizes, are unknown. Regardless of a species' mtDNA size, each plant cell contains 100 to 150 mtDNA copies (26). There are cases of mtDNA replication surges accompanying tissue differentiation.

The plant mitochondrial DNA encodes all three mitochondrial rRNAs, including a 5S rRNA (25). A comparison of proteins synthesized in vitro by plant mitochondria with those made by isolated animal and fungal mitochondria showed that plants produce polypeptides corresponding in size to the known mtDNA-encoded gene products in yeast and man: three cytochrome c oxidase subunits, one cytochrome b subunit, and at least two F1 ATPase subunits (25). However, no enzyme activity has actually been identified for these plant mitochondrial products.

Cytoplasmic male sterility (cms) is one very significant whole-plant character which has been correlated with mitochondrial inheritance. The phenomenon is widely used by seedsmen, but significant advances in understanding have been gained only in Zea and Nicotiana. Expression of cms is tissue-specific; it requires floral development. This effect of plant differentiation on expression of organelle-determined characters has hampered the study of cms. For complete coverage of this topic, the reader is referred to an excellent monograph (27).

For purposes of this discussion, cytoplasmic male sterility should be distinquished from viral effects, which are mechanically transmissible, and from nuclear genes conferring male sterility, which follow Mendelian genetics. Nuclear genes contribute to the expression of cytoplasmically-inherited male sterility; in grain crop seed production, each cms genome is combined with nuclear genes conferring restoration of fertility (22). Nuclear background can also influence the frequency of cytoplasmically-inherited reversion to fertility (22). Because the primary genetic determinant for the cms character is strictly maternally inherited, it seems that there is no male transmission of the relevant cytoplasmic genome. The physiology of male organ or gamete failure varies with the cytoplasmic genome. The contribution of any specific mtDNA sequence to the physiological or developmental breakdown of male gametogenesis is not yet known for any case.

Linkage of cms with disease susceptibility caused a renewed effort in plant mitochondrial genetics. In corn, Zea mays, a cytoplasm-linked susceptibility to Southern corn leaf blight contributed to a 1970 epidemic and severe crop loss because one single male-sterile cytoplasm was extensively used in seed production (22,27,28). The blight fungus, Helminthosporium maydis (Bipolaris maydis), produces a toxin which causes breakdown of the mitochondria from the disease-susceptible 'T' male-sterile cytoplasm (22). Only the mitochondria are affected; plastids from the same cytoplasm are unaffected by the toxin (29).

Physical characterization showed that mtDNAs of different sterile and fertile corn cytoplasms differ more than do their chloroplast DNAs (22). Further examination revealed that the genetically variable 'S' male-sterile cytoplasm contains episome-like small mitochondrial DNAs in addition to the larger mtDNAs typical of corn. These small DNAs have palindromic repeat termini; they disappear from native isolations of mtDNA concurrently with plant reversion to cytoplasmically-inherited male fertility (30). The time required to observe this event is about a plant generation. These episomal sequences are present in the fertile corn mtDNA, but their presence on separate mtDNA molecules in the sterile genotype apparently affects male gamete development (30). The rearrangements found in the fertile 'S' revertants' mtDNA, along with apparent loss of their episomal mtDNA, are suggestive of transposition and nonhomologous recombination mechanisms (22).

Corn plants with male-sterile 'C' and 'T' cytoplasms and their nuclear-gene restored fertile counterparts translate some different proteins on mitochondrial ribosomes (31). This alteration of mitochondrial translation products (presumably representing mtDNA transcripts) suggests nuclear regulation of mitochondrial gene expression in this system. All this experimental evidence strongly correlates mtDNA characteristics with the male-sterile trait in corn. Due to obligate maternal transmission of cytoplasmic male sterility, it is still difficult to separate mitochondrial influence from any cryptic plastid contribution to the character, and causality is not yet proven in the cms-mitochondria system.

The linkage of disease susceptibility to male sterility has also been used to explore mitotic variation of corn mitochondrial inheritance. Efforts to obtain male-sterile, blight-resistant plants from tissue cultures selected for toxin resistance showed that though variation could be generated during tissue culture growth, the linkage was difficult to break. Toxin-resistant variants were also male-fertile (29). Toxin-sensitive lines which were fertile could be recovered (32), but the desirable recombinant is still elusive.

In plant populations descended from cells containing heterologous mixtures of Nicotiana cytoplasms derived from cell fusions, mitotic recombination of mtDNAs, as inferred from restriction fragment changes, occurred concurrently with modification of floral cms phenotype (33,34).

The phenomenon of plant cytoplasmic male sterility and nuclear restoration of fertility offers an interesting example of the interaction of nuclear and organellar genetic systems. It is widely observed that a recurrent backcrossing program between different species within the same genus uncovers incompatibility between the cytoplasmic genome of one species and the nuclear genome of the other. A frequent nonlethal result is cytoplasmic male sterility. For hybrid breeding programs, nuclear genes which specifically restore male fertility to the cytoplasm can be obtained from the cytoplasmic parent species. Parents contributing cytoplasmic male sterility and nuclear restorer genes have been identified in many crops (cf. 35-39). In Nicotiana, the nuclear restorer genes are located in the nucleolus organizing regions (40). How ribosomal RNA transcription in the nucleus could affect mitochondrial gene expression is unknown. It would be interesting to determine whether nuclear genes can affect mitochondrial DNA stability and recombination in plants.

At present, cytoplasmic male sterility is the only phenotype correlated with plant mitochondrial DNA. Selectable biochemical markers would advance the study of plant mtDNA genetics.

I.4. Chloroplast genetics

I.4.1. Introduction

The rest of this discussion concerns chloroplasts. Like mitochondria, chloroplasts are double-membrane-bounded and can be represented genetically by many chloroplast DNAs per plastid and many plastids per cell. Both types of organelles demonstrate autonomous transcription and translation. Both organelles grow by membrane accretion, but have not yet demonstrated heritable characteristics with regard to this phenomenon. Because there are no eukaryotes with plastids but without mitochondria, it becomes necessary to discriminate plastid from mitochondrial characteristics. These distinctions depend primarily on the identity of chloroplasts as the photosynthetic compartment, enclosing both the light and dark primary processes of photosynthesis. Facultative photoheterotrophy facilitates the genetic analysis of chloroplasts. Chloroplast-encoded characters will be discussed in some detail later.

I.4.2. Chlamydomonas

Chlamydomonas rheinhardtii provides the most instructive model of chloroplast genetics. In the early 1950s Sager chose this green alga as the simplest organism in which to study chloroplast genetics because this eukaryotic microorganism is unicellular, has a single chloroplast, can be grown on defined media and handled by bacteriological techniques, and has a simple sexual cycle (2). *Chlamydomonas* is homogametic, with gametes of the same size, and heterothallic, with two nuclear gene-determined mating types. Following gamete fusion, the zygote undergoes meiosis to produce four haploid products, or grows as a vegetative diploid. *Chlamydomonas* is a facultative heterotroph; acetate in the medium can substitute for photosynthetically fixed carbon.

Selection for resistance to antibacterial antibiotics yielded the first selectable markers with a demonstrably cytoplasmic pattern of inheritance: maternal bias in crosses and segregation in mitotic progeny. Some photosynthetically impaired mutants follow the same cytoplasmic pattern of inheritance. These therefore could be separated from Mendelian markers of similar phenotype. Following several such markers in crosses, evidence was obtained for linkage and recombination between them. The *Chlamydomonas* chloroplast system deserves review because it includes all the necessary elements for genetic analysis: genetic variation, formal meiotic and mitotic genetics, active recombination, and population phenomena. A healthy controversy persists over analysis of the system. For a complete treatment of the topic, the reader is referred to several contrasting reviews (2,3,41).

The chloroplast DNA (ctDNA) of *Chlamydomonas* is a circular molecule of molecular weight 200 megadaltons (2). It can be distinguished from nuclear DNA by its characteristic density. There may be 25 to 50 of these DNAs in a cell's single plastid (3). Plastid DNA replicates at a different time from nuclear DNA in synchronized cultures;

cytoplasmic mutagenesis is most effective at the time of ctDNA replication (42).

Mutant genetic markers include resistance to antibiotics such as streptomycin, spectinomycin, erythromycin and kanamycin. These variants have altered chloroplast-encoded ribosomal components (43,44). Because the plastid ribosomes are assembled from many different components encoded in either plastid or nucleus, isolation of antibiotic resistant phenotypes yields nuclear mutants as well (45). Acetate-requiring photosynthetically deficient mutants can also be mapped to chloroplast or nuclear genes, although photosynthetic biochemistry has not been analyzed genetically as often in Chlamydomonas as in the higher plants. Chloroplast protein synthesis is necessary for nuclear DNA replication (46).

Chlamydomonas usually exhibits uniparental inheritance of ctDNA from the mating type plus (mt+) parent in sexual crosses (2). Sager discovered that UV-irradiation of the mt+ gametes alters the outcome of a cross to yield a biparental pattern of inheritance. The UV effect is photorepairable. The radiation presumably prevents the nucleus (the larger target, and the site of the mt+ gene) of the 'female' gamete from expressing a ctDNA-specific endonuclease. In the normal course of events, methylation of cytosine residues occurs in mt+ ctDNA, does not occur in mating type minus (mt-) ctDNA, and is followed by enzymatic destruction of mt- ctDNA in the zygote, while mt+ ctDNA is protected by its pattern of methylation (47). Sager has discovered a mutant locus, mat-1, in 'paternal' mt- gametes which causes methylation and protection of mt- ctDNA and subsequent biparental inheritance of ctDNA markers (47). In the life cycle of the alga, the signal for activation of the maternal restriction-modification system is apparently aggregation of the gametes' flagella; protoplast fusion in Chlamydomonas results in biparental inheritance of chloroplast markers (48). When gametes fuse, their plastids also fuse in the zygote, as seen in electron micrographs (49). Thus the membranes of the organelles do not prevent organelle fusion and molecular exchange between heterologous ctDNAs in this system.

To examine the results of a ctDNA-marked cross, the investigator must grow mitotic populations from haploid or diploid progeny to observe segregation of ctDNA copies. Thus, analysis of meiotic events depends on mitotic events. The four haploid meiotic progeny of a zygote can be separated on a petri dish, their daughter cells spread, and so on until a mitotic pedigree has developed. Alternatively, mass cultures of haploid or diploid vegetative progeny can be grown up and tested for frequency of ctDNA genotypes remaining after several generations. These techniques give evidence for both reciprocal and nonreciprocal recombination between parental ctDNAs. The frequency of these events depends on the cell's physiological state and is affected by growth conditions and gamete pretreatments (50). Apparently the inheritance of ctDNA sequences in crosses is a function of DNA copy number, nitrogen starvation, modification-restriction enzyme status, and segregation mode in subsequent cell divisions. The picture is not clear enough for there to exist an undisputed mathematical description of genetic exchange and distribution processes.

In studies on mutagenesis and on segregation frequencies in vegetative diploids, an attempt can be made to correlate ctDNA copy number and copy distribution in mitosis with the rate of appearance of selectable markers. These studies suggested that the chloroplast genome can segregate without genetic exchange, because marker appearance is delayed and rarer than target theory predicts (51,52). A likely hypothesis proposes that the multiple copies of ctDNA within each plastid are physically associated in a small number of nucleoids, dependent on a membrane attachment point. There may be little exchange between nucleoids.

Mapping of chloroplast markers relative to each other in Chlamydomonas uses several different techniques: cosegregation, rate of segregation, and standard recombination mapping in crosses (3,53,54). Unfortunately, no deletion mapping is possible; all mutants retain ctDNA. However, different authors obtain different map distances and, rarely, different map orders for the same markers (3,53,54). An attachment point is felt important to all models of ctDNA exchange and distribution (3,53,54). Map function has been hypothesized to correspond to a diploid model (41), or to a complex populational model (3). Any realistic model must account for the number of ctDNAs per cell and possible asymmetric distribution to daughter cells of attachment points and nucleoids. The populational model, as with the work on yeast mitochondria, is drawn from phage genetics, postulating a panmictic pool and depending on observation of progeny genotype 'output frequencies' and inference about 'input frequencies' in the original zygote (3).

Chlamydomonas does possess several mitochondria; its mtDNA is present in about 40 copies, and is small as in vertebrates, weighing 16 megadaltons (3). There has been little success in its genetic analysis, due to obligate aerobic requirements of the alga.

Much remains to be clarified in the Chlamydomonas organelle genetic system. The regulation of nuclear-chloroplast cooperation, the characteristics of mitochondria, and a clear model of ctDNA exchange and distribution remain to be established. Although the single plastid suggested simplicity at the outset of these investigations, this is nevertheless a complex genetic system.

I.4.3. Higher plant chloroplast genetics

An even more complex organismal and cellular biology influences the genetics of higher plant chloroplasts. Sexual transmission of this organelle occurs in the context of floral and seedling development; plastid numbers per cell and their state of differentiation change with growth and development of different plant tissues. There are numerous examples of nuclear genetic effects on plastid inheritance.

Many plant species exhibit strict maternal inheritance of plastids in reciprocal sexual crosses. In these species, plastids are excluded from the generative cell of pollen, and the plastids of the egg contribute all the chloroplast genomes of the progeny (55). Such species have been used to study the cell lineage of female organogenesis (56) and nuclear gene-induced plastid mutagenesis (57). The simplest plastid phenotype to follow is a cell-autonomous albino; all early work used this single character to study chloroplast genetics.

In some plant genera, notably Pelargonium and Oenothera, plastids are inherited from both parents in sexual crosses. Here, sexual hybridization experiments illustrate the quantitative effects of maternal nuclear genotype, intracellular competition between plastids, asymmetric cell divisions in early embryo development, and hybrid nuclear genotype on plastid sexual inheritance. When an egg cell is fertilized by the male gamete, any genetically marked plastids contributed by the pollen parent are introduced into the cytoplasm of the egg, which has been conditioned by the nucleus of the maternal genotype. Pre-existing biochemical conditions in the egg can favor replication or survival of one chloroplast genome over the other. Thus, in *Pelargonium*, female nuclear genetic background can influence the apparent competitive ability of a pollen-introduced mutant plastid genotype (58). The absence of some expected classes of variegated progeny may be the result of asymmetric cell divisions early in embryo development. In angiosperms, the bulk of zygote cytoplasm is sequestered into the suspensor cell and thus eliminated from the developing seedling's cell lineage (49). Consequently, a chloroplast genotype present in miniscule proportion in the zygote has a smaller than proportionate probability of appearing in the progeny seedlings.

In *Oenothera*, interspecific crosses cause albino and virescent variegation in leaves of the progeny. This photosynthetic dysfunction presumably reflects a breakdown of cooperation between heterologous chloroplast and nuclear genotypes; the variegation results from somatic segregation of the two parental plastid genotypes in the hybrid. Consistent differences in competitive ability of different chloroplast genomes permitted their ranking on the basis of apparent replication rates; nuclear genotype clearly affected plastid intracellular replicative competition (59,60).

Plastid fusion has not been observed in higher plants (49). No evidence has been obtained for genetic recombination between marked parental plastids in Oenothera or Pelargonium sexual progeny. Thus a primary genetic phenomenon of paramount importance in all other genetic systems is striking by its absence in the higher plant plastid. We do not know today whether plastid DNA interaction is prevented by membrane phenomena which prevent organelle fusion, or is simply not observed due to a lack of appropriate genetic markers for chloroplast DNA.

The random process of somatic segregation of plastids is modulated by the multiplicity of plastid genomes and organelles. The rate of plastid segregation in cell divisions depends upon the number of plastids and their mode of distribution in mitosis, and on the number of ctDNAs per plastid and their distribution in chloroplast budding. Michaelis (61) derived a rate function for segregation based on an average plastid number, but this model needs modification as both plastid number and ctDNA multiplicity fluctuate throughout the 100 or so cell divisions in the plant life cycle. The situation becomes yet more complex if a ctDNA lesion impairs molecular replication, plastid budding, or cell division (62).

Genetic studies to date have depended on plastid variants which survive the test of molecular, organellar and cellular fitness, but are photoheterotrophic. Plant cells are facultative photoheterotrophs. The mosaic organism can tolerate partial loss of photosynthetic function because it mimics normal developmental pathways, in which nongreen tissues such as stem and petal parenchyma and roots depend on translocated photosynthate.

States of plastid differentiation are as numerous as tissues. Chromoplasts, amyloplasts and chloroplasts provide striking examples of organelle differentiation. The carotenoid biosynthetic pathway, which occurs within chromoplasts, is apparently specified completely by nuclear genes, as is starch synthesis in amyloplasts (49). Only in chloroplast development has extensive ctDNA participation in organelle phenotype been demonstrated. These differentiated plastid types, and the undifferentiated proplastid, are all interconvertible *in vivo* (63). The mode of regulation of plastid differentiation and multiplicity is unknown.

Chloroplast DNA multiplicity, organelle number and size vary with plant cell differentiation. The DNA content of a given plastid varies directly with its size (64), and plastid size varies directly with nuclear ploidy (64). Chloroplast number per cell ranges from a high of 650 in a sugar beet cotyledon cell (64) to zero in a haploid alfalfa stomate -- the latter is a rare case of terminal differentiation (65). Chloroplasts range in diameter from a single thylakoid, 0.5 micron in an apical meristem, to 5 microns in a leaf cell (66).

Additional genome substructure affects the analysis of ctDNA inheritance. The plastid DNA is clustered in a few regions of the organelle. These nucleoids number from one to several per plastid, varying proportionally to organelle size; they may contain more than twenty DNA molecules each. Their distribution in organelle division could be asymmetric and membrane-dependent (49,67). Chloroplast DNA can be isolated associated with membrane fragments. More study is needed on these attachment points, their mode of division and distribution and their participation in ctDNA replication processes. We do not presently understand the role in plastid genetics of different multiplicities at the different hierarchical levels of plastid genome organization. The high number of ctDNA molecules is believed to be a conservative force constraining sequence divergence (49), but the fact of plastid DNA evolution shows that some nucleotide sequence change is possible despite these features.

Physical analysis of ctDNA *in vitro* has supplied information not available through transmission genetics (68). CtDNA is present in higher plants as a fairly homogeneous population of 100 megadalton circles. A single, usually inverted, repeat sequence includes some of the ctDNA ribosomal cistrons. Restriction site mapping yielded a circular map, on which the few known sequences for plastid-encoded proteins and RNAs can be located (68). Special features of higher plant ctDNA include the presence of about twenty ribonucleotide residues and the appearance in native preparations of ctDNA oligomers. The latter observation suggests that homologous recombination events may occur between ctDNAs *in vivo* (68). Chloroplast DNA polymerase and RNA polymerase are different from the corresponding nuclear enzymes; they are probably nuclear-encoded, as in other organelle systems (2). The origins of replication of ctDNA have not been mapped.

After allowing for the ribosomal cistrons, the ctDNA should still encode about 100 proteins. Considering this wealth of sequence diversity, a relative paucity of genes have been described. Most of these genes require some form of differentiation to be expressed; most cooperate with nuclear-encoded gene products in chloroplast function. The known loci and their variants will be briefly summarized.

All the ribosomal RNAs and transfer RNAs needed for message translation have been mapped. Loci have been described accounting for the 23S, 16S, 5S, and 4.5S rRNAs, and a sufficient number of tRNAs to translate the normal genetic code (68). Chloroplast rRNAs share extensive homology with those of *E. coli* (69). Variants in the plastid rRNAs are not known, but variants have been found in chloroplast-encoded ribosomal proteins. A maternally-inherited streptomycin-resistant tobacco mutant has altered plastid ribosomal protein patterns (70). No variants are known in plastid tRNAs; perhaps the cell could not survive mistranslation caused by a suppressor-type chloroplast tRNA mutation.

The most thoroughly studied plastid gene encodes the large subunit of ribulose bisphosphate carboxylase/oxygenase (RuBPCase). The holoenzyme performs the primary dark reaction of photosynthesis, fixing gaseous CO_2 into carbohydrate in the chloroplast stroma. It also initiates photorespiratory catabolism of fixed carbon when O_2 serves as its substrate. Eight identical large subunits of the enzyme, which include the active site, combine in plastids with eight small subunits, the latter having regulatory properties. Early evidence for maternal inheritance of electrophoretic variants of the large subunit (LS) came from interspecific hybridization in *Nicotiana* (71). Maternally-inherited species-specific variants of the LS have indicated the maternal parentage in ancestral hybridization of allopolyploid species in several genera (*Nicotiana*, 72; *Triticum*, 73). The sequence coding for the LS has been cloned from ctDNA (74). It appears as a single copy on the plastid DNA (68). Regulation of RuBPCase expression during plastid development and of its relative efficiency in different genotypes has received attention because of its importance in photosynthesis. In C4 plants, the different leaf cells typical of Kranz anatomy exhibit differential expression of the gene; bundle sheath cells do, and mesophyll cells do not, transcribe RuBPCase LS mRNA (75), although they contain the same ctDNAs (76).

The expression of the RuBPCase LS gene is coordinately regulated with production of small subunits (SS) from nuclear genes. The SS gene has also been cloned (77,78). The small subunit is translated as a precursor with a 'transit sequence' of 44 hydrophobic amino acid residues at the N-terminus needed for transport from the cytoplasm, across both plastid membranes, and into the chloroplast stroma (79). Some nuclear ploidy series of grasses show altered RuBPCase enzymatic properties (80, 81); whether the nuclear gene responsible for this effect encodes the SS or is some other locus remains to be established.

A second ctDNA locus currently receiving attention encodes a thylakoid lamellar protein. This gene product was originally identified by comparing plastid translation products of etioplasts and greening chloroplasts (82). The protein appeared to be synthesized as a 34.5-kilodalton precursor and posttranslationally processed to a 32-kilodalton protein within the chloroplast (83). Recent work identified this ctDNA sequence as the gene responsible for maternally-inherited triazine herbicide resistance in weeds of field crops in Europe and Canada (84-87). Thus a second chloroplast gene has been identified which contributes to agronomic performance.

A third group of plastid genes includes polypeptides of known function which are synthesized in isolated chloroplasts but which have not yet been mapped to a ctDNA sequence. Three subunits of the multimeric membrane ATPase, coupling factor 1 (CF1) are encoded on plastid genes by the criteria of synthesis on chloroplast ribosomes (88) and maternal inheritance (92). Species-specific electrophoretic variation in these polypeptides was correlated with maternally-inherited resistance or sensitivity to a CF1-binding fungal toxin, tentoxin, in Nicotiana (89-92). This provides a third example of agricultural relevance of plastid genes. Another plastid protein identified by isolated organelle synthesis is the apoprotein of cytochrome f (68).

Maternally-inherited mutants deficient in chloroplast development have been collected by plant geneticists over the years. It is reasonable to assume these represent ctDNA lesions, but so far physical studies of their DNA have found no restriction map differences, suggesting only that no large rearrangements have occurred in the ctDNA of the pleiotropic mutants. A primary genetic defect in their photosynthetic biochemistry is hard to assign because assembly of entire compartments within the plastid is impaired. Genetic loss of any component of a structural assembly in the chloroplast could result in apparent deficiency of all the proteins and pigments of the complex, both nuclear and ctDNA-encoded. Another unassigned, apparently temperature-sensitive plastid genotype has been briefly described (93-95).

As mentioned above, ctDNA is big enough to code for 100 proteins. At least 90 unidentified polypeptides are labeled in isolated organelle protein synthesis, but we presently know nothing about their functions in the plastid or their respective code locations on ctDNA (68). The function of a large proportion of ctDNA remains to be described.

I.5. Experimental introduction

I.5.1. Theoretical argument

Classical genetic analysis consists of genetic marker selection, sexual hybridization and progeny analysis. Cytoplasmic genes exhibit distinct behavior in such an analysis, showing aberrant inheritance in the organismal sexual cycle and marker segregation in mitotic cell divisions. What constitutes sexual hybridization of an organellar genome? The DNA molecules of heterologous parental organelles must interact to produce progeny cell lines with observably recombined sequences. The absence of genetic recombination phenomena in higher plant chloroplasts seriously limits growth of our knowledge of this genome. Within a single genotype, plastid genes have been identified by compartmentation of their transcription and translation or by detailed characterization *in vitro* of extracted nucleic acids. Given a pair of alleles at a single ctDNA locus, an observer could detect sexual inheritance or mitotic segregation, but not ctDNA recombination. Some ctDNA loci have been identified by comparing markers between species and observing their transmission genetics; in *Oenothera*, different species' plastids can be combined into the same cell by sexual hybridization, but the heterologous chloroplasts exhibit no spontaneous organellar hybridization.

Thus there is no readily apparent technique for combining genetic markers of interest into the same functional plastid genome *in vivo*. If such a phenomenon were available, one might construct a genotype with more selectable markers in order to better examine details of ctDNA genetic behavior. It is possible that heterologous chloroplast fusion and DNA recombination do occur spontaneously *in vivo*, but at a low frequency or within specific small ctDNA regions. If that were the case, we could not observe it because of the paucity of known genetic markers in any experimental system. It might be that for lack of a well-marked plastid genetic map, we cannot recognize recombination between ctDNAs. The ability to construct recombinant plastid genotypes would signify more than advances in fine-structure mapping; it also would permit the creation of useful ctDNA recombinants for agricultural plant breeding.

I.5.2. Somatic hybrids

Construction of cytoplasmic genotypes in plants is most readily approached via the route of protoplast manipulation. Plant protoplasts, viable single cells enzymatically stripped of cell walls, have been widely used during the past decade for physiological and genetic studies. Somatic hybrids produced by fusion of two different protoplasts or uptake of subcellular particles by protoplasts have been the object of intensive and competitive scientific activity. Only outstanding examples of these techniques and applications will be covered here, as the literature on this topic is voluminous. For excellent reviews of methods and results in cytoplasmic hybridization by protoplast manipulation, the reader is referred to the references (96-99).

Whether the parentage of the somatic hybrid combines intergeneric or interspecific genomes, the operational problems include selection of the hybrid against a background of unfused and homotypic fused cells. An optimal result would also include subsequent regeneration of the hybrid into a sexually mature plant for further meiotic inheritance tests and for analysis of genes which require tissue differentiation for their expression. In 1974, there was only one such hybrid plant reported (100). By 1981, nearly twenty such successful hybrids had been reported (99-103).

Intergeneric fusion-generated hybrids were laboratory curiosities which survived through only a few cell divisions (104-106) until the recent announcements of successful plant regeneration from two different intergeneric somatic hybrids. One of the these fusions combined potato and tomato genomes (107), and the other used Arabidopsis and

Brassica (108). One outcome of this procedure is illustrated by cell and plant lines containing bits of one parental genotype introgressed into the other in the hybrid, as in a fusion reported between carrot and parsley (109).

Inter- and intraspecific plant cell fusions, especially in the *Solanaceae*, have been much more successful than intergeneric experiments. The first report of a plant somatic hybrid regenerated into a sexually competent plant was the work of Carlson (110). In this instance, media requirements of the somatic hybrid, less stringent than those of its parental species, permitted selection of an interspecific *Nicotiana* hybrid *in vitro*. The next publication of successful regeneration of a plant derived from a protoplast fusion described a photoautotrophic hybrid within *N. tabacum*, selected by complementation between nuclear albino genes in the parents (111). This technique has been used elsewhere in the *Solanaceae*; nuclear albino complementation permitted selection and regeneration of a green somatic hybrid in *Datura innoxia* (112). By a combination of these techniques, somatic hybridization of sexually incompatible *Petunia* species was possible (113). The transfer of disease resistance between sexually incompatible *Nicotiana* species by protoplast fusion illustrates the relevance of this technique for plant breeding (114).

As success at somatic hybrid selection and regeneration increases, analysis of these plants can become more sophisticated. For thorough reviews of examples and analysis, see the references (98,99). The genetic analysis of plants arising from cell fusion can use markers from any of the cell's genomes: nuclear, plastid or mitochondrial. These may behave independently and require different analytical techniques. One example of nuclear gene inheritance in a somatic cell fusion is provided by the albino complementation scheme mentioned above to select somatic hybrids. This result implies additive fusion of nuclear genomes. When this is the case, proof of meiotic transmission of both parental genotypes from the hybrid is desirable.

A second trait receiving attention in somatic hybrids is cytoplasmic male sterility (cms). The available evidence links cms with mitochondrial DNA (section I.3.3). Transfer of cytoplasmic male sterility by protoplast fusion has been reported for inter- and intraspecific hybrids in Petunia (115,116) and in Nicotiana (117,118). In the most molecular of these studies, the generation of different floral morphologies in the somatic hybrids was correlated with the appearance of novel, presumably recombinant mtDNA restriction fragments (34).

Chloroplast segregation in somatic hybrids can be followed by any of the few markers known on ctDNA (section I.4.3): electrophoretic variants of the RuBPCase LS, ctDNA restriction maps, tentoxin resistance, drug resistance, and albinism. Unfortunately only the latter two markers are selectable in early states of hybrid cell growth. Some studies of somatic hybrids examined the plastid genotype in regenerated plants. Sexual progeny of the first *Nicotiana* hybrid produced by Carlson (110) were shown to have the RuBPCase LS of only one parental chloroplast type (119). When that work was later repeated (120), some first-generation hybrid plants of each parental RuBPCase LS genotype were found (121). In the historic wide hybridization between potato and tomato by protoplast fusion, analysis of RuBPCase LS showed some plants with potato, and some with tomato, LS electrofocusing patterns (107). A similar result was obtained later when *Nicotiana tabacum* and *N*. *rustica* were the parents of a somatic hybrid; some plants of each parental chloroplast type were recovered (122). In another recent study, inheritance of a streptomycin resistance marker was correlated with ctDNA restriction patterns in plants derived from a *Nicotiana* interspecific fusion experiment (101).

From these results, the expectation developed that heterologous plastids in a cell fusion would always rapidly segregate into cytoplasmically homogeneous lines; the heterozygous cytoplasmic condition would be short-lived. This, if true, would necessarily limit the chance of plastid genome interaction. However, this is not always the case, as shown below.

I.5.3. Outstanding published examples

The most complex genetic combinations of nuclear and cytoplasmic markers in a cell fusion are possible in *Nicotiana*, due both to the availability of a series of cytoplasmic male-sterile alloplasmic substitution lines (27), and to the ready regenerability from culture of this genus. Several published studies examined all three possible genomes in *Nicotiana* somatic hybrid plants.

In the French study already mentioned (34), protoplasts of a wild-type tobacco variety were fused with those of a male-sterile *N. debneyi* cytoplasmic substitution line. No selection for hybrids was imposed. The regenerated plants were scored for morphological characters intermediate between the parents; these alone represented the parental nuclear contributions to the hybrid. After plants were grown, the ctDNA restriction map of each individual was shown to be that of one or the other parent (123). The cytoplasmic male-sterile character segregated independently from the ctDNA and was correlated with mtDNA (34). Their failure to cosegregate implies a lack of linkage between cms and ctDNA within a system without observable plastid recombination, and lends support to the assignment of cms as a mtDNA character.

A Soviet scientist, Yury Gleba, fused plastid-linked albino with nuclear albino cells in two types of experiments (124). In combinations within *N. tabacum*, he obtained a high proportion of variegated plants. Using a male-sterile *N. debneyi* cytoplasmic substitution line homozygous for a nuclear albino marker and a *N. tabacum* plastid albino genotype, Gleba recovered and maintained plastid variegation in fusion plants (124). He correlated the color phenotype of the leaves with the RuBPCase LS electrophoretic markers; the variegated plants showed a combination of parental LS genotypes. The observation of variegated plants implies that somatic segregation of the heterologous plastids in a cell fusion need not prevent recovery of the heterozygous cytoplasmic state, provided that visual plastid markers permit its identification. Furthermore, the cosegregation of the albino character with the LS trait implies a stable linkage between these two genes. The plastid variegation was transmitted to sexual progeny of the somatic hybrids (124). Unfortunately, meiotic transmission of the nuclear marker was not tested and could have confused sexual progeny test results. Finally, all the plants which reached sexual maturity, including the variegated individuals, were male-sterile. Again, cms apparently did not cosegregate with plastid markers.

In the most recent reported thorough analysis of *Nicotiana* somatic hybrids, both inter- and intraspecific combinations could give variegating plastid markers in the fusion plants (102,103). Plastid albino tobacco mutant cells were fused with a nitrate reductase-deficient nuclear tobacco mutant (102). The variegated plant recovered could not be tested for any ctDNA markers other than the green/albino allele pair. When the nitrate reductase-deficient cells were fused with protoplasts of a N. suaveolens cytoplasmic substitution line, some somatic hybrid plants exhibited a mixture of parental chloroplast markers for RuBPCase LS electrophoretic pattern and tentoxin reaction (103). In the latter case, no visual marker discriminated between parental plastids. Here too, it was observed that male sterility and plastid markers do not cosegregate in fusion products.

These experiments overcome sexual barriers to cytoplasmic hybridization by somatic cell fusion. Other relevant techniques include protoplast uptake of subcellular particles, and pre-treatment of protoplast fusion partners to selectively inactivate genomes. Protoplasts can take up isolated nuclei (125,126), chromosomes (127), or plastids (128,129). In none of these experiments was a transplanted organelle shown to function in a regenerated plant. Subprotoplasts containing subsets of the cell's several genomes may be used for partial cell fusions in the future (96). Because whole protoplast fusions have been more successful than organelle uptake, techniques have emerged to selectively inactivate parental genomes in fusions between intact protoplasts, both in the fusion product and in the surrounding population of unfused and self-fused parental cells. X-irradiation pretreatment has been used to select against one parental cell type and to uniparentally eliminate nuclei in a cell fusion (117,130,131). X-irradiation of one parental protoplast genotype was held responsible for introgression of a few genes into the genome of the other parent in the somatic hybrid (109). Iodoacetic acid inactivation of one parental cell line was used to select against that parent in a fusion experiment (131). These techniques have proven successful in narrowing the range of possible outcomes in protoplast fusions and selections.

The preceding discussion of organelle genetics suggests that the study of organelle behavior in plant somatic hybrids could contribute experimental methods to manipulate those genomes for agronomic benefit. That premise led to the design of a well-marked cellular hybridization in *Nicotiana*. Experiments performed according to this design and their results are covered in the following chapters of this thesis.

MATERIALS AND METHODS

II.1. Experimental design

Cell fusions within the genus Nicotiana have genetic consequences for nuclear, mitochondrial and chloroplast genomes (see section I.5.2). To explore those consequences, a series of protoplast fusion experiments which included contrasting genetic markers from all three of these cell genomes was designed. The different chloroplast genomes paired together in these somatic hybrids were specially chosen to detect and select for ctDNA recombination.

Genetic marker combinations which were constructed used variants collected and kindly supplied by tobacco breeders and cytogeneticists. These initial seed stocks were of three types: nuclear albinos, cytoplasmic albinos, and male-sterile Nicotiana cytoplasmic substitution lines, all three within N. tabacum nuclear backgrounds. From these stocks, cytoplasmic male-sterile, nuclear albino homozygous genotypes were constructed by sexual crossing. Protoplasts of these cytoplasmically-marked, nuclear albino genotypes were fused with cytoplasmic albino N. tabacum protoplasts;

the genotypic construction of these somatic hybrids is shown in Figure 1.

Protoplast mixtures were treated to induce cell fusion, cultured, and regenerated into plants. Green individuals were visually selected; these were expected on the basis of complementation between nuclear and cytoplasmic albinos in the somatic hybrids. The cytoplasmic albino parent contributed a nuclear gene for plastid greening to the hybrid, while the nuclear albino contributed cytoplasm-substituted plastids which were genetically capable of greening in an *N. tabacum* nuclear background. Both cytoplasmic and nuclear albino parental genotypes and their homotypic fusion products could be selected against visually.

Green and variegated plants were recovered from these fusion preparations; they were analyzed for nuclear and cytoplasmic genotypes. Besides the obvious use of these genes in the selection of the somatic hybrid, each genome was analyzed separately, as described below.

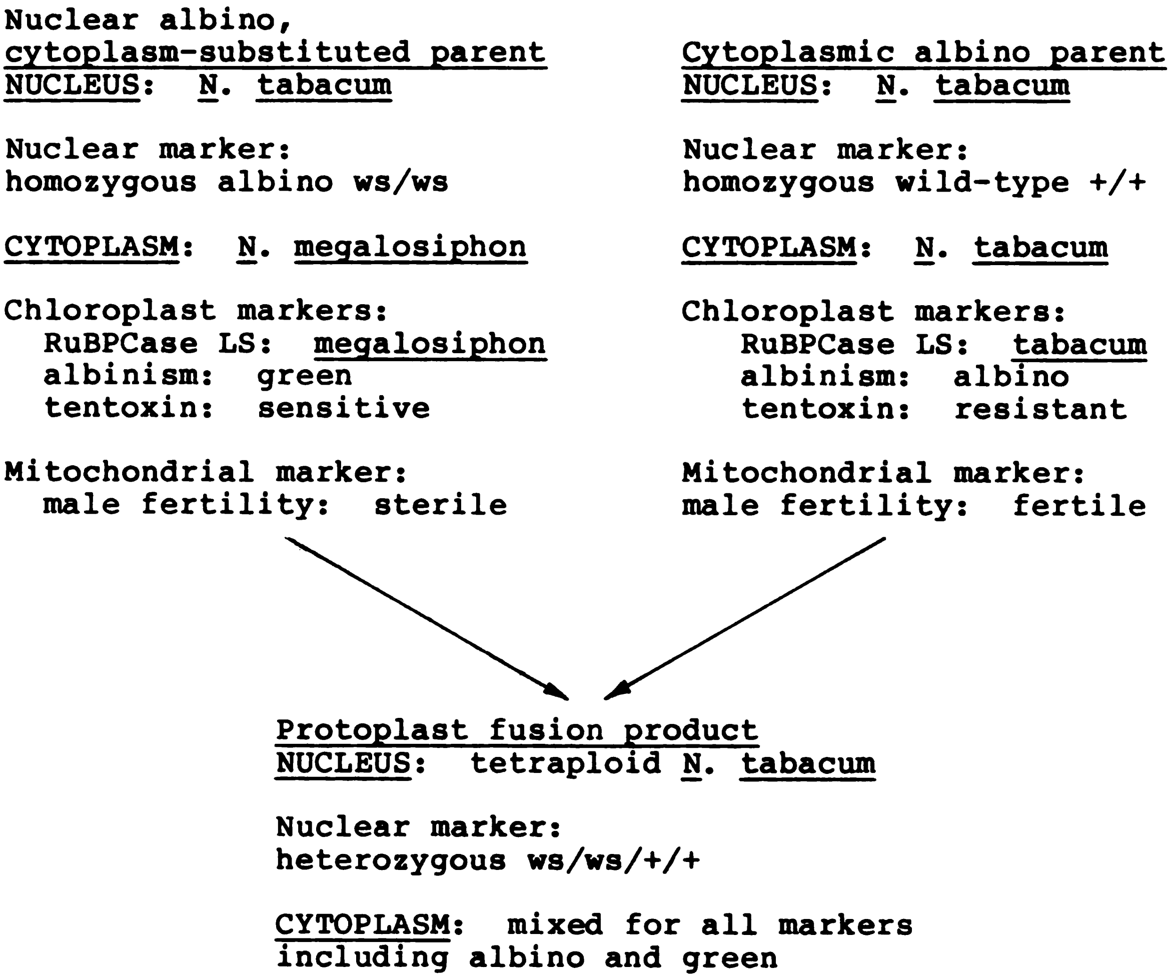
Meiotic transmission of nuclear albino alleles was tested in sexual progeny of the fusion plants. Visual observation also contributed information about additive morphological traits in the somatic hybrid. Ploidy of fusion plants gave information about the fate of the nuclei in the original cell fusions.

Because the nuclear albino, cms parent contributed a different species' cytoplasm from the *N. tabacum*, cytoplasmic albino parent, both mitochondrial and plastid genomes of the protoplast fusion parents differed in species-specific characters. The male-sterile character contributed to the fusion by the cytoplasmic substitution line, nuclear albino parent was followed by observation of the floral morphology in sexually mature fusion plants. This marker can be taken as an indicator of mitochondrial inheritance (see section I.3.3).

Three plastid markers were studied: the green/albino character, the electrophoretic focusing pattern of Fraction I protein, which differs between the cytoplasms used, and tentoxin reaction, representing ctDNA-encoded Coupling Factor 1 subunit differences (see section I.4.3). Other plastid markers in this system of contrasting species' cytoplasms are the ctDNA restriction endonuclease digestion patterns. These were beyond the scope of this investigation and their use was deferred until after the experiments reported in this thesis.

Recovery of green and variegated hybrid plants in a system with greater than one ctDNA marker permits examination of ctDNA markers for linkage. A different protoplast fusion experiment was designed to directly select for ctDNA recombination. In this experiment, protoplasts of two cytoplasm-linked albinos were fused; these parents differed as one was from a *N. megalosiphon* cytoplasmic substitution line, and the other was in *N. tabacum* cytoplasm. Both parents were in *N. tabacum* nuclear

Figure 1. Somatic hybridization plan using complementation between nuclear and cytoplasmic albino markers to select the somatic hybrid



Nuclear albino,
cytoplasm-substituted parent
NUCLEUS: *N. tabacum*

Nuclear marker:
homozygous albino ws/ws

CYTOPLASM: *N. megalosiphon*

Chloroplast markers:
RuBPCase LS: *megalosiphon*
albinism: green
tentoxin: sensitive

Mitochondrial marker:
male fertility: sterile

Cytoplasmic albino parent
NUCLEUS: *N. tabacum*

Nuclear marker:
homozygous wild-type +/+

CYTOPLASM: *N. tabacum*

Chloroplast markers:
RuBPCase LS: *tabacum*
albinism: albino
tentoxin: resistant

Mitochondrial marker:
male fertility: fertile

Protoplast fusion product
NUCLEUS: tetraploid *N. tabacum*

Nuclear marker:
heterozygous ws/ws/+/+

CYTOPLASM: mixed for all markers including albino and green

Figure 2. Somatic hybridization plan designed to select for ctDNA recombination

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Cytoplasm-substituted, cytoplasmic albino parent

NUCLEUS: *N. tabacum* +/+

CYTOPLASM: *N. megalosiphon*

Chloroplast markers:
  albinism: albino 1
  RuBPCase LS: *N. megalosiphon*
  tentoxin: sensitive

Mitochondrial marker:
  male fertility: sterile

N. tabacum cytoplasm, cytoplasmic albino parent

NUCLEUS: *N. tabacum* +/+

CYTOPLASM: *N. tabacum*

Chloroplast markers:
  albinism: albino 2
  RuBPCase LS: *N. tabacum*
  tentoxin: resistant

Mitochondrial marker:
  male fertility: fertile

Protoplast fusion product
NUCLEUS: tetraploid *N. tabacum* +/+/+/+

CYTOPLASM: mixed for all markers including albino 1 and albino 2

Select for green ctDNA recombinants

backgrounds. Visual selection for green regenerants attempted to employ positive selection for ctDNA recombination. The genetic construction of this somatic hybrid is diagrammed in Figure 2.

These experiments permit examination of the genetic consequences of somatic hybridization in *Nicotiana* for nuclear, plastid and mitochondrial genomes. Special attention is paid to the inheritance of plastid characters. This chapter describes the development of materials and techniques to perform the somatic cell hybridizations in Figures 1 and 2. The results of these experiments are discussed in Chapter III of this thesis.

II.2. Construction of parental materials

II.2.1. Seed sources

P. Carlson obtained seed of cytoplasmic male-sterile tobaccos in the varietal background cv. 'Hicks Broadleaf' from L. Burk, USDA-ARS, Oxford, N.C. These original stocks will be referred to as Hicks cms. The cytoplasmic substitutions from *N. megalosiphon*, *N. bigelovii*, *N. suaveolens*, and *N. undulata* into *N. tabacum* were supplied, as well as the *Nicotiana tabacum* cv. 'Hicks Broadleaf' (*N. tabacum* cytoplasm) maintainer line. These genotypes were grown in the greenhouse and their floral morphologies were confirmed to be as published (see Table 1).

Nuclear albino markers were obtained from L. Burk by P. Carlson. Selfed seed from heterozygotes of these genotypes were obtained: 'Sulfur', Su/+ in cv. 'John Williams Broadleaf', 'white seedling', ws/+ in white burley background, and 'yellow seedling', ys/+ in an undetermined varietal background.

This author obtained cytoplasmic albino markers from several sources. S. Wildman, UCLA, supplied a cytoplasmic albino mutant (herein called SamV) in the background cv. 'Turkish Samsun'. D. Gerstel, NCSU, contributed Seltman's V in the background of a coral-flowered Red Russian variety. L. Burk supplied DPl (herein called wvc) in cv. 'Xanthi nc', NC95V in NC 95 background, C298Vl in mixed background, and MSB21V. These are spontaneously-occurring mutants from breeder's stocks and are otherwise normal, male-fertile _N. tabacum_, except MSB21V. The latter is a cytoplasmic albino which K. Chen isolated in _N. megalosiphon_-cytoplasm substituted _N. tabacum_ cv. 'Burley 21', and is male-sterile. All of these cytoplasmic albinos were supplied as selfed seed from variegating (mixed wild-type and albino plastids) plants, with the exception of MSB21V, which requires pollination by a male-fertile Burley 21 _N. tabacum_ maintainer line.

This information is summarized in Table 1.

Table 1. Initial seed stocks: sources, references, descriptions

GENOTYPE	SEED SOURCE	REFERENCE	DESCRIPTION
Hicks cms:	L. Burk, USDA-ARS, Oxford, NC		
(*N. megalosiphon*)	"	(132)	stigmatoid anthers
(*N. bigelovii*)	"	(132)	petaloid anthers
(*N. plumbaginifolia*)	"	(132)	empty anthers
(*N. suavolens*)	"	(132)	stigmatoid anthers
(*N. undulata*)	"	(132)	petaloid anthers
N. tabacum cv. 'Hicks' maintainer line	"	(132)	male-fertile
Nuclear markers:			
'Sulfur' Su/+ selfed	L. Burk	(133)	nuclear, semidominant cv."J.W. Broadleaf'
'white seedling' ws/+selfed	"		nuclear recessive in white burley
'yellow seedling' ys/+ selfed	"		nuclear recessive unknown background

Table 1, (cont'd).

GENOTYPE	SEED SOURCE	REFERENCE	DESCRIPTION
Cytoplasmic albinos:			
wvc (DP1)	L. Burk	(134)	cytoplasmic albino in cv. 'Xanthi nc'
NC95V	"	(134)	cytoplasmic albino in cv. 'NC95'
C298V1	"	(134)	cytoplasmic albino associated with virus
MSB21V	"	(134)	cytoplasmic albino in cv. 'Burley 21' *N. megalosiphon* cytoplasm male-sterile stigmatoid anthers
N. tabacum cv. 'Burley 21'	"		Maintainer line for MSB21V, male-fertile
SamV	S. Wildman UCLA	(135, 136)	cytoplasmic albino in cv. 'Turkish Samsun'
Seltman's V	D. Gerstel NCSU	(137)	cytoplasmic albino in cv. 'Red Russian'

All plants were grown in pots in the greenhouse under ambient illumination. Genetic stocks were maintained as perennials and propagated by shoot cuttings or by cutting back existing plants, or treated as annuals and reproduced by self or cross seed when appropriate. All cross-pollinations were made by hand. Pollinations were easily made, especially with the cms genotypes which often had exserted stigmas. Careful tagging identified the many different crosses performed (Section II.2.2). Pollen was stored in gelatin capsules in dessicators in the freezer when necessary. Approximately 30 days were required for seed maturation in the greenhouse. Seeds were planted in the laboratory under lights and selected seedlings transplanted to the greenhouse when needed.

II.2.2. Nuclear albino plants

The Hicks cms lines and the nuclear albino stock lines (the latter as seed from selfed heterozygotes) were planted in the greenhouse; Su/+ individuals were identified visually as their chlorophyll levels are lower than those of wild-type plants (138). The following discussion of sexual crosses follows the convention that the female parent is written first; the symbol ⊗ denotes a self-pollination.

Five to seven wild-type individuals from the ws/+ ⊗ and from the ys/+ ⊗ populations were grown to maturity and allowed to self-pollinate. That selfed seed was planted in soil as a progeny test to identify the heterozygous individuals. Plants showing albino seedlings among their progeny were identified as nuclear albino heterozygotes, retained, and used as pollen parents during the transfer of the nuclear albino markers into the Hicks cms lines.

Transfer of the nuclear albino markers into the Hicks cms lines was accomplished during two sexual generations of the tobacco genotypes used. In the first generation, the Hicks cms lines were pollinated with pollen from the nuclear albino heterozygotes. The resulting seed was planted and comprised the second generation. Five to seven progeny in the case of the recessive nuclear markers, or three plants in the case of Su/+, were grown to maturity from each of the eighteen combinations of Hicks cms lines and nuclear albino markers. The nuclear albino heterozygotes identified in the preceding generation were again used to pollinate these plants. This progeny test was necessary to identify albino heterozygotes among the cms plants in the case of the two recessive markers. Heterozygous cytoplasm-substituted plants thus identified were used to generate a quantity of seed from the cross: Hicks cms nuclear albino heterozygote X nuclear albino heterozygote. This crossing scheme gave 25% nuclear albino seedlings among the 100% cytoplasmic substitution progeny. This plan was followed with all five cytoplasmic substitution lines plus the maintainer line as females, using the two recessive nuclear albino heterozygotes ws/+ and ys/+ and the semidominant nuclear

Figure 3. Crossing plan* generating homozygous nuclear albino cytoplasmic substitution lines

GENERATION	FEMALE	CROSS OR PROGENY TEST	MALE
1	Hicks cms +/+ (*N. megalosiphon* cytoplasm)	ws/+ ⊗ seed grown; 5 - 7 plants ⊗; progeny test identifies ws/+ individuals X	ws/+
2	Hicks cms ws/+ (*N. megalosiphon* cytoplasm)	grow 5 - 7 progeny and cross as females X ws/+; progeny test identifies Hicks cms ws/+ individuals X	ws/+
3		1/4 Hicks cms ws/ws (*N. megalosiphon* cytoplasm)	

* Hicks cms (*N. megalosiphon* cytoplasm) and ws/+ nuclear albino heterozygote genotypes are shown for purposes of illustration. The same plan was followed with all five Hicks cms lines and their maintainer line as female parents, and with heterozygotes of all three nuclear albino markers as male parents. Progeny tests were necessary with the two recessive nuclear albino markers ws and ys, but not with the semi-dominant albino marker Su.

marker Su/+ as male parents. No progeny test was needed to identify the Su/+ heterozygotes. No double mutants were constructed. The crossing scheme is diagrammed in Figure 3.

II.2.3. Cytoplasmic albino plants

The seed from variegating cytoplasmic albino plants, as obtained from other researchers, was planted in the greenhouse and variegated individuals were selected. Construction of pure albino lines required exploitation of the known developmental pathways of tobacco. The following discussion employs the convention that the three ontogenetic tissue layers in a plant apical or axillary meristem are written, in the order describing descent from the surface to the pith, as: LI (epidermis), LII, LIII (pith).

Variegated plants were pruned to encourage the growth of -WW and -WG sectors. This was accomplished by observing the color pattern on leaves and cutting the stem above any leaf showing a quarter of its area or more of the appropriate pattern. GWW and WWW chimeras are completely albino in leaf phenotype, as the epidermis in tobacco is colorless. They can be distinguished one from the other by occasional tiny green sectors which occur in a GWW leaf when the epidermis supports a rare periclinal mitosis and inserts green tissue into the LII during development (139). -WG chimeras are white around the periphery of the leaf and green in the midrib region, although the pattern created by the flexible leaf ontogeny is somewhat variable (140). When a leaf showed a quarter of more of its area in either of these chimeral patterns, the stem above it was cut off. The axillary shoot which grew out above the subtending conspicuously patterned leaf usually showed a chimeral pattern approximating that of the leaf. Then further pruning of this new mericlinal shoot could be performed to stimulate growth of an appropriately located axillary bud, for example one associated with an entirely chimeral leaf arising from the chimeral sector. When such -WW or -WG shoots were obtained they were used for further isolation of the albino genotypes.

Seed capsules were collected from naturally self-pollinated -W- chimeras of all five cytoplasmic albinos, except in the case of the MSB21V genotype, in which case a Burley 21 maintainer line was the pollinator. The LII gives rise to the gametes in tobacco; this was originally demonstrated with the wvc genotype by Burk (139). Such seed capsules gave 100% albino progeny; any exceptions caused the seed lot to be discarded.

II.2.4. Nuclear albino shoot cultures

Seed from crosses generating homozygous albino Hicks cms lines was surface-sterilized and planted *in vitro*. The protocol for this operation was as follows. A small quantity of seed was wrapped in a Miracloth bag and tied with a rubber band. This package was placed into a 10% (v/v) Chlorox solution (equivalent to 0.525% hypochlorite), containing 0.01% SLS (sodium lauryl sulfate, technical grade), for five minutes with stirring. The package was opened and fastened across the mouth of a flask. Several hundred ml of 95% ethanol, followed by 500 ml of sterile distilled water, were poured through the seeds. The seeds were then picked off the Miracloth with a forceps and aseptically planted on solid medium R5 (See Table 2). Seed was germinated in the light and the homozygous albino individuals were transferred to fresh R5 plates. Thereafter they were grown as plantlets in the light on R5 in deep petri dishes (Falcon 1005, 2 X 10 cm) and transferred approximately monthly as shoot cuttings.

Sterile plantlets from ws/ws homozygous nuclear albino Hicks cms lines showed multiple, small green leaf sectors. To study the genetic stability of the ws marker _in vitro_, white areas and green sectors of the sterile plantlets were excised and planted on regeneration medium (0.3/10, see Table 2) and plantlets derived from them were examined for pigmentation. Early regeneration stage buds were always white. Usually all expanded leaves from such regenerants had the same low proportion of green to white tissue as did the original seedling-derived homozygotes. Some plants derived from green sectors showed increased proportions of green tissue but such plants never survived transfer to soil culture. Predominantly albino plants derived from white

Table 2. Tissue culture media*

	R5 Shoot culture	3/.3 Callus growth	0.3/10 Shoot regeneration
Hormones:			
Indoleacetic acid	0	3 mg/L (17 uM)	0.3 mg/L (1.7 uM)
Kinetin	0	0.3 mg/L (1.4 uM)	0
2-isopentenyl adenine	0	0	10 mg/L (49 uM)
Vitamins:			
Pyridoxine HCl	0.5 mg/L	0	0
Nicotinic acid	0.5 mg/L	0	0

All three media contained the following, in milligrams per liter:

NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$MnSO_4.H_2O$	16.8
$ZnSO_4.7H_2o$	10.6
H_3BO_3	6.2
KI	0.83
$NaMoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Thiamine	1.0
Inositol	100
Sucrose	30,000
Agar	9,000

pH before autoclaving 6.0

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* Modified from Murashige and Skoog (147).

regions of Hicks cms ws/ws plants were again subjected to regeneration from white leaf tissue and the resulting plants were used for subculture on medium R5 and for protoplast experiments. Control experiments with protoplasts from this material never produced any green plants. It is assumed that green sectors on ws/ws plantlets represent a transient epigenetic escape from the albino condition, and that the white seedling homozygotes are genetically stable in mitosis. Such ws/ws homozygotes have never been studied through meiosis.

'Yellow seedling' homozygous individuals (Hicks cms ys/ys) never grew beyond the cotyledon stage on R5 medium. Callus was sometimes produced on the seedlings. It is possible that a nutritionally supplemented medium would support the growth of this albino genotype as sterile plants, but this was not explored experimentally. This marker was never used for protoplast experiments.

Su/Su homozygotes are known to produce revertant sectors which are genetically stable. Such sectors can be distinguished by coloration and can be produced by several different genetic mechanisms (141). Macroscopic sectors of dark green color on Su/Su plantlets were avoided when subculturing or making protoplasts from shoot cultures.

All six Hicks lines including the maintainer line were cultured as homozygous albino plantlets; all had the same performance <u>in vitro</u> with respect to plantlet growth rate and frequency of sectoring. It was necessary to choose several genotypes for protoplast experiments. Preliminary experiments in our laboratory had shown that the *N. megalosiphon* derivative, the *N. bigelovii* derivative, and the *N. undulata* substitution grew more rapidly than the others as callus on medium 3/.3 (see Table 2) and regenerated most readily on a high-cytokinin tobacco regeneration medium (142). In contrast, the *N. suaveolens* and the *N. plumbaginifolia* derivatives grew poorly and regenerated little in standard media.

A survey of the evolutionary relationships and known cytoplasmic characters within the sixty-member genus *Nicotiana* showed that *N. megalosiphon* and *N. suaveolens* are within the same taxonomic section Suaveolentes (143). A series of tentoxin reaction studies in sexual hybrids showed that these two species probably share cytoplasmic parentage (92). Because the *N. megalosiphon* cytoplasmic substitution performed better than the *N. suaveolens* derivative in tissue culture regeneration assays (142), the *N. megalosiphon* derivative was chosen to represent this cytoplasm to contrast with tobacco in protoplast fusion experiments. This fusion parent pair also provides a clear parallel to the protoplast fusions between *N. tabacum* and *N. megalosiphon*-cytoplasm substituted cytoplasmic albinos. The sensitive tentoxin reaction of *N. megalosiphon* and *N. suaveolens* differs from the resistant reaction of *N. tabacum*; however, Coupling Factor I subunits from these three species could not be differentiated on focusing electrophoretic gels (91). They do differ in their RuBPCase LS electrophoretic pattern (91,144).

Nicotiana undulata cytoplasm, though arising in a different taxonomic section from N. tabacum, shares the character of tentoxin resistance with tobacco (145). This cytoplasmic trait caused it to be dropped from the list of protoplast fusion parents.

The N. plumbaginifolia cytoplasmic substitution derives from the same taxonomic section Alatae as does the putative cytoplasmic parent of the ancestral hybridization which led to the present-day allopolyploid species N. tabacum (72,92,143). The N. plumbaginifolia cytoplasm differs from N. tabacum in its reaction to tentoxin; N. plumbaginifolia is sensitive to the CF I-binding toxin, whereas N. tabacum is resistant (92). However, the N. plumbaginifolia cytoplasmic substitution line regenerated with less efficiency than did the N. megalosiphon, N. bigelovii and N. undulata derivatives in preliminary studies (142). Its taxonomic relationship to N. tabacum and its poor tissue culture performance led to the rejection of the N. plumbaginifolia cytoplasm derivative for protoplast work.

The N. bigelovii cytoplasm is distinct from N. tabacum and its precursors by its position in a different taxonomic section; chromosome pairing in F1 hybrids between section Genuinae and section Bigeloviana is virtually nil, suggesting a relatively distant ancestral divergence (143).

N. bigelovii has a sensitive reaction to tentoxin, contrasting with the resistant *N. tabacum* response (145). Its RuBPCase LS electrophoretic pattern is distinct from both *N. tabacum* and *N. megalosiphon* (144). The *N. bigelovii* cytoplasmic substitution line responded with vigorous regeneration in standard tobacco media (142). The *N. bigelovii* cytoplasmic derivative was chosen as a second genetic contrast to the *N. tabacum* cytoplasm in planned protoplast fusions.

II.2.5. Cytoplasmic albino shoot cultures

Seed from -W- chimeras of cytoplasmic albinos was surface-sterilized and planted on R5 medium as described above. Vigorous sterile cytoplasmic albino plantlets could be obtained more rapidly with other explants, as described below.

Pith from -WW genotypes was excised aseptically from stem segments. The stem was surface-sterilized in 10% Chlorox, 0.01% SLS for five minutes and immersed briefly in 95% ethanol, followed by a rinse in sterile distilled water. The pith was dissected out with a scalpel and placed onto tobacco regeneration medium (0.3/10, see Table 2). The plantlets obtained were observed carefully for several months of transfer on medium R5 to confirm their pure albino status. Any cultures showing wild-type green, chimeral or variegated appearance were discarded.

Some cytoplasmic albino cultures were started from leaf explants. Leaves were surface-sterilized by a five-minute dip with stirring into 10% v/v Chlorox with 0.01% w/v SLS, followed by a brief immersion into 95% ethanol and extensive rinsing in sterile distilled water. Leaf discs were cut with a cork borer (0.5 cm) from -WW leaves or from the white margin of -WG leaves, where no green LIII tissue would be present. These albino leaf discs were planted aseptically on regeneration medium (0.3/10, see Table 2). The first crop of plants which are regenerated from such explants, appearing within six weeks without intervention of extensive callus growth, are usually derived from the LII or LIII and do not represent epidermal derivatives (146). Such plants were subcultured for at least six transfers on medium R5 to confirm their cytoplasmic albino homogeneity; again, any variegating, chimeral, or wild-type lines were discarded.

The cytoplasmic albino genotypes, when grown as plantlets in sterile culture, developed some green pigmentation although expanded leaves could readily be distinguished from the wild-type. The growth rate of cytoplasmic albino plants in vitro was less than that of wild-type, green sterile plantlets. The different cytoplasmic albino markers exhibited different severity of pigment reduction; in order from the most green to the most bleached under laboratory conditions, they ranked: Seltman's V - MSB21V - SamV - NC95V - wvc. To confirm the albino cytoplasmic marker in these tissue cultures as being homogeneous, a number of tests were performed. Plants were carefully observed to attempt to detect rearrangements from a green LI; no evidence was found for wild-type epidermis, or the chimera was discarded. Adventitious roots from explanted shoot cuttings were subjected to callus induction on medium 3/.3 and the callus was transferred to regeneration medium (see Table 2). This would give plants derived from the LIII of the original sterile albino plants. All such plants were albino, or the stock culture was discarded.

C298V1 was not completely albino as isolated *in vitro*. Additionally, it appeared to carry a virus contaminant. Samples of the sterile plants were ground in a mortar and pestle and rub-inoculated with carborundum onto the leaves of *N. tabacum* cv. 'Xanthi nc' plants. The test showed an easily transmissible virus but gave systemic symptoms on the host which gives local lesions with tobacco mosaic virus (TMV). TMV therefore was ruled out. The virus was transmissible in ordinary culture manipulations and contaminated other albino stock cultures. C298V1 was not used any further after this discovery and sterile lines of it were discarded. NC95V was not used for protoplast experiments; sufficient different cytoplasmic albino lines were available without it.

II.3. Protoplast fusion

II.3.1. Protoplast isolation and culture

Preliminary experiments determined a protocol for isolation of leaf protoplasts from sterile plantlets of these albino tobacco genotypes. The tender shoot culture leaves were cut sterilely into strips several millimeters wide and incubated with slow rotation in a mixture of commercially available cell-wall degrading enzymes. This crude enzyme solution had first been cleared by prefiltration or centrifugation, and sterilized by passage through an 0.45 micron Nalgene filter. Observations showed that a mixture of 3% w/v Cellulysin and 0.9% w/v Macerozyme (both from Calbiochem) would release the protoplasts within five hours. A milder treatment with 1% Cellulysin and 0.1% Macerozyme would release the protoplasts within a 12 to 16 hour period. This latter overnight enzyme incubation was used throughout the fusion experiments. An osmoticum of 0.6 $\underline{M}$ mannitol was required to protect the protoplasts from plasmolysis and bursting during preparation and handling. Buffers (0.1% w/v MES and 50 mM glycine, pH 5.7) were withdrawn from the enzyme incubation without apparent ill effect on the resulting protoplast preparations. Leaf debris was removed in a filtration step using Miracloth.

Sterile albino tobacco leaf protoplasts thus prepared were abundant and of varying size; many were large and highly vacuolate, making use of a hemocytometer impracticable. Albino cells of all sizes proved to be relatively light in density and were impossible to sediment with a centrifuge. In drop culture, they floated throughout the medium. A flotation method was devised to wash and collect these cells.

In preliminary experiments, albino protoplasts could be collected by flotation in 25% w/v sucrose, but these cells never grew when plated on culture medium. The iso-osmotic density gradient described by Harms and Potrykus for protoplast isolation (148,149) proved to be of too low viscosity to be practical for room temperature work; the polyethylene glycol (PEG) and dextran gradient described by Kanai and Edwards for protoplast flotation (150) was considered too damaging to these cells. PEG is applied specifically as a fusogen in the genetic experiments described in section II.3.4. A discontinuous density gradient of Ficoll, a sucrose polymer of low osmotic strength, was designed to collect the protoplasts and to separate them from the enzymes. The optimal version of this flotation step, as used for genetic experiments, is as follows: Protoplast media were made up to 25%, 8% and 2% w/v Ficoll (400DL, Sigma) and filter-sterilized (0.45 micron Nalgene). The enzyme-protoplast mixture was combined 1:1 with the 25% Ficoll solution and layered under 6 ml of 8% and 1 ml of 2% Ficoll in a 50 ml centrifuge tube. Centrifugation at 50xG for 45 minutes at room temperature in a swinging bucket rotor floated most of the albino protoplasts to the 8%-2% or the 2%-air interface, where they were collected with a wide-bore Pasteur pipette (Blood bank dropper, A.H. Thomas). The brown color characteristic of the crude enzyme mixture remained in the lowest layer of the discontinuous density gradient. Protoplasts could be collected in this manner with population densities averaging 10^6 cells per ml (see Figure 4).

Following the publications of Binding (151) and Schieder (152), V47 medium was extensively tested for culture of these albino tobacco protoplasts (see Table 3). In a series of experiments, protoplasts were prepared and plated on V47 salts plus varying sugars and hormones (J media, Table 3). Growth or division of protoplasts was seen only once in many trials with cells isolated and plated on these media. Albino tobacco protoplasts could not be successfully cultured on media with Binding's V47 salts.

An adaptation of Nagata and Takebe's tobacco protoplast culture medium (153) with the complex additives of Kao and Michayluk (154) had been devised by R. Malmberg in our laboratory for culture of tobacco protoplasts (155). This medium D3 (see Table 3) proved successful for culture of albino tobacco leaf protoplasts. All genotypes showed cell wall formation and cell divison within a week after isolation and plating in D3 (see Figure 5). To quantify cell density and plating effectiveness, measured aliquots of dilutions of the protoplasts as isolated were plated on thin

Table 3. Protoplast culture media

	V47 Salts(151) mg/L	N+T Salts(153) mg/L
NH_4NO_3	280	825
KNO_3	1466	950
$CaCl_2.2H_2O$	735	220
$MgSO_4.H_2O$	986	1233
KH_2PO_4	68.5	680
$MnSO_4.7H_2O$	3.89	168
$ZNSO_4.7H_2O$	1.44	10.6
KI	2.49	0.83
H_3BO_3	0	6.2
$NaMoO_4.2H_2O$	0.097	0.25
$CuSO_4.5H_2O$	0.015	0.025
$CoCl_2.6H_2O$	0.010	0.030
Na_2EDTA	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
	J Series	D3(155)
Thiamine HCl	1.0	1.0
Nicotinic acid	0.5	0
Pyridoxine HCl	0.5	0
Inositol	100	100
Hormones(153)	1.5 - 1.0	1.0
2',4'-D	3.0	3.0
NAA	1.0	1.0
6-BA		
Casamino acids vitamin-free(154)*	0	250*
Coconut water deproteinized(154)*	0	20 ml/L*
Sugars		
Sucrose	varied	17 g.
Glucose	varied	0
Mannitol	varied	82 g.
Agar		
pH before autoclaving	6.0	6.2

* Filter-sterilized

Figure 4. Density gradient centrifugation to collect albino protoplasts. Wvc cells in enzyme before centrifugation, at left, were collected at the top of the gradient after centrifugation, right.

Figure 5. Albino protoplasts growing in medium D3. MSB21V cells formed a cell wall (asymmetric cells) and divided (two-cell figure).

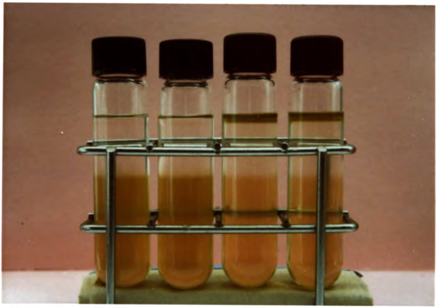


Figure 4.

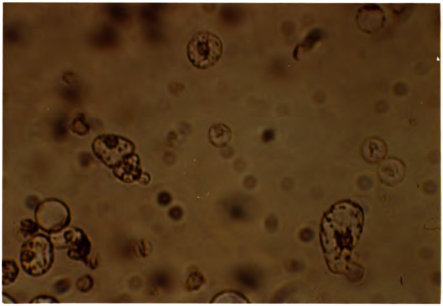


Figure 5.

layers of solid culture medium in gridded petri dishes (Falcon 3030, 2 mm grid) and their dead, surviving, and dividing numbers were counted over several grid squares at 200X magnification in an inverted microscope. Some protoplasts floated in the remaining liquid medium and counting on several focal planes was necessary. Most cell death occurred within 24 hours, so all counts were made after 24 hours of culture. Cell density ranged from 10^5 - 10^6 cells per ml, and plating efficiency ranged up to 10% in these experiments. Plating at cell densities less than 10^4 per ml caused culture death.

Good preparations of nuclear and cytoplasmic albino protoplasts were prepared from sterile shoot culture leaves using the overnight enzyme incubation period, Ficoll flotation wash, and plating over D3 medium solidified with 0.5% agar in 60x15 mm Petri dishes. Cultures were incubated in dim light at high population densities (10^5 cells per ml). Cell divisions (Figure 5) occurred by 7 days of culture.

II.3.2. Protoplast regeneration

The growth of albino protoplast cultures beyond their first few divisions in the high-osmoticum D3 medium was investigated in preliminary experiments. After two weeks on D3, the soft protoplast aggregates were spooned and spread sterilely on a series of media with varying hormone supplements. A month later, these plates were subcultured onto regeneration medium (0.3/10, Table 2) with varying sucrose content. Results showed that exposure to the high cytokinin hormone additions of medium 0.3/10 in the first protoplast transfer had a strong effect in promoting early regeneration from such cultures. Other hormone variables had inconclusive results in these tests. Many cultures were lost due to inviability or contamination of the protoplast preparation. Lowered sucrose during plant regeneration lowered the yield of regenerated colonies. Plantlets appeared from the 75th day after protoplast isolation and more frequently thereafter in the successful cultures. Regenerated plants could be grown as shoot cultures on medium R5 (Table 2), but not all could be recovered. Culture performance was extremely variable with respect to growth and regeneration per petri dish.

These experiments showed that all albino genotypes could be regenerated from protoplasts isolated and plated on D3, transferred to 0.3/10 (0.5% agar), and passaged repeatedly on medium 0.3/10 thereafter. No wild-type plants were ever regenerated from the albino protoplast cultures in these experiments. An estimated maximum of 5×10^3 plantlets per original plate of 5×10^5 protoplasts could be recovered after 5 - 6 months. By this time the culture would occupy about 100 petri dishes.

II.3.3. Protoplast fusion technique

The high pH - high Ca^{+2} treatment used to induce cell fusion in early reports (156) failed in preliminary experiments on albino protoplasts. Fusion treatments with polyethylene glycol (PEG) induced cell aggregation but resulted in a high frequency of cell death. The number of cells per aggregate varied with cell density during the PEG treatment. A high density of 10^6 protoplasts per ml caused high multiple aggregates which always died. Low density protoplasts (10^3 - 10^4 per ml) failed to make cell contact during PEG treatment; fusion frequency was very low. Intermediate densities, about 10^5 cells per ml, yielded dead cells, unfused cells, binary aggregates and some high multiple-cell aggregates.

Osmotic measurements were made in an attempt to rationalize performance of the cells in different media and fusion treatments. Readings on a thermocouple psychrometer (Wescor 33-T) compared sucrose and mannitol solutions, raw and autoclaved, with PEG (Baker 4000) solutions and culture media. Small effects due to sugar autoclaving and media salts, each equivalent to an increment of 0.1 M manitol, were noted, as well as information relating the osmotic strengths of the PEG and mannitol solutions. A fusion protocol was devised on the premise that cell fusion would be aided by slight cell plasmolysis, followed by relief of this osmotic stress during removal of the PEG.

Cells prepared in D3 (Table 3) were mixed 1:1 with 56% w/v PEG (Baker 4000) made up in 50 mM $Ca(NO_3)_2$ and 50 mM glycine, pH 5.7. The resulting 28% PEG - half D3 solution caused observable two-cell aggregations and the development of flat cell appression surfaces within five minutes. Higher cell multiples were aggregated in longer exposure times, depending on cell density. Increasing proportion of cell death occurred with longer PEG exposures. The cell preparation was diluted with D3 medium after ten minutes; this would lower the osmotic strength of the solution. Cell appressions were still visible after this step.

A discontinuous Ficoll gradient similar to that used after the enzyme treatment (section II.3.1) was devised in order to separate the cells from the damaging PEG solution and to collect them in sufficient density for successful plating. As finally established, this Ficoll wash of the protoplast fusion preparation was performed as follows.

The fusion preparation was mixed 1:1 with 25% w/v Ficoll in D3 and overlayered with 8% w/v Ficoll in medium D3. Gentle centrifugation at 50xG for 30 minutes floated the protoplasts to the 8% - air interface. Protoplasts could be recovered at a density of 10^5 - 10^6 per ml. A maximum of 20% of the PEG-treated cells could be recovered in this way. These cells included appressed cells in about the same proportion as observed during the PEG treatment. These PEG-exposed, Ficoll-floated cells could be successfully cultured on D3 followed by 0.3/10, as described for untreated albino protoplasts in section II.3.2.

No obvious fusion figures had been observed other than the two-cell appression type. A microscopic assay for cell fusion was devised. One-day-old protoplast fusion cultures were sampled, mixed on a slide with a little 0.1% acridine orange in water, and observed with UV-fluorescence in a high-power compound microscope. Fusion preparations were examined after 24 hours, before any wall formation or cell division were observed. Preparations showed about 1% binucleate protoplasts (Figure 6). These figures were interpreted as surviving cell fusions. The 1% fusion rate was considered a worthwhile compromise between the population dependence of protoplast growth and the high proportion of cell death in more rigorous PEG treatments. This fusion protocol was used for genetic experiments with the albino tobacco protoplasts.

II.3.4. Fusion experiments

Protoplast fusion experiments combined different albino tobacco genotypes using the methods optimized in the preceding sections. In each experiment, two albino leaf protoplast cultures were enzymatically isolated and collected by flotation in a Ficoll gradient according to the procedure outlined in section II.3.1. The two cultures were diluted with medium D3 to 5 X 10^5 viable cells per ml as

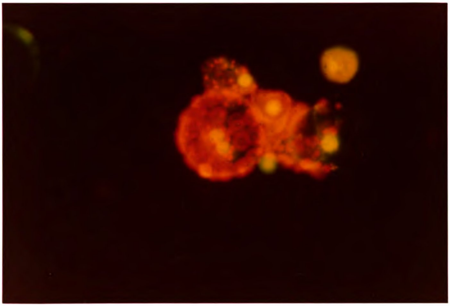


Figure 6. UV-fluorescent micrograph of a fusion product. Acridine orange-stained, PEG-treated cultures 24 hours after plating showed 1% binucleate cells; debris is visible.

suggested by a quick visual estimate of cell population density and viability, mixed 1:1, treated with PEG, and collected by Ficoll flotation as described in section II.3.3. This preparation was then cultured on D3 in the light for two weeks, and subsequently repeatedly subcultured at monthly intervals on medium 0.3/10 in the light. This sequence yielded regenerated plantlets (section II.3.2). Within each experiment, each plated one-milliliter aliquot of PEG-treated cells was numbered. All tissues and plants subcultured from that plate carried the same identifying number. Control plates of unfused cultures of each albino were also prepared and subcultured from each fusion experiment.

Cultures derived from fusion treatments combining nuclear and cytoplasmic albino cells, or different cytoplasmic albinos, were grown under lights at 1500 lux. Wild-type green callus and green regenerated plants were visually selected during repeated passages of the fusion cultures on medium 0.3/10 in the light. Recovered plants were subcultured onto medium R5 (Table 2) until shoot cultures were verified as to phenotype and until they had rooted. The plants were then washed of agar, transplanted into soil with a gradual acclimatization to low humidity, and grown up in the greenhouse.

II.4. Analysis of somatic hybrids

The fusion experiments combined contrasting genetic characters representing the nuclear, mitochondrial and plastid genomes (section II.1). Genetic analysis following recovery of green plants used leaf, root and floral tissues. The basis for selection, leaf greening ability, was the first character examined. Leaf variegation and sectoring were noted. Leaf material was consumed in the RuBPCase LS electrophoretic assay discussed below. Roots were used for chromosome number determinations. Floral development permitted scoring of anther morphology and the collection of genetic data through sexual crosses with the fusion plants.

Nuclear inheritance in the fusion plants was studied in several tests. The chromosome number of the somatic hybrids was examined in the compound light microscope using root tips pretreated with 0.1% w/v colchicine in water for five hours in the dark, fixed overnight in 3 parts ethanol: 1 part glacial acetic acid (v/v) in the cold, hydrolyzed in 1N HCl at 60° C. for 30 minutes, and stained with acetocarmine, propiocarmine, or Feulgen stain (157). Pollen mother cell meiotic squashes were made for plants exhibiting normal anther development. Floral buds of various sizes were collected into 6 parts ethanol: 3 chloroform: 1 glacial acetic acid (v/v/v), fixed overnight at room temperature, transferred to 70% ethanol, and stained with acetoorcein (158). Chromosome counts were made on meiotic figures under the microscope and from photographs and projected slides of such figures. For a quick test of nuclear ploidy in the somatic hybrids, their stomatal size was compared to haploid and diploid tobacco controls. Lower epidermal strips were peeled from expanded leaves, mounted in water, and measured for stomatal length and width under the microscope.

Meiotic transmission of nuclear albino markers in the somatic hybrid was studied by crossing the fusion plants as females to a nuclear albino heterozygote pollen parent. Progeny seedlings were grown in the laboratory under lights and scored for albino characters. Female fertility and seed viability in these crosses with fusion plants contributed information on their nuclear euploidy.

Cytoplasmic male sterility, which probably represents a mitochondrial DNA character (section I.3.3), was analyzed. Fusion plant floral morphology was examined and compared with parental phenotype (Table 1). Repeated floral flushes were observed from shoot cuttings of the fusion plants to attempt to detect somatic variation in floral characters.

Plastid markers included pigmentation, a leaf protein electrophoretic phenotype, and seedling tentoxin reaction. Regenerated plants were observed for color, variegation, and sectoring behavior. Determination of plastid contribution to RuBPCase electrophoretic pattern was extensively investigated. Isolation of the protein was attempted according to various published procedures (159-161). Leaves were homogenized in the cold with a blendor or mortar and pestle in buffer solutions containing 0.1-0.2 M NaCl, 1-5 mM EDTA, 0-10 mM $MgCl_2$, 10-50 mM Tris HCl pH 7.4, 0.2% w/v insoluble polyvinylpolypyrrolidone (PVPP, Sigma), and 10-80 mM mercaptoethanol. 3-10 mg/ml dithiothreitol (DTT) replaced mercaptoethanol in some of these experiments. The homogenate was squeezed through cheesecloth and clarified by centrifugation at 17-20 kG for up to 30 minutes. The supernatant was passed through a Sephadex G25 column and eluted with the same buffer minus DTT and PVPP. The column retarded phenolics; the protein front was collected. This eluate was concentrated under N_2 in an Amicon pressure ultrafiltration system and dialyzed against NaCl-free buffer. Repeated trials with this method showed that Fraction I protein from genotypes with substituted cytoplasms could not be precipitated by withdrawing the salt during dialysis of the leaf extract.

Rabbit antiserum to spinach Fraction I protein (the gift of Dr. N. Tolbert) was used in attempts to collect the various tobacco Fraction I proteins by immune precipitation. As in published procedures (162,163), leaf extracts prepared as described above were mixed in varying proportions with antiserum, held for 30 minutes at 4° C., and in some experiments held overnight at 4° C. These preliminary experiments were also unsuccessful at collecting the protein of the different Nicotiana genotypes by precipitation. A published procedure for collection of spinach Fraction I protein by selective precipitation of leaf extracts with PEG and Mg^{+2} ion (164) did not work for these tobacco extracts. Salt fractionation experiments showed that collecting the protein precipitate from the leaf extract between 35% and 50% of saturation with ammonium sulfate, $(NH_4)_2SO_4$ (AS) gave appreciable purification of Fraction I protein, as determined by band intensification in SDS gel electrophoresis of the redissolved isolate.

Leaf proteins collected between 35% and 50% of saturation in AS could be resuspended in extraction buffer to a maximum protein concentration of 10 mg/ml. The apparent relative intensity of the pI bands of interest varied with the preparation. Addition of Mg^{+2} and HCO_3^- ions, the enzyme's cofactors, to retard endogenous proteolytic attack (165) resulted in protein insolubility. Storage of protein preparations in the freezer always caused progressive insolubility. The carboxymethylation procedure of Kung (166) did not yield reliable results in preliminary experiments; problems were encountered with the generation of extraneous bands and with dilution in the column treatment after carboxymethylation.

Procedures for dissociating electrofocusing gels were adapted from published sources (166) and recommendations by coworkers (167). Ampholyte source and concentration, buffers and running conditions were varied in attempts to optimize band separation and focusing in the RuBPCase LS region. Methyl red, spinach RuBPCase (Sigma) and commercially available acetylated cytochromes (U.S. Biochemicals) were used as pI markers in some gels.

In the final version of this assay, protein preparations were isolated in the cold as follows. Five grams of deveined leaf tissue were scissored, frozen and pulverized in liquid nitrogen, thawed to 4° C., and ground to paste in a mortar and pestle with 0.5 g PVPP and 50 mg DTT in 10 ml of buffer containing 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris HCl, pH 8.0. This paste was squeezed through four layers of cheesecloth and centrifuged at 17 kG for 20 minutes. The supernatant was made up to 35% AS by the addition of saturated ammonium sulfate, pH 8.0, and allowed to stand 30 minutes at 4° C. After centrifugation at 12 kG for 15 minutes, the supernatant was made up to 50% AS; this precipitate was again collected by centrifugation and was redissolved in 0.2 ml of the extraction buffer. This crude protein isolate was dialyzed against the extraction buffer for one hour to reduce AS concentration, and applied to the gel. With this procedure, four different protein samples could be prepared in 8 hours.

Dissociating electrofocusing polyacrylamide gels were cast of 6.5% acrylamide (36 acrylamide: 1 bis by weight) with 8 M urea and 2% v/v Triton X-100 (reagents from BioRad). Ampholytes totaled 5% by volume, 3:1:1 (pH 5-8:4-6.5:3-10) (Sigma or BioRad) and 1.5 mg/ml each arginine, asparagine and lysine HCl (all from Sigma). Gels were polymerized with Temed and $(NH_4)_2SO_2$ (BioRad).

Gels were prerun at 200-300 V for an hour between the upper (anode) buffer of 0.4% v/v triethanolamine and the lower (cathode) buffer of 0.2% H_2SO_4. After protein loading, gels were run overnight with cooling at voltages between 200-700 V and with current decreasing from 15 to 5 mA per gel. Gel field exposure totalled 4000-6000 volt-hours.

Gels were washed in 20% v/v methanol, 5% v/v glacial acetic acid to fix and to prevent stain precipitation. They were stained for two hours in the same solution containing 0.1% w/v Coomassie brilliant blue R and 0.5% w/v $CuSO_4.5H_2O$, and destained in the same wash solution. Gels were photographed with transillumination to heighten contrast.

Another plastid-determined character analyzed was the tentoxin reaction of seedlings from the somatic hybrids (section I.4.3). Following published techniques (92,103), 100-200 seeds of each genotype were placed in petri dishes on filter paper moistened with 20 ug/ml tentoxin (Sigma), or with water as a control, and germinated in the light. Remnant seed from progeny tests of the fusion plants was tested alongside seed from green plants with the parental cytoplasmic genotypes as a control. The dishes were rewatered as necessary with the appropriate solution. After germination, seedlings were scored for yellow, toxin-sensitive, or green, toxin-resistant cotyledons. Toxin reaction was unambiguous under these conditions.

The final chapter of this thesis details the recovery of somatic hybrids from protoplast fusion experiments using the design of Figure 1, the failure of the selection for ctDNA recombinants planned in Figure 2, and the analysis of the hybrid plants obtained for nuclear, plastid and mitochondrial genomes.

RESULTS

III.1. Recovery of complementing fusions

Mixtures of nuclear and cytoplasmic albino protoplasts were subjected to bulk fusion and regeneration treatments. Cells of the *N. megalosiphon* cytoplasmic substitution homozygous for the nuclear albino markers ws or Su, or the *N. bigelovii* cytoplasmic substitution homozygous for ws, were fused in varying combinations with cytoplasmic albino *N. tabacum* genotypes Seltman's V, wvc, or SamV. A total of thirteen different fusion experiments were prepared and plated. Each fusion experiment produced four to six plates of medium D3, each with a one-milliliter aliquot of fusion-treated cells. Each D3 plate was numbered on the day of plating. All subcultures and plants descended from that original plate were identified by that same number. These cultures were passaged on medium 0.3/10 in the light. Green plants were selected among regenerating plantlets.

Complementation between parental nuclear and cytoplasmic albino genomes was expected to yield green fusion-derived plants (see Figure 1). The first green plant recovered was plated during the study comparing culture regeneration media. This first plant was potted in soil and transferred to the greenhouse six months after the date of that protoplast fusion experiment. A subsequent series of fusion experiments using the newly developed regeneration protocol produced a series of green plantlets which were found from four months to a year after the protoplast fusion plating date. If green plantlets were not recovered after five months in culture, green callus sectors were visually selected and propagated on regeneration medium. This callus selection proceure also yielded green regenerants. All green plantlets were grown for several passages as shoot cultures on medium R5, permitting confirmation of their phenotype during shoot multiplication and rooting. Green plants grown to seven to fifteen internodes were transplanted to soil and grown up to sexual maturity in the greenhouse. These putative somatic hybrids have been maintained and propagated by shoot cuttings to the present day. The eight different numbered groups of green plants recovered from these fusion experiments are described in section III.3.

Thirteen protoplast fusion experiments were plated; seven perished due to culture contamination or inviability. Six experiments produced growing cultures on four to five plates of D3 per experiment. Monthly subculture of these numbered plates on regeneration medium for up to a year led to as many as one hundred plates of tissue bearing any one identification number at a given time. During regeneration and selection of the series of fusion experiments, as many as a thousand petri dishes of fusion-treated materials required monthly subculture, making this a labor-intensive stage of the experiment.

Green plants and callus were visually selected at every stage of regeneration and shoot culture from the fusion experiments. A high proportion of false positive selections either lost their green phenotype or failed to survive culture. The visual selection system employed had limited effectiveness. The high volume of cultures propagated during regeneration was necessary due to inadequate discrimination between mutant and wild-type phenotypes during regeneration. Shoot cultures of selected plantlets could be scored more successfully than could regenerating buds. Expanded leaf color and growth rate of plantlets subcultured on R5 provided the final tissue culture selection.

Markers varied in their interference with the visual selection. The cytoplasmic albinos all had dark green regenerating shoot apices. This permitted no discrimination of fusion plants from one of the parents during the early regeneration stage. Experiments using the Su marker showed an overall yellow-green color of callus and regenerants. Callus selection proved helpful in fusion experiments using the Su marker. The difficulties experienced with the visual selection system apparently are due to the requirement for leaf cell differentiation for phenotypic expression of the hybrid genotype. This led to a delay in the exertion of selection pressure, resulting in excessive populations. A positive biochemical selection for a tissue culture phenotype could perform better in this situation.

Each successful fusion experiment produced green plants from one or two numbered cultures. Up to a dozen green plants were recovered within any one successful numbered culture. These plants derived from the same initial protoplast fusion culture carried the same identification number; they might represent either independent fusion events, or the descendants of only one fused cell. The final and most rigorous selection step was selection for photoautotrophy during plant transfer to the greenhouse. The time required from the date of plating of the protoplast fusion preparations to recovery of sexual offspring from the mature green plants was about 12 to 18 months.

III.2. Failure to recover selected recombinant fusions

In other fusion experiments, protoplasts of the cytoplasmic albino *N. tabacum* genotypes wvc or SamV were mixed and PEG-treated with protoplasts of the cytoplasmic albino, *N. megalosiphon* cytoplasm-substituted genotype MSB21V. These cell fusion preparations were then subjected to regeneration treatment under lights. It was predicted that green plants visually selected in these cultures would represent the products of genetic recombination between the two different plastid genotypes in somatic hybrid cells (see Figure 2).

Seven fusion experiments combined the two types of cytoplasmic albino cells. Some of these experiments were performed while techniques were being developed; some were made during the series of fusion experiments already mentioned, and some were prepared later. As found in the other fusion experiments, over half of the cultures perished due to inviability or contamination. Plates from the three experiments which survived were repeatedly subcultured on regeneration medium in the light. A prolonged attempt to select green plants from these cultures yielded none. The cytoplasmic albino genotypes employed were difficult to discriminate from the wild-type during the regeneration treatment; this added to the difficulty of selection. Green callus selection was attempted in some of these experiments. Selected plantlets always reverted to albino phenotype on subculture in medium R5.

The final fusion experiment combining different cytoplasmic albino cells was followed quantitatively to measure these negative results. Four months after protoplast fusion and plating on D3, 15,000 plantlets regenerated from 0.3/10 were scored as parental cytoplasmic albino phenotype and discarded. A small number of these plantlets had also been grown on R5. At the fifth month of subculture an additional 10,000 plantlets were scored negative. These figures were estimated by counting the number of plates and counting representative plates. This particular experiment was terminated after 5 months; earlier experiments had been carried under selective conditions for as long as 8 months.

Although these numbers are from only one experiment, they may be taken as roughly representative of the performance of any one growing protoplast fusion culture under these growth conditions. Results of these selections suggest that the frequency of ctDNA recombination in somatic hybrids, if it does occur at all, is too low to detect among the 75,000 plantlets regenerated from all three viable fusion preparations. An examination of the experiments reported in section III.1 shows that six viable fusion preparations gave recovery of eight distinct putative somatic hybrids. Taking the number of plantlets scored in the experiment described above as representative of all such fusion experiments, this would be equivalent to eight somatic hybrids recovered per 150,000 plantlets examined. The number of plantlets scored in these attempts to select ctDNA recombinants is of roughly the same order of magnitude. By analogy with these complementation experiments, five or six somatic hybrids should have been present in the cultures derived from fusion of two different cytoplasmic albinos. CtDNA recombination cannot be frequent in the somatic hybrid cells (as has been observed before, see section I.5.3). An improved selection scheme for recovery of a product of ctDNA recombination in somatic hybrids, without the examination of a greater volume of material, will require either early selection for the somatic hybrids, or the use of some as yet unknown causative agent to increase the frequency of the recombination event.

III.3. Analysis of hybrid plants

III.3.1. Introduction

Table 4 introduces the hybrid plants recovered by visual selection. These plants appear to represent a variety of possible outcomes of the somatic hybridization process. Green plants resulted from several fusion experiments using the *N. megalosiphon* cytoplasm ws/ws nuclear albino parent. A variegated plant was recovered using the *N. bigelovii* cytoplasm, but not with the *N. megalosiphon* cytoplasm parent. The fusions utilizing the Su marker gave two very different results. One plant seems to have completely lost one parental nucleus and the other cytoplasm; the other plant shows obvious sectoring for the hybrid nuclear marker and gave some evidence for cytoplasmic sectoring. Contrasting results were also obtained for inheritance of the cms character in this series of experiments; both fertile and sterile anatomies were found. The numbered cultures yielding more than one green plant gave an opportunity to examine these individuals for clonal

Table 4. Somatic hybrids recovered by visual selection*

Hybrid No.	Expt. No.	Plate No.	Number of Plants	Parents	Phenotype**
1	2	83	5	(M)ws/ws + Selt	white leaf sectors
2	"	86	3	" " "	like #1
3	4	113	2	(B)ws/ws + wvc	mf anatomy but self-sterile V plant recovered
4	5	130	2	(M)Su/Su + wvc	mf anatomy and self-fertile
5	"	133	7	" " "	Sulfur leaf sectors V plant lost
6	9	179	1	(M)ws/ws + wvc	like #1
7	"	182	1	" " "	like #1
8	13	349	1	(M)ws/ws + wvc	like #1 but no sectors

* Abbreviations: (M), *N. megalosiphon* cytoplasm; (B), *N. bigelovii* cytoplasm; Selt, Seltman's V; mf, male-fertile; V, variegated.

** Male-sterile, cms phenotype unless otherwise mentioned.

Figure 7. Somatic hybrid #1 and its parents. A shoot culture of plant #1 is shown at top; the protoplast fusion parents were *N*. *megalosiphon* cytoplasm-substituted ws/ws, lower left, and plastid albino Seltman's V, lower right.

Figure 8. Somatic hyrid #3 and parental genotypes. Shoot culture of #3 is shown at top; *N*. *bigelovii* cytoplasm-substituted ws/ws Hicks line at lower left, and wvc plastid albino at lower right, were protoplast fusion parents.

Figure 7.

Figure 8.

Figure 9. Somatic hybrid #4 and parental genotypes. Two shoot cultures of plant #4 are shown at top; it was derived from fusion of protoplasts of *N. megalosiphon* cytoplasm, Su/Su plants, lower left, and wvc plastid albino, lower right.

Figure 10. Somatic hybrid #5 and its parents. Hybrid #5, selected from the upper left plate, and its *N. megalosiphon* cytoplasm, Su/Su (lower left) and wvc (lower right) parents. Another regeneration plate is shown at the upper right.

Figure 9.

Figure 10.

Figure 11. Selection of somatic hybrid #6. The culture regeneration plate at upper left yielded somatic hybrid #6; upper right plate has no hybrids. The parental *N. megalosiphon* cytoplasm, ws/ws parent is at the lower right; wvc is at the lower left.

Figure 12. Hybrid #1 potted in soil. The plant is autotrophic; small sectors are visible.

Figure 11.

Figure 12.

Figure 13. Somatic sectoring for the nuclear marker in a plant of somatic hybrid #5. Single events caused the appearance of +/+ and Su/Su sectors on Su/Su/+/+ plants from the somatic hybrid plant family #5.

origin. These developments and further analysis are discussed below.

III.3.2. Macroscopic leaf characters

Hybrid plants were selected based on leaf color. The final in vitro selection stage is illustrated in Figures 7 through 11 for five plants. These photographs show dark green (or medium green, in the case of Su/+) plantlets and regenerating callus contrasting with the background of parental phenotype tissues in plants. The first plant recovered to the greenhouse is shown in Figure 12 as potted in soil. This plant #1 showed small white leaf sectors. These sectors were excised, sterilized and explanted onto regeneration medium in an attempt to recover the sector genotype as plantlets for further study. In almost all cases, green plantlets grew out of other layers of the explanted leaf lamina. It was not possible to rescue these sectors in this way. Cell lineage mapping was attempted by carefully recording the position and size of white leaf sectors on plants of the #1 family for a total of over 200 leaf nodes scored. These records show no relation of the rather random leaf sector pattern to phyllotaxy, to floral anatomy or to meiotic test results. The white sectors on these plants were always relatively small and never assumed dominance in a meristem. Similar sectoring behavior was observed on other plants (#2,6,7) derived from the fusion of the same parental genotypes, and at a lower frequency in their sexual progeny. Sectors of different leaf textures, some with restricted growth, were also observed. Leaf sectoring was especially prominent on the plants of group #5, regenerated from a fusion experiment using the semidominant nuclear marker Su. Figure 13 illustrates a larger than usual display of Sulfur phenotype sectoring on a plant from group #5. Whole plant wild-type pigment phenotypes were also recovered in this group.

In the analysis of somatic sectoring in nuclear and cytoplasmic heterozygotes, one must consider the different consequences of nuclear and organellar segregation events on the mitotic pedigree displayed in the plant shoot. The nuclear marker is theoretically present in two doses in the tetraploid fusion plant genome (see Figure 1). Segregation for the dominant or semidominant markers could occur following mitotic recombination between homologous or homeologous chromosomes, or following a nondisjunction event in mitosis. More phenotypes can be distinquished with the semidominant marker, but in any case these events lead to contiguous single-phenotype sectors of unique origin. Twin spots of contrasting phenotype may be visible in the case of the semidominant marker, but both arise from a single genetic event. Such events can recur elsewhere in the plant, but each occurrence yields a single-event macroscopic clone (see Figure 13). This pattern created by nuclear segregation contrasts visibly with the pattern generated by the many segregating copies of organelle genomes. Organelle segregation leads to a multiple-event pattern in which pure cell lineages appear to arise <u>de novo</u> in many closely related cell lineages. When revealed by a color phenotype, this multiple-event pattern is accurately termed variegation.

Two different plantlets showing this variegated phenotype were recovered during <u>in vitro</u> selection of somatic hybrids; one was found in cultures of hybrid #3, and the other in cultures of hybrid #5. Their appearance as variegated selections is illustrated in Figure 14 which shows the plantlet from culture #5. These two plantlets were propagated as shoot cuttings. Early somatic segregation yielded mostly albino tissue in the case of #5. Axillary buds subtending variegated leaves of the shoot cultures were explanted onto shoot culture medium to propagate the variegated phenotype. This process is demonstrated with the plantlet from hybrid #3 in Figure 15. Of these two variegated somatic hybrid selections, only one survived subculture and potting into soil. The variegated plant from family #5 was lost to contamination and to segregation before the potting stage. Figure 16 shows the recovered variegated plant from hybrid #3 as potted in the greenhouse; the multiple-event pattern is evident.

After a year of growth in soil, several additional variegated shoots and one albino shoot have developed from this plant but neither of these slower-growing phenotypes

Figure 14. Variegated plantlet from cultures of somatic hybrid #5. This plantlet was later lost.

Figure 15. Propagation of variegated plantlet from cultures of somatic hybrid #3. The axillary bud grew into a variegated plant.

Figure 14.

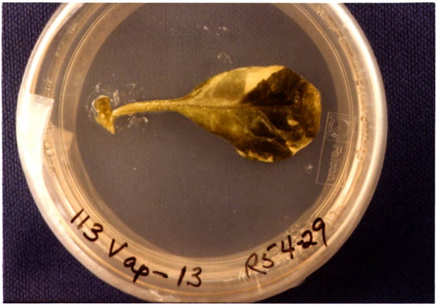


Figure 15.

Figure 16. Variegated plant of somatic hybrid #3 potted in soil. The multiple-event plastid variegation pattern is evident; 113 was the original protoplast plate number.

Figure 17. Somatic hybrid #1 displaying the cms floral phenotype. The stigmatoid anther development is typical of the *N. megalosiphon* Hicks cms line.

Figure 16.

Figure 17.

has yet flowered. All floral and meiotic data are from the first, green plant grown from this culture. The variegated and albino shoots arise from growth which mitotic records show coming from the crown of the original selected green plantlet. This observation makes sense considering that the first few leaf nodes developed from the regenerating meristem should contain residual mixed cytoplasm. Future efforts involving hybrid plant cytoplasms might profitably concentrate on this region of the regenerated plant anatomy. It was also possible to propagate the variegated shoot culture of #3 by explants of the finely variegated leaves onto regeneration medium, but this technique yielded few variegated plantlets.

III.3.3. Floral characters

The onset of flowering in these plants permitted meiotic analysis for the nuclear albino marker and observation of male and female fertility. Design of the crosses testing nuclear marker transmission was affected by the hybrids' floral anatomies and by their anticipated nuclear genotypes.

The floral phenotypes of the parents and fusion plants are as follows. Plants with the *N. megalosiphon* cytoplasmic substitution have accessory stigma and style development below the ovary, in a position in the floral whorl which indicates they replace the anthers. Plants with the *N.*

bigelovii cytoplasmic substitution have petaloid floret development from the same position. Environmental effects cause an additional phenotype, variable petal dissection, in the *N. bigelovii* cytoplasmic substitution line. Normal fertile *N. tabacum* are borne freestanding on slim filaments attached between the gynoecium and petal base. Table 5 presents the results of observations on the floral phenotypes of the somatic hybrids recovered. All fusion plant phenotypes fall clearly into the parental male-fertile or male-sterile anatomical classes. Both male-sterile and male-fertile phenotypes were recovered from fusion experiments using the *N. megalosiphon* cytoplasmic parent with *N. tabacum*. The only plant arising from a fusion combining the *N. bigelovii* and *N. tabacum* cytoplasms, #3, is male-fertile as with the *N. tabacum* cytoplasmic phenotype.

Plants exhibiting male-sterile floral development often showed further development of the accessory female structures into partially complete ovaries attached at the base of the primary gynoecium. Variably extreme pistil hypertrophy and splitting impeded pollination of the male-sterile fusion plants. One plant, #6, abscised most flowers prematurely; seedling data could not be collected from this plant. Seed set on some fusion plants had poor germination (see section III.3.4).

Although anatomically male-fertile, plant #3 did not set seed when self-pollinated. It never showed the split corolla typical of its *N. bigelovii* cytoplasmic parent.

Table 5. Cytoplasmic parentage, floral phenotype, and suggested mitochondrial genotype of the somatic hybrid plants

Parent cytoplasm and floral phenotypes	Fusion plant #	Fusion plant phenotype	Mitochondrial assignment
N. megalosiphon (stigmatoid anthers) + *N. tabacum* (normal anthers)	1,2,5,6,7,8	Stigmatoid anthers	*N. megalosiphon*
N. megalosiphon (stigmatoid anthers) + *N. tabacum* (normal anthers)	4	Normal anthers	*N. tabacum*
N. bigelovii (petaloid anthers) + *N. tabacum* (normal anthers)	3	Normal anthers	*N. tabacum*

Plant #4 exhibited both normal anther development and complete self-fertility. Acetocarmine staining showed that the pollen produced on plant #3 averaged less than 25% viable compared to tobacco controls, while plant #4 had a relative pollen viability of 91%. Self-fertilized seed was used for seedling assays on plant #4. All other fusion plants displayed the stigmatoid anther cms phenotype of their N. megalosiphon cytoplasmic parent. Figure 17 shows the cms floral phenotype of a plant from somatic hybrid family #1.

It is noteworthy that no variation in floral phenotype was found in any plant or group of plants regenerated from the same original protoplast fusion plate. For example, the seven members of fusion plant group #5 had strongly similar stigmatoid anther development. The correspondence of the fusion plant floral anatomies with the parental cytoplasmic phenotypes, and the evidence correlating mtDNA with the cms character (see section I.3.3), prompt the conclusion that the respective parental mitochondria are represented unaltered in fusion plants of similar phenotype. However, this analysis assays only one putative mitochondrial trait. Unfortunately, this is the only known whole-plant mitochondrial character. Parental mitochondrial constitutions in the fusion plants are assigned based on floral phenotype in Table 5, with the caveat that analysis of only one character cannot rule out genetic interaction between parental mitochondria in these somatic hybrids.

III.3.4. Nuclear genes

The fate of the parental nuclei in the somatic hybrids was investigated by progeny tests and ploidy determinations on the fusion plants. Meiotic transmission of the parental wild-type and mutant nuclear albino alleles was investigated by pollinating the fusion plants with pollen from a diploid tobacco plant heterozygous for the appropriate nuclear albino allele. Seedlings germinated from at least four successful crosses were scored for the albino character. These crosses can be written: fusion plants #1,2,3,6,7,8, (females) x (male) ws/+; and, plant #5 (females) x (male) Su/+. The expected nuclear genetic constitution of the fusion plants was a tetraploid heterozygote, receiving two mutant alleles from the nuclear albino parent and two wild-type alleles from the cytoplasmic parent (see Figure 1). True male fertility was not anticipated in the fusion plants due to expected high ploidy and to the use of the cms marker. Plant #4, which was male-fertile and self-fertile, produced abundant viable selfed seed which was used in this progeny test.

The expected genetic ratios resulting from this cross can be calculated. Table 6 presents the expected percentage of the lethal homozygous albino class, calculated assuming maximal or minimal exchange at synapsis with euploid disjunction in meiosis. Progeny expectations for hexaploid and octoploid female plants are included in Table 6 because of the possibility of regenerating plants from multiple cell fusions or from endopolyploidized cell colonies. The effect of possible aneuploidy in the fusion plants on these predicted progeny percentages in unknown. Any chromosome structural rearrangements in the fusion plants should decrease the recovery of the lethal albino class; high ploidy, aneuploidy, and rearrangements all should reduce female and male fertility. Although the ws marker is used as an example in Table 6, the calculated theoretical expectations are valid for Su or any other selectable marker.

The results of the progeny tests are shown in Table 7. The analysis of these data focuses on the frequency of the homozygous lethal class of progeny. The top half of Table 7 lists the observed frequency of the albino lethal class of progeny seedlings from test crosses made on fusions using the recessive nuclear marker ws. The observed frequency of this progeny class varied from 0% to 10% for different somatic hybrids. Plants #1,2,3 and #7 clearly show meiotic transmission from the fusion plants of nuclear albino and wild-type alleles originating from both somatic hybrid parental nuclei. Plant #6 was too infertile for any conclusions about its nuclear genotype to be drawn from the progeny tests, other than the judgment of aneuploidy. Progeny test results for plant #8 suggest that this somatic hybrid has lost the parental nuclear albino alleles. Based on overlap between the predictions of Table 6 and the

Table 6. Progeny test expectations*

	Percent albino lethal progeny: Range**		
Female genotypes	Minimum crossing over	---	Maximum crossing over
4N: + + ws ws	8.3%	-----------------	10.7%
6N: + + + + ws ws	0.0%	-----------------	2.7%
6N: + + ws ws ws ws	20.0%	-----------------	25.5%
8N: + + + + ws ws ws ws	1.4%	-----------------	3.8%

* Pollen parent, ws/+; euploid progeny outcomes only. The ws locus is used as an example only.

** The actual percentage of albino lethal progeny falls between the two values shown, depending on map position of the indicator locus.

Table 7. Results of progeny tests for nuclear markers in somatic hybrids

Nuclear marker	Pollen parent	Fusion plant	Seedlings scored:*				% Lethal	2SE	Female fertility**
			G	W					
ws	ws/+	1	1631	181			10.0%	±1.4	8%
"	"	2	2105	161			7.6	1.1	15
"	"	3	2077	38			1.8	0.6	42
"	"	6	42	0			-0-		1
"	"	7	3868	106			2.7	0.5	99
"	"	8	4050	1			0.0	.02	81
			DG	LG	YG	Y	% Y	2SE	
Su	Su/+	5-1	94	154	122	71	16.1	±4.6	
"	"	5-2	40	73	68	26	12.6	2.3	
"	"	5-3	7	24	22	3	5.4	3.0	
"	"	5-4	45	151	93	14	4.6	2.4	
"	"	5-5	65	130	101	45	13.2	3.7	
"	"	5-6	108	160	105	64	14.6	3.4	
"	"	5-7	75	158	100	33	9.0	3.0	
		5	434	850	611	246	11.5%		6%
Su	self	4	4361	0	0	0	0.0%		87%

* Phenotype abbreviations: G, green; W, white; DG, dark green; LG, light green; YG, yellow-green viable; Y, lethal yellow.

** Female fertility is calculated as average number of seedlings germinated per capsule threshed and planted, divided by 1000 as an estimate of diploid selfed seedling yield, and converted to a percentage.

observed values of %W ± 2SE in Table 7, nuclear genotypes of the somatic hybrids can be assigned. By this criterion, hybrid plants from families #1 and #2 follow the expectation for tetraploids, and hybrids #3 and #7 could be either 6N or 8N. Plant #8 cannot have any of the hybrid nuclear genotypes listed in Table 6; its high fertility suggests a diploid +/+ nuclear genotype.

The lower half or Table 7 lists progeny test results for crosses on fusion plants made using the Su nuclear marker. Plant #5 represents an example of multiple recoveries of a single somatic hybrid. Seven photoautotrophic plants were regenerated from a single protoplast fusion plate; they were analyzed separately in this progeny test as part of the assessment of the difference or similarity between these plants. Because this marker is semidominant, intermediate classes of progeny were counted.

The range of percentages of recovered homozygous lethal progeny seedlings varied from 4.6% to 16.4% in plants of group #5. Plants #5-1 and #5-2, the first two listed in the table, had a wild-type, dark green leaf phenotype but showed a higher average percentage of homozygous progeny than did the other five plants which had heterozygous phenotype, yellow-green leaves. There is overlap between the observed %Y ± 2SE from the plants in family #5, but they are significantly different by the Chi square test (df=6, $P<.005$). Plants #5-2 and #5-7 follow the expectations for a tetraploid segregation, while the %Y ± 2SE values for the other plants in this family fall variously into the 4N, 6N, or 8N expectation ranges, or between them.

Intermediate phenotypic progeny classes were examined in the case of this semidominant nuclear marker. A tetraploid female parent would be expected to produce four classes of offspring in a ratio varying from 1:5:5:1 to 3:11:11:3, with minimal or maximal crossing-over, respectively, in meiosis. Gradations of leaf color were observed in the seedling populations, but environmental variation made scoring of the seedling phenotypes difficult. The intermediate class seedling results in Table 7 have a high degree of uncertainty.

The progeny test results for somatic hybrid family #5 reported in Table 7 lack the symmetry of these predicted ratios; mutant progeny classes are underrepresented. The proportion of homozygotes can be tested to compensate for this asymmetry. The observed value of 31.8% exceeds the range predicted for the tetraploid, but is closest to the expectation calculated with maximal crossing over. Plants in family #5 with ploidies greater than 4N explain the failure of the tetraploid model to predict the number of progeny classes and their ratios.

All seven plants within somatic hybrid family #5 produced some albino offspring in this test, indicating meiotic transmission of both parental marker alleles. All displayed a consistent cms phenotype. All showed leaf sectoring and a reduced female fertility characteristic of aneuploids. Although these plants gave different results in this progeny test, they could represent different aneuploid outcomes of a single nuclear fusion followed by chromosome rearrangement, loss and endopolyploidization.

Plant #4 is unique in the fusion plant analysis. It was regenerated from a different original plate, but from the same experimental protoplast preparation, as plant group #5. The parental genotypes for this fusion were these: *N. megalosiphon* cytoplasm-substituted nuclear albino homozygote Su/Su, and *N. tabacum* nuclear wild-type +/+ cytoplasmic albino. Plant #4 differs strikingly from all the plants in group #5. Although constructed using the semidominant marker Su, somatic hybrid plant #4 showed wild-type, dark green leaves without any somatic sectoring. Its unexpected male fertility and self-fertility led to the use of selfed seed for progeny tests. Systemic fungal contamination in the seed capsules and germinating seed populations lowered effective seedling yield from plant #4, but enough data were collected to give a good female fertility index and a significant rating of 0.0% homozygous lethal progeny for this plant. Pollinating plant #4 with pollen from a Su/+ male parent would be more directly comparable to the other crosses discussed here; still, the lack of any phenotypic expression of the semidominant nuclear marker in the regenerated plant or its selfed offspring is sufficient evidence to conclude that the parental Su allele has been completely lost from this plant's nuclear genotype. Further, the conclusion that this plant must be a euploid, diploid individual can be drawn from its male and female fertility.

A rough assessment of the somatic hybrids' nuclear euploidy can be made based on a ranking of the plant fertility ratings of Table 7. Plants with 80% or better relative female fertility include #4, #7 and #8. As argued above, the complete loss of the Su marker and good male and female fertility of plant #4 indicate a diploid, +/+ genotypic assignment for that plant. Though male-sterile, plant #8 also did not transmit the nuclear albino marker to its progeny. Its single albino seedling among four thousand might have resulted from a de novo dominant mutation, or from a paternal haploid. Plant #8 thus could also be a +/+ diploid. Plant #7, however, showed appreciable meiotic transmission of the nuclear albino marker. With a homozygous seedling progeny percentage of 2.7%, it could be hexaploid or octoploid with respect to the ws locus. Its female fertility indicates euploidy, but other tests would be necessary to resolve its nuclear genotype.

Only one plant had intermediate female fertility in this test. The 42% seedling yield of plant #3 is the sole value in the 20%-70% range of female fertility in these results. The low but significant proportion of albino seedlings from plant #3 supports a prediction that it is probably hexaploid or octoploid with respect to the ws locus.

Low female fertilities in the range 6%-15% characterize plants #1, #2 and #5. These three plant families all showed a 2%-15% range of homozygous albino progeny. Plant #6 gave such poor seedling yield that it could not be scored in any seedling tests. The degree of female infertility in the fusion plants may be taken as an indication of their relative aneuploidy. Thus, plant #6 is severely aneuploid, contrasting with plants #4, #7 and #8, which are euploid by this criterion.

The diploid chromosome number for tobacco is 2N=48. These somatic hybrids were expected to combine the nuclei from two diploid parents. Chromosome number changes following fusion could arise by incomplete addition of the parental nuclei, or during the tissue culture phase of the experiment. Thirty root tip mitotic chomosome counts from cells of somatic hybrid #1 showed an average chromosome number of 2N=83, with a range from 69 to 97. Plant #1 is therefore subtetraploid by this assay. It was difficult to obtain an unobstructed spread of the many small tobacco chromosomes in these somatic hybrids and chromosomes were often overlayered. Several counts each were made on a minimum of five cell division figures, however all chromosome counts reported here probably err on the low side. Meiotic pollen mother cell squashes of plant #3 showed that at meiosis II, N averaged 62, with counts ranging from 37 to 82. This average figure suggests that plant #3 may have a ploidy between 5N=120 and 6N=144. Recall that segregation of the albino marker in seedlings from this plant (Table 7) indicated a 6N or 8N segregation; 5N was not calculated. It is possible, too, for chromosome loss to occur during aneuploid plant growth. Meiosis II counts from plant #4 showed an average of N=25 chromosomes. Meiosis I counts of plant #4 ranged lower, averaging 40 for the diploid value. This also serves as a reminder that meiotic disjunction can act to regularize the apparent ploidy of an aneuploid somatic hybrid's progeny.

When stomatal size was examined, haploid plants averaged 25 by 20 microns in stomatal length and breath; diploid control plants had measurements of 35-40 by 25-30 microns. Stomata from plant #7 averaged 50 by 40 microns, significantly larger than the value obtained for the diploid controls. Stomata from plant #3 averaged 57 by 42 microns, suggesting an even higher ploidy for this plant. Stomata from plants #4 and #8 averaged roughly the same measurements as the diploid control, supporting their assignment as 2N, +/+ nuclear genotypes.

It appears from this analysis that two nuclear genotypic results of the cell fusion procedure can be recovered with this system of markers: additive nuclear outcomes of various ploidies and proportions, as in plants #1,2,3,5,6 and 7, and wild type nuclei alone, as in plants #4 and #8. Results discussed in this section illustrate the different classes of nuclear genotypic results in the somatic hybrids. Plants #1 and #2 represent additive nuclear fusion to give roughly tetraploid nuclei with genotype ws/ws/+/+. Plants #3,5 and 7 represent other outcomes of nuclear addition with increased ploidy and variable marker dosage. Though plant #6 was severely infertile, it is included in this group on the basis of leaf sectoring for the nuclear marker. Only plant #7 of this group had good female fertility. A conclusion that nuclear genomes partially or completely combined in the somatic hybrid is supported by findings of somatic sectoring, maternal transmission of the nuclear albino marker, and raised ploidy in plants #1,2,3,5,6 and 7. In contrast, plants #4 and #8 appear to retain only one parental nucleus; both are approximately diploid, fully female-fertile, and did not transmit the nuclear marker in meiotic progeny tests. Neither showed sectoring for the marker allele.

Thus it appears that the consequence of somatic hybridization for the nuclear genome are variable. These results suggest that the initial event of nuclear fusion may or may not occur, and that it can be followed by subsequent changes in the hybrid nucleus. This analysis of the nuclear outcome in somatic hybrids could be improved with a different ploidy design or by the inclusion of a greater number of markers and individuals.

III.3.5. Plastid characters

Following scoring of leaf and floral characters and progeny tests, the somatic hybrids were subjected to electrophoretic analysis and seedling assays for two plastid-encoded chloroplast proteins, with the objective of determining the hybrids' plastid genotypes.

Leaf protein preparations enriched for RuBPCase were isolated from the fusion plants and subjected to electrofocusing on dissociating gels. This procedure is described in detail in section II.4. These experiments showed that crystallization of the enzyme by lowering salt concentration did not work for the cytoplasm-substituted genotypes; poor results were also obtained in preliminary attempts at specific immune precipitation of the protein. A crude differential ammonium sulfate precipitate gave better results but problems were encountered with loss, modification and progressive insolubilization of the electrofocusable LS polypeptides. Gel conditions were progressively modified to optimize the display of the LS bands of interest.

During this stage of hybrid analysis, 8 isolation techniques were used in a total of 24 protein isolation experiments. The protein preparations were analyzed in 28 gel runs yielding over 40 stained polyacrylamide gels. Isoelectric point standards (methyl red, spinach RuBPCase, and acetylated cytochromes) proved that the pI bands of interest, the RuBPCase LS triplet, lie in the pI region 5 - 6. Control experiments verified that the LS pattern displayed by the Seltman's V or wvc cytoplasmic albino genotypes in variegated or chimeral leaves is the same as in other, green N. tabacum varieties. Other controls confirmed the phenotypic differences between the LS triplet patterns of the N. megalosiphon and N. bigelovii cytoplasmic substitution lines and the N. tabacum cytoplasmic genotypes.

A typical good electrophoretic gel displayed the parental RuBPCase LS polypeptides as a triplet in the pI region 5 - 6. The N. megalosiphon triplet is displaced toward a more basic pI, and the N. bigelovii triplet is displaced toward a more acidic pI. All genotypes have N. tabacum nuclear genotypes, encoding the N. tabacum SS, a doublet in the acidic pI region 3.5 - 4.0 (see Figure 19).

With this procedure, the pI region 3 - 6 is relatively uncontaminated by other stainable entities. Other leaf proteins in the crude isolate, and their degradation and crosslinking products, stain in other regions of the gels. Protein samples of the parental cytoplasmic genotypes were isolated and run concurrently as standards with each experimental comparison of fusion plant genotypes. The relative concentration of the pI bands of interest in different isolates, and the appearance of shadow bands, smearing and background staining in the gels, varied considerably from experiment to experiment. Plant stresses from insects, insecticides, temperature extremes, plant age and aneuploidy contributed to the deterioration of extractable phenotypic gel patterns. Associated protease and phenolic oxidase activities hamper analysis of this abundant plastid genotypic marker.

All electrophoretic analyses of green leaves from fusion plants displayed parental phenotypes; no unusual results were obtained. All had the N. tabacum SS doublet. None showed the N. tabacum LS triplet, which was linked to the albino plastid trait in the parental genotype. The N. megalosiphon plastid marker was observed in LS triplet electrofocusing patterns from plants #1,4,6,7 and #8, and from all plants of family #5. White leaf sectors of plant #1 also gave the N. megalosiphon pattern. No successful displays of LS phenotype were obtained with samples from plant #2 or from variegated leaf material of plant #3. Green leaf tissue from plant #3 gave its parental N. bigelovii LS electrophoretic pattern. Figure 18 shows a typical gel. This particular gel resulted in a N. megalosiphon LS phenotypic assignment for the plastids of somatic hybrids #1 and #5 when extracts of these two plants were compared with the parental genotypes. Figure 19 shows an idealized representation of the results of all fusion plant analyses for the RuBPCase LS electrophoretic plastid marker. This figure combines the results of many different gels. The results of this analysis for RuBPCase LS in the fusion plants are included in Table 8, but related tests of

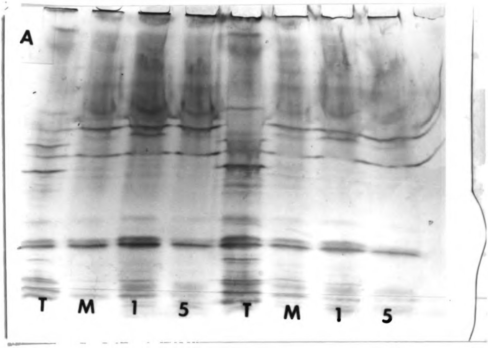
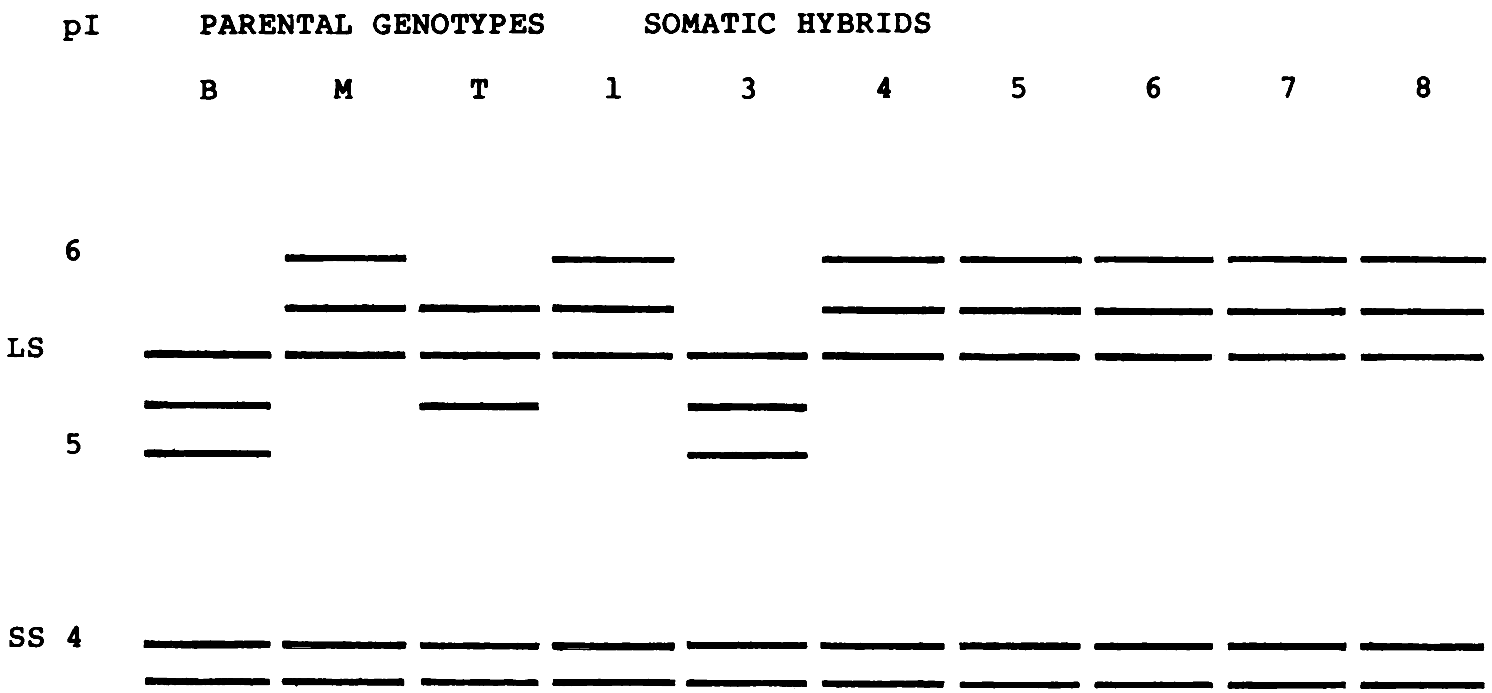


Figure 18. Gel isoelectric focusing assay for RuBPCase LS plastid genotypes of hybrids #1 and #5. This example shows the *N. tabacum* (T) and *N. megalosiphon* cytoplasm-substituted (M) parents electrophoresed next to extracts from somatic hybrids #1 and #5, which resemble the M parent. The A has no meaning here.

Figure 19. Gel electrophoretic assay for plastid genotype*



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* Abbreviations: T, *N. tabacum*; M, *N. megalosiphon* cytoplasmic substitution Hicks line; B, *N. bigelovii* cytoplasmic substitution Hicks line; LS, large subunit, and SS, small subunit of RUBPCase. All genotypes involved have a *N. tabacum* nucleus and SS. Protein samples from hybrid plants were run alongside parental samples to compare LS patterns, which are a plastid DNA marker.

another plastid marker, tentoxin resistance, should be discussed first.

Remnant seed from progeny tests of the somatic hybrid plants was assayed for tentoxin reaction and compared to parental genotypes with and without the toxin treatment. Techniques are detailed in section II.4. Resistance or sensitivity of the seedlings to tentoxin is a phenotypic marker for species-specific, ctDNA-encoded subunits of plastid Coupling Factor I; tobacco is resistant, whereas these related species' cytoplasms are sensitive to the fungal toxin. All genotypes were green when germinated without toxin, except where nuclear marker segregation gave a mixture of mutant and wild-type phenotypes. Seed of toxin-sensitive _N_. _megalosiphon_ and _N_. _bigelovii_ cytoplasmic substitution lines gave yellow seedling leaves when germinated in the toxin, while two _N_. _tabacum_ varieties gave green seedlings in the toxin treatment. Readings on some fusion plant progeny seed lots were made more difficult by their characteristic poor germination or by fungal contaminants. However, a reading could be made in all cases where seed germinated. Plant #6 gave such poor seed yield that it could not be included in this test (Table 7). Seed germination from plant #2 was sparse but sufficient for a limited toxin sensitivity assay. Nuclear marker segregation was evident in seed lots from plant #5 in the water control, and in both control and toxin treatments of seedlings from a self-pollinated Su/+ _N_. _tabacum_. All fusion plants tested gave sensitive seedling responses to the toxin, indicating the presence of _N_. _megalosiphon_ or _N_. _bigelovii_ plastids. Loss of the _N_. _tabacum_ ctDNA-encoded resistance was correlated with loss of the linked albino plastid marker of the parental cytoplasmic genotype.

Table 8 summarizes the results of analysis for three plastid characters in the somatic hybrids: color, RuBPCase LS, and tentoxin reaction. From the results abbreviated in the table, it is evident that the parental plastid genotypes were recovered intact, without any recombination between the three plastid markers tested. The albino _N_. _tabacum_ plastid appeared to be lost entirely, with the single exception of variegated segregants from plant #3; these exceptional plants have not yet been analyzed for any other genetic markers. Two plants each could not be scored for one marker. The overall picture permits confident assignment of the somatic hybrids' plastid genotypic outcomes.

III.4. Summary and conclusions

Somatic hybridization experiments combining contrasting nuclear, plastid and mitochondrial markers yielded the array of hybrid plant genotypic outcomes shown in Table 9. Selection for albino complementation in these experiments resulted in the recovery of eight somatic hybrids. From zero to two hybrids were obtained per successful protoplast fusion experiment. When more than one plant was regenerated

Table 8. Results of analysis of somatic hybrids for plastid markers*

GENOTYPE	COLOR	RuBPCase LS	TENTOXIN REACTION	PLASTID GENOME ASSIGNMENT
Parents:				
N. tabacum	albino	T	resistant	T
N. megalosiphon	green	M	sensitive	M
N. bigelovii	green	B	sensitive	B
Hybrids:				
1	green	M	sensitive	M
2	green	---	sensitive	M
3	green	B	sensitive	B
4	green	M	sensitive	M
5	green	M	sensitive	M
6	green	M	---	M
7	green	M	sensitive	M
8	green	M	sensitive	M

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* Abbreviations: T, *N. tabacum*; M, *N. megalosiphon*; B, *N. bigelovii*. The dashes indicate no data for that test. Refer to Table 4 for the parentage of the eight somatic hybrids.

Table 9. Summary of somatic hybrid analysis for nuclear, plastid and mitochondrial characters

PARENTS	NUCLEUS*	PLASTID*	MITOCHONDRIA*
T	T	T	T
M	M	M	M
B	B	B	B
SOMATIC HYBRIDS			
1	M+T	M	M
2	M+T	M	M
3	B+T	B	T
4	T	M	T
5	M+T	M	M
6	M+T	M	M
7	M+T	M	M
8	T	M	M

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* The parental cell genotypes are abbreviated: M, *N. megalosiphon* cytoplasm-substituted nuclear albino; B, *N. bigelovii* cytoplasm-substituted nuclear albino; T, plastid albino *N. tabacum*. Nuclear inheritance is based on progeny tests, fertility and ploidy data; plastid inheritance is based on three chloroplast characters; and mitochondrial inheritance is based solely on the cms character.

from the same protoplast plate, these plants all gave similar nuclear, plastid and mitochondrial results. These results suggest that under these experimental conditions, only one fusion product was recovered per plate; the plants are grouped accordingly. When different petri dishes plated within the same protoplast fusion experiment are compared (Table 4), the recovery of different genetic outcomes in plants #4 and #5 contrasts with the recovery of similar outcomes in plants #1 and #2, and in plants #6 and #7. The eight somatic hybrid genotypes displayed in Table 9 are the products of independent fusion events by virtue of their separate plating at the one-celled stage. The types recovered represent several different possible outcomes of the cell fusion experiments combining marked nuclear, plastid and mitochondrial genomes.

Somatic hybrids were selected by complementation between a nuclear albino marker in one parent and a plastid albino marker in the other cell parent to yield green plants. The nuclear albino parent contributed N. megalosiphon wild-type plastids and cms mitochondria, and the plastid albino parent contributed a wild-type nucleus and male-fertile mitochondria. Due to selection for green regenerants, any cell hybrids retaining only an albino parental nuclear genome, or only the albino plastid genome, were lost. The mitochondrial marker was unselected, and both types were recovered in all possible somatic hybrid cell types. Within the limits imposed by the selection strategy, all possible combinations of nuclear, plastid and mitochondrial genotypes are represented in the somatic hybrids.

A large group of hybrid plants appear to retain a mixed nuclear genome in company with *N. megalosiphon* plastid and mitochondrial markers. Plants #1,2,5,6 and 7 all fall into this class. The green tissue of hybrid plant #3 also shows a mixture of parental nuclear contributions, but combines the *N. tabacum* male-fertile mitochondrial marker with the other parent's *N. bigelovii* plastids. Plant #8 retains the *N. megalosiphon* plastids and mitochondria, but appears to transmit only the other parent's nuclear markers. Plant #4 shows a combination of *N. megalosiphon* plastid markers with *N. tabacum* mitochondria, but only one parental nucleus. These four hybrid groups represent all the possible autotrophic assortments of the three genomes examined. We must conclude that under these experimental conditions, independent segregation of the nuclei, plastids and mitochondria leads to recovery of all classes of somatic segregants.

Some green genotypes might be argued to represent escapes from the nuclear albino stocks by somatic genetic exchange or mutation. However, the hybrid plants with different species' plastids and mitochondria can only represent segregants from cell fusions; in fact, such plants cannot be constructed in any other way at present. A reasonable conclusion is that plants with homologous plastids and mitochondria obtained in these experiments result, as do other classes of somatic hybrids, from independent mitotic segregation of the three genomes in somatic hybrids.

No evidence was obtained for plastid recombination, either between selected markers, or between unselected markers in confirmed somatic hybrids. Selection against one plastid species mitigates against its recovery, except in the form of variegation; this proved difficult to obtain, and the sole example brought to greenhouse culture was not extensively analyzed. These variegated shoots from somatic hybrid #3 provide material for further work, as there is a selectable plastid recombinant seedling phenotype between the albino, tentoxin-resistant _N. tabacum_ plastid and the green, toxin-sensitive _N. bigelovii_ plastid. The visual marker facilitates vegetative propagation of the mixed plastid condition, and seed from variegated shoots can be collected when this plant flowers.

A higher cell fusion percentage and a more efficient selection scheme might permit a quantitative analysis of the behavior of different cytoplasmic genotypes in such experiments; however, Table 9 contains too few plants to draw any conclusions about the compatibility or segregation rates of the cytoplasmic genomes involved. The analysis of cell genome inheritance in somatic hybridization experiments like those reported here could be improved after discovery of more and better mtDNA markers, and by the inclusion of more nuclear loci in the analysis scheme. High nuclear ploidy in the resultant somatic hybrid genotypes could also be avoided with a different experimental design. Where high ploidy results, as with these experiments, one can proceed to use a pollinator which causes maternal haploids to obtain reduced ploidy in the next generation. *Nicotiana africana* has been reported to produce maternal haploids on tobacco (168).

These experiments created an assortment of plant cell organellar mixtures by *in vitro* manipulation. As described in Chapter I, we presently know little about plant cytoplasmic genetics. Improved techniques for obtaining, maintaining and deliberately rearranging plant cell component mixtures will improve our ability to manipulate plant plastid and mitochondrial genomes. Benefits of such progress can be anticipated in both the genetic and agronomic arenas.

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