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STUDIES OF TRANSMISSION AND RECOMBINATION OF *OENOTHERA* PLASTOMES

BY

Wan-Ling Chiu

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ABSTRACT

STUDIES OF TRANSMISSION AND RECOMBINATION OF *OENOTHERA* PLASTOMES

By

Wan-Ling Chiu

The likelihood of genetic recombination between higher plant chloroplasts was examined using *Oenothera*, a genus which exhibits biparental plastid transmission. Recombination was not detected among 10,000 progeny from crosses between plants carrying different plastid mutants.

The transmission abilities of the four *Oenothera* plastome types were compared in a constant nuclear background. In crosses between plants carrying mutant and wild-type plastids, the frequency of variegated progeny ranged from zero to 56%. From this frequency and the extent of variegation, it is apparent that both the efficiency and the timing of onset of plastid multiplication after fertilization are plastome-dependent.

Three autonomous replication sequences from the *Chlamydomonas* chloroplast genome were used to probe for homologous sequences in *Oenothera* cpDNA. Differences in hybridization patterns exist among different plastome types. An attempt was also made to test if cyanobacteria can serve as a heterologous system for the functional analysis of cpDNA origins of replication.

This thesis is dedicated to Drs. W. Stubbe and F. Schötz

for their delicate work on

***Oenothera*.**

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INTRODUCTION

The plastid is an essential organelle for the normal function of plant cells. It is responsible for aspects of amino acid biosynthesis and fatty acid synthesis in addition to photosynthesis (reviewed by Kirk and Tilney-Bassett, 1978).

The genome sizes of plastids range from 120 to 217 kb (reviewed by Palmer, 1985). Genes for about 50 chloroplast proteins, 4 different rRNAs, and 30 different tRNAs have been located on the chloroplast genome (Shinozaki *et al.*, 1986). Sequencing of the entire plastid genome (plastome) of tobacco (Shinozaki *et al.*, 1986) and liverwort (Ohyama *et al.*, 1986) has revealed a number of large unidentified open reading frames. Thus, the functions of many genes on the plastome remain to be determined.

Nevertheless, the great majority of genes for chloroplast proteins are nuclear-encoded (Kirk and Tilney-Bassett, 1978). Hence, the development and the normal function of chloroplasts require a close interaction between the genes of the two compartments. Most studies concerning the interaction between the nuclear and plastid compartments have concentrated on the development of photosynthetically active chloroplasts. It is known that the mutation of genes encoding chloroplast components in one

of the two genomes can block the expression of genes in the other (e.g. Mascia and Robertson, 1978). A physiological disturbance of the chloroplast can also affect the expression of nuclear genes encoding chloroplast proteins (Burgess and Taylor, 1987; Mayfield and Taylor, 1987). The close regulation of the interaction between the two genomes is especially obvious when chloroplast genome is placed in a foreign nuclear background. A phenomenon called hybrid bleaching caused by incompatabilities between nuclear and chloroplast genomes is well documented in the genera of *Oenothera* (Stubbe, 1959) and *Pelargonium* (Metzlaff *et al.*, 1982).

Since plastids are indispensible parts of a plant cell, some mechanism is required to ensure the presence of at least one plastid per cell during cell growth and division (reviewed by Boffey, 1984; Possingham, 1984). Clearly, the precision of this process is not equivalent to that of the nucleus, where DNA replicates only once during the cell cycle and where the mitotic spindle assures an equal partition of the genetic material.

Experiments using inhibitors of protein synthesis indicate that nuclear gene products are required for both chloroplast division and cpDNA synthesis (Hashimoto and Murakami, 1983; Heinhorst *et al.*, 1985). Inhibitors of chloroplast protein synthesis affect these two cellular processes to a very limited extent. The presence of cpDNA in chloroplast mutants lacking ribosomes also supports the concept that the processes of plastid multiplication are mainly controlled by the nuclear genome

(Scott *et al.*, 1982). On the other hand, Schötz (1954, 1974) studied plastid transmission in the genus *Oenothera* and suggested that the plastome itself determines the intrinsic multiplication rates of the plastid (reviewed by Kirk and Tilney-Bassett, 1978). According to these studies, both nuclear and plastid genomes can affect the process of plastid multiplication. The interactions between these two compartments in plastid multiplication have not been directly studied, although they must exist.

The characterization of mutations is a basic genetic technique for the identification of gene function. A large number of chloroplast mutants have been isolated (reviewed by Börner and Sears, 1986). Unfortunately, the characterization of plastid mutants has yielded only limited information regarding the function of plastid genes. The site of the genetic lesion is generally difficult to locate, and many of the chloroplast mutations have the same chlorophyll-deficient phenotype and lack photosynthetic activity. The methods used for characterizing mutations in prokaryotes have not been applied to chloroplasts for several reasons. First of all, there is no reliable means yet available to genetically transform the chloroplast. Thus, wild-type or mutant DNA of a chloroplast cannot be returned to the chloroplast to directly test its function. Secondly, recombination between different plastomes has not been observed to occur naturally except in the sexual crosses of the unicellular green alga, *Chlamydomonas* (Gillham, 1978). Therefore, a mutation of a higher plant plastome can neither be characterized by complementation with a wild-type gene nor through classical genetic

mapping. For these reasons, many gene functions in chloroplasts have been studied only in model systems such as unicellular algae or photosynthetic bacteria.

The genus *Oenothera* is uniquely suited as a system for the study of nuclear-chloroplast interactions with regards to both chloroplast development and plastid multiplication. First, plastids *of Oenothera* are transmitted from both parents (biparental transmission) in sexual crosses (reviewed by Kirk and Tilney-Bassett, 1978). Hence, two types of plastids can be brought into the same cell to allow the study of their interactions and to provide an opportunity for recombination events between their plastomes. Second, broad interspecific crosses are possible (Reviewed by Cleland, 1972). The interactions between plastid and nuclear genomes can be studied in a wide variety of plastome-genome combinations. Third, due to extensive chromosomal translocations, certain haploid genomes of *Oenothera* are transmitted as intact complexes during crosses (reviewed by Cleland, 1972). This facilitates the rapid movement of plastids into a desired nuclear background (Stubbe and Harrmann, 1982). Plastome-genome incompatability and differential plastid transmission in this genus have both been well documented (reviewed by Kirk and Tilney-Bassett, 1978). However, in neither case have the the phenomena been characterized at the molecular leval.

The experiments in the first chapter of this thesis were designed to recover wild-type plastome recombinants from crosses between *Oenothera* plants carrying only mutant plastids in their germ layers. The underly-

ing motive for this line of experimentation was the desire to associate regions of the plastome DNA with certain plastid-determined traits, in particular, plastome-genome incompatability and differential plastid transmission.

In order to understand the cooperative interaction between the two genetic compartments in the process of plastid multiplication, the roles of plastid and nuclear genomes should be examined more carefully. In Chapter 2 of this thesis, the transmission abilities of different plastome types of *Oenothera* were examined in a constant nuclear background to exclude the effect of nuclear variability and to concentrate initially on the role of plastome itself in this process.

Much of the data concerning *Oenothera* plastid transmission can be explained by postulating that the plastome exerts control over the multiplication of the plastid (Schötz, 1974). However, no plastid gene products appear to be essential to this process (Scott et al., 1982). This apparent inconsistency could be resolved if some chloroplast DNA structure, such as the origin of cpDNA replication, were the major plastid determinant for the differential plastid transmission observed in *Oenothera*. Chapter 3 of this thesis presents some preliminary efforts towards assessing this hypothesis. DNA hybridization was used in an attempt to see if the frequency and the distribution of cpDNA sequences that may be important for cpDNA replication correlates with the various transmission abilities of *Oenothera* plastids. In order to be able to analyze the functional requirements for a cpDNA origin of replication,

several heterologous systems were tested for their abilities to recognize a chloroplast DNA replication origin from *Chlamydomonas*.

CHAPTER 1

RECOMBINATION BETWEEN CHLOROPLAST DNAs DOES NOT OCCUR IN CROSSES OF *OENOTHERA*

INTRODUCTION

Recombination between chloroplast markers has been observed in the unicellular green alga, *Chlamydomonas* (reviewed by Gillham, 1978). In that organism, recombination between chloroplast DNAs (cpDNA) has facilitated the analysis of chloroplast gene function (Gillham, 1978; Mets and Geist, 1983). With higher plants, investigations into recombination have relied primarily on experiments using somatic hybrids generated from protoplast fusion. Most regenerated somatic hybrid plants carry plastids from only one parent or the other, and initially no recombination between different plastid genomes (plastomes) was detected (Chen *et al.*, 1977; Douglas *et al.*, 1981; Maliga *et al.*, 1981; Flick and Evans, 1982; Schiller *et al.*, 1982; Fluhr *et al.*, 1983, 1984). The first example of cpDNA recombination in higher plants was recovered by selection from a population of 1.9×10^5 calli resulting from cell fusions between two *Nicotiana* lines (Medgyesy *et al.*, 1985). In earlier somatic cell fusion experiments, the limited sample sizes and the lack of good selectable markers may account for the failure to detect chloroplast

recombination.

An attempt was made to search for cpDNA recombinants in sexual crosses of *Oenothera*. In this plant, plastids are often transmitted at a high frequency from both parents in sexual crosses (Schötz, 1954, 1974). Thus, two different plastid types can be combined in the zygote (Meyer and Stubbe, 1974) where there should be ample opportunity for plastid fusion and plastome recombination. An earlier report (Kutzelnigg and Stubbe, 1974) indicated that plastome recombination had never been recovered in crosses of *Oenothera*. However, the great utility of cpDNA recombination as a tool for the study of higher plant chloroplast genetics compelled us to look further for such events.

Oenothera further recommends itself to this type of study for the following reasons: 1) 200-500 seeds can be obtained from a single cross, thus providing a large offspring sample size. 2) Within each *Oenothera* subsection, the plants are generally interfertile, allowing great flexibility in crossing strategies (Stubbe and Herrmann, 1982). Within the subsection *Euoenothera*, Stubbe (1960) has defined five basic plastome types based on genetic criteria, which can be distinguished from each other also on the basis of DNA restriction fragment patterns (Gordon *et al.*, 1982). 3) The five plastid types have different abilities in transmission when placed in competition with other plastid types (Schötz, 1954, 1974). A "strong" transmitting plastome type in the paternal parent results in a higher percentage of progeny carrying plastids from both parents. 4) A wide variety of plastome mutants is avail-

able (Stubbe and Herrmann, 1982).

The experimental plan was to cross plants carrying different plastome types, with each type marked by a mutation that determines a white or pale green phenotype and by a distinguishable cpDNA restriction pattern. The progeny were analyzed for normal green sectors, which would suggest that recombination had occurred between the two mutant loci, resulting in a wild-type segregant.

MATERIALS AND METHODS

Plant Material. Seeds for the various *Oenothera* plastid mutants (Table 1) were kindly provided by Prof. Wilfried Stubbe at the University of Düsseldorf. For each of the mutants, the Roman numeral indicates the plastome type, while the Greek letter is the mutant designation. As determined by Hallier (1980) and summarized by Stubbe and Herrmann (1982), the plastome I mutants used here and the plastome III-gamma mutant have defects in photosystem I; plastome mutant II-beta has an abnormal ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) activity. The II-iota and IV-alpha mutations appear to be different from some of the other mutations based on ultrastructural characteristics (Kutzelnigg et al., 1975a,b); the IV-alpha mutation affects photosystem I, while the II-iota mutation is pleiotropic (R.G. Herrmann, personal communication).

Crosses and Selection of Presumptive Recombinants. Since most chloroplast mutations of higher plants involve photosynthesis-related

defects, they would be lethal if grown in the homoplasmic condition in the greenhouse or in the field. In order to obtain sexually mature plants carrying these defective plastids, the white tissue is maintained in combination with photosynthetically-competent green tissue in chimeric plants. Crosses were made between such plants, having a periclinal variegation pattern and carrying one of eleven different *Oenothera* plastome mutations in their L2 tissue layer (Table 1). For any given leaf, this tissue layer is continuous with the germ line of the flower occurring at the same node (Kutzelnigg and Stubbe, 1974). Thus, it is possible to recognize flowers that should have germ lines containing only mutant plastids. The mutants of plastomes I, II, and III were maintained with wild-type plastome IV in the variegated plants, while mutant IV-alpha was in a plant carrying wild-type plastome I. Seeds were surface-sterilized with 20% commercial bleach (Big Chief, Sodium hyperchlorite 5.25%), and germinated on 0.8% agar medium without growth regulators. Seedlings in the cotyledon stage were examined under a dissecting microscope for green sectors on the cotyledons. Seedlings having green sectors were propagated through shoot culture on NT agar medium (Nagata and Takebe, 1971) with hormone concentrations described by Stubbe and Herrmann (1982). Green shoots were selected during each transfer. When adequate numbers had been propagated, roots were induced by placing the shoots on medium containing 15 ug/ml naphthalene acetic acid. The plants were then transferred to soil and grown in the greenhouse.

Isolation of Chloroplast DNA. Mature leaves were homogenized in 6% (w/v) sorbitol, 6 mM EDTA, 1 mM ascorbic acid, 3 mM cysteine, 0.3% (w/v) polyvinyl pyrrolidone (PVP), 0.1% (w/v) BSA, pH 7.5. The homogenate was filtered through one layer of 100 um mesh gauze and two layers of Miracloth (Calbiochem). Following differential centrifugation and a wash with the same medium lacking PVP, the chloroplasts were purified by sucrose gradient centrifugation. The chloroplasts were lysed with 1 mg/ml pronase (Sigma) and 1% (w/v) sarkosyl (Sigma). The chloroplast DNA was purified by CsCl gradient centrifugation.

Analysis of Chloroplast DNAs. cpDNAs were digested with Bam HI restriction endonuclease (BRL) and compared by agarose slab gel electrophoresis.

RESULTS

Selection of presumptive plastome recombinants. The number of seedlings examined from each cross of the first field season is shown in Table 1. Only eleven seedlings out of 7671 had small green sectors on the cotyledons and all of these variegated progeny came from the cross between III-gamma (maternal) and IV-alpha (paternal). The green sectors could have arisen from recombination between the two mutant plastomes, simple complementation, or transmission of a wild-type plastome from green tissue of the variegated parents. As a control, the parental plants had been self-pollinated. The offspring of the self crosses were examined: 18 out of 379 seedlings issuing from the III-gamma self-pollination were solid green; the self-pollination of the IV-alpha

Table 1. Phenotypes of progeny from crosses of plastome mutants (first season).

The nuclear genotype is *Oenothera hookeri* str. Johansen (AA), except as noted: [a]albilaeta (AB) and [b]albirubata (AB). Mutants of plastomes I, II, and III were grown in the field in combination with wild-type plastome IV. The one mutant of plastome IV was grown in combination with wild-type plastome I. The crosses were performed as described by Stubbe and Herrmann (1982). Seeds were surface sterilized and germinated on sucrose-supplimented agar medium. The seedlings were scored about two weeks after germination for the coloration of their cotyledons.

Table 1.

Parental plastid type M x P	Number of progeny			
	White	Green	Variegated	Total
IV-alpha x I-zeta	384	0	0	384
IV-alpha x I-eta	471	0	0	471
IV-alpha x I-nu	625	0	0	625
IV-alpha x I-chi	425	0	0	425
IV-alpha x I-omega	825	0	0	825
IV-alpha x I-psi[a]	8	0	0	8
IV-alpha x II-iota[b]	294	0	0	294
IV-alpha x II-beta[a]	34	0	0	34
IV-alpha x III-gamma	811	0	0	811
III-gamma x I-zeta	348	0	0	348
III-gamma x I-eta	274	0	0	274
III-gamma x I-chi	855	0	0	855
III-gamma x I-nu	95	0	0	95
III-gamma x I-omega	490	0	0	490
III-gamma x I-psi[a]	24	0	0	24
III-gamma x II-epsilon	178	0	0	178
III-gamma x II-iota[b]	224	0	0	224
III-gamma x II-beta[a]	338	0	0	338
III-gamma x IV-alpha	957	0	11	968

parent yielded 2 variegated seedlings out of 462 total. These results suggested the 11 presumptive recombinants could be due to transmission from the dark green wild-type tissue of either parent. The reciprocal cross (IV-alpha X III-gamma) would be more likely to yield a high frequency of progeny inheriting plastids from both parents (Chap. II), and yet no green sectors were observed. This also suggests that inadvertent transmission of wild-type plastids from the chimeric background might have occurred.

Attempts were made to establish the 11 variegated seedlings resulting from the III-gamma X IV-alpha cross as shoot cultures. Two cell lines were lost due to contamination, and in a third the green sector did not persist. DNA was isolated from the eight remaining presumptive recombinants for further analysis.

Chloroplast DNA Analysis. Chloroplast DNA was isolated from the plastome mutants III-gamma and IV-alpha as well as from wild-type plastomes III and IV. Using the BamHI enzyme, their restriction patterns were compared. As shown in Figure 1A, no major DNA alteration can be detected between either mutant and its wild-type counterpart. Since chloroplast DNA from the mutant plants was less readily available, cpDNA from the wild-type plastomes was used for most of the subsequent restriction pattern comparisons.

Recombinants derived from the III-gamma X IV-alpha cross would be expected to have plastome DNA with DNA fragment patterns similar to plas-

Figure 1. CpDNA restriction fragment patterns.

CpDNA was digested with BamHI and electrophoretically separated on 1.0% agarose gels. A, DNA restriction fragment patterns of wild-type and mutant plastomes III and IV. Lanes 1 and 2 contain wild-type and mutant cpDNA from plastome III of *O. grandiflora*. Lanes 3 and 4 contain wild-type and mutant cpDNA from *O. ammophila*. B, cpDNA restriction fragment patterns of eight presumptive recombinants and wild-type plastomes I, III, and IV. Lanes a-h contain cpDNAs from the eight presumptive recombinants.

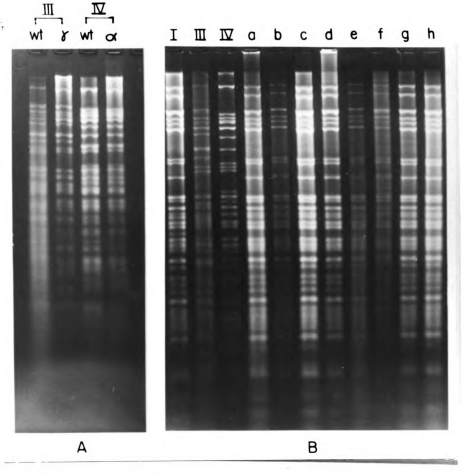


Figure 1.

tome III, plastome IV; or a combination of the two. In contrast, if the green plastids came from the transmission of wild-type plastomes from the parental plants, either plastome I (maintained with IV-alpha) or plastome IV (maintained with III-gamma) should be detected. It is clear from Figure 1B that all eight presumptive recombinants have the same Bam HI digestion patterns as plastome I. Apparently, they all resulted from the inclusion of cells containing wild-type plastids in the germ line of the paternal plant.

In the second field season, crosses between five different plastid mutants were performed. A significant frequency of progeny were pure green or variegated (Table 2). Judging by the transmission patterns of wild-type *Oenothera* plastids (Chapter 2 of this thesis), these green plastids also can be traced back to one of their chimeric parental plants. The pure green progeny probably obtained their wild-type plastids from the maternal side, whereas, the green plastids in the variegated progeny could be from either parent, depending on the type of plastome they represent (refer to Chapter 2 of this thesis). The plants that probably had carried wild-type plastids together with mutant plastids in their germ lines were indicated with asterisks in Table 2. Almost all of the crosses involving mutant IV-alpha resulted in some variegated or green progeny due to the inclusion of wild-type plastids in the germ layer. Plastid mutant IV-alpha was maintained as a chimera with wild-type plastome type I. Since plastome type I can transmit itself very successfully when contributed by either parent (Chapter 2 of this thesis), it is not surprising to find the contaminated wild-type

Table 2. Phenotypes of progeny from crosses of plastome mutants (second season).

The variegated parental plants carried the plastome mutants indicated in the maternal (M) and paternal (P) parents. The green plastids of the chimeric parents were of the plastome type IV, except for the plants which contained mutant IV-alpha. The latter carried wild-type plastome type I plastids.

Table 2.

Parental Plastid type M x P	Number of progeny White	Green	Variegated	Total
I-eta x II-epsilon	447	0	0	447
I-eta* x III-gamma	279	7	0	286
I-eta x IV-alpha*	449	0	3	452
I-zeta x II-epsilon	212	0	0	212
I-zeta x III-gamma	17	0	0	17
I-zeta x IV-alpha*	35	0	1	36
II-epsilon x I-eta	176	0	0	176
II-epsilon* x I-zeta	592	17	4	613
II-epsilon x III-gamma	10	0	0	10
II-epsilon x IV-alpha*	51	0	1	52
IV-alpha x I-eta	162	0	0	162
IV-alpha* x II-epsilon	21	16	0	37

(*) indicates the parental plants that may have carried wild-type plastids in addition to the indicated mutant plastids in their germ lines.

plastome type I in some progeny of crosses involving mutant IV-alpha. Wild-type plastome type IV was the wild-type plastid in the variegated plants carrying the rest of the plastid mutants. As plastome type IV has a very low transmission frequency when contributed from the paternal side (Chapter 2 of this thesis), the green progeny in crosses I-eta x III-gamma and II-epsilon x I-zeta are likely to be wild-type plastome type IV contributed from the maternal side. In the face of such a high background, no attempt was made to screen all of these variegated progeny as before in order to recover true recombinants from the progeny of this field season.

DISCUSSION

For the majority of higher plants in which plastids are transmitted only from the maternal side, the opportunity for different chloroplast DNAs to recombine is rare. Although chloroplasts with different genetic markers can be brought together into the same cell by fusion of somatic cells, here too is little opportunity for recombination. The segregation of chloroplast markers is usually complete at the stage when a plant is regenerated, and, hence, the fusion products generally display only one of the parental chloroplast types (Chen et al., 1977; Douglas et al., 1981; Maliga et al., 1981; Scowcroft and Larkin, 1981; Flick and Evans, 1982; Schiller et al., 1982; Akada *et al.*, 1983). Variegated plants containing persistent mixed chloroplasts within individual cells were found in some fusion experiments (Fluhr et al., 1983; Gleba *et al.*, 1985), yet there was no evidence for recombination between chloroplast DNAs (Fluhr et al., 1984). If recombination does occur between chloroplast DNAs but

at a low frequency, the sample sizes of most somatic fusion experiments were probably too small to permit detection.

Indeed, by using a large sample size (2 X 10^3 to 2 X 10^4 heterokaryons) and four selectable markers, one somatic hybrid line of *Nicotiana* containing a recombinant plastid type was recovered (Medgyesy *et al.*, 1985). In this single recombinant plastome, at least six recombination sites are indicated from the physical map constructed from the restriction patterns of the cpDNA.

In all but one of the crosses from the first field season described here, the slower-multiplying plastids were carried by the female parent which contributes larger numbers of plastids. The faster multiplying plastids were transmitted through the pollen. Thus, in most crosses, 25-50% of the offspring should contain chloroplasts from both parents in the cotyledon (Schötz, 1954; Sears, 1983; Chapter II of this thesis). Despite ample opportunity for different plastomes to recombine, only 11 out of 7500 progeny contained green sectors. According to the analysis of their cpDNA restriction patterns, these green sectors contained only plastids derived from the wild-type tissue of the chimeric plant.

Taking the results of this experiment at face value, it would appear that recombination between chloroplasts in *Oenothera* is very rare, much less than 0.05%. Since the sites of the mutations in these particular chloroplast mutants have not been mapped, the low frequency of chloroplast recombination may be caused by close linkage of these mutations on the plastome. This is unlikely, for most of the mutants have

distinctly different lesions, based on physiological (Hallier, 1980) and/or ultrastructural (Kutzelnigg et al., 1975a, b) criteria.
Since heteroplasmic cells are not rare in the young seedlings of *Oenothera* (Schötz and Heiser, 1969), and extensive cpDNA exchange between two plastomes has been demonstrated for *Nicotiana* (Medgyesy *et al.*, 1985), the largest barrier for the recovery of recombinant plastome must result from the low frequency of plastid fusion. Only in the zygote of *Chlamydomonas* has chloroplast fusion been demonstrated both microscopically (Cavalier-Smith, 1970) and genetically (reviewed by Gilham, 1978). Among the higher plants, electron microscopic evidence indicates that plastid fusion may occur in *Mimosa pudica* (Esau, 1972) and *Hosta* (Vaughn, 1981). In cell fusion experiments, the use of PEG to initiate the fusion of cell membranes might have facilitated the fusion between plastid membranes as well.

From a critical viewpoint, it is not clear that the experiments described here would have been able to detect recombination even if it did occur. The number of cell divisions which occurred to produce the cotyledons may not be adequate to allow the segregation of the rare recombinant cpDNA molecules and the plastids containing these recombinants (Michaelis, 1967). Without a selectable plastid marker (e.g. antibiotic resistance), it is particularly important to allow a sufficient number of cell divisions to take place before screening for green sectors. Rather than scoring cotyledons directly, it would have been preferable to induce callus formation from the cotyledon and regenerate plants from such calli. In fact, this method has been used to recover

the scarce paternal plastids from the progeny of sexual crosses of *Nicotiana* (Medgyesy *et al.*, 1986).

In conclusion, the apparent absence of recombination between different plastomes may be due to the limited number of cell divisions allowed before screening for such an event. On the other hand, the rarity of cpDNA recombination may be due to the lack of chloroplast fusion in nature. The highly conservative nature of the chloroplast genome could be one consequence of the low frequency of recombination between chloroplast DNAs (Sears, 1980, 1983).

CHAPTER 2

PLASTID INHERITANCE IN *OENOTHERA*: ORGANELLE GENOME INFLUENCES THE EXTENT OF BIPARENTAL TRANSMISSION

Introduction

Maternal inheritance is the rule for plastid transmission in approximately two-thirds of the angiosperms thus far examined (reviewed by Kirk and Tilney-Bassett 1978; Sears 1980). In these cases, the absence of plastids of paternal origin in the progeny is usually correlated with the physical exclusion of plastids from the pollen generative cell or with the degeneration of plastids during pollen maturation (Hagemann, 1979; Whatley, 1982; Connett, 1987). In contrast, some genera, such as *Oenothera* and *Pelargonium*, have generative cells that contain numerous plastids, and exhibit high frequencies of biparental plastid inheritance.

Studies on plastid transmission in *Pelargonium* have concentrated on a nuclear gene locus *Pr* (for *p*lastid *r*eplication) (reviewed by Kirk and Tilney-Bassett, 1978). Plastid inheritance patterns in crosses between *Pelargonium* cultivars are predominantly under the control of the *Pr* alleles of the female parent. The proportion of offspring inheriting plastids from only the maternal parent, from only the paternal parent, or from both parents, depends on the maternal genotype at this locus. A

number of possible mechanisms for *Pr* controlled plastid transmission have been suggested by Tilney-Bassett and Birky (1981). In all cultivars studied, *Pelargonium* plastids themselves also have some effects on the transmission patterns: white plastids usually have very poor transmission rates in comparison with wild-type green plastids.

In contrast, a consistent maternal predominance in plastid inheritance has been observed in *Oenothera*, as studied through interspecific crosses. Schötz (1954, 1968, 1974, 1975) compared the transmission abilities of wild-type green plastids from 28 species of *Oenothera* by using them as the female parent in crosses in which the male parent carried one of several mutant plastids. For each cross, Schötz reported a variegation rating, which was defined as the percentage of white tissue out of all the seedlings. Based on his extensive studies, Schötz concluded that the variegation rating of each cross was dependent on the types of plastids contributed by both parents. Three classes of plastids in the subsection *Euoenothera* were identified: strong, medium and weak, with respect to their ability to compete with the plastids contributed by the other parent (Schötz 1968, 1974, 1975). Although a strong maternal bias is consistently seen, a higher frequency of biparental progeny occurs when a female carrying a "weak" plastome type is crossed with a male carrying a "strong" plastome.

Independently, Stubbe categorized the *Oenothera* plastomes according to plastome-genome compatability (Stubbe, 1959). After performing interspecific crosses and observing a range of hybrid bleaching due to

plastome-genome incompatibilities, Stubbe classified haploid nuclear genomes of *Oenothera* species into three major groups: A, B, and C, which in combination make six basic types of diploid nuclear genomes (AA, AB, BB, BC, CC, AC). Plastomes were classified into five groups, namely type I through type V (Stubbe 1959), according to the compatibility of each plastome in association with the six basic nuclear types of the subgenus *Euoenothera*. Subsequent work has shown that these five groups are distinguishable in their chloroplast DNA restriction patterns (Gordon *et al.* 1982). According to Schötz' study, the strong plastids in his classification correspond to types I and III, medium to type II and weak to type IV (Schötz, 1968).

An effect of the *Oenothera* nuclear background on plastid transmission has been noted (Kemper, 1958; Schötz, 1974, 1975). Schötz (1974, 1975) performed crosses in which the pairs of plastids being compared remained constant but the hybrid genome varied. Although the relative order of transmission abilities was the same for the plastome types, the variegation ratings changed markedly in different hybrid nuclear backgrounds. Furthermore, a plastid tended to transmit itself better in a cross in which the hybrid genome of the progeny was the same as its native nuclear background. From both the *Pelargonium* and *Oenothera* studies, it is clear that the effect of the nuclear genome cannot be neglected when analyzing the plastid transmission pattern. However, based on Schötz' studies (1954, 1968, 1974, 1975), it is widely accepted that *Oenothera* plastids themselves are the major determinant in the process of plastid transmission, with the nuclear genome having only a minor effect

(reviewed by Sager, 1972; Hagemann, 1975; Kirk and Tilney-Bassett, 1978).

In order to explore the process of plastid transmission and elucidate the control of plastid multiplication at the molecular level, a greater understanding of the processes governing plastid transmission is required. In this report, the transmission abilities of *Oenothera* plastid types I through IV are compared systematically in a constant nuclear background, to determine more clearly the differences in transmission that are attributable to the plastome type alone.

The experiments described here differ from previous experiments in that: (1) all the crosses were performed in a constant nuclear background, to focus on the role of the plastome in determining transmission, (2) reciprocal crosses were performed, to allow us to exclude marker effects and more accurately assess the maternal predominance, (3) all seeds were germinated and scored in a sterilized condition on sucrose-supplemented agar medium, to extend the life time of predominantly white seedlings and to allow full expansion of their cotyledons.

Material and Methods

Plant material and crosses. All four plastid types (I-IV) used in our crosses are photosynthetically competent in plants of nuclear type AA (Stubbe, 1959). Crosses were performed with the plants of *Oenothera hookeri* str. Johansen, having a nuclear type A_1A_1 as defined by Stubbe (1959), and carrying a representative of one of the four basic wild-type

plastomes of the subgenus *Euoenothera*: plastome I, native plastid of *O. hookeri* str. Johansen; plastome II, *O. suaveolens* Grado.; plastome III, *O. lamarckiana*; plastome IV, *O. atrovirens*. Plastome V was not used because it is not viable in this nuclear background due to severe plastome-genome incompatibility (Stubbe, 1959). Seven spontaneous plastome mutants from different *Oenothera* species (Stubbe and Herrmann 1982) were used in the test crosses. Among them, I-zeta (isolated from *O. hookeri* str. Johansen) and I-beta (isolated from *O. hookeri* std.) have defects in photosystem I or cytochrome f complex, respec tively while I-eta (isolated from *O. elata*) has defects in both of these two photosynthetic complexes. Mutant II-gamma (originating from *O. suaveolens* Grado.) has a defective photosystem II whereas III-gamma (originating from *O. grandiflora* has a defective photosystem I. The mutations in II-epsilon (derived from *O. suaveolens* Fünfkirchen) and IV-alpha (derived from *O. ammophila*) have not been characterized.

Since the plastid mutants are chlorophyll-deficient and are not able to support photosynthesis, each mutant is maintained through the propagation of variegated plants that carry wild-type plastids along with the mutant plastids. Flowers having only mutant plastids in their germ lines can be recognized by the variegation pattern of the leaf at the same node: periclinal chimeras, which should contain only mutant plastids in the L2 tissue layer of these leaves, were used (Kutzelnigg and Stubbe 1974). Thus, reciprocal crosses were made between variegated (white plastid donor) and wild-type plants. These crosses are referred to as <u>White x Green</u> when the female parent donated the white plastids or

Green x White when the male parent provided the mutant plastids in the cross.

Germination and scoring of the seedlings. Seeds were surface sterilized with 20% bleach and germinated on MS basal medium (Murashige and Skoog, 1962) with 0.3% sucrose and 0.8% agar. Seedlings were scored for variegation after their cotyledons were fully expanded. The frequency of biparental plastid transmission is presented with a 95% confidence interval estimated by assuming a normal distribution of frequencies (Steel and Torrie, 1980). When the mutant plastids were contributed by the female, the area of the green sectors was also estimated as a fraction of cotyledons for each variegated seedling. Schötz (1954, 1974, 1975) and Kemper (1958) combined the areas of cotyledons and primary leaves to determine a variegation rating; however, we have not included the first pair of leaves in our estimation. The primary leaves are derived from the apical meristem which is composed of only a small number of cells within the embryo and hence represents a much smaller population of plastids from the zygote as compared to the cotyledons.

Results

Progeny from reciprocal crosses between *O. hookeri* str. Johansen carrying different wild-type and mutant plastids were scored for biparental inheritance, operationally defined as the appearance of variegation, that is, green and white sectors in the same seedling. Since all the mutant plastids were maintained in periclinal variegated plants, precautions were taken to avoid contamination of the germ line by green plastids introduced by aberrant cell division in neighboring green tissues

(Tilney-Bassett, 1986). We have found that such contaminating green cells introduced from a neighboring cell layer tend to give rise to egg cells with only wild-type plastids. Flowers containing these green plastids will produce some pure green progeny in the otherwise White x Green crosses. Pure green progeny were detected in three out of thirty-three White x Green crosses. Thus, for these crosses in which solid green progeny were detected, the results were examined by comparing with the same crosses from a second field season. Although it is easy to recognize the unexpected solid green progeny in White x Green crosses, progeny derived from eggs with mixed plastids (Stubbe, 1957; Gleba et al., 1985) rather than pure mutant plastids would be difficult to detect. However, when the results from two field seasons were compared, the frequency of biparental transmission from one season was within the 95% confidence interval of the other. These data have been summed and are presented in Tables 3 and 4.

White x Green crosses. The plastid inheritance patterns of crosses between female parents carrying one of seven different mutant plastids and male parents carrying one of four different wild-type plastids are shown in Table 3. With a constant pollen source, the frequency of transmission of the male plastid varied, depending on the plastid type in the female parent. When a wild-type plastome type I was contributed from the male parent, the progeny always included variegated seedlings, although the percentage of progeny carrying plastids from both parents varied, ranging from 9.7% to 40.9%. The transmission of wild-type plastome type III from the male parent was very similar to the transmission of plastome

Table 3. Biparental transmission of chloroplasts in White x Green crosses[a]

CROSSES		TRANSMISSION				Abundance of paternal plastids in
Female	Male	Maternal	Biparental	N	%Biparental[b]	BP progeny[c]
I-beta	I	52	36	88	40.9 ± 5.2	n.m.*
I-zeta	I	93	10	103	9.7 ± 2.9	0.30
I-eta	I	86	23	109	21.1 ± 3.9	0.30
II-epsilon	I	88	32	120	26.7 ± 4.0	0.47
II-gamma	I	132	47	179	26.3 ± 3.3	0.40
III-gamma	I	159	44	203	21.7 ± 2.9	0.42
IV-alpha	I	84	56	140	40.0 ± 4.1	0.56
I-beta	III	66	20	86	23.3 ± 4.6	0.38
I-zeta	III	141	8	149	5.4 ± 1.9	0.25
I-eta	III	346	76	422	18.0 ± 1.9	0.23
II-epsilon	III	260	23	283	8.1 ± 1.6	0.23
II-gamma	III	286	86	372	23.1 ± 2.2	0.28
III-gamma	III	160	61	221	27.6 ± 3.0	0.25
IV-alpha	III	61	62	123	50.4 ± 4.5	0.43
I-beta	II	392	25	417	6.0 ± 1.6	0.19
I-zeta	II	101	0	101	0	—
I-eta	II	201	4	205	2.0 ± 1.0	0.06
II-epsilon	II	100	2	102	2.0 ± 1.4	0.13
II-gamma	II	127	5	132	3.8 ± 1.7	0.16
III-gamma	II	160	5	165	3.0 ± 1.3	0.09
IV-alpha	II	104	17	121	14.0 ± 3.2	0.28
I-beta	IV	264	1	265	0.4 ± 0.4	0.20
I-zeta	IV	164	0	164	0	—
I-eta	IV	372	0	372	0	—
II-epsilon	IV	99	0	99	0	—
II-gamma	IV	296	2	298	0.7 ± 0.5	0.16
III-gamma	IV	192	5	197	2.5 ± 1.1	0.13
IV-alpha	IV	230	1	231	0.4 ± 0.4	0.06

[a]The nuclear background of both female and male parents was *O. hookeri* str. Johansen (nucler type A_1A_1). Variegated female plants, centaining only mutant plastids in the germ line were crossed with lines carrying green wild-type plastomes I, II, III, or IV.

[b]The biparental transmission frequencies are presented with a 95% confidence interval estimated by the normal approximation (Steel and Torrie, 1980).

[c]Average total area of tissue in the biparental progeny containing paternal plastids as a fraction of the two cotyledons.

*n.m. = not measured.

type I in the frequency of biparental plastid inheritance and it was always present in some fraction of the progeny of each cross. When the male plant carried wild-type plastome type II, the frequency of seedlings with both plastid types ranged from non-detectable to 14%. Plastome type IV contributed from the pollen could be detected in progeny from only four out of seven crosses, with the highest frequency of progeny with biparental plastid inheritance being 2.5%.

Figure 2 shows a comparison of the transmission abilities of the four wild-type plastids with the female parent held constant. The biparental transmission frequencies can be ranked in the order I>III>II>IV in crosses involving five out of seven mutant plastids. In crosses where III-gamma and IV-alpha were contributed from the female parent, plastome type III from the male parent gave a higher frequency of biparental progeny than did plastome type I.

Except for crosses in which wild-type plastome type IV was contributed from the male parent, the frequency of biparental plastid transmission and the average size of the green areas in the cotyledons of the biparental seedlings are highly correlated (Table 3). The overall correlation coefficient, r=0.853, is significant at the 99% confidence level. The extent of variegation in the progeny from crosses in which male parent contributed wild-type plastome type I or III is illustrated in Figure 3. Although the frequencies of biparental plastid inheritance are similar in these crosses (Table 3 and Figure 2), when plastome type III is carried by the pollen, smaller green sectors are seen in the

Figure 2. Frequency of biparental transmission in White x Green crosses.

Progeny from crosses between *Oenothera hookeri* str. Johansen plants carrying one of seven different mutant plastids and plants carrying one of four wild-type plastids were scored for biparental inheritance of plastids. The bars indicate the biparental transmission frequencies from crosses grouped according to the maternal plastid types. The mutant designations are abbreviated as follows I-b for I-beta, I-z for I-zeta, I-h for I-eta, II-e for II-epsilon, II-g for II-gamma, III-g for III-gamma, IV-a for IV-alpha.

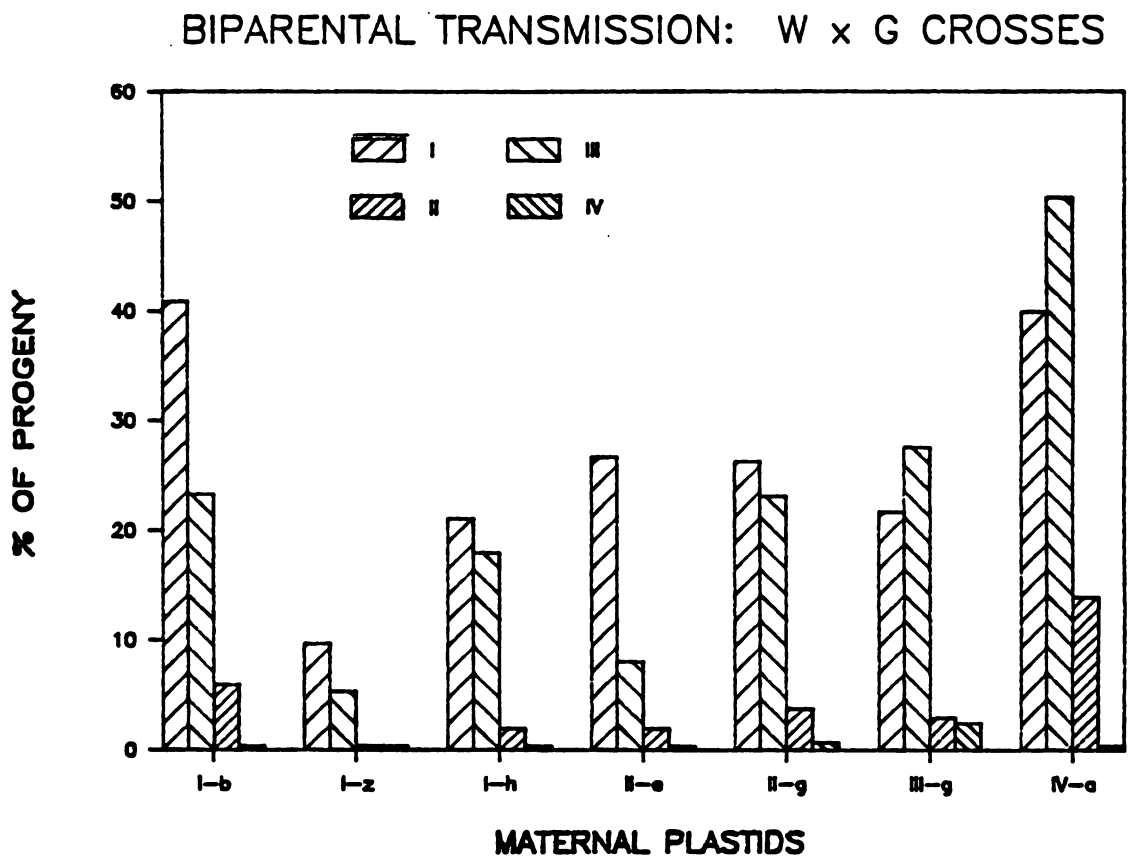
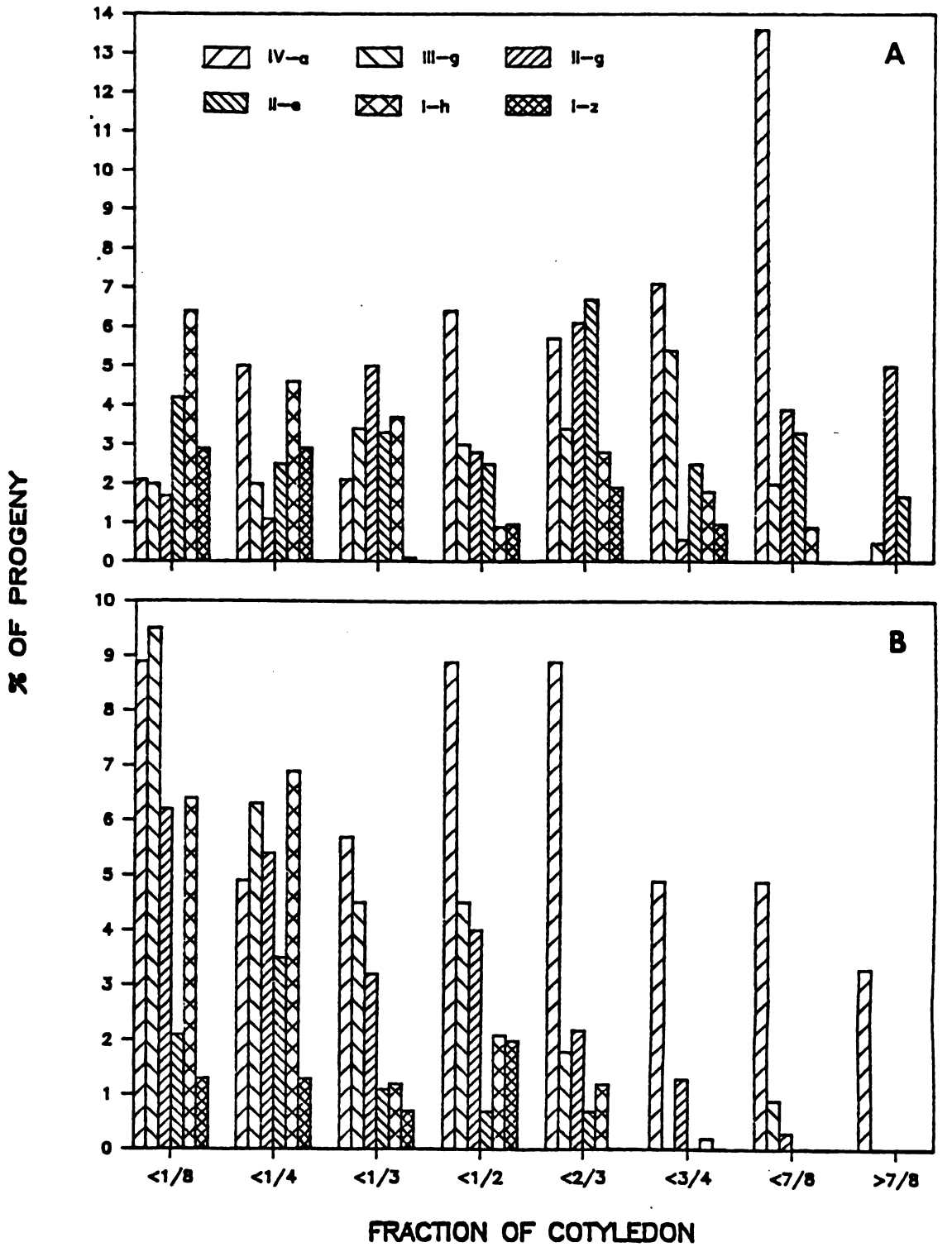

BIPARENTAL TRANSMISSION: W x G CROSSES
% OF PROGENY
60
50
40
30
20
10
0
I
II
III
IV
I–b
I–z
I–h
II–e
II–g
III–g
IV–a
MATERNAL PLASTIDS

Figure 3. Abundance of tissue containing paternal plastids in variegated progeny from White x Green crosses.

In the crosses illustrated, either wild-type plastome type I (A) or type III (B) were used as paternal plastid. The area of green tissue was scored as a fraction of the total area of the two cotyledons. The height of the bars show the percentage of progeny exhibiting the degree of variegation, which is indicated on the X-axis. The abbreviations of the mutants are the same as in Figure 2.


IV–a
III–g
II–g
II–e
I–h
I–z
A
B
% OF PROGENY
<1/8
<1/4
<1/3
<1/2
<2/3
<3/4
<7/8
>7/8
FRACTION OF COTYLEDON

variegated progeny than when plastome type I is contributed by the pollen.

Green x White crosses. For most of the crosses listed in Table 3 reciprocal crosses were performed. Biparental transmission frequencies of these crosses are presented in Table 4. When wild-type plastome I or III was contributed by the female parent, the frequency of biparental seedlings in the progeny was low. In contrast, the presence of a wild-type plastome IV in the female was associated with a very high incidence of biparental plastid transmission. Wild-type plastome II in the female parent of the corresponding crosses resulted in a wide range of biparental transmission depending on the plastid-type contributed by the male parent. These data are illustrated in Figure 4, where they are grouped according to the plastid type of the male parent. With the male parent constant, the biparental plastid transmission frequencies vary according to the plastid type of female parents.

Statistical analysis of biparental transmission frequencies. The biparental plastid inheritance frequencies listed in Table 3 and 4 were transformed into arcsine and subjected to a randomized complete-block analysis (Steel and Torrie, 1980). Results of these analyses are presented in Table 5. The F ratio for all three terms: the maternal, paternal, and the interaction between these two terms, are significant. Since the nuclear background is constant throughout the experiment, the plastome type is the only variable in both maternal and paternal terms. In both sets of crosses, the variance attributable to the type of paternal plastome is greater than that attributable to the type of maternal

Table 4. Biparental transmission of chloroplasts in Green x White crosses[a]

CROSSES		TRANSMISSION			
Female	Male	Maternal	Biparental	N	%Biparental
I	I-beta	167	2	169	1.2 ± 0.8
I	II-gamma	9	0	9	0
I	III-gamma	83	7	90	7.8 ± 2.8
I	IV-alpha	206	0	206	0
III	I-beta	246	19	265	7.2 ± 1.6
III	I-eta	89	20	109	18.4 ± 3.7
III	II-gamma	96	4	100	4.0 ± 2.0
III	III-gamma	219	21	240	8.8 ± 1.8
III	IV-alpha	224	2	226	0.9 ± 0.6
II	I-beta	145	22	167	13.4 ± 2.4
II	I-eta	7	9	16	56.3 ± 12.4
II	II-gamma	85	0	85	0
II	III-gamma	106	49	155	31.6 ± 3.7
II	IV-alpha	171	2	173	1.2 ± 0.8
IV	I-beta	135	37	172	21.5 ± 3.1
IV	I-zeta	215	104	319	32.6 ± 2.6
IV	I-eta	80	87	167	52.1 ± 3.9
IV	II-gamma	173	39	212	18.4 ± 2.7
IV	II-epsilon	216	38	254	15.0 ± 2.2
IV	III-gamma	107	136	243	56.0 ± 3.2
IV	IV-alpha	142	0	142	0

[a]The nuclear background of the plants are the same as in Table 1.

Figure 4. Frequency of biparental transmission in Green x White crosses.

The bars indicate the biparental transmission frequencies from crosses grouped according to the paternal plastid types. The abbreviations of the mutants are the same as in Figure 2.

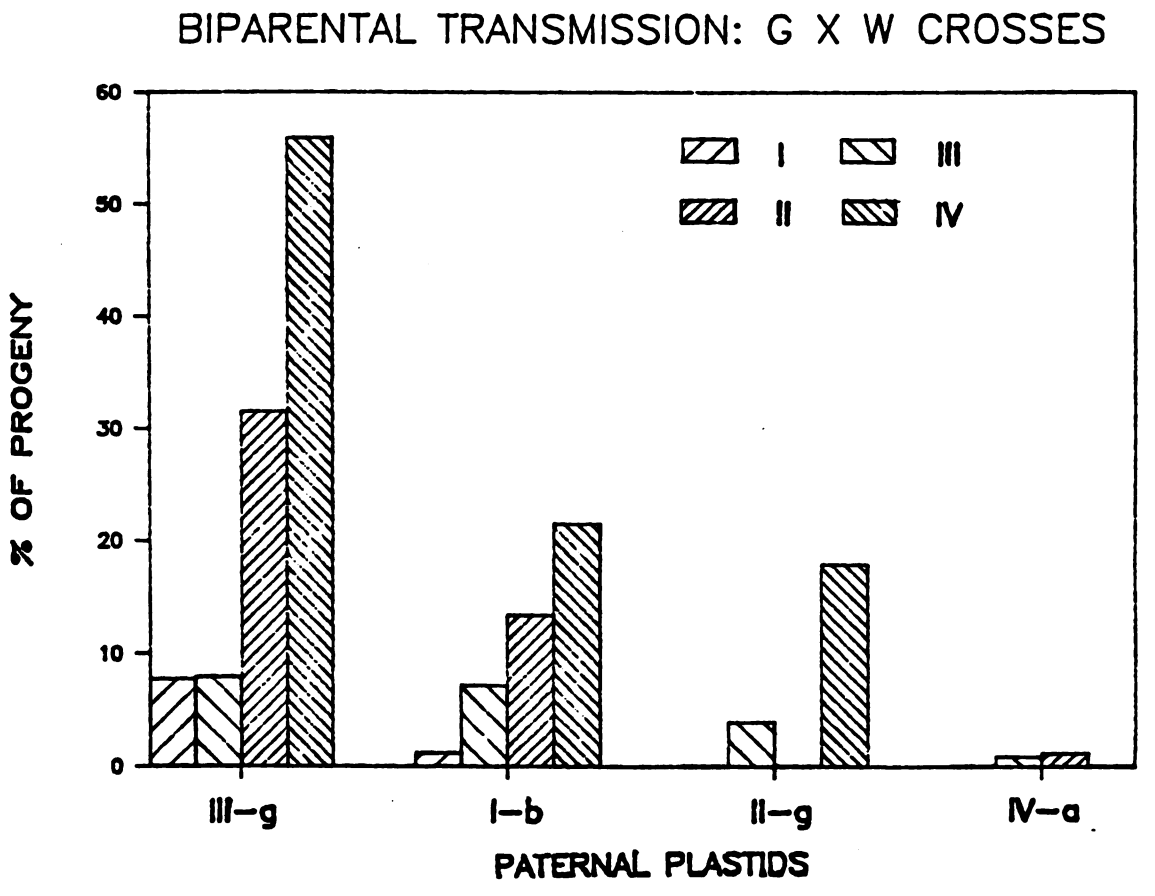

BIPARENTAL TRANSMISSION: G X W CROSSES
% OF PROGENY
60
50
40
30
20
10
0
I
II
III
IV
III–g
I–b
II–g
IV–a
PATERNAL PLASTIDS

Table 5. Analysis of variance of biparental transmission frequencies

	White X Green			
	d.f.	MS	F	σ^2p/σ^2m
Maternal	6	156.38	7.21**	
Paternal	3	1216.72	56.11***	5.1
M X P	18	21.69	5.16**	
Error	∞	4.21		

	Green X White			
	d.f.	MS	F	σ^2p/σ^2m
Maternal	3	376.94	5.62*	
Paternal	4	769.31	11.76**	2.9
M X P	12	65.41	12.28**	
Error*	∞	5.30		

*, P < Ø.Ø5; **, P < Ø.Ø1; ***, P < Ø.ØØ1
*Theoretical variance for arcsine transformation

plastome in the cross.

Comparisons of different mutant plastids. Crosses were performed in which the female contributed one of three different plastome I mutants. The result was three distinguishable transmission patterns (Figure 2). The transmission abilities of the three plastids when contributed from the female parent are in the order of I-zeta>I-eta>I-beta.

When III-gamma and I-eta were contributed by the male parent, these two mutants gave a high frequency of biparental progeny (Table 4). But when contributed by the female parent, their transmission abilities were relatively low, comparable to the two plastome II mutants (Figure 2). Similarly, when mutant I-beta was used as the maternal plastid, as high a frequency of paternal plastid transmission occurred as when mutant IV-alpha was donated by the maternal parent (Figure 2). However, when mutant I-beta was contributed from the paternal side, it showed a much higher transmission ability than did mutant IV-alpha (Table 4).

Discussion

Oenothera is known to transmit plastids from both parents in crosses, but not all crosses result in a high frequency of biparental inheritance (reviewed by Kirk and Tilney-Bassett, 1978). The frequency of biparental transmission can be influenced by both nuclear and plastid genomes, but the relative importance of these two components is not clear. Studies of *Pelargonium* have demonstrated a strong nuclear control of the plastid transmission patterns (Tilney-Bassett, 1979; Tilney-Bassett and Birky,

1981). The experiments presented here have demonstrated that, in *Oenothera*, plastid transmission depends on the types of plastome and the interactions between the two types of plastids contributed by both parents. These effects are superimposed on an intrinsic maternal predominance.

In this report, the transmission abilities of four major plastid types of subgenus *Euoenothera* are compared in a constant nuclear background. The four wild-type plastids show very different transmission abilities in the nuclear background of *Oenothera hookeri* str. Johansen (nuclear type A_1A_1). Plastome type I, the most successful plastome, was transmitted to some fraction of the progeny in all crosses. Plastome type III appeared to have a transmission ability very close to that of plastome type I, but plastome types II and IV showed much weaker transmission abilities when compared to the others. Hence, in this nuclear background, the relative transmission abilities of the four representative plastomes are in the order of I>III>II>IV. These results agree with Schötz' conclusions from comparisons made in various nuclear backgrounds (Schötz, 1974).

How can the great variation of biparental plastid transmission be explained? Several hypotheses for the variation of plastid inheritance have been suggested. The simplest explanation is a variation of input: gametes with different plastid types carry different numbers of plastids. However, if the biparental transmission frequency simply reflects the input numbers of plastids from both parents, one would con-

clude that the male gametes that produce almost no biparental progeny should contain no or very few plastids. Yet the same pollen can be crossed to a different female parent and make a significant contribution to the plastid population of the progeny (Table 3 and 4). Likewise, if the female parent is held constant (such that the plastid input from the female parent is constant), different frequencies of biparental transmission are observed when the plastid type of the pollen is varied. Furthermore, Schötz (1954) found that plastid numbers did not differ significantly in the egg cells of different *Oenothera* species, yet different frequencies of biparental progeny were obtained when these plants were crossed with a constant pollen source. In short, differential input of plastids in the gametes cannot be the major cause of the plastid-dependent variations in *Oenothera* plastid transmission.

A second hypothesis is that differential destruction of plastids and/or plastid DNA destruction occurs during fertilization, perhaps through a DNA modification/restriction system in the zygote, as postulated by Sager and Kitchen (1975). According to this theory, critical events would occur shortly after gamete fusion. However, our observations suggest that the extent of plastid transmission from the male parent is affected not only by the events of fertilization, but also by later events. As shown in Table 3 and Figure 4, crosses in which wild-type plastome I or plastome III is contributed by the male parent produce similar frequencies of biparental progeny, suggesting that they are equally successful in their initial transmission (or survival) within the fertilized egg. However, as shown in Figure 3, biparental progeny

that receive wild-type plastome I from the male parent tend to have much larger green sectors than those that receive wild-type plastome III from the pollen. This suggests that the critical stage for the determination of the degree of maternal predominance extends beyond the fertilization event. Furthermore, an electron microscopic study of zygote development in *Oenothera* did not indicate degeneration of organelles from either parent following fertilization (Meyer and Stubbe, 1974). Thus, the differential destruction hypothesis seems unlikely for this plant.

Another hypothesis suggested by Sager (1972) as well as Tilney-Bassett and Birky (1981) to explain genetic data of biparental plastid inheritance is the gene conversion model. This hypothesis suggests that recombination between different plastomes in the zygote frequently occurs and the subsequent DNA repair is biased through the use of the "strong" plastome as template. Existing data do not support this theory: (1) plastid fusion is infrequent in higher plants (reviewed by Sears, 1980), and (2) even under conditions designed to select infrequent cpDNA recombinants, none have been recovered from biparental progeny of *Oenothera* (Kutzelnigg and Stubbe, 1974; Chiu and Sears, 1985).

A fourth hypothesis to explain the differential transmission of *Oenothera* plastids was originally proposed by Renner (1924, 1929) and also advocated by Schötz (1954). This hypothesis postulates that different plastids have different intrinsic rates of multiplication. According to this hypothesis, the faster replicating plastid in mixed cells will be propagated at the expense of the slower one, due to a copy

number limit on the plastids in each cell (Kirk and Tilney-Bassett, 1978). Consequently, the frequency of the slower multiplying plastid in subsequent cell generations will be decreased. The competitive advantage of the faster plastid will exist until the two types of plastids sort out completely. Schötz and Heiser (1969) analyzed the plastid content of heteroplastidic mesophyll cells of variegated progeny and concluded that the crosses which produced a higher variegation rating also had mixed cells with a higher frequency of plastids of paternal origin. Although this is not direct evidence for differential multiplication of plastids in mixed cells, all the genetic studies reported thus far are consistent with this hypothesis.

Our data support the hypothesis that the differential transmission of *Oenothera* plastids is caused, in part, by plastid-dependent differences in multiplication. First, the analysis of variance suggests that, with the nuclear background held constant, the transmission of plastids is dependent upon the plastid types of both parents and the interaction between these two types of plastome (Table 5). Secondly, the plastid types that had the highest transmission frequencies when contributed from the male parents were also the most successful when contributed from the female parent. Thirdly, with the plastid from the male parent constant, the White x Green crosses that produced the highest frequencies of biparental seedlings usually gave rise to the largest sectors of green tissue in those biparental seedlings (Table 3), which is an indication of earlier plastid segregation. The number of cell divisions required for complete sorting-out is dependent on the total number of segregation

units and the input ratio of one plastid type to the other in the mixed cell (Michaelis, 1967). Assuming a constant plastid input at fertilization, the dependence of biparental transmission frequency on plastome type must be achieved by the relative multiplication abilities of the two types of plastid within the mixed cells. Finally, this model can partially explain the absence of plastids from the male parent in all of the progeny of some crosses: a plastid contributed by the pollen may not be detected if it has a double disadvantage of a significantly lower input as well as a lower multiplication efficiency compared to that of the maternal plastid.

However, differences in multiplication rates alone cannot explain all the observed differences in plastid transmission. If the multiplication rates were the only determinants, then one would expect to see the same biparental transmission frequencies for all crosses in which the green and white plastids have similar multiplication rates. In fact, crosses with the "stronger" plastids and their corresponding mutants (e.g. I-zeta x I) resulted in higher biparental transmission frequencies than equivalent crosses with "weaker" plastids (e.g. IV-alpha x IV). These results could be explained if the initiation of multiplication of the plastid from the paternal parent is generally delayed in comparison to the plastids from the maternal side and if the period of delay depends on the plastid type (Meyer and Stubbe, 1974; Hagemann, 1976). In general, plastids from the male parent in young zygotes are smaller, and they do not contain as many starch grains as plastids derived from the female (Diers, 1963; Meyer and Stubbe, 1974). It is possible that male

plastids take a longer time than female plastids to accumulate factors required for multiplication. The higher variance values attributable to the paternal plastome type compared to that of maternal ones (Table 5) lend support to this hypothesis.

If a lag period does in fact precede multiplication of paternal plastids in the zygote, this would explain some of the discrepancy between biparental frequencies and the extent of variegation in certain crosses. Crosses in which the mutant I-zeta came from the maternal side and wild-type plastome I or III was contributed by the male parent provide an example. In these crosses, the biparental transmission frequencies were low, but the sizes of green sectors in the biparental progeny were comparable to those crosses with higher frequencies of biparental transmission (Table 3). Evidently, the two measurements are sensitive to two related but separable phenomena, and we suggest that there are two stages that affect the transmission pattern: the first stage determines whether any plastids of paternal origin will be transmitted to the progeny, and the second stage determines the extent of variegation.

Early embryogenic development in *Oenothera* offers support for the idea that there might be two different stages determining the transmission pattern. The first division of the zygote is asymmetrical. The much larger of the two daughter cells develops into the suspensor, and the smaller terminal cell develops into the main body of the embryo (Renner, 1915). Since the mixing of plastids can be very limited in the zygote (Meyer and Stubbe, 1974), a delay in the multiplication of paternal

plastids compared to that of maternal plastids before zygote division may greatly decrease the probability of their entering the terminal cell and hence lower the observed frequency of biparental progeny. In later stages of embryo development, however, the relative success of the two different types of plastids in multiplication determines the relative proportion of the two different types of plastids in mixed cells, which, in turn, determines the extent of variegation in the seedling. The differences between wild-type plastome type I and III when contributed from the paternal side, as illustrated in Figure 3, could result from differential plastid multiplication in the second stage.

In *Pelargonium*, mutant plastids generally do not transmit as well as wild-type plastids (Kirk and Tilney-Bassett, 1978). However, Schötz' (1975) studies of *Oenothera* suggested that plastid mutants and their corresponding wild-types have comparable transmission frequencies. In our experiments, several plastid mutants showed differences in transmission compared with the wild-type plastomes. In one case, three mutant type I plastomes showed three distinguishable transmission abilities in crosses with wild-type plastids. The plastid mutant I-zeta is very similar to the wild-type plastome I in its transmission ability, and in fact, it was isolated from *O. hookeri* str. Johansen, the same source as the wild-type plastome I representative. The mutants I-beta and I-eta have much lower competitive abilities compared with I-zeta when contributed by the female parent. These mutants were isolated from *O. hookeri* standard (nuclear type A_2A_2) and *O. elata* (nuclear type A_1A_1) respectively. Although it is possible that the mutations carried by the

plastomes affect their competitive abilities, the variation in transmission abilities could also reflect differences inherent to the various plastids that have been grouped as plastome type I.

In most cases, the relative competitive ability of a plastid does not depend on whether the plastid is contributed by the female or the male parent. Crosses involving plastid mutants III-gamma, I-beta and I-eta are exceptions to this rule. These three plastid mutants showed greater competitive abilities when contributed by the male parent than by the female parent. One explanation for this difference is that some physiological functions of the maternal plastids could be important for efficient self-multiplication and hence, a high degree of maternal predominance.

Although our experiments employed *Oenothera*, the concepts defined by this investigation may be pertinent to plastid transmission in higher plants in general. Even in plants such as *Nicotiana* and *Epilobium*, considered to inherit plastids in a strictly maternal fashion, trace amounts of plastid DNA from the male parents can be detected in the progeny (Medgyesy *et al.*, 1986; Schmitz and Kowallik, 1986).

In this report, we have confirmed the significant role of the plastome in the process of plastid transmission. Some of the patterns of plastid transmission can be explained in terms of intrinsic competitive abilities of the plastids, but neither competition nor input bias can account for all of the observations. The manner in which the plastome

participates in this process remains to be determined, as does the role of the nuclear genome in the same process. Certainly, lower input of paternal plastids alone cannot explain the predominance of maternal transmission: events occurring after fertilization, particularly competitive multiplication of plastids, must also be considered.

CHAPTER 3

INDIRECT APPROACHES TO CHARACTERIZE CHLOROPLAST DNA ORIGENS OF REPLICATION

Introduction

Plastids are not created *de novo*. That is, they can originate only from preexisting plastids (reviewed by Possingham and Lawrence, 1984). The propagation of plastids involves two major events: chloroplast DNA replication and plastid division. Most enzymes involved in these two steps are likely to be nuclear encoded (Scott *et al.*, 1982). However, the plastome itself has a strong influence on plastid transmission and multiplication (Schötz, 1974; Chapter 2 of this thesis). The plastome may affect these processes through the contribution of some cofactors or via special recognition sequences, such as the origin of DNA replication (ori/rep).

Chloroplast DNA origins of replication have been located in *Chlamydomonas* (Waddell *et al.*, 1984) and *Euglena* (Ravel-Chapuis *et al.*, 1982) through electron microscopic localization of DNA replication intermediates named D-loops, where D stands for displacement. The DNA sequences of these cpDNA replication origins reveal some common features: they are both highly A+T rich and able to form large stem-loop structures (Wu et al., 1986; Koller and Delius, 1982). In higher plants,

replication of chloroplast DNA has been shown to initiate from two sites 7.1 kb apart (Kolodner and Tewari, 1975). However, these replication origins have not been characterized further, presumably due to the low frequency of replication intermediates that can be observed.

An alternative way to identify the origin of chloroplast DNA replication would be through a functional assay of DNA sequences, testing for those that can act as initiation sites for DNA synthesis. Since no means is available currently to transform the chloroplast, it is not yet possible to test a putative origin of replication *in vivo*. For this reason, heterologous systems have been used to test the function of putative DNA replication origins. Both yeast (Ohtani *et al.*, 1984; Overbeeke *et al.*, 1984) and *Chlamydomonas* (Rochaix *et al.*, 1984) have been used as heterologous systems to aid the search for a chloroplast origin of replication.

Sequences that can promote autonomous DNA replication in yeast are named ARS (autonomous replication sequence) (Struhl et al, 1979). The frequency of ARS's found in the yeast genome coincides with the abundance of replication intermediates observed in the chromosome (Beach *et al.*, 1980; Chan and Tye, 1980) and they are postulated to represent yeast nuclear DNA replication origins. However, whether ARS's are normal DNA replication origins in yeast chromosomes is still open to question (Walmsley *et al.*, 1984).

Several sequences with ARS properties have been found in the chloroplast genome by transforming yeast with *E. coli* plasmids that contain cloned cpDNA fragments. At least seven such DNA regions have been identified in the *Chlamydomonas* chloroplast genome: three have been found in *Petunia* (de Haas *et al.* 1986), two are known in *Nicotiana*, and at least one exists in *Chlorella* (Yamada *et al.*, 1986). These chloroplast DNA sequences all contain the 11 bp consensus sequence common to the yeast ARS.

Another heterologous system has been used by Rochaix *et al.* (1984), who searched for sequences that can promote DNA replication in the nucleus of *Chlamydomonas*. ARC (autonomous replication in *Chlamydomonas*) activity has been found in four fragments of *Chlamydomonas* chloroplast DNA (Rochaix, 1984). Two partially conserved elements of 19 and 12 bp are found in these four ARC containing sequences.

None of these ARS and ARC sequences found in chloroplast genomes actually coincide with the observed initiation sites of chloroplast DNA synthesis recognized by D-loop mapping, although some are nearby (Vallet and Rochaix, 1985; Yamada *et al.*, 1986). The actual role of these ARS or ARC sequences *in vivo* is still unknown. Nevertheless, in the *Chlamydomonas in vitro* cpDNA replication system, a fragment containing ARS and ARC together with one of the cpDNA replication origins (*ori*A) served as a better template for DNA synthesis than *ori*A alone (Wu et al., 1986). Also, a maize cpDNA fragment that incorporated the highest amount of nucleotides under the direction of partially purified pea chloroplast DNA polymerase also contained ARS and ARC consensus

sequences (Gold et al., 1987).

If autonomous replication sequences in yeast (ARS) or in *Chlamydomonas* (ARC) do enhance the binding of DNA replication enzymes, their presence near the cpDNA replication origin may facilitate the replication of the plastid genome. To test this hypothesis, three ARC sequences from the *Chlamydomonas* chloroplast genome were used to probe for homologous sequences in three types of *Oenothera* plastomes that showed differential transmission abilities (Schötz, 1974; Chapter 2 of this thesis).

Despite the failure of yeast and *Chlamydomonas* to recognize the ori/rep of the chloroplast, there are examples in which the true ori/rep could be recognized by a different organism. Heterologous systems have been successfully used to characterize essential DNA sequences within the bacterial chromosomal DNA replication origin (oriC). Zyskind *et al.* (1983) identified the replication origin of the marine bacterium *Vibrio harveyi* through functional analysis of DNA replication in *E. coli*. Comparison of *Vibrio harveyi* with five moderately related bacteria from the family of Enterobacteriaceae revealed clusters of conserved sequences composing the replication origin.

Instead of using eukaryotic transformation systems such as yeast or *Chlamydomonas* to test the function of cpDNA ori/rep, a prokaryotic system is a more reasonable choice, since chloroplasts are almost certainly of prokaryotic origin. Cyanobacteria, with their presumed evolutionary relationship to chloroplasts (reviewed by Whatley and Whatley, 1981;

Doolittle, 1982), would seem to be the best candidate for an *in vivo* heterologous system to functionally analyze a chloroplast DNA replication origin.

In the experiments described here, plasmids carrying a *Chlamydomonas* chloroplast DNA replication origin (*oriA*) were introduced into three different strains of cyanobacteria either by triparental mating or by transformation to test if this chloroplast DNA origin of replication can support autonomous replication in these heterologous systems.

Material and Methods

Isolation of chloroplast DNA from Chlamydomonas. A strain of *Chlamydomonas reinhardii* cc-1615 (from the culture collection at Duke University), lacking a cell wall (*cw-15* mutation) was used as a source of chloroplast DNA. Chloroplast DNA was isolated according to Rochaix (1984) with some modifications. One to four liters of *Chlamydomonas* cell culture were grown in Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965). Stationary cultures were harvested by centrifugation at 3,000xg for 5 minutes. The pellet was resuspended in NET (15 mM NaCl, 100 mM EDTA, 50 mM Tris, pH 9.0) buffer at a final density of 1 x 10^9 cells/ml. Predigested pronase (2 hours at 37°C, 10 min. at 80°C) was added to a final concentration of 0.6 mg/ml. The solution was mixed in the cold for 10 minutes and 10% SDS was added to a final concentration of 1.2%. The solution was incubated at 50°C. Additional pronase (half the amount of the first addition) was added after one hour. After 2 to 2 1/2 hours of incubation, the lysate was cooled on ice and extracted with

phenol/chloroform. After ethanol precipitation of the nucleic acid, the high molecular weight nucleic acid was spooled on a glass rod and immersed in cold 70% ethanol for 10 minutes. After drying the nucleic acid on the glass rod in air, the total nucleic acid was resuspended in TE (10 mM Tris, 1 mM EDTA) buffer and treated with RNase (50 ug/ml, 37°C for one hour). The solution was extracted with phenol/chloroform and the DNA was spooled as before. The concentration of DNA was adjusted by measuring optical density at 260nm (A_{260}) and diluting the DNA by adding 3-5 ml TE buffer for every 10-15 A_{260} units. Bisbenzimide was added to a final concentration of 100 ug/ml. CsCl was added to the solution and the concentration was adjusted to a refractive index of 1.3980. After an overnight centrifugation at 45,000 rpm in a vTi65 rotor, four bands could be resolved. The species of DNA bands from top to the bottom are chloroplast, mitochondrial, ribosomal and nuclear DNA, respectively (Rochaix, 1984).

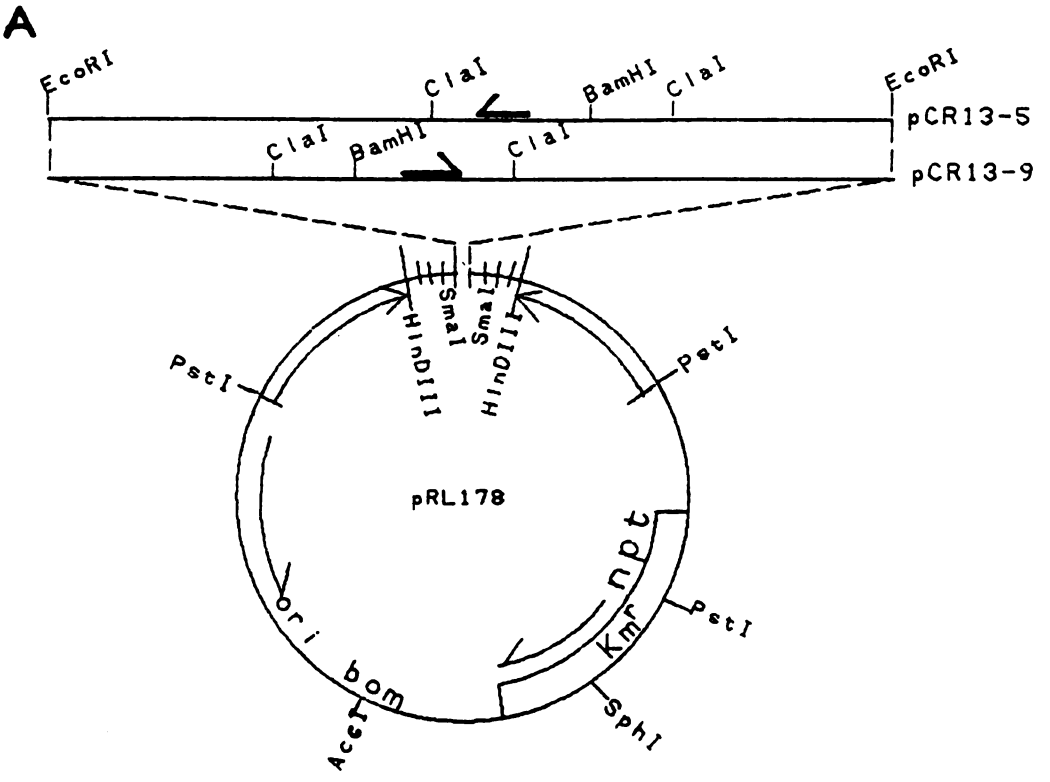
DNA blot hybridization. Chloroplast DNA from three major types of *Oenothera* plastids were digested with the restriction endonucleases BamHl and EcoRl. The fragments were separated on an agarose gel and transferred onto nitrocellulose paper. Plasmids carrying ARC sequences (pCAl, pCA2, pCA3, pCA4) were obtained from Dr. J.-D. Rochaix. Each ARC-containing fragment (ARCl, 153 bp; ARC2, 414 bp; ARC3b, 257bp) was cut out from the vector, pJD2, after digestion with Hind III and SalI. These fragments were separated from the vector on an agarose gel and were then removed from the gel by electroelution. Each ARC-containing fragment was ethanol-precipitated, redissolved and then labeled with ^{32}P-CTP by nick

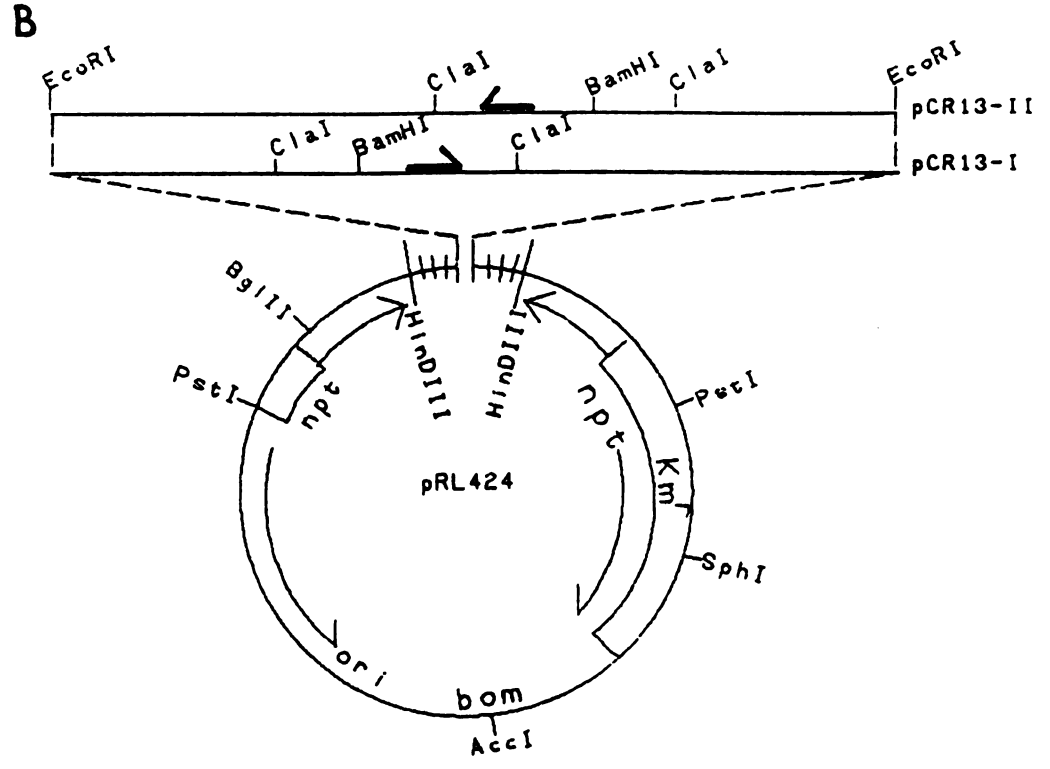
translation (Maniatis *et al.*, 1982). The hybridization and washing procedures were performed in low stringency (Maniatis *et al.*, 1982) such that the three ARCs can cross hybridize with each other.

Cloning of the chloroplast DNA origin of replication from *Chlamydomonas*. Two plasmid vectors, pRL178 and pRL424 (Elhai and Wolk, submitted) were used in this study. These vectors are based on the replicon of pBR322. The common features of these plasmid vectors include a symmetrical polylinker for cloning and the kanamycin resistance gene from Tn5 as a selectable marker. A 5.6 kb EcoRI (R-13) fragment that contains a *Chlamydomonas reinhardii* chloroplast DNA origin of replication (Waddell et al., 1984) was isolated through agarose gel electrophoresis as described previously and was cloned between the EcoRI sites of cloning vector pRL178 and pRL424 (Figure 5). This 5.6 kb fragment was also subcloned as 3.6 kb and 2.0 kb fragments by using the single BamHI site inside of the R-13 fragment and the two BamHI sites on the polylinker.

Bacterial strains and growth conditions. The unicellular cyanobacterium *Synechococcus R2* and the filamentous cyanobacterium *Anabaena* strain M131 were used for the conjugation experiments. These strains were obtained from C.P. Wolk (MSU). *Synecocystis* PCC 6803 was obtained from L. McIntosh (MSU) and was used for transformation. All cyanobacterial strains were grown in liquid in either BG-11 (Rippka *et al.*, 1979) or eight-fold diluted A.& A. (Hu *et al.*, 1981) medium supplemented with 10 mM $NaNO_3$. These cultures were maintained under constant light (1500 lx), shaken (100 rpm) at 30°C.

Figure 5. Maps of the plasmids carrying *C. reinhardii* cpDNA *ori*A. The 5.6 kb EcoRI (R13) fragment of *C. reinhardii* cpDNA, which contains one initiation site of cpDNA replication (*ori*A) was cloned into the vectors pRL178 (A) and pRL424 (B) in both possible orientations (clone names indicated at right). The thick arrow inside of R13 indicates the position and the direction of the initiation of DNA synthesis at *ori*A. The regions between the two HinDIII sites of both vectors contain multiple cloning sites (MCS). There are two SmaI (AvaI) sites in the MCS of pRL178 but none in the MCS of pRL424. The thin double-sided arrows indicate the inverted repeat regions within the vectors. The *bom* (*b*asis *o*f *m*obility) region is required in cis for conjugal mobilization. The *npt* (*n*eomycin *p*hospho*t*ransferase) gene confers resistance to kanamycin, neomycin, and some other aminoglycosides. *ori* represents the replication origin of the plastid vector. The directions of transcription and DNA replication are indicated by half-arrows.


A
EcoRI
ClaI
BamHI
ClaI
EcoRI
pCR13-5
ClaI
BamHI
ClaI
pCR13-9
HinDIII
SmaI
SmaI
HinDIII
PstI
PstI
pRL178
ori
bom
AccI
Kmr
npt
PstI
SphI


B
EcoRI
ClaI
BamHI
ClaI
EcoRI
pCR13-II
ClaI
BamHI
ClaI
pCR13-I
BglII
HinDIII
HinDIII
PstI
PstI
npt
npt
Km
pRL424
ori
bom
AccI
SphI

Conjugation of plasmids into cyanobacteria. Conjugation of plasmids into cyanobacteria was performed according to Wolk *et al.* (1984). Overnight cultures of *E. coli* strains containing conjugal plasmid (RP4 [Km^r Tc^r Ap^r]) or helper plasmid (pDS4101 [Ap^r]) with or without test plasmids (containing chloroplast DNA fragments [Km^r]) were diluted 40-fold and regrown for 4 hours. Each strain was harvested by spinning 1.5 ml of the cultures in microfuge tubes for one minute. The cell pellet was resuspended in 1.5 ml of LB medium. 0.75 ml of *E. coli* cells containing the conjugative plasmid (RP4) and the same volume of cells containing the test plasmid were mixed and centrifuged together. 60 ul of LB was added to the pellet. At the same time, cyanobacterial cells were prepared. 45 ml of a growing culture was centrifuged at 2000 rpm for 10 minutes. The cell pellet was resuspended in 1 ml of BG-11 liquid medium. A series of dilutions from 1 to 10^7-fold was made from this cell suspension. 5 ul of each *E. coli* mating mixture was added separately to 5 ul of cyanobacterial cells from each dilution. 2 ul of each *E.coli*/cyanobacteria mixture was spotted onto a nitrocellulose filter placed on a BG-11 agar plate. These plates were incubated at 30°C with continuous illumination. After 24 hours, the filter was transferred to a BG-11 agar plate containing 2 ug/ml of kanamycin. In the case of Anabaena M131, 10 ug/ml of neomycin was used. Since neomycin phosphotransferase confers resistance to either kanamycin or neomycin, the choice of antibiotics is determined by the sensitivities of the organisms.

Transformation of Synechocystis 6803. The transformation of *Synechocystis* was performed according to a modified procedure of Grigorieva and Shestakov (1982). A 30 ml culture of *Synechocystis* 6803 was started in BG-11 liquid medium at an optical density of A_{730} = 0.05-0.15. The culture was grown to A_{730} = 0.37. The cells were harvested by centrifugation at 6,000 rpm at room temperature for 10 minutes. The pellet was resuspended in fresh BG-11 at an optical density (A_{730}) of 2.5, which corresponds to a cell concentration of about 2 X 10^8/ml. 0.3 ml of cell suspension was mixed with 1-2 ug of the plasmid DNA to be tested. The transformation mixture was incubated at 34°C for 2-4 hours with occasional shaking. 100 ul of the transformation mixture was plated onto nitrocellulose filters placed on BG-11 agar plates which were then incubated at 34°C for 18-20 hours. The filters were transferred to BG-11 plates with either 5 or 10 ug/ml of kanamycin.

Isolation of total DNA from *Synechocystis* PCC 6803. DNA from *Synechocystis* was isolated according to a base lysis procedure (Kuhlemeier, 1981). A 500 ml culture of *Synechocystis* 6803 was grown to stationary phase with aeration. The cells were spun down and the pellet was washed once with 10 ml of NE solution (0.12M NaCl, 0.05M EDTA), and again with 10 ml of lysis buffer (23 mM Tris, pH 8.0; 10 mM EDTA; 50 mM glucose). The cell pellet was resuspended in 1 ml lysis buffer plus 0.5 ml lysozyme (10 mg/ml). The suspension was incubated at 37°C for one hour. 0.5 ml of fresh 10% SDS was added at the end of the first hour and the incubation was continued for another hour. 0.25 ml 5M NaCl was added to the cell lysate and the suspension was mixed gently and left over-

night at 4°C. The total nucleic acids were spun down at 10,000xg for 30 minutes. The pellet was dissolved in TE buffer and was then extracted with phenol/chloroform. Total nucleic acids were precipitated with ethanol and resuspended in TE.

Results

Detection of sequences homologous to ARCs in *Oenothera* cpDNA.

The fragments of *Chlamydomonas* chloroplast DNA that support autonomous replication in the nucleus of this alga were used as probes to see if homologous sequences exist in *Oenothera* chloroplast DNA. Two probes, one carrying ARC1 and the other ARC2, both hybridized to similar fragments of *Oenothera* cpDNA that had been digested with EcoRI (Figs. 6B and 7B). The ARC1 hybridized to three EcoRI fragments of plastome I and III DNA with approximate sizes of 4.8, 3.9 and 2.5 respectively (Fig. 6B). The same probe also had hybridized weakly to a 1.6 kb DNA fragment from plastome III. Only the 4.8 kb fragment of plastome IV could be detected when probed with the ARC1-containing fragment. The ARC2 probe hybridized with two fragments of plastome I (4.8 kb and 3.9 kb), three fragments of plastome III (4.8 kb, 3.9kb and 1.6kb), and two fragments of plastome IV (4.8 kb and 1.6 kb). ARC3b is located inside the gene encoding the chloroplast 23s rRNA. A fragment containing this region hybridizes strongly with the 4.8 kb fragment (Fig. 6B). This 4.8 kb EcoRI fragment recognized by all three probes is located inside of the inverted repeat region of the *Oenothera* chloroplast genome (Gordon *et al.*, 1981). The 1.6 kb EcoRI fragment which hybridized to the ARC1 and ARC2 probes is only present in plastome types III and IV (Fig. 6B).

Figure 6. Hybridization of ARC1 element to *Oenothera* cpDNA.

A. Agarose gel electrophoretic patterns of *C. reinhardii* (C), *Oenothera* plastome type I, III, and IV cpDNA digested with EcoRI.

B. Autoradiograms of Southern hybridizations of the DNAs described above probed with the 153 bp fragment containing ARC1, which is located inside of EcoRI fragment 13 (R13) of *C. reinhardii* cpDNA. The numbers indicate the sizes (in kb) of the bands hybridized by the probe.

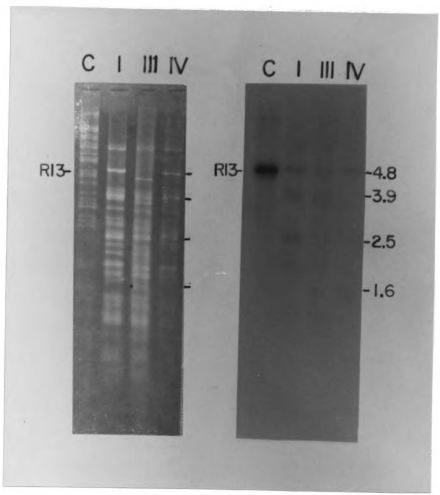


Figure 6.

Figure 7. Hybridizations of ARC2 and ARC3b elements to Oenothera cpDNA.

Autoradiograms of Southern hybridizations of the cpDNA described in Figure 6 with the ^{32}P-labelled fragments carrying ARC3b (A) and ARC2 (B). ARC3b and ARC2 are located inside of EcoRI fragments 24 and 18, respectively, of *C. reinhardii* cpDNA. The sizes of the bands hybridized with the probes are indicated in kb.

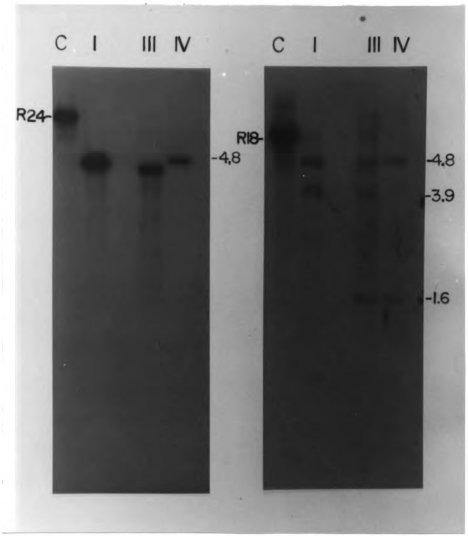


Figure 7.

Testing autonomous replication activity of *oriA* in cyanobacteria

Conjugation of *an oriA*-containing plasmid into cyanobacteria. The 5.6 kb R-13 fragment that contains the *oriA* of *Chlamydomonas* cpDNA was first cloned into the EcoRI site of the vector pRL178 and was then transferred into cyanobacteria by conjugation. The two clones containing the R-13 fragment in the two possible orientations are designated as pCR13-5 and pCR13-9 (Fig. 5a).

A broad-host plasmid pRL153 (Km^r) was used as a positive control for the triparental mating. There is phenotypic evidence on replication of this plasmid in *Anabaena* M131, *Synechococcus* R2, and several other cyanobacteria (T. Thiel, personal communication). Spots containing the highest concentration of cyanobacteria always showed some growth despite the presence of kanamycin or neomycin. In one conjugation experiment, using Synechococcus R2, cell growth could be observed with pCR13-9 or pRL153 as the test plasmid, even at the level of 10^7-fold dilution (14 colonies from each strain). This represents a frequency of of 7 x 10^{10} resistant colonies/ml of conjugation mixture. pCR13-5 gave a slightly greater number of resistant colonies (10^{11}/ml) than the other two plastids, but the size of the colonies was smaller. However, when several individual colonies were streaked onto fresh BG-11 agar plates with 2 ug/ml of kanamycin, no kanamycin-resistant colonies were recovered. In the control, cells receiving no test plasmid did not produce single colonies on the conjugation plate.

Only one or two colonies of *Anabaena* M131 were observed on the spot of ten fold dilution after receiving pCR-5 or pCR-9. The positive control,

pRL153 produced colonies at a 1000-fold dilution of *Anabaena*. None of the colonies receiving pCR-5 of pCR-9 could be recovered after streaking on fresh plate containing 5mg/ml of neomycin.

Conceivably, the high concentration of kanamycin resistant *E. coli* surrounding the cyanobacteria might protect Km-sensitive cyanobacteria against kanamycin leading to apparent Km-resistant colonies on the conjugation plate, which would be unstable upon transfer. If so, early elimination of *E. coli* following conjugation should reduce the occurrence of these false positives. In order to eliminate the *E. coli* shortly after conjugation, lysogens were constructed using a strain of phage lambda carrying the temperature sensitive repressor, cI_{s57}. Shifting to the non-permissive temperature should kill unwanted *E. coli* by inducing the phage to enter its lytic cycle. To this end, all *E. coli* strains involved in the earlier conjugation experiment were infected with phage lambda cI_{s57} at 30°C. Temperature-sensitive lysogens were verified by selecting the *E. coli* colonies that could grow at 30°C but not at 37°C

Following the conjugation procedure described earlier, the temperature was raised to induce the lytic cycle of phage lambda. Upon raising the temperature to 42°C, all strains of *Synechococcus* R2 grew much faster on the conjugation plate. However, no differences could be observed among strains receiving pCR13-5 and pCR13-9, or the negative control pRL178. All these strains gave rise to several colonies at 10^6-fold dilution of *A. nidulans* R2 cells.

Restriction of incoming DNA by endogenous nucleases of the cyanobacteria is one possible reason for the failure of the *Chlamydomonas* chloroplast *oriA* containing plasmid to survive after transfer (Wolk *et al.*, 1984). To partially overcome the possible restriction problem, the R-13 fragment was transferred into a vector, pRL424, that has fewer sites that can be recognized by AvaI; an isoschizomer of which is found in *Anabaena* M131. The restriction specificities of enzymes from *Synechococcus* R2 are unknown. Clones of R-13 in pRL424 in both possible orientations were designated as pCR13-I and pCR13-II (Fig. 1). These two plasmids and their subclones (pCR13-36.4, pCR13-36.6, pCR13-20.1, pCR13-20.2) were sent into *Synechococcus* R2 and *Anabaena* strain M131 by triparental mating. As a control, pRL424 alone was also sent into the same strains.

In each case, growth of the cyanobacteria on antibiotic-containing media occurred only at spots where high concentration of the recipient cyanobacterial cells were present. All of these clones supported the growth of cyanobacteria on the conjugation plate to a similar extent. The difference in efficiency between these clones and the vector alone was less than ten fold. Several colonies were transferred to fresh BG-11 agar plates. None of these clones gave rise to single colonies in the presence of antibiotic.

Transformation of cyanobacteria with *oriA*-containing plasmids. All the clones containing portions of the R-13 fragment of *Chlamydomonas reinhardii* chloroplast DNA were used in an attempt to transform the unicellular cyanobacterium *Synechocystis* strain PCC6803. A plasmid, pKW1189

(Km^r), which can integrate into *the Synechocystis* 6803 chromosome through homologous recombination (McIntosh, personal communication) was used as a positive control. Results from the first of these experiments are listed in Table 6. pRL178 carrying the 5.6 kb R-13 fragment from the *Chlamydomonas* cpDNA in both orientations (pCR13-5, pCR13-9) did not transform *Synechocystis* 6803 with a frequency higher than the vector alone, whereas many colonies were formed from the positive control (pKW1189). The same fragment cloned in pRL424 (pCR13-I) gave a slightly higher frequency of colonies than did the vector alone. When pCR13-II, which contains the insert as pCR13-I but in different orientation, was used, the transformation frequency was as high as with the control plastid. However, it was noticed later that this pCR13-II plasmid preparation was mixed with the positive control plastid in a ratio of 5:1.

DNA isolated from the wild type strain of *Synechocystis* 6803 and the kanamycin resistant colonies receiving the mixture of pCR13-II and the positive control, pKW1189, were compared by southern hybridization using both plasmids as probes. The hybridization indicated the presence of only pKW1189 integrated into the chromosomal DNA of Synechocystis 6803 through homologous recombination but there was no indication of pCR13-II in the cell (data not shown).

In the subsequent transformation experiments, neither pCR13-I and pCR13-II isolated from different plasmid preparations nor their subclones (3.6 kb and 2.0 kb) showed any transformation frequencies higher than the

Table 6. Transformation of *Synechocystis* PCC 6803

Plasmid tested	Number of colonies	
	5ug/ml Km	10ug/ml Km
pCR13-5	0	0
pCR13-9	7	1
pRL178	7	5
pRL153	3	0
pCR13-I	37	42
pCR13-II*	>1000	>1000
pRL424	10	9
pKW1189	>1000	>1000

*Contained 15% of pKW1189

background of the vector alone.

Discussion

Chlamydomonas ARC sequences themselves are not the origin of cpDNA replication (Rochaix et al., 1985), however, the fact that they can support autonomous replication in the nuclei this green alga (Rochaix *et al.*, 1984) and the localization of one of the ARC sequences (ARC1) near the *oriA* of *Chlamydomonas* chloroplast DNA suggest that ARCs may play a role in facilitating the binding of proteins that participate in the process of DNA replication (Wu *et al.*, 1986). ARC1 was used to probe maize cpDNA but no hybridization signal was detected under relatively permissive conditions (Gold *et al.*, 1987). However, sequence analysis did show a 19 bp consensus sequence in the presumed maize cpDNA replication origin.

In this report, fragments carrying three ARC sequences were used to probe for the homologous sequences of *Oenothera* chloroplast DNA. Under non-stringent hybridization conditions, these three fragments can cross hybridize among themselves, and furthermore, one EcoRI fragment (4.8 kb) within the inverted repeat region of the *Oenothera* cpDNA was recognized by all three fragments (Fig. 6 and 7). Two other EcoRI fragments (3.9 kb and 1.6 kb) of plastome type III were recognized by the probe containing ARC2. The same probe also lit up the 3.9 kb EcoRI fragment of plastome type I and the 1.6 kb fragment of plastome type IV. Whether or not these differences in the ARC hybridization signals among plastome types correlate with the differential transmission abilities of plastids (Chapter 2

of this thesis) still requires further investigation. The relationship between the appearance of ARS/ARC homologous sequences and the efficiency of chloroplast DNA replication cannot be drawn until more ori/rep sequences of chloroplast DNA from higher plants are characterized and a good functional assay for cpDNA replication becomes available.

Neither yeast nor *Chlamydomonas* can utilize the observed cpDNA ori/rep from *Chlamydomonas* (Vallet and Rochaix, 1985; Yamada *et al.*, 1986). It is understandable why these system did not recognize the cpDNA ori/rep: the line of descent leading to eukaryotic nuclei is very different from that leading to chloroplasts (Fox *et al.*, 1980). On the other hand, chloroplast and cyanobacteria are much more closely related (reviewed by Gray and Doolittle, 1982; Woese, 1987). Thus, an attempt was made to use an evolutionally more related system to test for the ori/rep function of cpDNA. Three cyanobacterial strains, one filamentous and two unicellular, were used in order to cover a wide evolutionary spectrum within the range of existing cyanobacteria.

Plasmids containing one of the *Chlamydomonas* cpDNA replication origins were introduced into cyanobacteria either through triparental mating or transformation. None of the tested oriA containing plasmids gave stable antibiotic resistant colonies. In the case of *Synechococcus* R2, both the vector alone and the vector with an inserted oriA fragment supported the growth of the bacteria on the conjugation plate to a similar extent. However, these colonies did not survive transfer to fresh antibiotic-

containing plates.

When *Synechocystis* 6803 was transformed with a plasmid carrying homologous sequences of its chromosome (pKW1189), the transformation frequency was very high, due to the frequent stable integration through homologous recombination. However, when the *oriA* containing plasmids were used to transform the same strain, the frequencies were very low (Table 6). In most experiments, the transformation frequency was not higher than the vector alone. It seems that *Chlamydomonas* chloroplast *oriA* is unable to support the independent replication and/or maintenance of plasmids in all three strains of cyanobacteria tested.

For a number of reasons, chloroplasts of rhodophytans (red algae) and crytophytans are thought to be the direct descendants of cyanobacteria (Whatley and Whatley, 1981; Gray and Doolittle, 1982). However, whether the chloroplasts of green algae and higher plants arose directly from the cyanobacteria or from some other relative is still unknown (Woese, 1987). As far as the evolutionary distance is concerned, the chloroplast ori/rep of red algae might be expected to have a better chance to function in blue-green bacterial system.

Other factors besides evolutionary distance must also be considered. The stable inheritance of a plasmid in a growing bacterial cell involves two separate processes: replication and partitioning (Nordström, 1985). Even if the cyanobacterial cells can recognize the green algal chloroplast origin of replication, the proper partitioning of foreign plasmids is

equally important for the maintenance of such plasmids. The chloroplast ori/rep sequences alone may not be sufficient for the proper segregation of the DNA molecules. In the experiments described here, the inability to maintain stable antibiotic-resistant colonies could be explained if plasmids carrying the chloroplast ori/rep were unable to segregate properly following cell division.

CONCLUSION

The multiplication and development of chloroplasts require coordinated expresion of nuclear and chloroplast genes. The study of nuclear-plastid interactions in higher plants is limited because no reliable way currently is available to manipulate higher plant chloroplasts.

DNA recombination is a basic tool in genetics. Recombination between chloroplast genomes has never been recovered in sexual crosses of higher plants, since plastids of most angiosperms are maternally inherited. The first chapter of this thesis described experiments designed to test the likelihood of plastome recombination in *Oenothera*, a genus in which frequent transmission of paternal plastids can occur. Crosses between *Oenothera* plants carrying only mutant plastids in their germ lines were performed and the progeny were screened for a wild-type phenotype. In the first field season, only one out of twenty crosses between different plastome mutants yielded progeny with green sectors. However, analysis of restriction enzyme digestion patterns revealed that these green sectors were all derived from the wild-type tissue of the paternal plant. In the second field season, more green plastids originating from wild-type tissue were observed but no true recombinants. Thus, recombination between chloroplast DNAs of *Oenothera* was not detected in these experiments. Selectable genetic markers and a higher number of cell

division cycles may be required to recover infrequent recombinants.

Both nuclear and plastid genomes can influence the extent of biparental plastid transmission in *Oenothera*. However, the degree of control by each genetic compartment has never been examined separately. In Chapter 2 of this thesis, the transmission abilities of four out of the five major plastome types of *Oenothera* (I-V) were analyzed in a constant nuclear background by assessing both the frequency of biparental inheritance and the extent of variegation in the progeny. Reciprocal crosses were performed with four wild-type plastids and seven white plastid mutants. The frequency of biparental plastid transmission ranged from 0 to 56% depending on the plastid types involved in the crosses. The transmission abilities of the four representative wild-type plastids appear to be in the order of I>III>II>IV in the nuclear background of *O. hookeri* str. Johansen. In general, variegated seedlings from crosses which produced a higher frequency of biparental plastid transmission also had larger sectors containing plastids of paternal origin ($r = 0.853$). Although the transmission abilities of most *Oenothera* plastid mutants are comparable to the wild-type plastids, three mutant plastids derived from species having different type I plastids showed three distinguishable transmission patterns. This study confirms the significant role of the plastome in the process of plastid transmission and possibly in plastid multiplication.

However, the hypothesis that the different plastid types multiply at different rates can only partially explain these results. According to

this investigation, the time of onset of plastid division after zygote formation may also be plastome dependent. It is known that no chloroplast gene product is required in the maintenance of the normal plastid DNA level. Hence, the observed dependence of plastid transmission on plastome type may be caused by a special cpDNA structure, such as the origin of replication. Current investigations are directed towards an understanding of differential plastid transmission at the molecular level. These involve the characterization of initiation sites of cpDNA synthesis in different plastome types. Also, the importance of nuclear–plastid interactions in the process of plastid transmission is being further addressed by measuring the transmission efficiencies of plastids in different nuclear backgrounds including their native ones.

The cpDNA origin of replication is the most likely region where the control of plastid multiplication might take place. Three DNA fragments that can support autonomous replication in *Chlamydomonas* were used to probe for homologous sequences in *Oenothera* chloroplast DNA. Some differences in the hybridization signals could be observed among the three plastome types examined. However, since the function of ARC elements is still unknown and no *Oenothera* cpDNA replication origin has been mapped, the significance of the observed DNA hybridization differences relative to plastid multiplication cannot be assessed at this time.

Since there is still no method available to directly transform higher plant chloroplasts, a functional analysis of the cpDNA origin of replication in its natural enviroment cannot be achieved. In order to

look for a heterologous system to functionally analyze the cpDNA replication origin, *E. coli* plasmids containing a *Chlamydomonas* cpDNA origin of replication (*ori*A) were transferred by conjugation into three strains of cyanobacteria. None of these plasmids carrying *ori*A could be propagated and/or maintained in any of the cyanobacterial strains tested. It is possible that the evolutionary distance between blue-green bacteria and chloroplasts is still too great for the cpDNA replication origin to function. Alternatively, the *ori*A region of Chlamydomonas may not have carried enough information for stable maintainance of the replicon.

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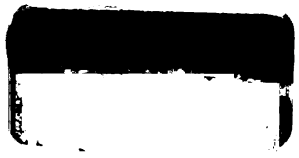


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