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Mitochondrial inheritance in *Medicago sativa*

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MITOCHONDRIAL INHERITANCE IN MEDICAGO SATIVA

by

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ABSTRACT

Numerous studies show that plastid DNA is inherited biparentally in alfalfa (Medicago sativa L.). Mitochondrial DNA has been shown to be inherited in a uniparental-maternal fashion in a limited number of sexual crosses. This study investigated the inheritance of mitochondrial DNA in 54 alfalfa progenies from crosses between two cytoplasmic male sterile maternal plants and 54 paternal plants, representing each of the eight basic germplasm groups of M. sativa. Cloned mitochondrial DNA fragments from M. sativa were used as hybridization probes to identify polymorphisms in mitochondrial DNA for Eco RI restriction sites. Polymorphisms were analyzed for patterns of mitochondrial DNA inheritance. All progenies displayed the Eco RI mitochondrial DNA restriction pattern of the maternal parent, indicating that maternal mitochondrial inheritance predominates in alfalfa. The same progenies were also examined for their plastid DNA inheritance pattern. Plastid DNA displayed a mixed inheritance pattern, with both uniparental-maternal and uniparental-paternal inheritance. Variation observed between paternal parents for plastid transmission was absent for mitochondrial transmission.

INTRODUCTION

Much of the genetic information of a plant is found in the DNA of the nucleus, but plant cells also contain DNA in cytoplasmic organelles, the mitochondria and plastids. These organelles and their genomes are replicated and inherited independently of the nucleus. Although uniparental-maternal cytoplasmic inheritance appears to predominate among animals and in most plant species, biparental inheritance of organelles has been shown to occur in a variety of plant species. Organelle inheritance has been studied in many species ranging from unicellular algae to angiosperms using cytological, genetic and molecular techniques. Most research has concentrated on the plastids for a number of reasons. The large size of this organelle relative to mitochondria makes discerning differences between maternal and paternal plastids simpler in whole-plant and ultrastructural studies. The availability of visible plastid mutants, especially those causing chlorophyll deficiency, has also allowed comprehensive genetic analysis. The greater number of copies of the plastid DNA molecules per cell and the ease of chloroplast DNA isolation compared to mitochondrial DNA, also make this organelle attractive to study at the molecular level. Additionally, it appears that there may be more variation in the manner of plastid transmission between and within species than is seen for mitochondria. There are still many gaps in our knowledge of organelle inheritance both in terms of the evolutionary basis of inheritance patterns, and in the mechanisms affecting

organelle transmission.

Cytoplasmic Traits: Biology and Crop Improvement

The role of cytoplasmic genes in crop improvement and plant development is just beginning to be recognized. Certain cytoplasmically inherited traits are presently utilized in breeding programs (Harvey et al., 1972; Bosemark, 1983; Smith, 1989a). These traits display a non-Mendelian inheritance pattern, with the trait usually inherited only through the maternal parent. One such maternally inherited, economically important trait is cytoplasmic male sterility (CMS), which is associated with a mutation in the mitochondrial genome of maize (Zea mays) (Levings and Pring, 1976) and sorghum (Sorghum bicolor) (Dixon and Leaver, 1982). The CMS phenotype is used commercially to produce F_1 hybrids in these and other crops by preventing self-fertilization of the female parent in production of hybrid seed. A second important cytoplasmic trait is resistance to the S-triazine herbicides, known in most cases to be due to modifications in the plastid membrane that prevents triazine binding (Robertson, 1985).

Organelle genomes also provide model systems for many phenomena in heredity due to their relative small size and simplicity (Birky, 1976). Cybrids (cytoplasmic hybrids) produced from somatic hybridization allow detection and examination of cytoplasmic genome exchanges and recombination between parental organelles (Gleba and Meshkiene, 1984). Finally, a more thorough

understanding of organelle inheritance patterns may help to answer some of the questions surrounding the evolutionary origin of mitochondria and chloroplasts. The importance of these organelles lies in the fact that they encode genes that are associated with energy generating functions of the cell.

Mechanisms Controlling Organelle Inheritance

Cytological analysis of gametogenesis and embryogenesis has revealed most of the information known about mechanisms of cytoplasmic organelle inheritance. Genetic and biochemical analyses of the control of organelle inheritance have been conducted for just a few species, however. Much of the literature in this area concentrates on plastids, although these mechanisms may also apply to the mitochondria. Regardless, any mechanism affecting plastid inheritance is not necessarily influencing mitochondrial inheritance simultaneously. There are three general mechanisms recognized to insure uniparental organelle inheritance. These are (1) physical exclusion of one parent's (usually paternal) organelles from the zygote, (2) degradation of the organelle or organellar DNA in the gamete or zygote, and (3) replication of only one parent's organelles in the zygote or embryo. Variation exists within each of these mechanisms according to the level of stringency of the mechanism (that is, the ability of the method to eliminate one organelle type) and the stage of development of the gamete or the embryo for which the mechanism occurs (Whatley, 1982). These differences may parallel differences in the morphology

of the gametophytes and the physiology of the fertilization event between species, both of which are also quite variable in the plant kingdom (Sears, 1980; Whatley, 1982). As a result there are many ways that uniparental organelle inheritance can occur at different stages of development in the gamete and the embryo.

Evidence exists for each of the three basic mechanisms throughout the plant kingdom. Among the angiosperms, there is evidence that cytoplasmic organelles (especially plastids) are absent from the sperm cells of a significant number of species (Corriveau and Coleman, 1988; Hagemann and Schroder, 1989). This exclusion of paternal organelles from the sperm cells of the pollen grains can occur during microgametogenesis, presumably at the first haploid mitosis (Jensen, 1974). In lower plants, exclusion of organelles occurs when a portion of the cytoplasm of the sperm is discarded via an enucleated vesicle before fertilization (Whatley, 1982). Uniparental plastid inheritance can also occur when parental organelles degenerate. This is known to occur in some species of algae where paternal plastids "disappear" either before or after fertilization (Whatley, 1982). Maternal plastid degeneration has been reported in conifers (Neale and Sederoff, 1987). Among those angiosperm species that contain plastids in the generative cells, degeneration or alteration of plastids can also occur during pollen development or after fertilization (Vaughn et al., 1980; Miyamura et al., 1987). Finally, differences in replication rates of cytoplasmic

organelles may account for some of the variation observed in the frequency of biparental transmission among certain species. Competition between organelle types from different parents may exist where one organelle type is favored for replication in the zygote (Kirk and Tilney-Bassett, 1978; Chui et al., 1988). In the developing embryo, cytoplasmic organelle persistence can also be influenced by vegetative segregation due to random partitioning of organelles at cell division and differences in replication rates of organelles and organellar DNA (Birky, 1983). The latter would be due either to random forces, enhanced by differences in copy numbers present at fertilization, or nonrandom forces, such as selection, acting on one organelle type.

ORGANELLE INHERITANCE PATTERNS

Plastids

Plastid inheritance has been studied in only a few hundred of the roughly 400,000 species in the plant kingdom, or less than 0.1% of species. In 60 to 80% of species studied, results show that plastid inheritance is purely maternal (Kirk and Tilney-Bassett, 1978). However, not all of these studies have been conclusive since a low frequency of biparental or paternal transmission may not be detected if sample size of progenies is small (Smith, 1989a). In addition, some studies have relied solely on cytological analysis of gametophytes which may or may not be comprehensive. The following is a review of some of the research that has been conducted on the inheritance of plastids.

Algae

Plastid inheritance has been studied in approximately 50 algal species. Most of these studies involved light or electron microscopic observations of the gamete and zygote (reviewed by Sears, 1980; Whatley, 1982). Transmission of plastids appears to be mostly uniparental-maternal even though both gametes contain plastids in many algal species. In oogamous and anisogamous algae, the larger, and sometimes nonmotile, gamete is considered the female gamete; in isogamous species, the female gamete is identified by biochemical properties. The most frequently observed method for elimination of the paternal plastid from the zygote is disintegration either before or after gamete fusion. If

paternal plastids persist within the cytoplasm of the egg cell after fertilization, the fate of the organelles in the maturing zygote is uncertain. In many oogamous algae the paternal plastids are fewer and smaller than the maternal plastids and thus may not be able to persist in the zygote.

Chlamydomonas is one of the few organisms where the control of chloroplast DNA (cpDNA) inheritance has been studied extensively using genetic and biochemical analyses. This research began in the 1960s with the work of Ruth Sager who developed experimental methods for the study of cytoplasmic genetics in C. reinhardtii. In this isogamous green alga, both parents contribute equal amounts of cytoplasm to the zygote by complete fusion of the two gametes. Despite this, cpDNA markers are transmitted by the mt+ (maternal) parent to most meiotic progeny (Sager, 1972; Gillham, 1978). In less than 10% of sexual zygotes, cpDNA alleles are transmitted from both parents, and very rarely from the mt- (paternal) parent only. This behavior has been observed in three other Chlamydomonas species (McBride and McBride, 1975; Vanwinkle-Swift and Aubert, 1983; Lee and Lemieux, 1986). Studies with C. reinhardtii have also shown that the mt- cpDNA is selectively degraded soon after mating and before fusion of the two parental nuclei and the two parental chloroplasts in the zygote (Burton et al., 1979; Matagne and Mathieu, 1983). This degradation may be due to a nuclease activity encoded by a gene localized in the mt- genome (Matagne, 1987).

Bryophytes and Lower Vascular Plants

Among the bryophytes and non-seed bearing vascular plants, ultrastructural analyses indicate that cytoplasmic organelles are inherited in a uniparental-maternal manner. There are many accounts of observations made on the complex events leading to fertilization in these organisms, although often not in direct reference to organelle transmission. Reports on mosses and hornworts generally show that the plastids of sporogeneous cells have less complex lamellar systems than those of vegetative cells (Neidhart, 1979) possibly leading to their elimination after fertilization. This reduction in complexity is accompanied by a reduction to a single plastid in the spermatozoids (flagellated sperm). Monoplastidy in the sperm may arise due to fusion of all plastids into a single plastid or through continued mitotic divisions of the sporogenous cells, which are not accompanied by divisions of the plastids (Paolillo, 1984). Monoplastidy has also been reported in several genera of liverworts, although in Riccardia, sperm cell plastids are numerous (Paolillo, 1984). Reports on Marsilea (Myles, 1978), Pteridium (Sears, 1980) and Equisetum (Bednara and Rodkiewicz, 1985) show numerous plastids in the sperm cells. The mature eggs of bryophytes and ferns contain organelles in the cytoplasm although there is considerable variation in their number and distribution (Chopra and Kumra, 1988). These differences in parental plastid number could result in the dominance of maternal plastids in the sporophyte.

In the heterosporous fern Marsilea, most of the sperm cytoplasm is shed before fertilization is complete (Myles, 1978). This loss occurs in two steps, first when about half the cytoplasm containing numerous mitochondria is released from the sperm as it becomes motile. Later, as the spermatozoid penetrates the megaspore mucilage, it loses a posterior vesicle of cytoplasm containing all of the plastids and the nuclear envelope. The remaining single sperm mitochondrion in the egg cytoplasm degenerates before the first division of the zygote. Discarding a portion of the sperm cytoplasm prior to ferilization has been reported in bryophytes as well and is believed to enhance motility of the sperm in the archegonium (Whatley, 1982). There do appear to be exceptions however; a Marchantia species passes plastids from the sperm to the egg, but the subsequent fate of the plastids is unknown (Whatley, 1982). The only example of a variegated (green/chlorophyll-deficient chimera) fern, Scolopendrium vulgare, appeared to exhibit biparental plastid inheritance of a mutant phenotype in reciprocal crosses (Kirk and Tilney-Bassett, 1978).

Gymnosperms

In gymnosperms, the patterns of organelle inheritance appears to differ from that observed for any other group of plants. The best data exists for genera within the Coniferophyta. In all conifer species investigated to date, paternal plastids predominate in the progeny. Within conifers, studies exist for species within three families: Taxodiaceae, Cupresaceae, and Pinaceae. Studies on

plastid inheritance in the conifers can be divided into three sections based on the type of data collected or genetic marker used: ultrastructure, genetic or visible markers, and molecular markers.

Ultrastructure

Electron microscopic analysis of gamete development and fertilization in all conifer genera so far examined have revealed the presence of plastids in the pollen generative cell. In addition, there appears to be no mechanism for physical exclusion of paternal plastids from the egg cell or of paternal plastid degradation therein. Interestingly, elimination mechanisms do exist for maternal plastids from the zygote. As summarized by Chesnoy and Thomas (1971), maternal plastids are not incorporated into the pro-embryo of Biota orientalis and Chamaecyparis lawsoniana (Cupresaceae). In Cryptomeria japonica and Sciadopitys verticillata (Taxodiaceae) (Camefort, 1970; Chesnoy, 1973; Gianordoli, 1973) maternal plastids degenerate either before or after fertilization. In the family Pinaceae, ultrastructural observations have been made on Pinus nigra (Camefort, 1965), Larix decidua (Camefort, 1967) and Douglas fir (Pseudotsuga menziesii) (Thomas and Chesnoy, 1969). It appears that maternal plastids are not concentrated close to the egg nucleus in these species but are contained in a membrane-bound inclusion in the egg cell prior to their degeneration (Neale and Sederoff, 1987). Following fertilization, the zygote contains plastids originating only from the pollen parent (Chesnoy and Thomas,

1971).

Visible markers

The only conifer species in which plastid inheritance has been studied using visible markers is Cryptomeria japonica. Ohba et al. (1971) found a plastome mutation that resulted in a color variation and was transmitted by the male parent to 97% of progeny in normal green (G) x mutant (CD) crosses. (By convention, the maternal parent in crosses is listed first throughout this thesis.) More than 99% of the progeny from the reciprocal CD x G crosses displayed the normal green phenotype. Both crosses showed a very low frequency of variegated (or biparental) progeny. This supports the ultrastructural observations suggesting a strong paternal bias in this species.

Molecular markers

While ultrastructural studies predominate in cytoplasmic inheritance studies in lower plants, recent research in higher plants more commonly uses molecular techniques, such as restriction fragment length polymorphism (RFLP) analysis to study cytoplasmic organelle DNA inheritance. Two approaches have been used to study the inheritance of organellar nucleic acids in plants. The first method involves isolating plant organelle DNA, which is then cut with restriction enzymes and visualized under UV light after staining the DNA with ethidium bromide. The limiting step of this procedure is isolation of digestable organellar DNA without nuclear DNA contamination. Suitable protocols are available to

achieve this based on initial separation of organelles in density gradients followed by organelle lysis and DNA purification. A second and more rapid approach is to isolate total DNA from plant tissue and screen for organellar DNA polymorphisms by Southern hybridization with heterologous probes containing organellar DNA fragments. By both procedures, the restriction patterns of the parents and the hybrids are compared and the organellar content of the progeny determined.

Using the latter procedure, Neale et al. (1986) reported strictly paternal inheritance of plastid DNA in Douglas fir. They examined 212 full-sib progenies from three families and found that without exception progenies had the same restriction pattern as the paternal parent. They concluded that maternal plastid transmission was very rare. This result led Sederoff, Neale and co-workers to study plastid inheritance in two intraspecific crosses of coast redwood (Sequoia sempervirens), which led to confirmation of paternal plastid inheritance in this species as well (Neale and Sederoff, 1987). Using similar techniques, paternal inheritance of cpDNA has been observed in progeny from interspecific crosses in Pinus (P. contorta x P. banksiana, Wagner et al., 1987; and P. rigida x P. taeda, Neale and Sederoff 1989), Picea (Szmidt et al., 1988; and P. engelmannii x P. pungens, Stine and Keathley, 1990) and Larix (L. decidua x L. leptolepis, Szmidt et al., 1987). The only exception to strict paternal inheritance of cpDNA was found in a subset of progenies from hybrids of Larix that showed one

maternal and two combined maternal-paternal cpDNA restriction patterns. Given the variation that has been observed in patterns of plastid inheritance among angiosperms, it is not surprising that uniparental-paternal inheritance of plastids in conifers is not absolute and infrequently plastids are transmitted through the maternal parent.

Angiosperms Other Than *Medicago sativa*

Not surprisingly, plastid inheritance has been studied most extensively in the angiosperms. This may explain why the most variation in the mode of inheritance of plastids has been found in this diverse group of plants. As with the gymnosperms, studies on angiosperms can be classified as ultrastructural, genetic, or molecular. While plastid transmission is uniparental-maternal in the majority of angiosperms examined (Kirk and Tilney-Bassett, 1978) the number of species found to exhibit biparental plastid inheritance is increasing (Smith, 1989a).

Ultrastructure

Development of the microgametophyte in angiosperms involves two mitotic divisions. The first asymetrical division results in a large vegetative (tube) cell and a smaller generative cell. The vegetative cell elongates to form the pollen tube. The generative cell later divides inside the cytoplasm of the vegetative cell giving rise to two sperm cells, one of which may fertilize the egg.

Plastid exclusion from the generative cell can occur at the first mitotic

division of the microspore (Jensen, 1974). Ultrastructural investigations of pollen grains at this stage have provided information on the status of extranuclear elements in a number of species. In most species examined, plastids appear to be absent from the generative cell but present in the vegetative cell (Kirk and Tilney-Bassett, 1978). Hagemann and Schroder (1989) list 43 species for which electron microscopic observations have provided evidence for plastid elimination from the generative cell before the second mitotic division. For some of these, genetic data from sexual progenies corroborate this observation. For example, cytological examination of Beta vulgaris could not confirm plastids in the generative cell, a species known from genetic studies to transmit plastids uniparental-maternally (Kirk and Tilney-Bassett, 1978). The same is true for other species, such as, Mirabilis jalapa, cotton (Gossypuim hirsutum), and tomato (Lycopersicon esculentum) (Kirk and Tilney-Bassett, 1978; Hageman and Schroder, 1989).

The inability to detect plastids in the generative cell with this technique is not definitive proof of their absence, however. For example, Karas and Cass (1976) reported the absence of plastids in the generative cells of rye (Secale cereale), while Hageman and Schroder (1985) report the existence of cytological evidence for plastids in the sperm cells of this species. Furthermore, reports of biparental plastid inheritance exist for this species from genetic studies (Kirk and Tilney-Bassett, 1978) as well as RFLP studies (Soliman et al., 1987), yet

Corriveau and Coleman (1988) report the absence of plastid DNA from the generative cells using DAPI and epifluorescence microscopy (see below).

Such discrepancies can result from differences in serial sectioning of the pollen grains (Sears, 1980) or possibly inadequate sampling of intraspecific genetic variation (Smith, 1989a). Genetic data on plastid inheritance in Pisum sativum is scant and has been interpreted to indicate uniparental-maternal inheritance (Kirk and Tilney-Bassett, 1978). A recent study by Corriveau et al. (1989) analyzed P. sativum pollen from 13 pea lines (12 inbred) with fluorescent staining and microscopy. They found that the percentage of pollen grains containing plastid DNA in the generative cells ranged from 3% in one accession to 65 % in another. Such variation suggests biparental inheritance is more likely to occur in some pea accessions than in others. A thorough examination may be required to detect low frequency paternal transmission in this species.

In other species, however, the presence of plastids or proplastids is observed consistently in the generative cell. In such cases, the possibility for regular biparental transmission of the plastids exists. Hagemann and Schroder (1989) list some 33 species for which cytological studies suggest at least a few plastids in the generative cell of micropores. Again, in some of these, genetic data correlates well with cytological data.. For example, in Pelargonium zonale and two species of Oenothera, ultrastructural observations have shown many proplastids in the generative cells, and these species are well known to exhibit

biparental inheritance of plastids from genetic studies (Kirk and Tilney-Bassett, 1978).

The presence of plastids in the generative cell at this stage of pollen development does not guarantee transmission to the zygote, however. In some species, cytological studies suggest plastid elimination occurs in the generative cell during gametophyte maturation by degradation or dedifferentiation. Miyamura et al. (1987) examined pollen grains in wheat (Triticum aestivum) at successive stages of maturation using fluorescence microscopy. While plastid DNA was present in the generative cell after the first mitosis, it had disappeared from the sperm cells by the time maturation was complete, suggesting degradation of plastids therein. In another study, plastid organelles were reported in the generative cells of Hosta japonica (Hagemann and Schroder, 1989) but genetic studies have only provided evidence for uniparental-maternal plastid inheritance in this species (Kirk and Tilney-Bassett, 1978). Ultrastructural evidence also exists for plastid debilitation in the early stages of pollen development. Vaughn et al. (1980) compared plastids of albino rice (Oryza sativa) plantlets produced from anther culture with normal uninucleate pollen from Hosta japonica. In both cases plastids were reduced. Day and Ellis (1984) examined cpDNA from albino wheat plants, also produced from anther culture, using restriction analysis and found large deletions in the chloroplast DNA molecules. These changes could explain the albinism of plants

regenerated from pollen callus and the uniparental-maternal inheritance of plastids observed in these species (Day and Ellis, 1984).

Plastid nucleoids (a small localized subset of cpDNA molecules, containing 1 to 5, or more plastid genomes) are present in the generative cells of mature pollen grains of Mirabilis japonica and Pharbitis nil (Miyamura et al., 1987) but genetic studies have only provided evidence for uniparental-maternal inheritance of plastids in both of these species (Kirk and Tilney-Bassett, 1978). If mature microspores contain plastids, it is expected that some degree of biparental plastid inheritance would be observed in genetic studies. The lack of evidence for biparental inheritance could be due to inadequate sampling of intraspecific variation, since different plants were used for each kind of study. Alternatively, some other mechanism could exclude plastids from the zygote. Possibilities for late paternal plastid elimination exist, although these mechanism are difficult to identify. The first is a form of physical exclusion from the zygote where the sperm cytoplasm is discharged into the degenerating synergid while only the sperm nucleus enters the egg cell (Sears 1980; Hagemann and Schroder, 1989). This is similar to what was observed in some bryophytes and ferns (e.g. Marsilea) and may explain the uniparental-maternal inheritance of plastids observed in barley (Hordeum vulgare). Based upon ultrastructural analysis of barley pollen grains, Mogensen (1988) reported remnants of the sperm cytoplasm, including plastids and mitochondria, in the degenerating synergid

near the position where the sperm fuses with the egg. One sperm nucleus is simultaneously visible within the egg cell.

Another form of physical exclusion could conceivably occur in the zygote if the distribution of paternal and maternal organelles differ when this cell divides. Paternal plastids could become clustered and localized in the part of the zygote destined to become the suspensor, while maternal plastids predominate in the part of the zygote which gives rise to the proembryo (Kirk and Tilney-Bassett, 1978). The chance of this occurring may increase if maternal plastids outnumber paternal plastids in the zygote. Tilney-Bassett and Almouslem (1989) proposed this to at least partially account for variation in plastid inheritance patterns observed in Pelargonium. A third possibility is degradation of paternal plastids at a later stage, i.e. within the young zygote. There appears to be no direct evidence for this in angiosperms, although this has been shown to occur in Chlamydomonas (Burton et al., 1979; Matagne and Mathieu, 1983). All of these explanations would account for the observation that plastids are present in sperm cells but only maternal plastids are present in progeny.

A relatively new approach to study organelles in the male gametophyte uses a fluorescent DNA dye, 4',6-diamidino-2-phenylindole (DAPI), in conjunction with epifluorescence microscopy. This technique allows visualization of DNA aggregates (nucleoids) in the pollen cytoplasm (Coleman et al., 1986) and has been used in a number of recent studies of organelle inheritance. Corriveau

and Coleman (1988) screened pollen from 235 angiosperm species for the presence of plastid nucleoids using DAPI and epifluorescence microscopy. They detected plastid nucleoids in the generative or sperm cells of 43 species, nine of which have proven to transmit plastids biparentally in genetic studies. In four cases, genetic studies have not proven biparental inheritance. No genetic studies exist for the other 30 species, which may be good candidates for such experimentation.

One of these is Plumbago zeylanica. Extensive ultrastructural work by Russell (1984; 1985) examined sperm cell pairs within the egg cytoplasm at fertilization. He found that the sperm cell pairs are dimorphic in positioning and organelle content. One sperm cell (S_{vn}) is physically associated with the vegetative nucleus and contains very few plastids. The second sperm cell (S_{ua}) is not associated with the vegetative nucleus and contains numerous plastids. In 11 of the sperm pairs, organelle content was quantified. S_{nv} contained on average more than 25 mitochondria and an occasional plastid while the S_{ua} contained on average some 50 mitochondria and 25 plastids. S_{ua} was observed to fertilize the egg in 16 out of 17 cases examined. This suggests that this plant could frequently transmit plastids biparentally. In a small study of the egg cell, Russell (1986a) estimated it contained 14,000 mitochondria and 110 plastids. Furthermore, the difference in plastid number in the egg cell versus the plastid contribution of the sperm cell could lead to a maternal bias in

progeny.

In the zygote of Oenothera erythrosepala the number of maternal plastids is estimated to be between 25 to 29, which is at least twice the number of paternal plastids, estimated at 8 to 13 (Meyer and Stubbe, 1974). Biparental plastid inheritance occurs regularly in this species, but maternal inheritance predominates (reviewed by Kirk and Tilney-Bassett, 1978). Although differences in the initial contribution may explain this, other factors are believed to be significant; namely, the effect of the maternal and hybrid nuclear genotypes and differences in the rates of division of the two plastid types, which may be related to a nuclear-plastome interaction (Chiu et al., 1988; Corriveau and Coleman, 1990).

Based largely on ultrastructural observations, Hagemann and Schroder (1989) have suggested classifying plastid transmission in angiosperms into four categories. Type 1 (Lycoperscion-type) plants would include those plants for which plastids are excluded from the generative cells of pollen. Type 1 plants, such as species of Mirabilis and cotton, exhibit strict maternal inheritance of plastids. Type 2 (Solanum-type) plants would include those for which plastids are initially present in the generative cell but elimination occurs shortly thereafter, such as in Hosta. In these plants, biparental inheritance of plastids is a rare event. Type 3 (Triticum-type) plants are an extension of type 2, except that plastids are eliminated later in the fertilization process and biparental

inheritance may occur at a low frequency. Type 3 plants would include barley and Mirabilis japonica. In the final category are type 4 (Pelargonium-type) plants, which are known to transmit plastids biparentally. Examples of type 4 plants would be Oenothera and alfalfa (Medicago sativa).

Visible markers

Plastid inheritance patterns have been determined through genetic studies using visible markers for a large number of angiosperm species (see Kirk and Tilney-Bassett, 1978; Smith, 1989a). There are three types of mutations that have been used in these studies: chlorophyll-deficiencies, resistance to herbicides, and resistance to antibiotics. The most widely studied and the ones that will be discussed here are those mutations that cause chlorophyll deficiencies. Mutations in the chloroplast genome that cause pigmentation changes can be utilized relatively easily since they are associated with variegation of plant tissue. Variegated tissue contains cells with mutated and normal plastid types.

It is important to discuss the concept of vegetative segregation or "sorting-out" when considering the studies with visible plastid markers. Numerous genetically identical organelles are present within most higher plant cells, with each organelle containing numerous copies of the DNA molecules. Such a cell is termed homoplasmic. Replication of the organelles and the DNA is believed to be random and independent of nuclear DNA replication (Birky, 1983).

Furthermore, at cell division these organelles (both the mitochondria and plastids) are partitioned at random to the two daughter cells (Birky, 1978; 1983). When a mutation occurs in an organelle genome, it is possible that this mutation will be replicated. The result will be genetically different organelles within the same cell, which would then be considered heteroplasmic. During mitosis, the organelles will segregate at random so that progeny cells will either be heteroplasmic or homoplasmic. After a series of cell divisions three populations of cells, derived from one heteroplasmic cell, can exist: cells that are homoplasmic for the wild-type organelle, homoplasmic for the mutation, cells that contain both types of organelles. Sorting-out is complete when all dividing cells are homoplasmic. How quickly this occurs depends on the proportion of normal and mutant plastids, the original total number of organelles, and their rates of replication (Michaelis, 1967; 1976). Random partitioning of organelles at cell division is referred to as vegetative segregation and has been studied in several species using chlorophyll-deficient (CD) plastid mutants (Kirk and Tilney-Bassett, 1978; Smith, 1989a).

Sorting-out of variegated tissue gives rise to a variegated plant. Variegated plants carrying a CD mutation will contain up to three types of tissue: pure sectors of normal green tissue (G), pure sectors of mutant, usually white or yellow CD tissue, and possibly sectors of variegated tissue (V), again depending on what stage of development sorting-out is complete. Gametes

produced in pure sectors will contain only normal or mutant plastids while those produced on variegated tissue may contain both types of plastids (Kirk and Tilney-Bassett, 1978). To study plastid inheritance, reciprocal crosses are made between G and CD sectors of the same or different plants. If biparental transmission of plastids to a zygote occurs, it will be heteroplasmic, given there is no organelle degradation in the zygote. Sometimes crosses are made between G and V sectors and/or CD and V sectors with the variegated plant as the pollen parent. The coloration pattern of the resulting progeny is analyzed for the parental contribution of plastids. For example, in G x CD crosses, progeny with only G tissue will have received plastids from only the maternal parent, whereas progeny with only CD tissue will have recieved all plastids from the paternal parent, and V progeny will have received plastids from both parents. Generally, if biparental transmission of plastids occurs then the result of these crosses will be a combination of these three types of progeny (this is referred to as mixed inheritance), the proportions of which will vary between species, cultivars and reciprocal crosses (Kirk and Tilney-Bassett, 1978; Tilney-Bassett and Almouslem, 1989). Interpretation problems may arise if sorting-out is not complete on what appears to be pure tissue. For example, progeny from CD x G crosses will show a mixed inheritance pattern if biparental transmission of plastids occurs, whereas progeny from V x G crosses will result in the same inheritance pattern whether maternal or biparental transmission actually occurs.

Kirk and Tilney-Bassett (1978) suggest evaluation of self-progenies from variegated sectors to test if the gametes are pure.

Smith (1989a) compiled a list of 38 studies for which G - CD crossing experiments have shown that the plastids are transmitted only through the maternal parent. In cases where biparental inheritance of plastids has been shown to occur, the frequency of the paternal plastid input is variable and often quite low. Therefore, studies that examine adequate numbers of progeny from reciprocal crosses offer the best evidence for strict maternal inheritance (Smith, 1989a).

Genetic control of plastid inheritance has been extensively studied in species of Pelargonium and Oenothera, both of which exhibit biparental plastid inheritance. In Pelargonium, plastid transmission is believed to be highly dependent upon the genotype of the maternal parent (Tilney-Bassett, 1973; and Tilney-Bassett and Abdel-Wahab, 1982). Progeny show a mixed inheritance pattern after intercultivar reciprocal crosses between G and CD sectors. Families produced from different females exhibit two basic types of inheritance in G x CD crosses. Type I females produce families in which most progeny are pure G, less are V, and few are pure CD. Type II females produce families in which pure G and pure CD progeny occur at about the same frequency and V progeny are least frequent. The effect of the maternal genotype on plastid inheritance is unknown but may either directly or indirectly control plastid

replication in the zygote (Kirk and Tilney-Bassett, 1978). Others factors may contribute to the variability within each pattern such as plastid competition (G plastids may replicate faster than CD plastids), spatial distribution of plastids in the zygote, and numerical differences between the maternal and paternal plastid contribution (Tilney-Bassett and Almouslem, 1989).

In contrast to Pelargonium, maternal plastid inheritance predominates in Oenothera with no purely paternal transmission of plastids ever observed (reviewed by Kirk and Tilney-Bassett, 1978). Progeny from interspecific reciprocal G - CD crosses contain plastids from either the maternal parent only, or, less frequently from both parents. As previously stated, this is believed to be partly due to the greater number of plastids in the egg cell than in the sperm cell at fertilization (Meyer and Stubbe, 1974). A second factor is the effect of the plastid genome. Analysis of crossing experiments led to the classification of five plastid types (I-V) according to their variegation pattern in different nuclear backgrounds (Stubbe, 1959). These were later shown to be distinguishable by their cpDNA restriction patterns (Gordon et al., 1982). The highest frequencies of biparental plastid transmission occur when the maternal parent is carrying a weak plastome type and the paternal parent a strong plastome type. Conversely, the lowest frequencies of biparental plastid transmission occur when the maternal parent is carrying a strong plastome type and the paternal parent a weak plastome type. Intermediate frequencies occur with all other

combinations. These differences in plastid types are attributed to the differences in their rates of replication (Chiu et al., 1988). A third factor contributing to plastid transmission is the hybrid nuclear genome (Kirk and Tilney-Bassett, 1978). Progenies were examined from crosses in which the plastid types compared remained constant in variable hybrid backgrounds. The degree of variegation was affected by the genotype of the hybrid, although the relative strength of the plastid types remained the same (Kirk and Tilney-Bassett, 1978).

In these two genera where paternal plastids are transmitted to the zygote, the persistence of the plastids in the sporophyte depends largely upon either the maternal genotype (Pelargonium) or upon the plastome itself (Oenothera), possibly affecting plastid replication frequencies. Paternal plastids are believed to be at an initial disadvantage in both genera since they are entering a foreign cytoplasm and incompatabilities, whether with the nucleus or with the maternal plastid type, could influence plastid stability (Tilney-Bassett and Abdel-Wahab, 1982; Russell, 1986a).

Molecular markers

Restriction fragment analyses of cpDNA of parents and progeny have been used to determine the mode of plastid inheritance in a number of angiosperm species. This method has been used to show uniparental-maternal inheritance of plastids in progenies from interspecific crosses in Zea (Conde et al., 1979),

Oenothera (Hachtel, 1980), wheat (Vedel et al., 1981), Brassica (Ichikawa and Hirai, 1983), and soybean (Glycine) (Hatfield et al., 1985), and intraspecific hybrids of maize (Pring and Levings, 1978), sorghum (Pring et al., 1982), and Beta vulgaris (Mikami et al., 1984). Biparental inheritance of plastids has been confirmed using this method in Pelargonium (Metzlaff et al., 1981), barley x rye intergeneric hybrids (Soliman et al., 1987), and carrot (Daucus) (Boblenz et al., 1990), with trace amounts of cpDNA from the male parents detected in hybrids in Epilobium (Schmitz and Kowallik, 1986), and Nicotiana (Medgyesy et al., 1986). RFLP markers are inherited in a codominant manner and depend only upon the existence of differences in the DNA sequence of the genome and not upon the expression of an altered genotype. Once suitable markers are found, it is possible to efficiently examine a large number of hybrids. The percentage of parental organellar DNA in a given sample can be estimated by comparison to known standards, giving an approximation of the ratio of parental organellar DNA in the plant.

Medicago sativa

Plastid transmission in alfalfa is unique among angiosperm species investigated so far. Progeny produced from reciprocal crosses between G and CD sectors show a mixed inheritance pattern where progenies containing plastids from both parents (biparental) are common, but the majority contain plastids from only the paternal parent (Smith, 1989b).

Ultrastructure

Ultrastructural analysis and three dimensional reconstruction of generative and sperm cells of alfalfa pollen grains has been conducted to determine organelle content and morphological differences between three paternal lines (Zhu et al., 1990; Zhu et al., 1991). All generative and sperm cells observed were found to be morphologically similar and contain numerous plastids. Although variation in plastid number per generative cell existed between the three genotypes examined, no strong correlation could be made between plastid number and the known plastid transmission behavior of these genotypes (Zhu et al., 1991; Smith, 1989b). Thus, variation in plastid number in the sperm may not be a significant factor affecting variation in transmission patterns between genotypes. Plastid number and distribution in the egg may be more important in its effect on plastid inheritance patterns (H.L. Mogenson, pers. comm.).

Visible markers

Smith et al. (1986) demonstrated biparental inheritance of mutant CD plastids in progenies produced from reciprocal crosses. Further genetic analyses of families produced from 16 crosses using five G plants as maternal parents and four CD plants as paternal parents showed that the highest percentage of progeny within most families contained plastids from only the paternal parent, while the lowest percentages were among progenies containing

only maternal plastids (Smith, 1989b). Furthermore, plastid transmission frequencies were affected by both the maternal and paternal nuclear genotypes. Whereas maternal inheritance predominates among most Pelargonium crosses and depends largely upon the maternal genotype, paternal inheritance predominates among alfalfa crosses and is influenced by both parental genotypes.

Molecular markers

Analysis of cpDNA restriction fragment patterns from the parents and progeny of G x CD crosses showed that cpDNA from normal G tissue of sectored progeny displayed the maternal cpDNA restriction pattern, while cpDNA from CD tissue displayed the paternal cpDNA pattern (Lee et al., 1988; 1989). These studies proved biparental inheritance of plastids at the molecular level in alfalfa. Further RFLP studies of progeny from crosses among only normal green plants confirmed a paternal bias in plastid transmission (Schumann and Hancock, 1989; Masoud et al., 1990). Of 212 progeny from 16 crosses, Masoud et al. (1990) found that leaf samples extracted from 82% of the progeny exhibited cpDNA restriction patterns identical to the paternal parent while just 2% displayed restriction patterns identical to the maternal parent. The remaining 16% displayed the combined maternal-paternal (biparental) restriction pattern.

Mitochondria

Fewer studies have been conducted on mitochondrial inheritance in plants largely because of the difficulty in obtaining easily scored mtDNA markers (Grun, 1976; Gillham, 1978). CMS, which has been observed in a limited number of angiosperm species, does not allow for reciprocal crosses, and may be difficult to score. Restriction fragment analysis, which depends only on differences in base sequence between mtDNA molecules, has been used to predict mitochondrial inheritance patterns within and between species. Ultrastructural studies are often limited by the inability to distinguish between maternal and paternal mitochondria in the zygote but have been useful in determining the presence and quantity of mitochondria in the gametophytes, especially the male gametophyte (Russell, 1986a; Mogensen, 1988).

Lower Eukaryotes

There are few descriptions of mitochondrial inheritance in lower eukaryotes. Cytological studies may include observations on cytoplasmic organelles but generally comment only on the presence or absence of the organelle in the gamete or zygote. Mitochondrial inheritance has been shown to be uniparental-maternal in several species of fungi (Gillham, 1978). Based on this and the available data on mitochondrial inheritance in higher plants and animals, it is generally assumed that mitochondrial inheritance in the algae is uniparental-maternal. The possiblity of biparental mitochondrial inheritance should not be

entirely ruled out. For instance, in the oogamous green algae Oedogonium, Hoffman (1974) noted the presence of intact sperm mitochondria in the egg cell just after karyogamy, whereas sperm plastids were seldom observed and then appeared only as plastid remnants. Whether the mitochondria persist in the zygote has not been determined. A more detailed analysis has been conducted in Chlamydomonas. Sexual progenies from interspecific reciprocal crosses between C. smithii and C. reinhardtii displayed the mtDNA restriction pattern of the mt- (paternal) strain (Boynton et al., 1987). Thus, it appeared that opposite mating types were transmitting mitochondria and chloroplasts to progeny in this species. Another similar study examined C. eugametos and C. moewusii hybrids for mitochondrial transmission (Lee et al., 1990). The mtDNA restriction pattern of the fourth backcross of F_1 hybrids to one or the other parent was identical to the mt+ strain in every case examined, even while approximately 50% of the backcross to one parent and more than 25% of the backcross to the other parent were biparental for inheritance of chloroplast resistance markers. If appropriate markers could be found, it would be interesting to see the results on mitochondrial transmission in intraspecific crosses within these Chlamydomonas species.

Results from research on mitochondrial transmission in Bakers' yeast (Saccharomyces cerevisiae) may be applicable to mitochondrial genetics in other species, especially the models proposed for the effects of random drift on

vegetative segregation. Yeast can reproduce vegetatively in the haploid and diploid state by budding of daughter cells. Mating of haploid cells is controlled by a pair of Mendelian alleles (a,α). Opposite mating types will fuse pairwise to form diploid zygotes when mixed. Diploid cells undergo meiosis under certain growth conditions to produce a tetrad of haploid spores in which mating type alleles segregate in a 1:1 ratio. Antibiotic-resistant mitochondrial markers are used in transmission experiments. Biparental inheritance of mitochondria occurs in the zygote, however, in many crosses sorting out of mitochondrial alleles is so rapid that most zygote clones of diploid cells will be homoplasmic for one genotype or another, or more rarely, for a recombinant genotype after about 20 divisions (Birky, 1978; Gillham, 1978)). Zygote clones that are biparental for a particular mitochondrial genotype exhibit large variation in gene frequencies. Both of these events are probably due to a combination of factors: variation in frequencies of mitochondrial genomes in the parents affects variation in frequencies in the zygote; differences in cell division rates among the different types of zygotes (e.g. if homoplasmic cells divide faster than heteroplasmic cells); and random changes in gene frequencies occurring in the zygote (i.e. random drift) (Birky, 1983). Experiments in which cell division was delayed by starvation or use of temperature-sensitive mutants, resulted in an increase in the frequency of uniparental zygotes and an increase in the variance of gene frequencies (Thrailkill et al., 1980). This and other experiments

suggested that intracellular random drift due to random replication of mtDNA molecules was an important factor contributing to vegetative segregation (Birky et al., 1982). Intracellular random drift could eliminate a genotype present in low frequency, such as the cytoplasmic organelle contribution of the sperm to the egg (Birky, 1983).

Gymnosperms

Ultrastructural studies have revealed the presence of mitochondria in the pollen generative cell of conifer species within the families Cupresaceae, Taxodiaceae, and Pinaceae (Chesnoy and Thomas, 1971). As previously discussed, the events following fertilization in Pinaceae have been well documented. While maternal plastids degrade within a membrane bound inclusion, maternal mitochondria become localized near the egg nucleus. Furthermore, while the pollen appears to contribute some mitochondria to the zygote, they are outnumbered by maternal mitochondria. An RFLP study of 20 full-sib progeny from each of two loblolly pine (Pinus taeda) crosses indicated maternal inheritance of mitochondria (Neale and Sederoff, 1989). This occurs in spite of the fact that plastids were shown to be inherited paternally in interspecific crosses with loblolly pine as the paternal parent. Comprehensive ultrastructural studies have not been done for species in other conifer families, however, there are indications that mitochondrial inheritance may be different than in Pinus. Progeny examined from two crosses of coast redwood

(Taxodiaceae) displayed the mitochondrial DNA restriction pattern of the paternal parent (Neale et al., 1989). There are also indications that mitochondria are transmitted through the paternal parent in Calocedrus decurrens (Cupressaceae) (Neale and Sederoff, 1987).

Angiosperms Other Than *Medicago sativa*

Although there is relatively little data on mitochondrial inheritance in angiosperms, the few accounts that do exist suggest that many of the same strategies that promote uniparental-maternal inheritance of plastids are among the same mechanisms affecting mitochondrial inheritance, namely, exclusion, degradation and selection. A study by Mogensen (1988) noted the presence of mitochondria in barley sperm cells, however the entire contents of the cytoplasm remained within the degenerating synergid at fertilization. Thus, it appeared mitochondria, like plastids, were not transmitted to the zygote in this species. Sperm cells of spinach (Spinacia oleracea) also contained mitochondria but these organelles have been observed to slowly degenerate as the cytoplasmic volume of the sperm decreases (Wilms, 1985). Mitochondria are present in the generative and sperm cells of wheat (Miyamura et al., 1987), rice and Hosta (Vaughn et al., 1980), and tomato (Kadej and Kadej, 1985), but are observed to degenerate in the pollen before fertilization in these species. All of these plants are believed to transmit plastids strictly through the maternal parent. Finally, ultrastructural studies on Plumbago zeylanica (Russell, 1984;

1986a) showed that the S_{vn} contained on average 250 intact mitochondria while the S_{us}, which are most likely to fertilize the egg, contained approximately 50 mitochondria. The egg cell was estimated to contain some 14,000 mitochondria, greatly outnumbering either sperm cell's number of mitochondria, thereby decreasing the possibility of paternal mitochondrial persistence in the developing embryo.

Unlike with plastids, there are just two cases where biparental inheritance of mitochondria has actually been observed in angiosperms, both shown by RFLP analysis. The first study examined intergeneric hybrids of barley and rye. Rye, which has been shown to transmit plastids biparentally in intraspecific crosses, was the pollen parent in these crosses. The progenies examined displayed the biparental restriction pattern for both cpDNA and mtDNA (Soliman et al., 1987). In the second study, Erickson and Kemble (1990) reported a small percentage of progenies (10%) from crosses in Brassica napus were biparental for mtDNA restriction markers while maternal for cpDNA markers. The low frequency of observed biparental mitochondrial transmission among angiosperms could be partly due to the difficulty in sampling those progeny that contain some mitochondria from both parents. If differences in the maternal and paternal mitochondrial numbers in the zygote are significantly in favor of the maternal parent, then the frequency of biparental progeny would be low. Furthermore, among those progeny that are biparental, sorting-out and drift would occur and

the tissue that may contain paternal mitochondria would be hard to detect with RFLP analysis. With CD mutations, a 1% paternal plastid content would be easily visible in the coloration pattern of the offspring (Smith, 1989a); however 1% paternal mitochondria may be difficult to identify with RFLP analysis (Scowcroft and Larkin, 1981). On the other hand, it could be that the mechanisms promoting uniparental-maternal mitochondrial inheritance are more effective and hence few cases of biparental mitochondrial inheritance would actually exist.

Medicago sativa

Few studies exist on mitochondrial inheritance in species proven to transmit plastids biparentally. In higher plants, reports on the inheritance of mitochondria for plants known to transmit plastids biparentally presently exist only for rye (Soliman et al., 1987), the two species of conifer (Neale and Sederoff, 1989; Neale et al., 1989), and, as in the present study, alfalfa. Thirty hybrids from an interspecific reciprocal cross of alfalfa (M. sativa - M. falcata) were shown to have the maternal mtDNA restriction pattern (Schumann and Hancock, 1989). Ultrastructural studies are not included since they presently cannot definitively show uniparental or biparental inheritance. Two previous studies have posed a question regarding the pattern of mitochondrial inheritance in alfalfa. First, Zhu et al. (1991) confirmed the presence of mitochondria in the generative cell of alfalfa pollen grains using transmission

electron microscopy. Secondly, Fairbanks et al. (1989) documented the presence of paternal mitochondrial-associated RNA in F_1 progenies of sexual crosses.

RESEARCH OBJECTIVES

The objective of this study was to examine the mode of inheritance of mitochondria in alfalfa using RFLP analysis of mtDNA. This research compliments plastid research with alfalfa, giving a more comprehensive view of organelle transmission in this plant. Sexual hybrid plants and their parents were used for this investigation. Cloned mitochondrial DNA fragments from M. sativa were used as hybridization probes for identification of restriction fragment polymorphisms of mtDNAs from parental plants. Inferences regarding the inheritance of mitochondria in alfalfa and the significance of the results with other results from studies carried out on alfalfa and other angiosperm species are presented.

MATERIALS AND METHODS

Parental Plants and Sexual Hybrids

Two plants were used as the maternal parent in all crosses, both of which displayed cytoplasmic male-sterility. These are, 6-4, a plant selected from 'Saranac' by E.T. Bingham, Univ. of Wisconsin (Brown and Bingham, 1984; McCoy and Smith, 1983), and 4MN-D, a plant selected from the population 'MN1292cms' (D.K. Barnes, pers. comm.). 'Saranac' and '1292cms' were both derived from the Flemish cultivar 'DuPuits'. There are eight distinct sources of M. sativa-based alfalfa germplasm recognized in the US, representing most of the genetic diversity in present available cultivars (Barnes et al., 1977). All of the eight germplasm groups were represented in the plants used as pollen parents in this study (Table 1). Plants used in crosses were maintained year-round in the greenhouse under 24 h fluorescent illumination. Parental and progeny plants were grown in either metal flats or 21-cm conetainers.

Crosses in this study were performed by Steve Smith (Dept. of Plant Sciences, Univ. of Arizona) and M.A. Flynn (Dept. of Plant Sciences, Univ. of Arizona). Since the maternal parents used in the study have only male-sterile flowers, emasculation was not necessary. Pollinations were executed by hand using a metal spatula as described by Barnes (1980).

Leaf tissue was taken for RFLP analysis from a total of 54 hybrid progeny plants. Considering sorting-out of parental organelles and possible dilution

Table 1. Alfalfa germplasm groups and the representative cultivars used in this study.

Germplasm group†	Cultivar	No. plants used as pollen parent
Ladak	Ladak	7
M. varia	Travois	7
Turkistan	Lahonton	7
Flemish	DuPuits	7
Chilean	Chilean‡	7
Peruvian	Peruvian‡	4
Indian	Indian‡	7
African	CUF 101 Egyptian IIIA Sewa Ed-Damer (Sudan)	8
Total		54 hybrids

† After Barnes et al.,1977.

‡ Arizona seed sources.

effects resulting from a bias for the maternal organelles, tissue was collected from one or two adjacent stems on a single plant. Pollen production was previously studied in flowers produced on families following crosses between the two CMS plants and plants from each of four germplasm groups; African, Flemish, Ladak, and Turkistan (Smith et al., 1990). Certain plants used as the paternal parent were classified as maintainers of sterility and others as restorers of fertility, according to the pollen load of progenies produced from these crosses. Leaf tissue was taken from both restored and maintained progeny and analyzed separately.

Genomic DNA Library in Lambda Phage Enriched for mtDNA

DNA Isolation

DNA was isolated by differential centrifugation, a modification of a procedure described by Kemble (1987) and Douce (1985). Alfalfa seedlings were germinated and kept in the dark for 6 to 8 d at 21 C. All of the following steps and centrifugations were done at 4°C with prechilled equipment. Leaf and stem tissue (10 - 20 g) from all seedlings was combined and chopped with scissors into pieces approximately 2 to 4 mm^2 and placed in a Polytron homogenizer. Grinding buffer (5 mM ethylenediaminetetraacetic acid [EDTA], 10 mM N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid; 2-([Hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino) ethanesulfonic acid [TES] pH 7.2, 0.5 M mannitol, 0.2% defatted bovine serum albumin [BSA], 0.05% cysteine) was

added at 50 mL per 10 g tissue. Tissue was immediately homogenized for 8 s at 5.0 Polytron setting. The homogenate was filtered through 2 layers of cheesecloth plus 1 layer of miracloth and centrifuged at 1000 Xg for 10 min in 30 mL centrifuge tubes to separate nuclei and chloroplasts from mitochondria. The supernatants were transferred to fresh tubes and centrifuged for an additional 10 min at 1000 Xg to further remove any remaining nuclei and chloroplasts. The supernatants from this centrifugation were transferred to fresh tubes and centrifuged at 17000 Xg for 10 min to pellet the mitochondria. Supernatants were discarded and the pellets gently resuspended in 500 µL of lysis buffer (20mM EDTA, 50mM Tris-HCl pH 7.5, 3% sodium dodecyl sulfate [SDS]), transferred to Eppendorf tubes and incubated at room temperature for 1 h. Lysates were deproteinated by two extractions with an equal volume of equilibrated phenol:chloroform:isoamyl alcohol (25:24:1), and one extraction with an equal volume of chloroform. Nucleic acids were precipitated by adding sodium acetate to a final concentration of 0.25 M and 1 volume of isopropanol (2-propanol). This remained at -20°C for at least 2 h. Following precipitation, nucleic acids were pelleted at 13000 Xg in a microcentrifuge at 4°C for 30 min. Pellets were washed with cold 70% ethanol and centrifugation repeated for 10 min. Final pellets were air-dried and allowed to dissolve in 100 µL TE (10mM Tris-HCl pH 8.0, 1mM EDTA, pH 8.0). To the samples, 5 µL 1mg/mL ribonuclease A (RNase A) was added and incubated at 25°C for 1 h. Total

volume was increased to 500 µL and one phenol/chloroform/isoamyl alcohol extraction and one chloroform extraction was repeated as before. DNA was precipitated in sodium acetate and isopropanol, washed and centrifuged as described above. Pellets were dissolved in 50 to 100 µL TE. Test restriction endonuclease digestion of DNA was carried out in a total volume of 50 µL under the specific enzyme conditions required. Undigested and digested DNA was separated on 1% agarose gels in Tris-acetate buffer (0.04M Tris, 0.02M sodium acetate, 0.001M EDTA), electrophoresed at 50 V and stained with 0.5 µg/mL ethidium bromide. Gels were observed under 302 nm transmitted UV light.

Mbo I digestion

Undigested sample DNA (donor) was prepared for ligation with lambda vector, following the standard protocol described by Sambrook et al. (1989). Sample DNA was cleaved by partial digestion with restriction endonuclease Mbo I (New England Biolabs). Partial cleavage of high molecular weight DNA will produce a random set of overlapping fragments. The optimum enzyme concentration and digestion time was tested in a series of trial reactions and analysed electrophoretically. Approximately 20 µg DNA per reaction was digested for the amount of time and enzyme concentration that embraced the optimum cleavage conditions. Reactions were conducted in a total volume of 90 µL containing NEB enzyme buffer, 1X BSA and 0.2 units Mbo I. One quarter

of the reaction was removed every 10 min and placed at 65°C for 5 min. EDTA was added to 0.05 M and reaction was temporarily placed on ice. The DNA was either size-fractionated by gel electrophoresis or immediately precipitated in sodium acetate and isopropanol.

Size Fractionation

Size fractionation increases the efficiency of recombinant phage recovered per μg lambda arms used in the ligation and packaging reactions. *In vitro* packaging extracts will discriminate against recombinant DNA molecules that are too small or too large to be packaged if the DNA was not previously size fractionated. Digested sample DNA was size fractionated by electro-elution. The time points of all samples to be fractionated were combined. DNA was separated by electrophoresis at 50 V in a 0.5% agarose gel in TBE buffer (0.1M Tris, 0.1M boric acid, 2.5M Na_2 EDTA pH 8.3) at 4°C for approximately 2 h. The gel was stained with ethidium bromide and examined under UV light. Agarose containing the appropriate size DNA (9 to 23 kb), as indicated by lambda digested *Hind* III marker, was then removed. A well was enlarged in the gel and a dialysis tube membrane to collect DNA was inserted covering the entire well area. The agarose containing the appropriate size of DNA was placed on top of the membrane with a minimum amount of TBE buffer. Electrophoresis was allowed to continue until DNA in agarose had migrated into the buffer contained within the membrane (about 20 V for 30 min). Eluted DNA

was collected, placed in an Eppendorf tube and buffer-saturated butanol was added to remove ethidium bromide. The top layer was discarded and the butanol extraction repeated. DNA was precipitated in sodium acetate and isopropanol and centrifuged as before. The resulting pellet was air-dried and dissolved in TE. The quantity of DNA was determined to 3 to 5μg by visual estimation of fluorescence due to ethidium bromide.

Filling-In Reaction

In order to prevent joining of the partially cleaved donor DNA (which would otherwise be able to form multiple inserts), ends were "filled in" with the deoxynucleotides dA and dG before ligation to vector DNA. Staggered ends with overhanging nucleotides A and G remained. Reactions were conducted in a total volume of 50 μL containing ligation buffer (66mM Tris-HCl pH7.5, 7mM $MgCl_2$, and 100mM dithiothreitol [DTT]), 0.3 mM dATP, 0.3 mM dGTP, and 2 units DNA polymerase I, Klenow (United States Biochemical). The reactions were mixed with a pipet and left for 1 h at 25°C. To stop the reaction, the tube was placed in a 65°C water bath for 5 min then placed on ice. TE was added to bring up the volume to 0.5 mL and phenol/chloroform/isoamyl alcohol extractions were conducted. DNA was precipitated in sodium acetate and isopropanol and the resulting pellet was dissolved in 20 to 25 μL TE.

Vector DNA Preparation

Lambda DASH II (Stratagene) is a replacement vector used for cloning large fragments of genomic DNA and was used as the cloning vehicle. The lambda arms were prepared by Gabriel Meyer (Dept. Biochem, Univ. of Arizona) following the procedure described by Sambrook et al. (1989). The cohesive termini were annealed enzymatically with T4 DNA ligase (United States Biochemical) to form concatenated molecules. The reaction was stopped and gently extracted with phenol/chloroform/isoamyl alcohol, and ethanol precipitated. To remove the stuffer fragment, the lambda DNA was digested with restriction enzyme Xho I and fractionated by gel electrophoresis. The arms were recovered by electro-elution into dialysis membrane and ethanol precipitated. Arms were made compatible with donor DNA by filling-in with deoxynucleotides dC and dT. Staggered ends with overhanging nucleotides C and T remained.

Ligation of Vector and Donor DNA

A series of ligation reactions were conducted to obtain the optimum concentrations of vector and donor DNAs (an equal molar ratio of vector to donor is optimum), following the vector manufacturer's protocol. The ratio of lambda DASH II DNA to sample donor DNA varied in each reaction. The reactions contained ligation buffer, 0.6 mM ATP, 1 unit T4 DNA ligase in a total volume of 5 μL. Before addition of ligase, the mixture was incubated at 42°C

for 30 min then cooled to assure annealing of cohesive ends of vector DNA. Reactions were mixed with pipet and placed in 16°C water bath for 16 to 18 h.

Packaging Reaction

Packaging extracts were prepared by Gabriel Meyer following standard procedure described by Sambrook et al. (1989). Extracts were prepared from two strains each containing an integrated lambda phage with a different amber mutation interrupting the phage assembly in different steps. Both phages were heat inducible and contained a deletion that reduces the frequency of excision of the phage DNA from the chromosome. On mixing of the two extracts and the exogenous DNA (ligation reaction), the latter is packaged in phage particles that can be used to infect bacterial cells. A portion of the ligation reactions (1.5 μL) was mixed with the prepared extracts (5 μL sonic extract and 25-30 μL freeze-thaw lysate) and left at 25°C for at least 2 h (8 h maximum). Volume was increased to 0.2 mL with SM buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM $MgSO_4$, 2% gelatin) plus several drops (10 μL) chloroform. The quality of packaged ligation products were tested by plating on E. Coli host strain, TAP90. Libraries were obtained in the order of 10^4 to 10^6 plaque forming units per mL (pfu/mL) and 10^5 to 10^6 pfu/μg lambda DNA.

Screening the Lambda Library for mtDNA Clones

Plating

To prepare bacterial host strain for plating, cultures were started from a single colony and grown overnight with shaking at 37°C in LB media (5g/L bacto yeast extract [Difco], 10g/L bactotryptone extract [Difco], 10g/L NaCl pH7.2) supplemented with 0.2% maltose, and 15 µg/mL tetracycline. The cells were centrifuged at 1000 Xg for 10 min then resuspended in 0.5 volumes of 10 mM $MgSO_4$ to obtain an approximate OD reading of 0.5 at 600 nm. The cells were stored at 4°C for up to 7 d. Before plating, the phage were preincubated with 50 µL of E. coli (TAP90) for 20 min at 25°C to allow phage to attach to the cells. Three mL of LB top agar (LB media, 7g/L bactoagar [Difco], 2.5g/L $MgSO_4$) at 50°C was added and plated on LB plates (LB media, 15g/L bactoagar). Plates were inverted and incubated 16 to 18 h at 37°C and stored at 4°C. An arbitrary portion of the library was plated for mtDNA screening.

Radiolabeled Probe Preparation

Organelle DNA fragments were recovered and radioactively labeled for use as heterologous probes. Clones containing mtDNA fragments from several plant species (see Table 2 in results) were obtained from Jeff Palmer (Dept. of Biology, Indiana University). The clones were contained in various plasmids as the vector (in XL1Blue host strain). Plasmids were recovered by Chris Michalowski (Dept. of Biochem, Univ. of Arizona) using a plasmid miniprep

procedure. Plasmids containing cpDNA fragments from Vicia faba were obtained from Chris Michalowski. Organelle DNA fragments were separated from the plasmids by restriction endonuclease digestion with the appropriate enzyme (depending on the plasmid used) and fractionation on low-melting (FMC Bioproducts) 1.2% agarose gel in Tris-acetate buffer. Electrophoresis was carried out at 50 V for 1.5 h. The gel was stained with ethidium bromide and visualized under UV light. The desired mtDNA or cpDNA restriction fragment was excised from the gel, placed in an Eppendorf tube, and the agarose melted at 55°C. Alternatively, the digested plasmids were run on a 1% agarose gel, fragment of interest excised, and purified from the agarose with Gene Clean (Bio 101, Inc.), following instructions of the manufacturer. The mtDNA fragments were used to screen the library. The cpDNA fragments were used in Southern analyses.

The restriction fragments were labeled by random primer method (Feinberg and Vogelstein, 1983) in which the Klenow fragment is used to copy single-stranded DNA templates primed with random oligodeoxynucleotides. Labeled deoxynucleoside triphosphates (^{32}P) present in the reaction mixture are incorporated into the newly synthesized complementary strand. The fragment (20-100 ng DNA in 2-6 µL water so that the total volume of the reaction is 25 µL) was denatured at 85°C for 5 min then immediately placed on ice. To the tube was added 1 µL 10mg/mL BSA, 11.4 µL LS solution (440mM HEPES[4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid]-NaOH pH7.6, 44μM each dGTP, TTP, 110 mM Tris-HCl pH7.6, 11mM $MgCl_2$, 22mM 2-mercaptoethanol, oligodeoxyribonucleotide hexamers at 300μg/mL), 30μCi [α^{32} P]dATP at 3000 Ci/mM, 30μCi [α-^{32} P]dCTP at 3000 Ci/mM and 1 unit Klenow fragment. Reactions were incubated at room temperature for at least 4 h. The reaction mixture was run through a column containing Sephadex G-50 medium in 2XTE pH 7.5 to separate unincorporated labeled deoxynucleoside triphosphates from the newly synthesized partially labeled double-stranded DNA. The larger DNA fragments of high radioactivity (as measured with a Geiger counter) were collected and used as the probe. The labeled probe was stored in -20°C for up to two weeks. Before hybridization, the total volume of the probe was increased to 5 to 15 mL with hybridization buffer (0.24% dry milk, 50% formamide, 6XSSC [0.15M sodium chloride, 0.015M sodium citrate]) and denatured in 85°C for up to 15 min and immediately placed on ice to cool for 10 to 15 min.

Filter Preparation

Hybridization was carried out on filter replicas to which the plaques were blotted, as described by Sambrook et al. (1989). Primary screenings were done on 150 mm plates containing up to 10,000 plaques. Further screening of positive recombinants was done on 100 mm plates containing 50 to 300 plaques. Nitrocellulose filter circles (137mm, 0.45μm or 82mm, 0.45μm) were

layed onto the top agar surface and allowed to absorb moisture from the agar. Each filter was marked with a black ballpoint pen for identification and holes notched through the filter and agar to mark the orientation of filter to the plate. Two to three filters per plate were taken in succession with the first filter layed for 1 min, the second for 3 min, and the third for 6 min. Each filter was carefully removed and placed plaque side up in 1.5 mL (82mm) or 2.5 mL (137mm) denaturing solution (0.5M NaOH, 5M NaCl) for 1 min then removed and placed in 1.5 or 2.5 mL neutralizing solution (0.5M Tris-HCl pH 7.5, 3M NaCl) for 2 min twice. Filters were air dried and baked for 1 h at 85°C in a vacuum oven to crosslink the DNA to the nitorcellulose. Filters were washed by boiling in water and 0.1% SDS for 5 min to remove debris.

Hybridization

The filters were prehybridized by incubation at 42°C for 2 h in hybridization buffer at 10 mL per 5 filters in heat-sealed plastic bags. The hybridization solution was removed, the denatured radioactive label was added (5mL per 5 filters) and the bag resealed. Hybridization was carried out at 42°C for 10 to 18 h with agitation. The label was removed and the filters washed twice at 25°C with 2X SSC, 0.1% SDS for 15 min each, and twice with 0.1X SSC, 0.1% SDS for 15 min each. Filters were dabbed with paper towels, wrapped moist in saran wrap and taped to a used piece of x-ray film. For orientation, the tape was marked with radioactive ink (India ink containing ^{35}S radioactive

compound). Filters were exposed to Kodak X-OMAT film with an intensifying screen for 16 to 20 h at -70°C. Probe could be removed from the filters by boiling for 5 min in water and 0.1% SDS three times, each time replacing the water/SDS mixture. The filter could then be rehybridized to another labeled probe. Only filters from primary screens were reused. Filters for purifying screens were used for a single probe only.

Rescreening positive recombinants

Autoradiography of filters bearing DNA from plaques resulted in signals that were intense (dark spots) relative to a weak background. An intense positive signal appearing on duplicate filters and corresponding to a certain plaque was further investigated. Plaques producing positive signals were picked with a pipette and transferred to 500 μL SM with a drop of chloroform. Mixture was briefly vortexed and left at 25°C for 30 min before dilutions. Initially, the solution was titered by serial dilutions of 10^{-1} to 10^{-4} and 10 μL of each dropped onto plated soft top agar (100mm) containing the host bacterial cells. After the phage were absorbed into the agar, the plates were incubated at 37C for 16 to 18 h. Thereafter, 1 to 10 μL of 10^{-3} dilutions were plated on a single plate to obtain the desired number of plaques per plate. While primary screens were done at a high density pfu/plate to examine as many recombinants as possible, secondary (purifying) screens were done at low density to avoid picking more than one plaque. The entire screening procedure was repeated. Plaques were

transferred to nitrocellulose filters, processed and hybridized as described aboved. If a high frequency of positive signals was obtained (> 10%), one plaque corresponding to a positive signal was picked, diluted and plated to test for a pure culture. If all plaques in this screen produced a positive signal, the source of phage was plated at a high density for amplification of the single recombinant (pure culture).

Amplification

Pure cultures were plated to obtain an amplified phage stock. Ten µL of a 1:10 dilution of a plaque picked from the second screen was plated and incubated overnight at 37°C. Five mL SM was added to the plate and agitated for 2 h at 25°C to allow viral diffusion. The SM was collected into sterile 10 mL tubes and centrifuged at 1000 Xg for 15 min to pellet bacterial debris. SM was transferred to a fresh tube and 10 µL of chloroform added. Amplified stock was stored at 4°C.

DNA Extraction and Southern Hybridization

Lambda DNA Isolation

Prior to use in the Southern analysis, lambda DNA was isolated from amplified phage stocks (mtDNA clones) following a procedure described by Carlock (1986). Between 10^8 and 10^9 pfu were combined with 1 mL bacterial cells (overnight culture in 0.5 volumes $MgSO_4$ until OD at 600nm was 2) and adsorbed at 25°C for 20 min. The infected culture was added to 40 mL LB

media in a 200 mL flask and shaken for 16 h at 37°C. This mixture was then shaken with 0.3 mL chloroform for 15 min to kill the bacteria, and transferred to a 50 mL tube for centrifugation at 1000 Xg for 20 min. The supernatant was transferred to a fresh tube with 40 µg RNase A and 200 µg deoxyribonuclease I (DNase I) and incubated at 4°C for 1 h. To precipitate the lysate, 2.3 g NaCl and 4 g polyethylene glycol-8000 was added and dissolved completely before incubation on ice for 2 to 3 h. The precipitate was centrifuged as above and resuspended in 360 µL of a solution containing 50mM Tris-HCl pH8, 10mM EDTA and 33 µl of a solution containing 100mM Tris-HCl pH8, 100mM EDTA, 1% SDS. To deproteinate, 15 µg of proteinase K was also added and the sample was incubated at 37°C for 1 h. The solution was extracted with phenol/chloroform/isoamyl alcohol and the DNA precipitated with 2 to 3 M ammonium acetate and 2 volumes ethanol. The resulting pellet was resuspended in 75 µL TE and the DNA examined by gel electrophoresis. The DNA from the clones was further analyzed by restriction endonuclease digestion and fractionation by gel electrophoresis. One µL of DNA from selected clones was used in random primer labeling reaction for hybridization to Southern blots.

Plant DNA isolation

Total DNA was isolated from all parents and hybrids by a plant miniprep procedure (Dellaponta et al., 1983). Leaf tissue was cut with scissors and placed in liquid nitrogen. Tissue was either stored at -70°C or immediately

placed in a mortar and pestle that had been cooled with liquid nitrogen. One g leaf tissue was ground in liquid nitrogen to a powder and placed in a 30 mL sorvall tube with 15 mL extraction buffer (100mM Tris-HCl pH8, 50mM EDTA pH8, 500mM NaCl and 10mM mercaptoethanol) and 1 mL 20% SDS. Parafilm was placed over the tube which was shaken vigorously and incubated at 65°C for 10 min. Five mL 5 M potassium acetate was added and the lysate was shaken vigorously and incubated on ice for 20 min. The tubes were then centrifuged at 25000 Xg for 20 min at 4°C and the resulting supernatant was filtered through miracloth two times into fresh tubes. Ten mL ice cold isopropanol was added and the solution was mixed and placed at -20°C for 30 min. The tubes were again centrifuged at 20000 Xg for 15 min at 4°C. The resulting pellet was air dried and dissolved in 0.7 mL 50 mM Tris-HCl pH8, 10mM EDTA pH8, and transferred to an Eppendorf tube. Two phenol/chloroform/isoamyl alcohol extractions were conducted, followed by one chloroform extraction and the nucleic acids were precipitated in 0.25 M sodium acetate and isopropanol as described before. The pellets were washed with cold 70% ethanol, centrifuged for 10 min and allowed to air dry. The nucleic acids were resuspended in 100 µL TE. Restriction endonuclease digestion of total DNA was conducted in a total volume of 50 µL containing enzyme buffer and 1 µL 1mg/mL RNase A at 37°C. Additional enzyme was added and the total volume of the reaction increased 10 µL with buffer and

water after 4 h. An aliquot of the reaction was tested for complete digestion by agarose gel electrophoresis. If the digestion was not complete, additional enzyme, buffer, and water was added to the reaction. Total volume of complete digestions was reduced to 40 µL by monitoring volume in a vacuum. Complete digestions were fractionated on 20 cm 1% agarose gels at 20 V for 16 h, each well containing 7 to 10 µg DNA. Gels were photographed using an orange Wratten gelatin filter under 302 nm transmitted UV light and processed for transfer to nitrocellulose.

Southern Hybridization

Total restricted DNA from the gel was transferred to nitrocellulose by Southern blotting (Southern, 1975). Following electrophoresis, the gel was soaked in water for 2 h at 4°C to remove excess acid. The gel was then soaked in 25°C denaturing solution for 20 min with agitation, then in neutralizing solution twice, each time for 20 min. Whatman 3MM filter paper was arranged on a solid support larger than the gel and placed inside a dish containing 10X SSC. The filter paper was saturated with the buffer. The gel was placed upside down on top of the filter paper and nitrocellulose paper (previously wetted in water) was placed on the gel. All exposed filter paper surfaces were covered with Parafilm extending to the edge of the dish. Two additional sheets of filter paper were placed on top of the nitrocellulose sheet, followed by a stack of paper towels (approximately 8cm) and a light weight

compressing all layers. The transfer was allowed to proceed for 16 to 18 h. The blot was placed between paper towels and baked in a 85°C vacuum oven for 1 h. All blots were prehybridized by incubation at 42°C in 5mL hybridization buffer in a heat-sealable bag for 1 to 2 h and hybridized as previously described to a labeled mtDNA or cpDNA probe. Following hybridization, the blots were washed twice at 25°C with 2X SSC, 0.1% SDS for 20 min each then twice at 45°C with 0.1X SSC and 0.1% SDS for 15 min each. The washed blots were wrapped in saran wrap and exposed to Kodak XOMAT film with an intensifying screen for 4 to 48 h at -70°C. The probe could be removed from the blots by boiling for 5 min in water and 0.1% SDS three times, each time replacing the water/SDS mixture. The blots could then be rehybridized to another labeled probe. Autoradiograms were examined for mtDNA restriction fragment length polymorphism between parents and the mtDNA restriction pattern of the hybrids between them.

RESULTS AND DISCUSSION

Library and Clone Analysis

Initially, an attempt was made to isolate intact mitochondria and prepare a lambda library containing only mitochondrial DNA recombinants. The procedure involved loading crude mitochondrial pellets obtained through differential centrifugation onto percoll gradients (40 - 8%) and centrifuging samples at a high speed in an ultracentrifuge (37000 Xg). The resulting DNA from lysed mitochondria was extensively degraded. The protocol was varied by changing the tissue grinding conditions, EDTA concentrations in the buffers, and the amount of tissue. Degradation and probably nuclear or plastid contamination continued to be a problem. As a result of these difficulties, a lambda library enriched for mtDNA was constructed and screened with heterologous mtDNA probes.

An alfalfa DNA library (10^4 to 10^6 pfu/mL) was obtained and was analyzed for the number of recombinants. When total alfalfa DNA was labeled with ^{32}P-deoxynucleotide triphosphates, hybridized to a replicate filter of an arbitrary plating of the library (1000 plaques), and exposed to x-ray film, the autoradiogram showed a high percentage of recombinants. Fifty percent of the plaques displayed positive signals with varying degrees of intensity. Less than 5% gave very strong signals. Since the library had been made from DNA isolated by differential centrifugation, it was hoped that the library would be

enriched for mitochondrial DNA. This would lower the number of recombinants that would need to be screened to find low-frequency mtDNA clones. In an initial experiment 10,000 plaques were screened on duplicate filters with a combination of three probes (totaling 20 kb), 30 duplicate intense positive signals were observed. To know the extent of the enrichment, it would be necessary to compare this to a screen of a library prepared from total genomic alfalfa DNA that was not enriched.

Table 2 lists the heterologous mtDNA gene probes used to screen the library. Alfalfa clones were isolated that hybridized to these probes. Table 3 lists the purified alfalfa clones and estimated molecular weights of the insert sizes in kb. Alfalfa clones were referred to using the gene name from the original organism from which they were isolated. For example, alfalfa clones isolated that hybridized to the maize atpA gene are referred to as an alfalfa atpA clone. DNA was extracted from purified alfalfa mitochondrial clones and their restriction pattern analyzed electrophoretically. The alfalfa mtDNA was restricted with at least one of three enzymes: Eco RI, Bam HI, Hind III. Insert sizes were estimated by measuring the distance the fragments moved from the well and placing that on a regression line of lambda marker DNA (plotted logarithm of length to gel migration distance).

During the screening procedure, it was noticed that the same alfalfa clones that hybridized to the maize 18S/5S rRNA probe also hybridized to the

Table 2. Mitochondrial gene probes used to screen the alfalfa DNA library.†

Species	Gene ‡	Restriction fragment
Maize	atpA	4.2 kb HindIII
Maize	26S rRNA	14 kb BamHI
Oenothera	3' cob	1.5 kb EcoRI
Maize	18S/5S rRNA	6.0 kb BamHI
Oenothera	coxI	2.9 kb HindIII
Citrullus	5' ndh1	726 bp BamHI/HindIII

† Mitochondrial clones were obtained from Jeff Palmer, Dept. of Biology, Indiana University.

‡ Gene nomenclature: atpA encodes the alpha subunit of F_1 ATPase complex; 26S rRNA and 18S-5S rRNA encode ribosomal RNAs; cob encodes apocytochrome b of subunit II; cox I encodes cytochrome oxidase subunit I; ndh1 encodes subunit 1 of NADH dehydrogenase complex I.

Table 3. Alfalfa mtDNA clones isolated from a DNA library which was screened with the heterologous probes listed in Table 2.

Clone	Probe used	Estimated Insert Size (kb)†
1	atpA	13.4
2	atpA	10.4
3	atpA	18.6
4	26S rRNA	12.6
5	26S rRNA	13
6	cob	26
7	cob	24.2
8	cob	10.6
9	cob	10.4
10	18S rRNA, coxI	11.6
11	18S rRNA, coxI, ndh	14.8
12	18S rRNA, coxI, ndh	10.2
13	18S rRNA, coxI, ndh	11.6
14	ndh	5.2
15	ndh	11
16	ndh	11

†Insert size was estimated by linear regression of the logarithm of length against gel migration distance of lambda HindIII and BstEII marker and predicting for unknown fragment size. Standard deviation of estimates are within 1kb.

Oenothera cox I probe. Duplicate secondary screening lifts were hybridized with one of the pair to the 18S/5S rRNA probe and the second to the cox I probe and the same plaques produced positive signals on autoradiograms of both lifts. There were four such clones taken through three screenings. Three of these alfalfa clones also hybridized to the watermelon ndh 1 probe, as indicated by cross-hybridizing plaques during primary and secondary screening. This was further analyzed by Southern blotting of clone restriction fragments (Fig. 1). The ndh 1 probe hybridized to restriction fragments of two of the 18S/5S rRNA clones tested but not to the third clone, as expected from the screening results. Of the remaining three ndh 1 clones isolated, two did not cross-hybridize with the 18S/5S rRNA or cox I probes in the primary screens and the third clone produced a relatively light signal on an autoradiogram of a secondary screening lift hybridized to both the 18S/5S rRNA and cox I probes. These plaques did not hybridize with the Oenothera cob clone, as indicated by screening results. Also, there was no cross-hybridizing between atpA, cob and 26S rRNA clones. That is, autoradiograms of secondary screening lifts that produced positive signals with the maize atpA probe did not produce positive signals with the Oenothera cob probe, and vice versa. This also applied to the maize 26S rRNA probe. Furthermore, a Southern blot of restriction fragments of three of the cob clones did not cross-hybridize to the maize atpA probe. A Southern blot of digested alfalfa cob clones 6, 7 and 8, hybridized with the

Fig. 1. Southern blot of clones 10, 11, and 13 digested with Bam HI and hybridized in **A** with maize 18S-5S rRNA probe and in **B** with watermelon 5' ndh probe. Clones 10, 11, and 13 were originally isolated with the 18S-5S rRNA probe. Clones 11 and 13 were later found to hybridize with the 5' ndh probe. The 2.5 kb fragment contains a region of alfalfa mtDNA that is homologous to both the maize and watermelon gene probes.

Maize cob probe, is shown in Fig. 2.

It is likely that the cox I and 18S/5S rRNA genes are near one another on the alfalfa mitochondrial genome. (In all higher plants studied to date the gene encoding the 5S rRNA is closely linked to the 18S rRNA gene [Lonsdale, 1989].) They could be close enough on the genome that clones contain all or part of both genes. The insert sizes are large enough to contain both genes, but this would depend on the length of the intergenic spacer region between these two genes and the location of Mbo I restriction sites on the outside borders of the genes or within the genes. There is probably not an Mbo I restriction site between these genes, since all clones hybridizing to one gene probe also hybridized to the other gene probe. On the other hand, border sequences on the gene probes used could be homologous to border sequences of the same genes on the alfalfa mitochondrial genome, so that Mbo I restriction sites could exist between these genes and one could still see cross-hybridization with both probes. Even if this were true, however, the genes would still need to be in close proximity to each other for homologous border sequences to cross-hybridize. It is unlikely that there would be a high degree of homology between large intergenic regions. In the few species for which genetic and physical maps exist, most mitochondrial genes appear to be well dispersed on the genome (Lonsdale, 1989). If this is the case in alfalfa, then it is unlikely that the entire intergenic region would be cloned into a single

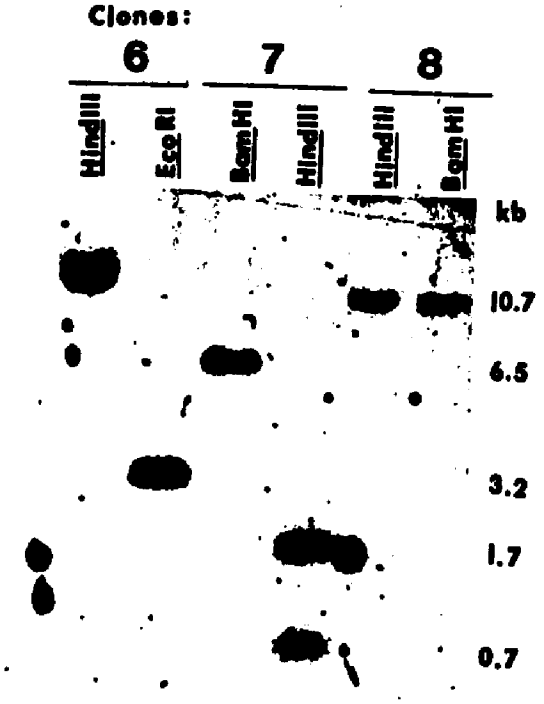

Fig. 2. Southern blot of digested clones **6**, **7**, and **8** hybridized to maize cob probe. These clones were isolated from the library with the maize cob probe. The Southern blot shows the fragments containing regions of the clones homologous to the cob probe.

recombinant. It is most likely that these two genes are physically close to one another without an Mbo I site between them. A Southern blot of digested fragments could distinguish the parts of the clones containing sequences homologous to each of the probes and the size of the restriction fragment(s) hybridizing to both probes could be further analyzed.

Likewise, the ndh 1 gene could be in relatively close proximity to these two genes on the alfalfa mitochondrial genome. Since some but not all clones cross-hybridized to all three probes then it is likely that the ndh 1 gene resides on one side of both genes but not between them. In addition, there must be an Mbo I site between them since the partial digestion produced some fragments containing all or part of all three genes and other fragments containing only the ndh 1 gene. Another possibility is that there may be more than one copy of the ndh 1 gene. It is known that the 18S/5S and 26S rRNAs are duplicated on the mitochondrial genome of wheat (Falconet et al., 1985). Other such duplications have been reported in other species (see Lonsdale, 1989). Finally, I did not test if the 26S rRNA probe would hybridize with the 18S/5S rRNA clones. It is possible that these genes are adjacent to each other on the alfalfa mitochondrial genome. In the maize N mitochondrial genome, the 26S rRNA gene lies between the cox I and the 18S/5S rRNA gene (all within approximately 60 kb) (Fauron and Havlik, 1989). However, it has been noted that the relative order of mitochondrial genes of higher plants does not appear to be conserved

(Gray, 1989).

Alfalfa mtDNA Restriction Fragment Polymorphism

Table 4 lists the parental plants examined for mtDNA polymorphism. The restriction pattern of 50% of the parental plants used in the crosses was examined. Approximately half of the paternal plants were not available for examination. Restriction fragment length polymorphisms unique to parental mtDNA were sought in individual plants. Alfalfa probes revealed two distinct Eco RI polymorphisms among the genotypes studied (Table 5). An atpA probe (clone 1) revealed a 1.6 kb Eco RI fragment unique to the maternal parents, while a 2.0 kb fragment characterized the paternal parents. Variability was also found with a cob probe (both clones 6 and 9). The maternal Eco RI pattern included a unique 9.9 kb fragment, while an 8.4 kb fragment characterized the paternal restriction pattern (Fig. 3). Exceptions to this were noted in 5 of the 25 paternal plants examined. Two plants (Ladak 25 and Travois M8) displayed the maternal Eco RI restriction pattern with these two probes. Indian M18 also displayed the maternal Eco RI restriction pattern but also contained a unique cob fragment at approximately 4.5 kb. Egypt IIID did not display either the maternal 1.6 kb or the paternal 2.0 kb atpA Eco RI restriction fragment. The final paternal plant to exhibit a unique Eco RI restriction pattern was Ladak M1. This plant displayed the maternal 1.6 kb atpA marker but the paternal 8.4 kb cob marker. In addition, Ladak M1 did not display the 5.5 kb cob marker

Table 4. Paternal plants examined with RFLP analysis along with the CMS maternal plants, 6-4 and 4MN-D.

Maternal Parents	Paternal Parents
6-4	CUFA
	Sewa D
4MN-D	Egypt 3A
	Egypt 3D
	Indian M5
	Indian M18
	Lahonton C
	Lahonton E
	Lahonton M27
	Ladak B
	Ladak 12
	Ladak 25
	Ladak M1
	Travois M8
	Chilean M5
	Chilean M12
	Chilean M18
	Chilean M24
	Chilean M30
	DuPuits B
	DuPuits E
	Peruvian M2
	Peruvian M10
	Peruvian M22
	Peruvian M24

Table 5. EcoRI hybridization patterns detected with alfalfa mtDNA MboI fragments.+

13 kb atpA probe		10 kb cob probe	
Maternal pattern	Paternal pattern	Maternal pattern	Paternal pattern
(kb)		(kb)	
		9.9	
			8.4
		5.5	5.5
3.9	3.9		
2.5	2.5		
2.4	2.4	2.4	2.4
	2.0		
1.6		1.6	
1.5	1.5		

+Sizes of hybridized filter-bound fragments detected by autoradiography were estimated by linear regression of the logarithm of length against the gel migration distance of lambda BstEII and HindIII markers and predicting for unknown length. Standard deviation of estimates are within 1 kb.

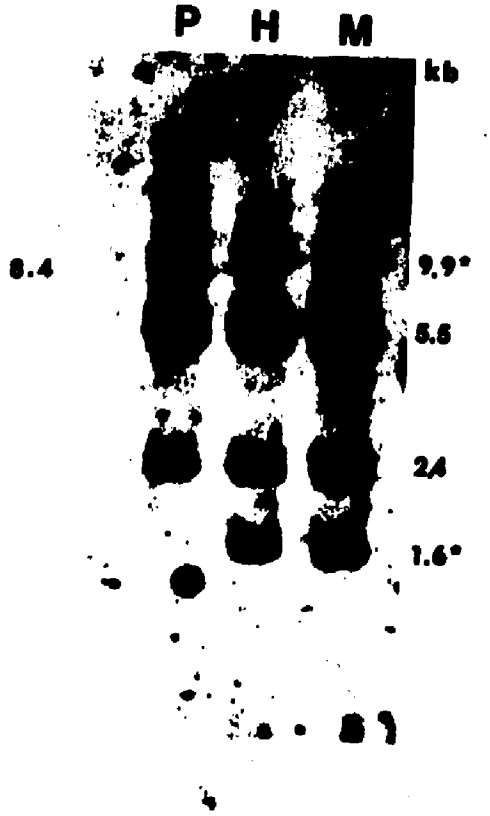

Fig. 3. Southern blot of Eco RI digested alfalfa DNA showing the polymorphism exhibited between parents when hybridized with an alfalfa cob mtDNA probe (clone 9). **Lane P** (paternal), CUFA ♂; **lane H** (hybrid), 6-4 x CUFA; **lane M** (maternal), 6-4 ♀.

characteristic of all the plants, but did exhibit a unique atpA marker at approximately 4.3 kb (Fig. 4). No polymorphism was found in Eco RI or Hind III digests of parental plants with an 18S/5S rRNA probe (clone 13) or a 26S rRNA probe (clone 5). The 18S/5S rRNA probe hybridized to a 10.3 kb Eco RI fragment in every genotype examined and the 26S rRNA probe hybridized to a 2.4 kb Eco RI fragment. Triple digests with Eco RI, Bam HI, and Hind III of parental plants (both maternal plants, and Lahonton E, Ladak 25, Ladak 12, Travois M8, and Peru M2) showed no polymorphism with the cob, atpA, or the 18S/5S rRNA probes. In conclusion, in all parental combinations examined but two, mtDNA RFLPs were identified. These provided codominant, heritable markers to distinguish the mtDNA genotypes of sexual progenies among these parents.

Inheritance of Mitochondrial DNA

A total of 54 F_1 hybrid progenies were analyzed for their Eco RI mtDNA restriction pattern (Table 6). Every progeny plant examined was the result of a cross made on one of two CMS plants as the maternal parent and one of 54 plants as the paternal parent. Tissue from each was kept and evaluated separately. This set of progenies includes a wide range of genetically diverse plants and each of the eight basic germplasm groups was represented about equally among the crosses. In every case, the restriction pattern of the F_1 progeny did not differ from that of the maternal parent (e.g. Fig. 3 - 5). Tissue

Fig. 4. Southern blot of Eco RI digested alfalfa DNA hybridized with an alfalfa atpA probe (clone 1). 6-4 is the maternal parent of three hybrids shown here. Indian M18, one of the paternal parents, has a restriction pattern identical to 6-4 with this enzyme and probe combination. Ladak M1 displays a unique 4.3 kb Eco RI band with this probe.

Table 6. Parental combinations made to produce hybrid progenies examined with RFLP analysis.

Paternal parents		
Crossed to ♀ 6-4		Crossed to ♀ 4MN-D
CUF A	Travois M4	Egypt IIID
Sewa B	Travois M6	Lahonton A
Sewa D	Travois M7	Lahonton C
Sudan D	Travois M8	Lahonton E
Egypt IIIA	Ranger M14	Ladak B
Egypt IIID	Ranger M19	Ladak 12
Egypt IIIE	Ranger M24	Ladak 25
Indian M4	Chilean M5	DuPuits A
Indian M5	Chilean M9	DuPuits B
Indian M9	Chilean M12	DuPuits C
Indian M18	Chilean M15	Dupuits E
Indian M24	Chilean M18	DuPuits G
Indian M26	Chilean M24	
Indian M29	Chilean M30	
Lahonton A	DuPuits B	
Lahonton C	DuPuits E	
Lahonton E	Peruvian M2	
Lahonton M27	Peruvian M10	
Ladak B	Peruvian M22	
Ladak 25	Peruvian M24	
Ladak M1		
Ladak M29		

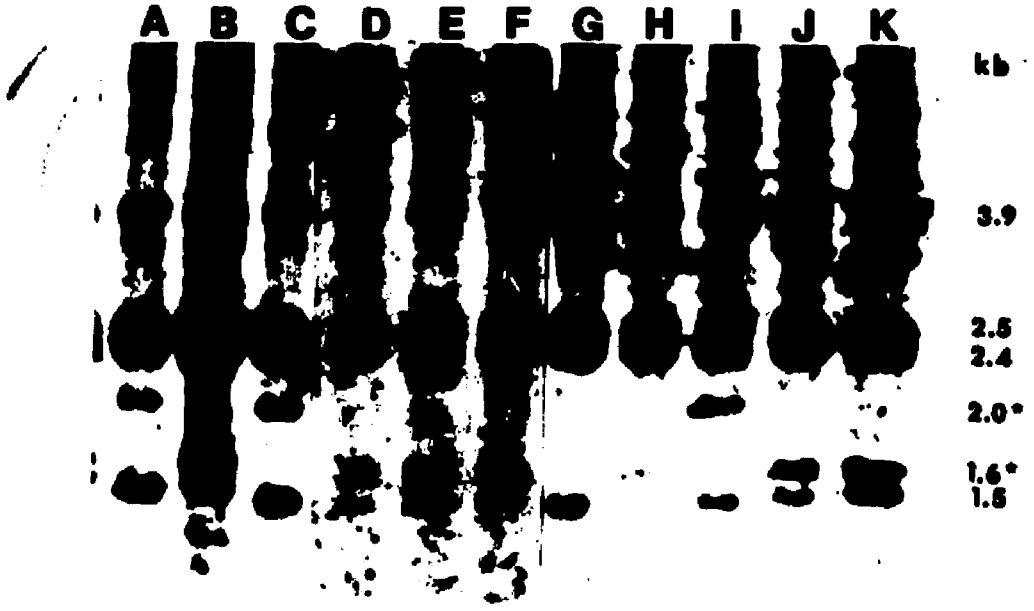

Fig. 5. Southern blot of Eco RI-digested alfalfa DNA hybridized with alfalfa atpA mtDNA probe (clone 1) showing maternal inheritance of mtDNA in alfalfa. Maternal and hybrid plants display the 1.6 kb band, while the paternal plants display the 2.0 kb band, with the exception of Egypt IIID ♂ (Lane G). **Lane A**, DuPuits E ♂; **Lane B**, 6-4 x DuPuits E; **Lane C**, Chilean M5 ♂; **Lane D**, 6-4 x Chilean M5; **Lane E**, 6-4 ♀; **Lane F**, 6-4 x Egypt IIID; **Lane G**, Egypt IIID ♂; **Lane H**, 4MN-D x Egypt IIID; **Lane I**, DuPuits B ♂; **Lane J**, 4MN-D x DuPuits B; **Lane K**, 4MN-D ♀.

was taken from stems containing male-fertile flowers as well as male-sterile flowers from different plants. In both cases, based upon RFLP data, mtDNA was shown to be maternally inherited and therefore the absence of the CMS trait could not be a result of the presence of paternal mitochondria in that tissue. Due to the sensitivity of this method, it can be stated with assurance that paternal mtDNA is absent or very rare in tissue from the sexual progenies examined. It has been estimated that 5 to 10% unique organellar DNA in a mixed sample could be easily detected by autoradiography of ^{32}P probed filters, and that 0.1 to 1% would be possible under optimum conditions (Scowcroft and Larkin, 1981).

The ability to detect paternal mtDNA depends on the frequency of paternal transmission to the progeny. Using a binomial model, it is possible to estimate the probability of finding progeny containing paternal mitochondria in 54 samples (trials) given that the rate of paternal transmission is some number π (Smith, 1988). Discounting the effect of sorting out, if the frequency of paternal transmission is 0.1, that is, 10% of the progeny from a particular cross contain at least 1 - 5% paternal mtDNA, then the probability of not finding progeny containing paternal mtDNA is 0.003. That is, the probability of seeing at least one sexual progeny with paternal mtDNA among 54 sampled progenies is 0.997. If the frequency of paternal transmission is 0.05 (5%), then the probability of finding at least one in 54 is 0.94. However, if the frequency is

0.01 (1%), then the probability of finding at least one in 54 is 0.42. Obviously, the lower the occurrence of paternal transmission, the higher the chance of not observing progenies containing paternal mtDNA in 54 samples. These numbers will be affected by sorting out of mitochondria in the tissue, which in turn largely depends on the maternal to paternal mtDNA ratio in the zygote. The higher the ratio, the sooner sorting out will be complete, favoring cells containing maternal mtDNA.

The question of recombination between interorganelle homologous mtDNA molecules should be addressed. Recombination would involve crossing over and possibly gene conversion. Molecular studies have shown that intergenomic recombination among mtDNAs has occurred in somatic hybrids of tobacco (Nicotiana) (Feges et al., 1990), Petunia (Rothenberg et al., 1985) and Brassica (Morgan and Maliga, 1987) by restriction or physical mapping of novel restriction fragments in the hybrids. Gene conversion among organellar DNA molecules would be more difficult to detect than recombination. Gene conversion would occur during random pairing of two DNA molecules carrying different alleles of a gene. By DNA-repair mechanisms, one allele may be converted to the other, resulting in unpredictable changes in the frequency of alleles (Birky, 1983). If low frequency paternal mitochondrial genes were being converted in this manner, then this would lower the frequency of the paternal allele in the progeny even more. If this were a random event, however, then

maternal mitochondrial alleles should also be affected. The RFLP markers used in this study may be subject to gene conversion in the progenies if recombination were occurring. The more markers used (until the whole mitochondrial genome is examined), the greater the ability to determine if this event were taking place. No evidence of organellar DNA recombination (e.g. novel restriction patterns) in the progenies was observed, however, and it is unlikely that gene conversion affected the results seen in this study.

Alfalfa cpDNA Polymorphism and Inheritance

Since it was known that plastids are inherited biparentally in alfalfa with a strong paternal bias (Smith, 1989b), the filter-bound Eco RI digestions were examined for cpDNA polymorphisms and inheritance patterns. The filters were probed with cpDNA from Vicia faba. A physical map of the fragments used is available (Michalowski et al., 1987). The probe containing Bam HI-fragment 3 revealed a polymorphic Eco RI restriction fragment between 5 and 6 kb (Fig. 6, 7). No Eco RI polymorphism was detected between parental plants with the Bam HI-fragments 12 and 17 or the Sal I-fragment 4 as probes. Using the Bam HI-3 probe, it was possible to distinguish the parental source of cpDNA in 17 hybrid plants (Table 7). Based on this, six progenies (35%) displayed the restriction pattern of the maternal parent while eleven (65%) displayed the paternal restriction pattern. Since the sizes of the polymorphic fragments were very similar, the parental source of the restriction fragment in the progeny was

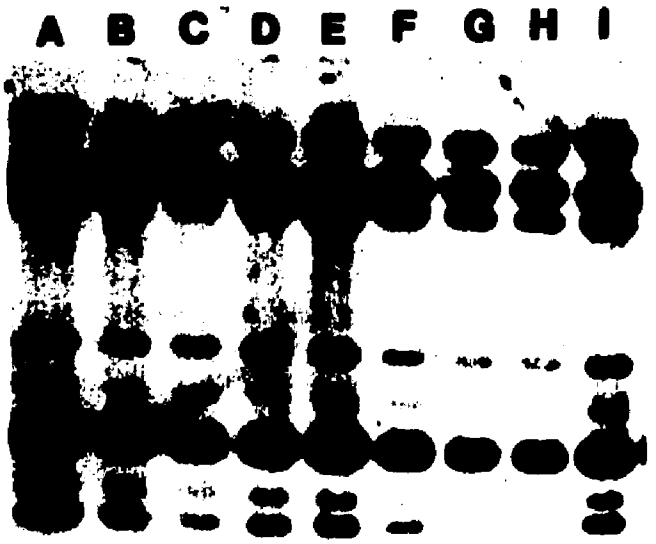

Fig. 6. The same Southern blot as in Fig. 5, hybridized to cpDNA from Vicia faba (Bam HI-fragment 3 and Sal I-fragment 4). **Lane A**, 4MN-D ♀; **Lane B**, 4MN-D x DuPuits B; **Lane C**, DuPuits B ♂; **Lane D**, 4MN-D x Egypt IIID; **Lane E**, Egypt IIID ♂; **Lane F**, 6-4 x Egypt IIID; **Lane G**, 6-4 ♀; **Lane H**, 6-4 x Chilean M5; **Lane I**, Chilean M5 ♂. The top polymorphic band is between 5 and 6 kb. The hybrids in lanes B, D, and F display the paternal RFLP marker; the hybrid in lane H displays the maternal RFLP marker.

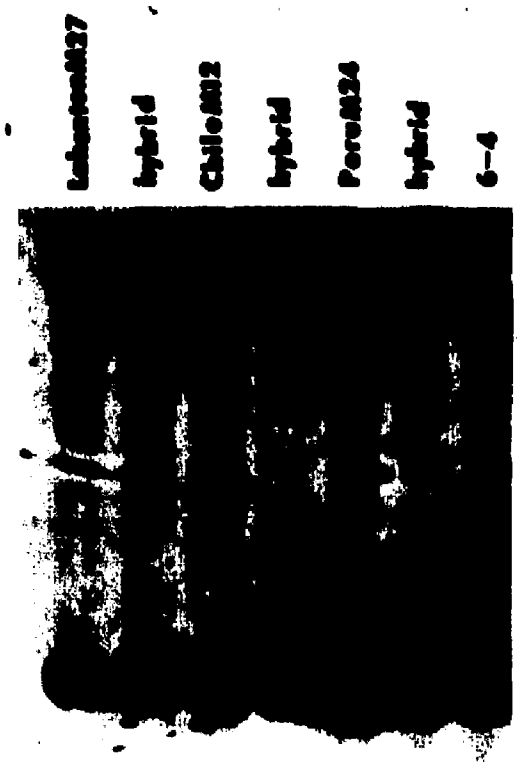

Fig. 7. Southern blot hybridized with cpDNA from Vicia faba (a portion of the Bam HI-fragment 3 clone) showing a mixed inheritance pattern for cpDNA in alfalfa. 6-4 is the maternal parent in all three hybrids shown. 6-4 x Peruvian M24 and 6-4 x Lahonton M27 display the paternal RFLP marker; 6-4 x Chilean M12 displays the maternal RFLP marker.

Table 7. Plant cpDNA inheritance as indicated by RFLP analysis.

Progeny	Inheritance	Fertility†
6-4 x Egypt IIID	Paternal	M
4MN-D x Egypt IIID	Paternal	M
6-4 x Indian M18	Maternal	?
6-4 x Lahonton C	Maternal	R
6-4 x Lahonton M27	Paternal	?
6-4 x Ladak B	Paternal	R
4MN-D x Ladak B	Paternal	M
6-4 x Ladak 25	Maternal	?
6-4 x Travois M8	Paternal	?
6-4 x Chilean M5	Maternal	?
6-4 x Chilean M12	Maternal	?
6-4 x Chilean M18	Maternal	?
6-4 x DuPuits B	Paternal	M
4MN-D x DuPuits B	Paternal	M
6-4 x DuPuits E	Paternal	M
4MN-D x DuPuits E	Paternal	M
6-4 x Peruvian M24	Paternal	?

† Male sterility is maintained (M) or male fertility is restored (R) in the hybrid progenies (Smith et al., 1990).

often difficult to classify. DNA from parental and progeny plants were subsequently digested with other enzymes (triple digests with Eco RI, HindIII, and Bam HI; and double digests with Eco RI and Msp I, Eco RI and Sau IIIA, Eco RI and Alu I, and Eco RI and Hind III) and analyzed by Southern blotting, in order to more readily distinguish the polymorphic sequence. Autoradiograms of the blots hybridized to the same probe did not detect polymorphisms between parents.

More data would be necessary to make inferences on a possible correlation between cpDNA inheritance and CMS in these plants. Pollen scores were available for just 10 of the hybrid plants for which the parental source of cpDNA could be determined (Table 7). Smith et al. (1990) found no correlation between plastid inheritance and CMS, and the little information obtained from this study appears to agree with that finding.

CONCLUSIONS

Organelle Inheritance in Alfalfa

This study provided no evidence for paternal inheritance of mitochondrial DNA to F_1 progenies based on analysis of mtDNA restriction fragment length polymorphisms. This result was somewhat unexpected since previous work by Fairbanks et al. (1989) described mitochondrial-associated RNAs that were present in F_1 progenies and the paternal parent but absent in the maternal parent. These RNAs were believed to be mtDNA encoded, and transmitted to progenies from the paternal parent. The RFLP analysis of this study provides convincing evidence, however, that mtDNA is not being transmitted to progenies through the paternal parent. The rationalizations proposed here to explain this discrepency are that the RNA studied by Fairbanks et al. (1989) is: (1) encoded by alleles in the nuclear genome of the paternal parent, or is expressed only in a particular genetic background as found in the paternal and progeny plants, and (2) is translocated to the mitochondria and cannot be separated from the mitochondria through sucrose gradients or RNase treatment.

I can readily extrapolate the results of my research to indicate that mitochondria are inherited strictly through the maternal parent in alfalfa. Previous ultrastructural work by Zhu et al. (1991) has shown that mitochondria do exist in the mature generative cell of the pollen grain in alfalfa. This

suggests that either immediately prior to or after fertilization, elimination of the paternal mitochondria may occur. There are several mechanisms that may promote uniparental-maternal mitochondrial inheritance in this situation. The first involves physical exclusion, whereby paternal cytoplasmic inclusions containing the mitochondria are released from the sperm just prior to fusion of the male and female gametophytes. This would be similar to what has been observed in barley (Mogensen, 1988), however, in barley only the sperm nucleus fuses with the egg. The exact manner in which the sperm and the egg fuse in alfalfa is still unknown; however, since plastids are inherited through the pollen (Smith, 1989b), obviously some cytoplasmic organelles from the sperm are included in the zygote. Although the mitochondria could become localized in the sperm and released independently of the plastids, it is possible that the entire contents of the egg and the sperm fuse. The ultrastructural studies by Zhu et al. (1990) report an equal distribution of both plastids and mitochondria in the mature generative cell, indicating no localization of cytoplasmic organelles or sperm cell dimorphism in relation to organelle content. Russell (1983) estimated a 15% reduction in sperm mitochondrial numbers in Plumbago at the time of expulsion of the sperm from the pollen tube through loss of cytoplasmic bodies. In any case, at least some mitochondria may be maintained and transmitted to the zygote in alfalfa. A further reduction in mitochondrial numbers through cytoplasmic discharge, however, could have an overwhelming

affect on the persistence of paternal mitochondria in the developing zygote in this case. Genetic data on plastid inheritance in alfalfa casts doubt on physical exclusion as the sole mechanism enforcing maternal mitochondrial inheritance.

The potential for paternal inheritance also depends upon the viability of mitochondria in the sperm. The condition of the mitochondria observed in alfalfa pollen is not known. Debilitation of pollen mitochondria could impair the ability of the organelle to function and replicate in the developing zygote, giving further advantage to the maternal mitochondria. There has been no study to test the physiological condition of pollen mitochondria; however, since paternal plastids are functional and perhaps necessary for the survival of the pollen, this suggests the mitochondria may also be competent.

A second possible mechanism for uniparental-maternal inheritance of mitochondria involves elimination of paternal mitochondria after fertilization. This could occur either by mitochondrial degradation in the zygote or by localization of paternal mitochondria in the portion of the zygote destined to become the suspensor. Degradation of paternal mitochondria in the zygote could involve an enzymatic response in which the maternal homologues are spared while paternal mitochondria degenerate. Evidence for preferential enzymatic degradation of cpDNA from mating type "mt-" in sexual zygotes of Chlamydomonas (Burton et al., 1979; Matagne and Mathieu, 1983) provides a basis for investigation of this phenomenon in higher plants. The strategy

employed by the algae could be based on protection of maternal cpDNA from enzymatic degeneration via DNA methylation activity (Sager et al., 1984).

If the maternal mitochondria so greatly outnumber the paternal contribution in the zygote, then drift during development could also lead to the virtual elimination of paternal mitochondria in the progeny (Birky, 1983). In three alfalfa genotypes studied by Zhu et al. (1991), there were significant differences in mitochondrial numbers per generative cell between genotypes, with mean mitochondrial number equal to 110.3 in one genotype, 54 in another, and 6.7 in the third genotype. There are no estimates of mitochondrial number in the egg cell; however, the numbers are likely to be quite large. Russell (1986a) estimates 14,000 mitochondria in the egg cell of Plumbago. Differences in mitochondrial volumes may also be a factor (Zhu et al., 1991; Russell 1986a). All offspring examined in this study were the result of crosses made using one of two plants as the maternal parent. Differences in the effect of the maternal parent due to differences in mitochondrial numbers in the egg could affect the outcome of mitochondrial inheritance in different crosses. Genetic studies on plastid inheritance in alfalfa has shown that there is a maternal and paternal effect on the frequency of paternal transmission (Smith, 1989b). Thus, there is some heritable aspect to plastid inheritance, which may involve a fixed number of plastids in a defined gametophyte, or an influence of the maternal and paternal genotypes on plastid replication. Likewise, there could be a maternal

factor influencing paternal mitochondria and mtDNA replication. In addition, Zhu et al. (1991) did not find a significant correlation between plastid number in the generative cell as determined by electron microscopy and the ability of the paternal parent to transmit plastids to the progeny according to the results of genetic studies, suggesting that other factors (e.g. genetic, physical) in the zygote are affecting plastid inheritance. Lastly, the results of this study agree with a previous study conducted by Schumann and Hancock (1989). The mtDNA restriction pattern of 30 hybrids from a M. sativa - M. falcata reciprocal cross were shown to be identical to the maternal parent, showing that variation for a mitochondrial inheritance pattern in this cross is negligible.

Finally, mitochondria may be inherited biparentally in alfalfa, but the conservative nature of mitochondrial DNA, especially in the coding regions, does not tolerate sequence differences among mtDNA molecules within a single cell. Plant mtDNA is believed to evolve slowly in sequence (Palmer and Herbon, 1988). Lonsdale et al. (1989) have suggested that plant mtDNA exists as a panmictic population (due to fusion of mitochondria) in recombinational equilibrium, which suppresses nucleotide sequence change through processes such as copy correction. The extent of mitochondrial fusion in sexually produced hybrids is unknown, but studies on cybrids obtained after protoplast fusion suggest that mitochondrial fusion could be a regular occurrence. Intergenomic recombination of mtDNA has been reported in somatic hybrids of

tobacco (Feges et al., 1990); Petunia (Rothenberg et al., 1985); and Brassica (Morgan and Maliga, 1987) upon examination novel restriction fragments in the hybrids. That this has not been observed in sexual hybrids could be due to the apparent low frequency of biparental mitochondrial inheritance. If biparental inheritance occurred, then homologous recombination and copy correction mechanisms could remove unique mtDNA sequences in the progenies, using the more common mtDNA (presumably maternal) as the template, thereby changing heteroplasmic cells to homoplasmic ones. The central question would then be why the copy correction mechanism doesn't work in the somatic hybrids.

Intergenomic recombination of cpDNA in somatic hybrids appears to occur less frequently than mtDNA recombination (Morgan and Maliga, 1987; Kemble et al., 1988; Thanh and Medgyesy, 1989). It has been suggested by Sager (1975) that uniparental-maternal inheritance of extra nuclear organelles is the primary constraint on recombination between organelle genomes. However, studies in some species that inherit plastids biparentally have not found evidence of recombinant plastid genotypes (Kutzelnigg and Stubbe, 1974; Chiu and Sears, 1985). Likewise, although not discussed, no evidence for interorganellar recombination in cpDNA in alfalfa has been detected in several cpDNA RFLP studies of sexual progenies (Lee et al., 1988; Lee et al., 1989; Schumann and Hancock, 1989; Masoud et al., 1990). The difference between

mitochondria and chloroplasts in this regard could be that mitochondria readily fuse and plastids do not. An increasing amount of molecular evidence suggests that these two organelles have very different evolutionary histories.

The most outstanding aspect of cytoplasmic organelle inheritance in alfalfa is the significantly high frequency of paternal plastid transmission. Such high frequencies of paternal plastid inheritance have not been observed in any other angiosperm species studied and may be rare among them. That mitochondrial inheritance follows a different pattern than plastid inheritance in the same plant poses some questions about the evolution of such a phenomenon. Most obviously, why does this plant inherit these two organelles differently? Tilney-Bassett (Kirk and Tilney-Bassett, 1978) has suggested that biparental inheritance of cytoplasmic organelles is the ancestrial form of organelle inheritance and that through either direct or indirect selection uniparental organelle inheritance developed, whether uniparental-maternal or uniparental-paternal. If this were true, we might expect that the same mechanism controlling uniparental inheritance would apply to both organelles. In alfalfa and possibly other angiosperms as well as some gymnosperms, it appears that the mechanisms controlling cytoplasmic organelle inheritance are different for each organelle. If mitochondria are evolutionarily older as organelles than the plastids, then a distinct strategy may have developed specific for mitochondrial inheritance earlier in evolutionary history. This may also explain why

uniparental-maternal inheritance appears to be nearly universal among all organisms. Accordingly, mechanisms later developed to regulate plastid inheritance in plants, which may or may not be the same which govern mitochondrial transmission.

Future Prospects for Research

Ultrastructural studies on the development of the pollen grain have provided a great deal of descriptive information on the morphology of binucleate and trinucleate pollen, including their cellular components. Transmission electron microscopy (TEM) can be used to visualize cellular organelles with very high resolution. In alfalfa, TEM could further help to answer some of the questions surrounding organelle inheritance. In the sperm for example, TEM analysis could ascertain whether localization of the mitochondria was occurring prior to fertilization and investigate signs of mitochondrial degradation.

Recent efforts have been made to isolate sperm cells in sufficiently large numbers for further characterization of their cellular determinants (Russell, 1986b; Hough et al., 1986; Dupuis et al., 1987; Cass and Fabi, 1988). These procedures involve an initial release of the sperm cells from the pollen (the various methods employed include osmotic shock, grinding, and treatment with cell wall degrading enzymes), followed by the separation from other organelles and cytoplasmic debris of the pollen tube by centrifugation. Access to sperm

cells has allowed further ultrastructural characterization (Wagner et al., 1989a), the study of cell surface components using monoclonal antibodies (Pennell et al., 1987), and analysis of protein content (Geltz and Russell, 1988). Although the quality of the isolated sperm can be assessed through ultrastructural evaluation, no direct test on sperm cell function is available (Roeckel et al., 1990). The next phase of sperm cell examination could involve molecular and biochemical analyses, including the cytoplasmic genomes. Probes specific to plastid and mitochondrial DNA could verify the quality and coding capacity of the organelle constituent of the male gamete. Studies on the mtDNA content of alfalfa sperm would help to support or refute some of the above models for organelle inheritance.

Manipulation of the female gametophyte for microscopic examination is impeded by the numerous surrounding sporophytic cells (Huang and Russell, 1989). Protocols for isolation of the megagametophyte are being improved in an effort to more fully characterize the egg cell (Wagner et al., 1989b; Huang and Russell, 1989). These studies are critical for physiological and biochemical analyses of the egg and fertilization processes. Quantitative data on the organelle distribution in the egg cell prior to fertilization is scarce, and would help to clarify some of the observations on organelle inheritance in alfalfa. Ultimately, fertilization and embryo development could be analyzed for cytoplasmic discharge, localization of organelles in the suspensor, and evidence

of organellar degradation. Other possibilities for research could include in situ hybridizations in the zygote with probes sensitive to paternal cytoplasmic organelles.

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