PLASTID INHERITANCE AND CHLOROPLAST DNA REPLICATION IN *OENOTHERA*

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ABSTRACT

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Plastids of Oenothera, the evening primrose, can be transmitted to the progeny from both parents. Previous studies suggested that the extent of biparental plastid inheritance and the competitive ability of plastids to multiply are determined primarily by the plastid genome (plastome) itself, with the nuclear genome having only a minor impact. These results are in apparent conflict with observations that plastid multiplication in land plants does not appear to require proteins encoded by the plastome. In this dissertation, the impact of the nuclear background on plastid transmission was reassessed. In crosses with diverse nuclear and plastome combinations, a good correlation was observed between compatibility of the particular plastome with the nuclear genome of the progeny.

In order to test the hypothesis that a non-coding function of the plastome, the origin of cpDNA replication, may be the plastome component that influences plastid multiplication, the origins of chloroplast DNA replication were located in two Oenothera plastomes. Two pairs of displacement loop (D-loop) initiation sites, each lying within the inverted repeats, were mapped to the Oenothera cpDNA molecule by electron micro-

scopic analysis of restriction fragments. The starting points of the two adjacent D-loops are approximately 5 kb apart, bracketing the 16S rRNA gene. No differences in the number and the location of replication origins were apparent between plastomes with the highest and the lowest transmission efficiencies.

Restriction analysis of several Oenothera plastomes revealed a highly variable region in the 16S rRNA-trnI spacer near one of the cpDNA replication origins. DNA sequences of the 16S rRNA-trnI spacer were obtained from several Oenothera plastomes to assess the possibility that local DNA sequence variation may affect the efficiency of replication initiation. Multiple copies of repeated sequences that have the potential to form stable stem-loop structures were found in this region. These secondary structures may interfere with the movement of the replication fork. A model based on the slipped strand mispairing of DNA during replication is proposed to explain the amplification of repeated sequences in this region.

This dissertation is dedicated in memory of

my loving aunt Jo-Mei Chiu

for her encouragment and support

throughout my school years

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GENERAL INTRODUCTION

Plastids are essential organelles of plant cells. Besides their role in photosynthesis, plastids function both in photosynthetic and nonphotosynthetic plant cells, such as roots, tubers, flowers and fruits, for the synthesis or storage of starch (amyloplasts), carotenoids (chromoplasts), amino acids, fatty acids, and lipids (reviewed by Thomson and Whatley 1980).

The many proteins required for the diverse functions of plastids are not all encoded by the plastid genome (plastome). Judging from genes deduced from the complete plastome sequences available from three land plants, Marchantia (Ohyama et al. 1986), tobacco (Shinozaki et al. 1986), and rice (Hiratsuka et al. 1989), and from the small genome sizes of plastomes (120-180 kb; reviewed by Palmer 1985), it is evident that plastomes encode proteins mainly of the photosynthetic apparatus and the transcription and translation machinery, with the majority of plastid proteins being nuclear-encoded. Nuclear gene products not only participate in the metabolic processes occurring in the plastid such as photosynthesis, and amino acid and fatty acid biosynthesis (reviewed by Kirk and Tilney-Bassett 1978), but also determine the developmental fate of plastids by regulating plastid gene expression (reviewed by Mullet 1988). The coordination of gene expression occurs in the other direction as well. Several studies have found that photo-oxidative damage of chloroplasts can affect the expression of photosynthetic genes in the

nucleus (reviewed by Taylor 1989). Also, some nuclear genes that encode chloroplast proteins have cis-acting regulatory elements that respond to the developmental stage of chloroplasts in the same cell (Aoyagi et al. 1988, Jefferson et al. 1987, Stockhaus et al. 1989). Apparently, the coordinated expression of nuclear- and plastid-encoded components is necessary to maintain the normal functions of plant cells. This thesis investigates one vital area of the plastome-genome interaction: the multiplication and transmission of plastids.

Since new plastids arise only from pre-existing plastids, mechanisms must exist to ensure the continuity of plastids in each daughter cell. Our current understanding of the mechanisms responsible for the multiplication and segregation of plastids in plant cells remains very limited. Studies using inhibitors of protein synthesis (Heinhorst et al. 1985) and investigations on plastid mutants that lack ribosomes (Walbot and Coe 1979, Scott et al. 1982) suggest that cpDNA synthesis is dependent on nuclear-encoded proteins and that plastids can multiply even in the absence of proteins synthesized in the plastids. By quantifying the amounts of DNA in individual chloroplasts during leaf development, several groups concluded that plastid division in land plants is not tightly coupled to cell division and that cpDNA synthesis does not immediately precede plastid division (Lamppa and Bendich 1979, Lawrence and Possingham 1986). Concordantly, the number of plastids per cell can increase from 10-15 in meristematic cells to several hundred in mature mesophyll cells, and the number of plastomes per plastid can range from a few in the proplastid to several hundred in chloroplasts of expanding mesophyll cells prior to the active stage of chloroplast

division (Lawrence and Possingham 1986, Bendich 1987). The multiple copies of plastomes are organized into several nucleoids that, through their attachment with the plastid membrane, may serve as basic units for the segregation of cpDNA (Kuroiwa et al. 1981, Hansmann et al. 1985, Lindbeck et al. 1987).

The continuity of plastids also depends upon the transmission of plastids from generation to generation. The inheritance of plastids in different land plant groups has been observed to be maternal, paternal, or biparental. Conifers inherit their plastids mainly from the paternal side (Neale et al. 1986, Neale et al. 1989, Szmidt et al. 1987, Stine et al. 1989), however, two smaller groups of gymnosperms, Ginkgoales and Cycadales, seem to transmit plastids only from the maternal parent (Whatley 1982). The majority of angiosperms inherit their plastids only from the maternal parents, due to the degradation or exclusion of plastids during male gamete development (reviewed by Hagemann and Schröder 1989). However, a high frequency of biparental (reviewed by Kirk and Tilney-Bassett 1978) or paternal plastid transmission (Schumann and Hancock 1989, Masoud et al. 1990, Bobenz et al. 1990) has also been observed in a few angiosperms. Even in some species thought to exhibit solely maternal plastid inheritance, a low frequency of paternal plastids has been detected in the progeny (Medgyesy 1986, Schmitz and Kowallik 1986). Apparently, the barriers that prevent transmission of plastids from one parent or the other are not absolute.

Genetic studies of plastid transmission in different plant species have suggested that both the nuclear genomes of the parents and the plastomes

can affect the pattern of plastid inheritance. For example, in Petunia hybrida, a species that exhibits maternal plastid inheritance, a paternal genotype was identified that enables the transmission of paternal plastids to a low percentage of the progeny (Cornu and Dulieu 1988). In alfalfa (Medicago sativa), a species that exhibits a high frequency of paternal and biparental plastid transmission, a particular maternal genotype was found to significantly increase the frequency of paternal plastid transmission (Masoud et al. 1990). Similarly, in Pelargonium, the fraction of progeny inheriting plastids from maternal, paternal, or both parents is influenced mainly by a nuclear gene on the maternal side (reviewed by Tilney-Bassett 1988). In contrast, in Oenothera, the evening primrose, the plastome has a major influence on the frequency of biparental plastid transmission (Schötz 1974, 1975, Chiu et al. 1988).

Organelle genetics can be thought of as a question of intracellular population genetics (Birky 1978). Factors that influence either the input ratio of plastids from the parents or the dynamics of the plastid population during embryo development should affect the output of plastids among the progeny (ie, plastid inheritance). In order to understand the mechanisms through which nuclear and plastid genomes may affect plastid transmission, both genetic and molecular approaches were taken in this dissertation to analyze factors that may influence plastid multiplication and transmission in Oenothera.

The genus Oenothera is a unique system for studying plastome-genome interactions in plastid multiplication and transmission. First, Oenothera exhibits a high frequency of biparental plastid inheritance.

Second, broad interspecific crosses are possible in this genus (reviewed by Cleland 1972). Hence, the interactions between different plastomes and between plastomes and nuclear genomes can be studied with a wide variety of combinations. Third, due to extensive chromosomal translocations, many haploid genomes of Oenothera are transmitted as intact chromosomal complexes. This allows the rapid transfer of plastids into a desired nuclear background in a few generations (reviewed by Stubbe and Herrmann 1982). Crosses between some strains of Oenothera result in a phenomenon known as hybrid variegation (reviewed by Kirk and Tilney-Bassett 1978) due to incompatibilities between nuclear and chloroplast genomes. Stubbe (1959) categorized Oenothera plastomes into five basic types (I, II, III, IV and V) according to their compatibility with the six major nuclear genome types (AA, AB, BB, BC, AC and CC). Results from a series of experiments conducted by Schötz (1954, 1974, 1975) suggested that the five basic plastome types of Oenothera have different abilities in competing with each other in crosses and that the nuclear genome has only a minor effect on plastid transmission. The plastome-dependence of both the efficiency and the onset of plastid multiplication after fertilization was confirmed by more controlled experiments (Chiu et al. 1988), in which the transmission efficiencies of Oenothera plastomes were compared in a constant nuclear background.

Since plastid multiplication does not appear to require proteins encoded by the plastome, the observed plastome-dependent variation in Oenothera plastid multiplication and transmission raises a paradox: nuclear-encoded genes are thought to play the most major role in plastid multiplication, and yet the plastome has a major influence on the

efficiency of plastid multiplication. This dissertation examines the hypothesis that the observed plastome-dependent efficiency of plastid transmission is at least partially dependent on the interaction between plastid and nuclear genomes. Thus, both nuclear and plastid components are critical to efficient plastid transmission. Since previous work confirmed the importance of the plastome in this process (Schötz 1954, 1974, 1975, Chiu et al. 1988), the first chapter of this thesis reassesses the degree to which the nuclear genome influences plastid transmission. Specifically, it examines how the relative efficiency of plastid transmission in various nuclear backgrounds relates to the concept of plastome-genome compatibility.

The remainder of this dissertation considers a second hypothesis that a non-coding function of the plastome, the origin of cpDNA replication, may be the plastome component that modulates the efficiency of plastid multiplication. In the second chapter, the cpDNA replication origins of two Oenothera plastomes that have the highest (type I) or the lowest (type IV) multiplication efficiency were localized by electron microscopy. These investigations examined the possibility that differences in the number or location of cpDNA replication origins are responsible for the observed variations in the efficiency of plastid multiplication. The third chapter focuses on a highly variable region near one of the Oenothera cpDNA replication origins to assess the possibility that local DNA sequence variation may affect the efficiency of replication initiation. A model based on the slipped strand mispairing of DNA is proposed to explain the elevated frequency of DNA length mutation in this region.

CHAPTER 1

PLASTOME-GENOME INTERACTIONS AFFECT PLASTID TRANSMISSION IN *OENOTHERA*

Introduction

The majority of angiosperms inherit their plastids only from the maternal parent. However, in some angiosperms, plastids are transmitted at a high frequency from both parents (reviewed by Kirk and Tilney-Bassett 1978) or predominantly from the paternal parent (e.g., Schumann and Hancock 1989, Masoud *et al*. 1990, Bobenz *et al*. 1990). Genetic studies of plastid transmission in different plant species have suggested that plastid inheritance can be affected either by the parental nuclear genomes (Cornu and Dulien 1988, Tilney-Bassett 1988, Masoud *et al*. 1990) or by the plastid genomes (plastomes) involved in the crosses (Schötz 1974, 1975, Chiu *et al*. 1988).

In *Oenothera*, plastids can be transmitted from both parents. In some interspecific crosses of *Oenothera*, biparental inheritance of the plastids can be observed as "hybrid variegation", with plastids from one parent failing to become fully pigmented in the hybrid nuclear background. This genetic dysfunction has been termed "plastome-genome incompatibility". The genetic studies of Stubbe (1959) on this subject led him to categorize the chloroplasts of the subsection *Oenothera* into five basic types (I, II, III, IV and V) according to their compatibilities

with the six major diploid nuclear genotypes (AA, BB, CC, AB, AC and BC).

The five plastome types of Oenothera also differ in their transmission efficiencies. Schötz (1954, 1974, 1975) studied the frequencies of biparental plastid transmission in crosses of a large number of wild-type Oenothera species with several strains carrying mutant plastids. He concluded that the five plastome types have differing abilities to compete with incoming plastomes in crosses and classified the five plastome types as strong (types I and III), intermediate (type II), or weak (types IV and V), according to their relative competitive abilities. The same relative transmission efficiencies were observed when plastome types I through IV were compared in reciprocal crosses in a constant nuclear background (Chiu et al. 1988). These studies, which span four decades, are the basis of the widespread belief that the efficiency of plastid transmission in Oenothera is determined mainly by the plastids themselves (Kirk and Tilney-Bassett 1978, Stubbe 1989).

Although Schötz (1974) pointed out that the nuclear genome can also influence plastid inheritance, the actual role of the nuclear genome in plastid transmission has not been defined in Oenothera. Since both plastome- and nuclear-encoded gene products are required for transcription and translation within the plastid (reviewed by Sugiura 1989), it is conceivable that critical processes such as plastid multiplication and transmission may also rely on the interactions between plastome and genome. In this study, crosses were performed to analyze the impact of both the plastid and nuclear genomes on plastid transmission.

Material and Methods

Plant material. *Oenothera* strains used in this study are listed in Table 1.1 All mutant plastids were maintained as periclinal chimeras in *Oenothera hookeri* str. Johansen (genotype $A^{j}A^{j}$) that carried wild-type plastids of plastome type IV, in addition to the mutant plastids (Chiu *et al*. 1988). All the strains heterozygous for chromosome complexes were provided by Prof. W. Stubbe (University of Düsseldorf, FRG).

Genetic crosses

Group 1: formation of hybrid genotype AC. $A^{j}A^{j}$ plants with mutant plastomes of types I, II, III, and IV were used as the maternal parent in this set of crosses. Pollen was obtained from the *Oenothera* hybrid *albicans/percurvans* ($A^{a}C^{p}$) with plastomes of type II, III, or IV, *Oenothera ammophila* ($A^{r}C^{p}$) with plastomes of type II or IV, *O. parviflora* ($B^{a}C^{s}$-IV) or *O. atrovirens* ($B^{p}C^{f}$-IV). Due to the pollen lethal factors carried by the A^{a}, A^{r}, B^{a} and B^{p} genome complexes, the pollen transmits only the type C chromosome complex (Cleland 1972), and hence, offspring of these crosses have the genotype AC, with A contributed by the egg and C by the pollen.

Group 2: formation of hybrid genotype AB. Crosses yielding progeny of AB-genotype were performed by pollinating *Oenothera* strains carrying a

Table 1.1. Oenothera strains used in the crosses

Oenothera species	Genome complexes[a]	Genotype[b]	Plastome type[c]	Source of plastid
O. hookeri str. Joh	^{h}Johansen.^{h}Johansen	$A^{j}A^{j}$	I-♀	O. hookeri str. Joh
O. hookeri str. Joh	^{h}Johansen.^{h}Johansen	$A^{j}A^{j}$	II-ε	O. suaveolens
O. hookeri str. Joh	^{h}Johansen.^{h}Johansen	$A^{j}A^{j}$	III-γ	O. grandiflora
O. hookeri str. Joh	^{h}Johansen.^{h}Johansen	$A^{j}A^{j}$	IV-α	O. ammophila
hybrid	albicans♀.percurvans♂	$A^{a}C^{p}$	II	O. suaveolens
hybrid	albicans♀.percurvans♂	$A^{a}C^{p}$	III	O. grandiflora
hybrid	albicans♀.percurvans♂	$A^{a}C^{p}$	IV	O. ammophila
O. ammophila	rigens♀.percurvans♂	$A^{r}C^{p}$	II	O. suaveolens
O. ammophila	rigens♀.percurvans♂	$A^{r}C^{p}$	IV	O. ammophila
O. parviflora	augens♀.subcurvans♂	$B^{a}C^{s}$	IV	O. parviflora
O. atrovirens	pingen♀.flectens♂	$B^{p}C^{f}$	IV	O. atrovirens
O. grandiflora	grandiflora.grandiflora	$B^{g}B^{g}$	III	O. grandiflora

[a] The name of each chromosome complex follows Cleland (1972). Haploid genome that can be transmitted only through one of the gametes is indicated with male or female symbol.
[b] Each haploid genome is represented by A, B, or C according to the compatability group to which it belongs (Stubbe 1959). The superscript letter is an abbreviation of the name of the haploid genome.
[c] The Roman numerals indicate the plastome type. If followed by a Greek letter, a plastome mutant is indicated.

type B chromosome complex with pollen from an A^jA^j plant carrying a mutant plastid. Due to the egg lethal factors carried by the C^f and C^s chromosome complexes (Cleland 1972), crosses using either O. parviflora (B^aC^s) or O. atrovirens (B^pC^f) as the maternal parent result in progeny with nuclear backgrounds A^jB^a or A^jB^p, respectively.

Germination and scoring of the seedlings. Seeds were placed in water in a beaker, and were shaken at 100 rpm under constant fluorescent light until they germinated. The seeds were surface-sterilized daily with a combination of 20% commercial bleach and 0.1% SDS until roots emerged (3 - 6 days). Due to the strong maternal bias in Oenothera plastid transmission, crosses in which mutant plastids were contributed by the maternal parents produced mainly white seedlings. For this reason, germinating seeds from White X Green crosses were placed on agar medium, as previously described (Chiu et al. 1988), while germinating seeds from Green X White crosses were transferred to soil.

Progeny with plastids from both parents were recognized by the variegation of their cotyledons. In each cross, the number of progeny with plastids from both parents was recorded, as well as the fraction of both cotyledons with pigmentation characteristic of the plastids inherited from the paternal parent.

Germination of Oenothera pollen in vitro. Mature Oenothera pollen grains were harvested from mature flower buds that were ready to open on the same day (judging from the size and color of the flower bud) by aspirating the pollen into a pasteur pipet. These pollen were sprinkled

onto a thin layer of 0.6% agarose, containing 10% sucrose, 0.01% H_3BO_3, and 0.02% $CaCl_2$ (modified from Corriveau and Coleman 1988) in a petri plate. After two hours of incubation at room temperature, the extent of pollen tube growth was recorded by placing a ruler under the petri plate and estimating the point to point distance between the base and the tip of several typical pollen tubes in a fairly homogeneous population.

Results

Haploid chromosome complex C facilitates the transmission of plastome type IV from pollen

If the efficiency of plastid transmission is influenced by the relative compatability between the plastome and the genome, an enhanced transmission of paternal plastids might be seen when the nuclear background of the progeny is more compatible with the paternal plastid than with the maternal plastids. In order to test this hypothesis, the relative efficiencies of plastid transmission in crosses producing progeny with A^jA^j or A^jC^p genotypes were compared. Genotype A^jA^j is associated with plastome type I in nature (Stubbe 1964). In the A^jA^j nuclear background, chloroplasts with plastomes I, II, and IV have normal pigmentation, while those with plastome III are periodically bleached (Stubbe 1959). Genotype AC is the natural nuclear background of plastome type IV. The relative compatabilities of the same plastome types in an AC nuclear background are in the order of IV $\geq$ II > III > I (Stubbe 1959). Due to plastome-genome incompatibility, AC-I plants were

not viable in the field and A^aC^p-III plants produced pollen with poor vigor. Thus, the analysis of paternal parents with an AC genotype could only include plastomes II and IV.

In the crosses detailed in Table 1.2, the maternal parent had the genotype A^jA^j and contained mutant plastids, representing one of the four basic plastome types. Wild-type plastids of plastome type II or IV were contributed by the pollen. Figure 1.1 shows a comparison of the crosses in which the paternal parent had an A^aC^p genotype (Panel B and C) with previous data, obtained using pollen from A^jA^j plants (Panel A). The transmission efficiency of paternal plastome type II in crosses producing progeny with the genotype A^jC^p (Panel B and C) is somewhat higher, but fairly comparable to that obtained from crosses producing genotype A^jA^j (Panel A). But for plastome type IV, crosses producing progeny with genotype A^jC^p have a much higher transmission efficiency of paternal type IV plastome than in the case for crosses producing progeny with the A^jA^j genotype.

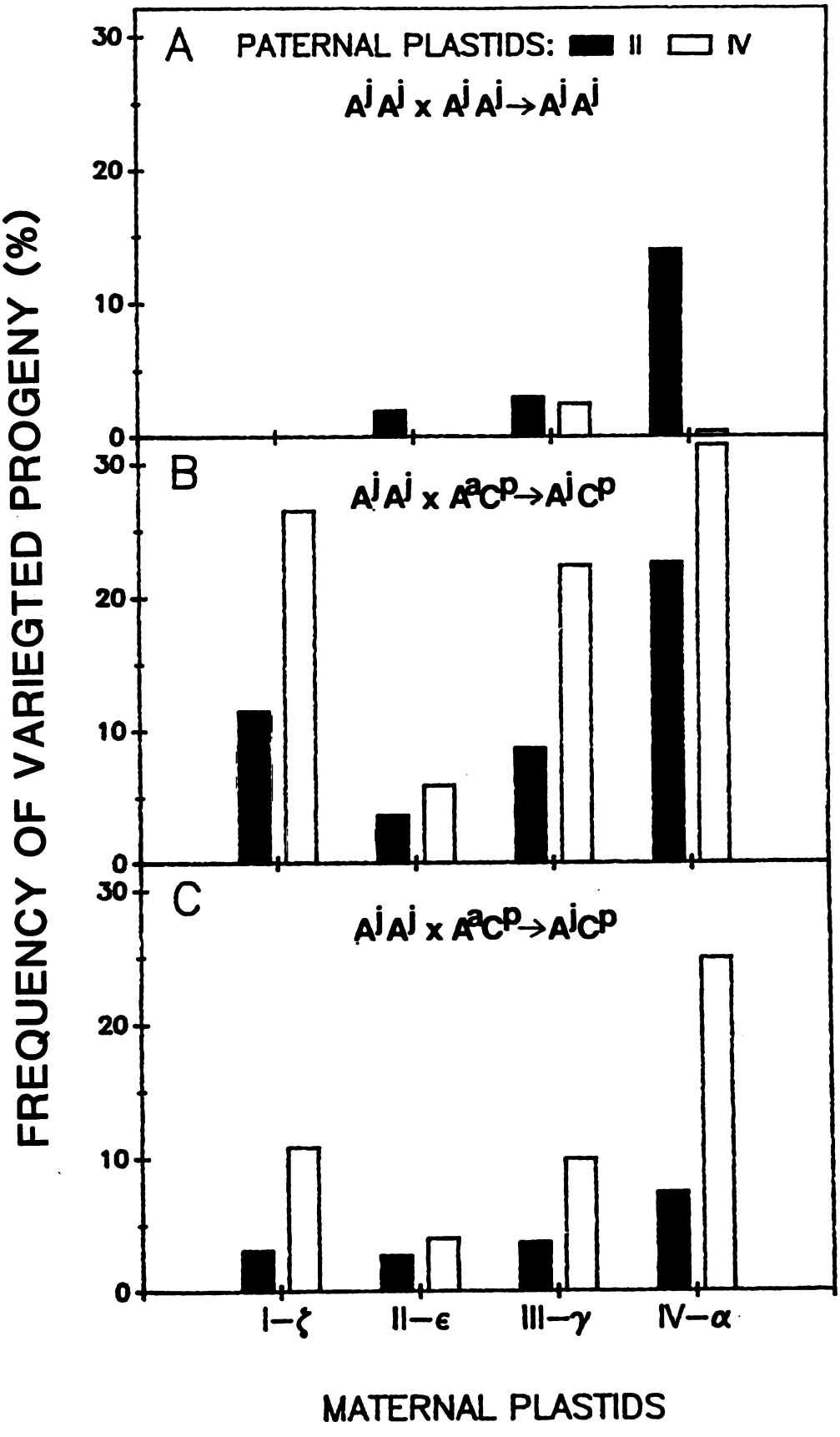
The frequencies of biparental plastid transmission from crosses performed in the first field season were higher than those obtained from the second field season (Table 1.2 and Figure 1.1B and C), but each set of data is internally consistent in terms of the relative transmission efficiencies of the different plastome types. Crosses in the first year were performed later in the season, and their higher frequencies of biparental plastid transmission may be due to the smaller maternal flowers produced late in the season (see Discussion).

Table 1.2. Plastid transmission in crosses producing progeny with AC genotype

Crosses		F_1 Hybrid	Transmission		Average area of green tissue in BP progeny
Maternal	Paternal	Genotype	N	%BP[a]	
Season 1					
Set 1					
A^jA^j-Iγ	A^aC^p-II	A^jC^p	96	11.5±3.3	0.18
A^jA^j-IIε	A^aC^p-II	A^jC^p	84	3.6±2.0	0.17
A^jA^j-IIIγ	A^aC^p-II	A^jC^p	138	8.7±2.4	0.15
A^jA^j-IVα	A^aC^p-II	A^jC^p	53	22.6±5.7	0.31
Set 2					
A^jA^j-Iγ	A^aC^p-IV	A^jC^p	132	26.5±3.8	0.29
A^jA^j-IIε	A^aC^p-IV	A^jC^p	136	5.9±2.0	0.13
A^jA^j-IIIγ	A^aC^p-IV	A^jC^p	67	22.4±5.1	0.11
A^jA^j-IVα	A^aC^p-IV	A^jC^p	133	31.1±4.0	0.29
Set 3					
A^jA^j-IIIγ	B^aC^s-IV	A^jC^s	131	6.9±2.2	0.13
A^jA^j-IVα	B^aC^s-IV	A^jC^s	30	10.0±5.5	0.17
Set 4					
A^jA^j-IIIγ	B^pC^f-IV	A^jC^f	21	0	—
A^jA^j-IVα	B^pC^f-IV	A^jC^f	29	0	—
Season 2					
Set 1					
A^jA^j-Iγ	A^aC^p-II	A^jC^p	162	3.1±1.4	0.14
A^jA^j-IIε	A^aC^p-II	A^jC^p	188	2.7±1.2	0.14
A^jA^j-IIIγ	A^aC^p-II	A^jC^p	190	3.7±1.4	0.13
A^jA^j-IVα	A^aC^p-II	A^jC^p	27	7.4±5.0	0.15
Set 2					
A^jA^j-Iγ	A^aC^p-IV	A^jC^p	176	10.8±2.3	0.15
A^jA^j-IIε	A^aC^p-IV	A^jC^p	99	4.0±2.0	0.20
A^jA^j-IIIγ	A^aC^p-IV	A^jC^p	101	9.9±3.0	0.18
A^jA^j-IVα	A^aC^p-IV	A^jC^p	173	24.9±3.3	0.24
Set 3					
A^jA^j-Iγ	A^rC^p-IV	A^jC^p	438	5.9±1.1	0.19
A^jA^j-IIε	A^rC^p-IV	A^jC^p	152	3.3±1.4	0.20
A^jA^j-IIIγ	A^rC^p-IV	A^jC^p	205	6.8±1.8	0.18
A^jA^j-IVα	A^rC^p-IV	A^jC^p	125	8.0±2.4	0.15

[a] The biparental (BP) transmission frequencies are presented with their standard deviations estimated by the normal approximation for the binomial population (Steel and Torrie 1980).

Figure 1.1. Frequency of progeny carrying plastids from both parents in White X Green crosses of Oenothera. The maternal parents had the genotype A^jA^j and the plastome type indicated on the X-axis. The pollen carried either plastome II (closed bars) or IV (open bars). (A) Both paternal and progeny genotypes were A^jA^j. (B) and (C) Paternal genotype was A^aC^p, and progeny genotype was A^jC^p. The data for (A) is taken from Chiu et al. (1988). The data for (B) and (C) are from Table 2 (season 1 and 2, respectively).


A
PATERNAL PLASTIDS: II IV
$A^iA^i \times A^iA^i \rightarrow A^iA^i$
B
$A^iA^i \times A^aC^p \rightarrow A^iC^p$
C
$A^iA^i \times A^aC^p \rightarrow A^iC^p$
30
20
10
0
I–ζ
II–ε
III–γ
IV–α
FREQUENCY OF VARIEGTED PROGENY (%)
MATERNAL PLASTIDS

The relative success of different maternal plastids also varied with nuclear background. In crosses producing genotype A^jA^j (Figure 1.1A), the transmission success of the maternal plastome was in the order of I-zeta > II-epsilon $\geq$ III-gamma > IV-alpha, as seen by the increasing frequency of plastid transmission from the male parent (judged by the frequency of variegated progeny). However, in crosses producing genotype A^jC^p, the relative success of maternal plastid transmission was in the order of II-epsilon > III-gamma > I-zeta > IV-alpha (Figure 1.1B and C).

To distinguish between effects on plastid transmission exerted by the genotype of the paternal parent as opposed to the genotype of the progeny, two strains, A^aC^p-IV and A^rC^p-IV, were used. These two strains carry different genotype A complexes (A^a vs. A^r) but the same C chromosome complex (C^p), which is the haploid genome that can be transmitted through pollen. According to the results from the second season (Table 1.2, Season 2), pollen plastids are transmitted at a lower frequency from A^rC^p pollen (Set 3) than from A^aC^p pollen (Set 2). Thus, the transmission of plastome type IV from pollen carrying the identical C genome can vary, depending on the genotype of the pollen parent. An attempt to include A^rC^p-II in these comparisons failed because plastome-genome incompatibility resulted in poor vigor of the A^rC^p-II plants and poor pollen tube growth.

Crosses with two other strains capable of contributing chromosome complex C from pollen, B^aC^s-IV and B^pC^f-IV, were also conducted to allow a comparison of the impact of different C genomes on plastid transmission. As shown in Table 1.2, the transmission frequency of

plastids from B^aC^s-IV pollen (Season 1, Set 3) was lower than that from A^aC^p-IV pollen (Season 1, Set 2). Crosses between A^jA^j strains and B^pC^f-IV produced very few seeds. As will be discussed later, this may have been due to limited pollen tube growth. No plastids from the paternal B^pC^f-IV parent were detected in a total of 50 progeny produced from these crosses (Season 1, Set 4). Despite the difficulty in obtaining seeds from some crosses, the results from crosses involving pollen carrying a C genome indicate that the presence of a C genome in the pollen or progeny does not ensure efficient transmission of plastome type IV from pollen. Furthermore, the diploid genome of the paternal parent may also affect the transmission efficiency of pollen plastids.

Relative efficiency of plastid transmission differs in reciprocal crosses that result in progeny with different hybrid genotypes.

In reciprocal crosses of those performed in season 2 of Table 1.2, the same A^jA^j plants carrying mutant plastomes I through IV were used as paternal parents while A^aC^p-II, A^aC^p-IV and A^rC^p-IV plants were used as maternal parents. Due to the egg lethal factors carried by chromosome complex C^p, only the A complexes are transmitted by the maternal parents. In these crosses, resulting in the hybrid nuclear background A^aA^j or A^rA^j, maternally- contributed plastome type II (Table 1.3, Set 1) was consistently transmitted at a higher efficiency than maternally-contributed plastome type IV (Table 1.3, Sets 2 and 3). The efficiencies of paternal plastid transmission were in the order of I-zeta $\geq$ III-gamma > II-epsilon > IV-alpha, in all three sets of crosses. Thus, the relative transmission efficiency was different from that

Table 1.3. Plastid transmission in AC X AA crosses[a]

Crosses		F_1 Hybrid	Transmission		Average area of white tissue in
Maternal	Paternal	Genotype	N	%BP[b]	BP progeny
Set 1					
A^aC^p-II	A^jA^j-Iζ	A^aA^j	164	75.0±3.4	0.34
A^aC^p-II	A^jA^j-IIε	A^aA^j	121	19.0±3.6	0.14
A^aC^p-II	A^jA^j-IIIγ	A^aA^j	93	61.3±5.0	0.20
A^aC^p-II	A^jA^j-IVα	A^aA^j	195	6.2±1.7	0.12
Set 2					
A^aC^p-IV	A^jA^j-Iζ	A^aA^j	127	82.7±3.4	0.45
A^aC^p-IV	A^jA^j-IIε	A^aA^j	184	69.0±3.4	0.24
A^aC^p-IV	A^jA^j-IIIγ	A^aA^j	201	85.6±2.5	0.30
A^aC^p-IV	A^jA^j-IV	A^aA^j	120	39.2±4.5	0.14
Set 3					
A^rC^p-IV	A^jA^j-Iζ	A^rA^j	235	89.4±2.0	0.45
A^rC^p-IV	A^jA^j-IIε	A^rA^j	187	76.5±3.1	0.29
A^rC^p-IV	A^jA^j-IIIγ	A^rA^j	209	85.6±2.4	0.35
A^rC^p-IV	A^jA^j-IVα	A^rA^j	196	31.6±3.3	0.25

[a] Reciprocal crosses of those listed in Table 1.2, season 2.
[b] see Table 1.2.

observed in reciprocal crosses resulting in progeny with genotype $A^{J}C^{P}$ but the same as that observed in crosses resulting in genotype $A^{J}A^{J}$ (Chiu et al. 1988).

The relative transmission efficiencies of plastome types I and III from the pollen are reversed in progeny with AB vs. AA nuclear backgrounds

Plastome type I is more compatible with an AA nuclear background than is plastome III, while the reverse is true with an AB nuclear background (Stubbe, 1959). The crosses shown in Table 1.4 resulted in hybrid genotype AB. In all three sets of crosses, mutant plastome III-gamma was transmitted from the pollen with a higher efficiency than was mutant plastome I-zeta. In crosses resulting in genotype $A^{J}B^{a}$ (Set 1) or $A^{J}B^{g}$ (Set 3), the relative efficiency of paternal plastid transmission was in the order of III-gamma > II-epsilon > I-zeta > IV-alpha, while in crosses resulting in genotype $A^{J}B^{P}$ (Set 2), the order of I-zeta and II-epsilon was reversed.

In variegated progeny, the abundance of tissue displaying the paternal plastid phynotype in individual progeny is an additional parameter that can be used to assess the competitive multiplication abilities of the various plastome types. In general, the abundance of paternal plastids in the cotyledons has a positive correlation with the frequency of biparental plastid transmission, especially when both values are high (see Tables 1.2, 1.3 and 1.4). In progeny with an $A^{J}B^{P}$ nuclear background, the fraction of white tissue was much higher when the pollen contributed mutant plastome III-gamma than when mutant plastome I-zeta

Table 1.4. Plastid transmission in crosses producing progeny with genotype AB

Crosses		F_1 Hybrid	Transmission		Average area of white tissue in
Maternal	Paternal	Genotype	N	%BP[a]	BP progeny
Set 1					
B^aC^s-IV	A^jA^j-Iζ	A^jB^a	218	77.1±2.8	0.14
B^aC^s-IV	A^jA^j-IIε	A^jB^a	129	82.2±3.4	0.25
B^aC^s-IV	A^jA^j-IIIγ	A^jB^a	108	87.0±3.2	0.25
B^aC^s-IV	A^jA^j-IVα	A^jB^a	23	53.8±10.4	0.16
Set 2					
B^pC^f-IV	A^jA^j-Iζ	A^jB^p	196	85.7±2.5	0.33
B^pC^f-IV	A^jA^j-IIε	A^jB^p	285	75.4±2.6	0.37
B^pC^f-IV	A^jA^j-IIIγ	A^jB^p	293	91.8±1.6	0.47
B^pC^f-IV	A^jA^j-IVα	A^jB^p	162	35.2±3.8	0.19
Set 3					
B^gB^g-III	A^jA^j-Iζ	A^jB^g	220	16.4±2.5	0.19
B^gB^g-III	A^jA^j-IIε	A^jB^g	50	24.0±6.0	0.10
B^gB^g-III	A^jA^j-IIIγ	A^jB^g	156	46.2±4.0	0.17
B^gB^g-III	A^jA^j-IVα	A^jB^g	203	8.4±2.0	0.11

[a] see Table 1.2.

was brought in through the pollen (Figure 1.2B). This was true even though the crosses producing these progeny had very similar frequencies of biparental transmission (91.8% vs. 85.7% in Table 1.4). The relative abundance of the two paternal plastids was reversed in progeny with an $A^{j}A^{a}$ nuclear background (Figure 1.2A), which is more compatible with plastome I.

Plastome-genome interaction can affect pollen tube growth

When pollen from Oenothera species with small flowers was used to fertilize strains with large flowers, seed development was always limited to the upper part of the capsule. This phenomenon was most likely due to the limited growth capacity of pollen tubes produced by the small flowered species. However, differences in the extent of fertilization could also be observed among pollen from strains with the same genotype but carrying different plastomes. An example of this can be seen in the seed capsules from $A^{j}A^{j}$ flowers that had been pollinated with pollen from $A^{a}C^{p}$-II or $A^{a}C^{p}$-IV plants (Figure 1.3). Judging from the development of the capsule and the location of viable seeds in the capsules, pollen from the $A^{a}C^{p}$-II plants could only fertilize eggs in the upper part of the capsule (Figure 1.3B).

The differential capacity for pollen tube growth among Oenothera strains was examined in vitro. Most pollen tubes from large flowered $A^{j}A^{j}$ strains carrying any of the four plastome types reached a length of 1 cm after two hours of incubation at room temperature. In the same period of time, pollen tubes from a strain with medium-sized flowers, $A^{a}C^{p}$-IV,

Figure 1.2. Frequency distribution of the degree of variegation in biparental progeny.

Crosses were performed between maternal parent (A) $A^{a}C^{p}$-IV or (B) $B^{p}C^{f}$-IV and paternal parent $A^{j}A^{j}$-I (closed bars) or $A^{j}A^{j}$-III (open bars). The X-axis indicates the fraction of cotyledon area occupied by the white plastids transmitted from the pollen parent, with the height of the bars showing the percentage of progeny in each group. The frequencies of biparental plastid transmission from these crosses are listed in Tables 3 and 4 respectively.

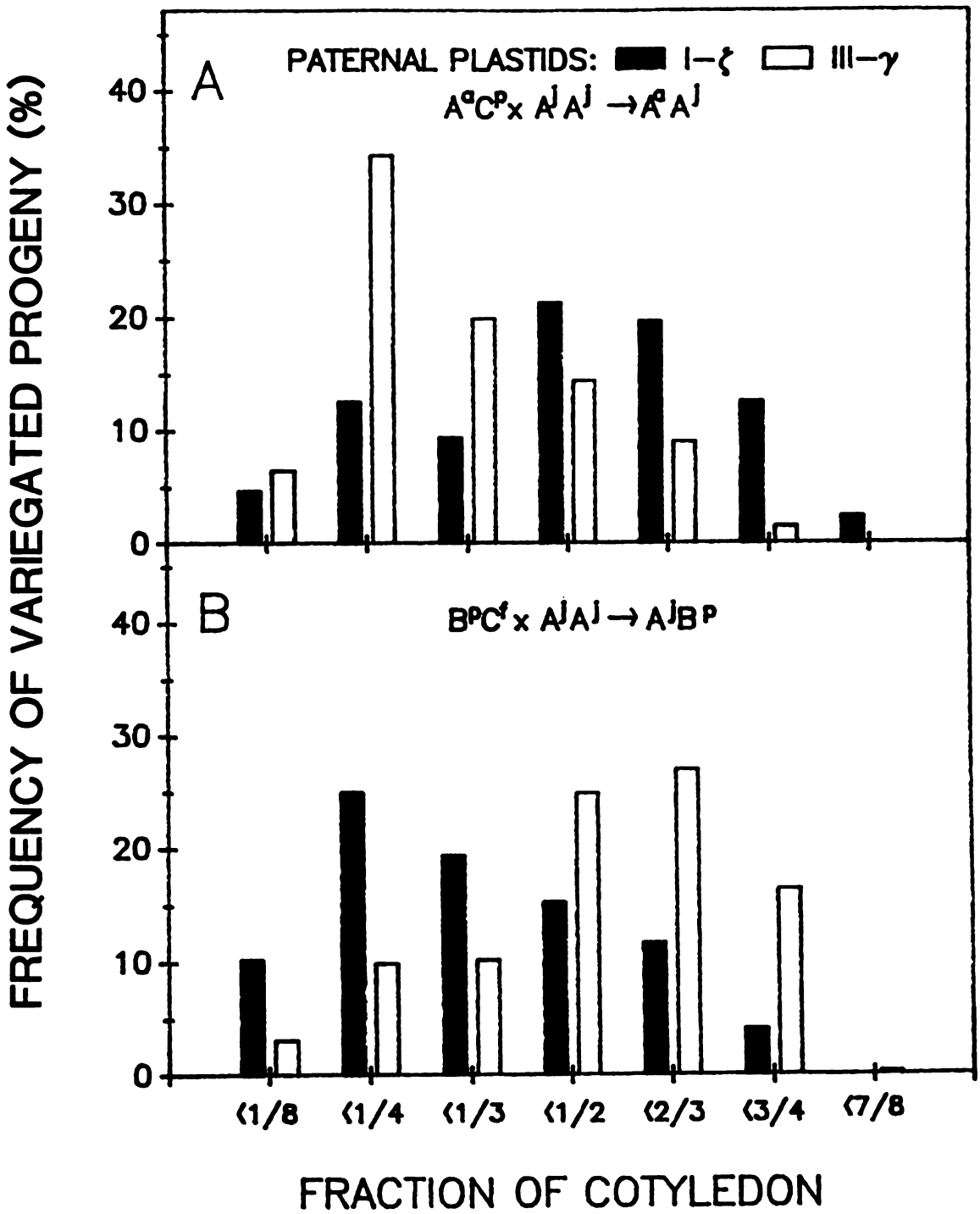
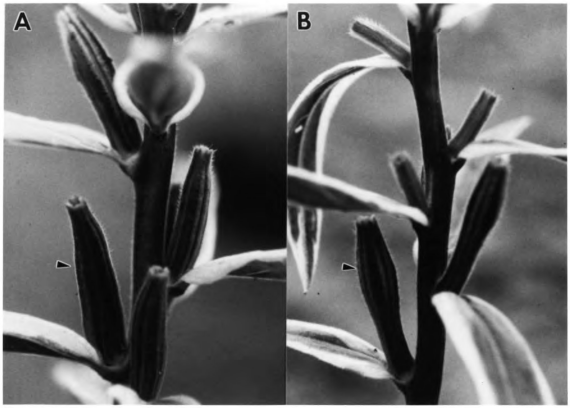

A
PATERNAL PLASTIDS: I–ζ III–γ
$A^aC^p \times A^jA^j \rightarrow A^aA^j$
B
$B^pC^f \times A^jA^j \rightarrow A^jB^p$
FREQUENCY OF VARIEGATED PROGENY (%)
0
10
20
30
40
⟨1/8
⟨1/4
⟨1/3
⟨1/2
⟨2/3
⟨3/4
⟨7/8
FRACTION OF COTYLEDON

Figure 1.3. Seed capsule development from crosses using A^aC^p-IV or A^aC^p-II pollen. The maternal parent was genotype A^jA^j with mutant plastids, and the paternal parent was either: (A) A^aC^p-IV (A) or (B) A^aC^p-II. The arrow in each panel indicates a well developed capsule.


A
B

reached a length of 0.5 cm, while pollen tubes from the same strain carrying plastome type II (A^aC^p-II) grew to only 0.2 cm. The latter is a length similar to that attained by pollen tubes from a small-flowered strain, A^rC^p-IV, over the same period of time. Conceivably, the Figure incompatibility between plastome type II and haploid genome C (Stubbe 1959) reduces the vigor of the pollen tube, although the pollen is nonetheless fertile.

Discussion

The pattern of plastid inheritance in Oenothera has been thought to be determined primarily by the plastome, due to different intrinsic rates of plastid multiplication associated with each plastome type (Schötz 1954, 1974, 1975; Chiu et al. 1988). The present study modifies this concept and stresses the importance of plastome-genome interactions in plastid transmission. The influence of both the plastid and nuclear genomes on the relative efficiencies of plastid transmission is evident either by comparing the frequency of biparental plastid transmission or by assessing the relative abundance of paternal plastids in the variegated progeny.

Although the plastome-genome compatibility is generally measured as the ability of plastids to become fully pigmented in a given nuclear background, this phenotype may serve as an indication of how well the two genetic compartments can cooperate with each other in a broader sense. Indeed, the relative efficiencies of plastid transmission

correlate rather well with the compatibilities of the plastomes with the nuclear background of the progeny. For example, the relative success of three maternal plastomes in crosses producing progeny with an AC genotype (II-epsilon > III-gamma > I-zeta; Table 1.2, Figure 1.1B, C) fell in the same order as the compatibilities of these plastomes with the AC nuclear background (Stubbe 1959). Similarly, the relative transmission efficiencies of paternal plastomes I-zeta and III-gamma were reversed in crosses producing progeny with AA or AB genotypes, as might be expected from their relative compatibilities with these nuclear backgrounds. An extreme example of the influence of plastome-genome compatibility on plastid transmission was reported by Stubbe (1963), who recovered progeny carrying only paternal plastids in Oenothera from crosses producing a hybrid genotype that was severely incompatible with the maternal plastid. The influence of plastome-genome compatibility on plastid transmission is independent of the photosynthetic ability of the plastids, since the correlation between transmission efficiency and compatibility applies to white mutant plastids as well as wild-type plastids. The correlation between the relative degree of compatibility and the relative efficiency of plastid transmission can be explained if the efficiency of plastid multiplication is dependent at least somewhat upon the cooperative interactions between plastome and genome.

Schötz (1974) noticed that the competitive ability of a plastome was highest in its native nuclear background. However, due to two major limitations in his experiments, Schötz did not observe the broad effect that plastome-genome interactions can exert on plastid transmission. First, because of the low viability of the mainly "white" progeny from

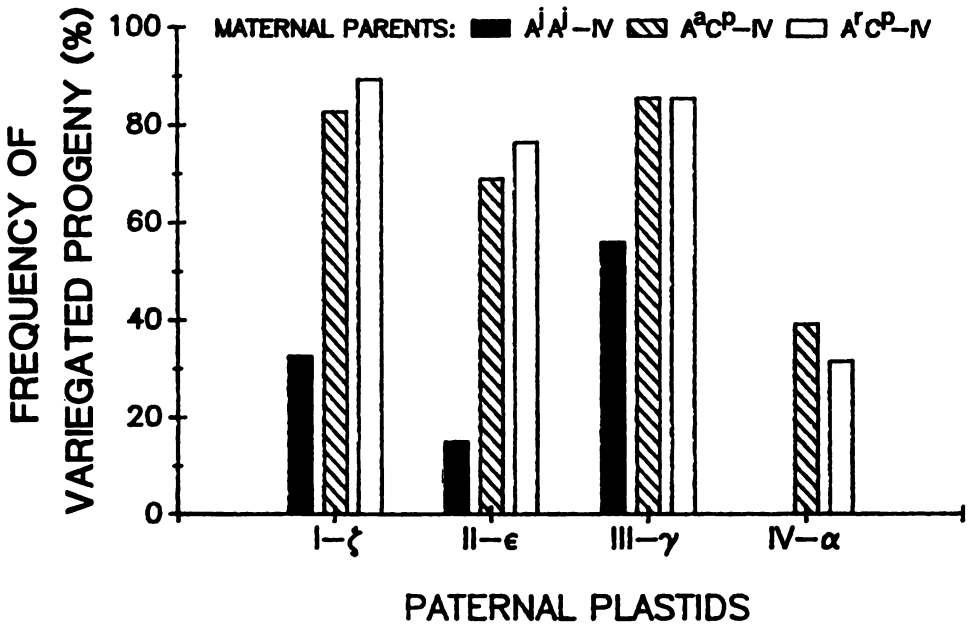
"White x Green" crosses (Schötz 1954), only "Green x White" crosses were performed in his subsequent studies (Schötz 1974, 1975). Since many of the chromosome complexes of Oenothera are only transmitted through either the egg or the pollen, reciprocal crosses often produce progeny of different genotypes. In the absence of reciprocal crosses, the impact of certain genotypes, especially the native nuclear background of plastome IV (the "weak" plastome), on plastid transmission was rarely examined. Second, due to the limitations in the available natural plastome-genome combinations, the transmission abilities of all plastome types were not compared simultaneously in the same nuclear background. In this study, the ability to compare results from reciprocal crosses and to compare the four Oenothera plastomes simultaneously in several different nuclear backgrounds made it possible to obtain a more complete view of the role of both plastid and nuclear genomes in plastid transmission.

In addition to its influence on the relative efficiency of plastid multiplication, the compatibility between plastome and genome may also affect plastid transmission through its influence on other steps in the process of fertilization, such as pollen tube growth. The growth capacity of the pollen in vitro is correlated positively to the size of the flowers from the pollen donor. However, the growth of the pollen tubes also can be affected by the plastome type carried by the plant: pollen from an A^aC^p strain that carried the less compatible plastome type II could grow only to a length that was half that of pollen carrying plastome type IV. The influence of the plastid evidently extends to pollen tube growth in vivo, since A^aC^p-II pollen could only

fertilize eggs on the upper part of the capsule when used to pollinate A^jA^j, a strain with large flowers (Figure 1.3). Poor pollen tube growth may also be responsible for the failure of seed production in crosses between A^jA^j and two even less compatible combinations, A^rC^p-II and A^aC^p-III. As discussed below, the vigor of pollen tube growth may have a significant effect on the input of paternal plastids to the zygote.

The impact of the maternal genotype on Oenothera plastid transmission appears to correlate with the size of the flower. Among the species used in this study, O. hookeri str. Johansen (A^jA^j) and O. grandiflora (B^gB^g) are strains with large flowers. The length of the style in these large-flowered strains is 7 to 9 cm. O. ammophila (A^rC^p), O. parviflora (B^aC^s) and O. atrovirens (B^pC^f) have small flowers and their styles are only 2 to 3 cm long. The flower of hybrid strain A^aC^p is of medium size with an average style length of 4.5 cm. In all cases, flower size is not significantly influenced by the plastome type of the plant. When a constant pollen source is used in crosses, a much higher frequency of paternal plastids is detected in the progeny when strains with small flowers are used as maternal parents, as compared to maternal parents with large flowers (Table 1.3, 1.4 and Chiu et al. 1988). The influence of maternal flower sizes on plastid transmission is illustrated by the three sets of crosses shown in Figure 1.4, where pollen was contributed from A^jA^j lines carrying mutant plastids and either A^jA^j-IV (large flower), A^aC^p-IV (medium flower), or A^rC^p-IV (small flower) was used as the maternal parent. Progeny from these crosses all have genotype AA and plastome type IV from the maternal parent. Although biparental plastid transmission occurred at similar frequencies with plants having small

Figure 1.4. Frequency of biparental plastid transmission in Green X White crosses of Oenothera. The paternal parents had the genotype $A^{j}A^{j}$ and the mutant plastome indicated on the X-axis. The maternal parents carried plastome type IV with $A^{j}A^{j}$ (large flower), $A^{a}C^{p}$ (medium flower) or $A^{r}C^{p}$ (small flower). Data for crosses involving $A^{j}A^{j}$-IV (closed bars) are from Chiu et al. (1988); data for crosses using $A^{a}C^{p}$-IV (hatched bars) and $A^{r}C^{p}$-IV (open bars) as maternal parents are from Table 3.


FREQUENCY OF VARIEGATED PROGENY (%)
100
80
60
40
20
0
MATERNAL PARENTS: $A^{i}A^{i}$–IV $A^{a}C^{p}$–IV $A^{r}C^{p}$–IV
I–ζ
II–ε
III–γ
IV–α
PATERNAL PLASTIDS

and medium flowers as the maternal parent, when crosses involved a large-flowered maternal parent, a consistently lower frequency of biparental plastid transmission was observed. A similar observation was made by Schötz (1974), who reported that a small flower variant of O. hookeri allowed the transmission of more paternal plastids than did the original strain. Conceivably, the differences in plastid heredity could be due to differences in the input of maternal plastids that correlate with the size of the flower. According to Schötz (1954), the strain with the smallest flowers did have slightly fewer plastids in the egg (between 14 and 26), but all other strains examined had similar numbers (between 20 and 30). These results led Schötz to conclude that differences in the input of maternal plastids should not have a major impact on plastid transmission.

This study agrees with Schötz, and find it more likely that the input of paternal plastids has a major impact on the final result of plastid transmission. For all Oenothera strains examined, 90 to 100% of the mature pollen generative cells contain plastid DNA aggregates (Corriveau and Coleman 1990, and personal communication). However, high frequencies of paternal plastid transmission are achieved only when the maternal parent is a small-flowered strain or when the paternal parent produces pollen capable of vigorous growth. The influence of maternal flower size and the growth capacity of pollen tubes on plastid transmission can be best explained if plastids degenerate in the generative cell as the pollen tube extends towards the egg. According to this hypothesis, when a slow-growing pollen tube progresses through a very long style, the time between pollination and fertilization is longer, and more plastids

in the generative cell would be degraded before fertilization. Plastid degeneration during pollen development is one of the mechanisms through which maternal plastid inheritance is achieved in several plant species (reviewed by Hagemann and Schröder 1989) including Epilobium (Schmitz and Kowallik 1987), a close relative of Oenothera. In Epilobium, plastids are inherited almost exclusively from the maternal side, but biparental transmission can occasionally be detected (Schmitz and Kowallik 1986).

At least 14% of the angiosperms surveyed contain plastid DNA in their pollen generative cells (Corriveau and Coleman, 1988) and should be able to transmit their plastids through the pollen. However, based on this study and earlier studies of plastid transmission in Oenothera (Schötz 1954, 1974, 1975, Chiu et al. 1988) and Pelargonium (Tilney-Bassett and Almouslem 1989), the final pattern of plastid inheritance depends not only on the input of the parental plastids but also on the plastome, the interaction between plastome and genome, as well as plastome-independent effects determined by the nuclear genome of the parental plants.

CHAPTER 2

ELECTRON MICROSCOPIC LOCALIZATION OF REPLICATION ORIGINS IN *OENOTHERA* CHLOROPLAST DNA

Introduction

Chloroplast DNA (cpDNA) replication in land plants is not coupled to the synthesis of nuclear DNA. Rather, the timing of cpDNA amplification is under developmental control (Lamppa and Bendich 1979, Lawrence and Possingham 1986). *In vivo* studies using inhibitors of protein synthesis (Heinhorst *et al*. 1985) and studies of mutant chloroplasts completely lacking ribosomes (Walbot and Coe 1979, Scott *et al*. 1982) suggest that all proteins essential for cpDNA replication and plastid multiplication are nuclear-encoded. Indeed, a search of sequenced land plant chloroplast genomes has not revealed any genes similar to those known to encode proteins involved in DNA replication, with the possible exception of the gene for single strand DNA binding protein (ssb) (reviewed by Umesono and Ozeki 1987, Sugiura 1989).

Despite the apparent dispensibility of plastid-encoded proteins in cpDNA replication, the plastid genome (plastome) appears to be critical in determining the efficiency of plastid multiplication, as judged by genetic studies of differential plastid transmission in the evening primrose, *Oenothera*. In crosses of *Oenothera*, plastids can be

transmitted from both parents to the progeny (reviewed by Kirk and Tilney-Bassett 1978). However, the frequency of biparental plastid inheritance is strongly influenced by the plastome types involved in a given cross (Schötz 1974, Schötz 1975, Chiu et al. 1988). Schötz (1974) suggested that the five major plastome types of subsection Oenothera (reviewed by Stubbe 1989) have different rates of multiplication and ranked them into three categories with regard to their abilities to compete with each other in crosses: "strong" (plastome types I and III), "medium" (plastome type II) and "weak" (plastome types IV and V). The results of comparing plastid transmission in a constant nuclear background lent support to this concept (Chiu et al. 1988).

Since plastid function depends on plastid gene products, it is essential that cpDNA replication keep pace with plastid division to ensure that both daughter plastids receive DNA. With this in mind, we have considered the possibility that differences at the cpDNA origin of replication could be responsible for the observed differences in plastid multiplication intrinsic to the plastome type. Variation in the efficiency of mitochondrial DNA replication has been suggested as an explanation for the differential transmission of mitochondria in baker's yeast, Saccharomyces cerevisiae (Piskur 1988, Piskur 1989). The mitochondrial genome of S. cerevisiae has eight potential DNA replication origins and mitochondria with some of these origins and the flanking regions deleted are transmitted less efficiently than wild-type mitochondria. Since the cpDNA of the five Oenothera plastome types differ from each other by a number of insertions or deletions (Gordon et al. 1982), it is possible that some of these length mutations might

affect the number of replication origins or the efficiency with which the origins are recognized by nuclear-encoded proteins and, consequently, plastid multiplication. As a first step to test this hypothesis, we have located the origins of cpDNA replication in two Oenothera plastomes that differ greatly in their transmission efficiencies.

Materials and Methods

Plant material. Oenothera hookeri str. Johansen, carrying its native plastome type I, or plastome type IV from O. atrovirens (Chiu et al. 1988), was used for this study.

CpDNA preparation. Young leaves (0.1-5 cm long) from actively growing plants were used for DNA isolations. CpDNA from 30 to 50 g of leaves was prepared according to the protocol described by Palmer (1982), with some modifications. Sucrose gradient centrifugation was omitted, and cpDNA was separated from nuclear DNA by two successive CsCl gradient centrifugations in the presence of the flourescent dye bisbenzimide (Sigma) at 0.01 mg/ml. The dye was removed by two extractions with isopropanol saturated with 5M NaCl, and the cpDNA was dialyzed against three changes of dialysis buffer (10 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl) at 4°C, using a dialysis apparatus and collodion membranes with an average retention of 25 kd (Schleicher & Schuell). Purified cpDNA was stored at -20°C in small aliquots until immediately before use. CpDNA was digested with restriction enzymes (GIBCO-BRL) in high salt buffer

(React 3) for two hours at room temperature and was then immediately processed for electron microscopic analysis.

Electron microscopy and data analysis. Spreading of DNA for electron microscopy was carried out according to Kolodner and Tewari (1975a). X174 DNA and its RF form (obtained from GIBCO-BRL) were used as internal standards for single-stranded and double-stranded DNA, respectively. Grids were shadowed with platinum-palladium (80:20) and reinforced with a thin layer of carbon. The samples were examined using a Philips 201 electron microscope. The electron micrographs were enlarged and the contour of each DNA molecule was traced on a digitizing table (Calcomp 91480) connected to an IBM XT PC computer. The length of each DNA fragment was calculated and converted to kb according to the internal standards, using software written by the Michigan State University Computer Center and Jeff Elhai (Michigan State University).

Results

Oenothera cpDNA replication is initiated through the formation of multiple displacement loops

The preparation procedure yielded a relatively high frequency of intact chloroplast DNA molecules. In the best preparation, nearly a quarter of the intact cpDNA molecules contained displacement loop (D-loop) replication intermediates, which result from unidirectional synthesis of one daughter strand (Kornberg 1980). Frequently, more than one D-loop was observed on the same DNA molecule. These D-loops lay either within

several kb (Figure 2.1A) or quite far apart (est. 30-50 kb) from each other (Figure 2.1B,C). Very rarely, D-loops could be observed at four sites on the same molecule, as two distant pairs of D-loops.

D-loop initiation sites are located in both copies of the inverted repeats

In order to localize the sites of D-loop initiation, cpDNA was digested with SalI and PstI immediately prior to processing for the electron microscope. Digestion of Oenothera cpDNA with SalI or PstI yields 11 and 12 fragments, respectively (Figure 2.2). Except for minor DNA fragment length differences, the restriction maps for these two enzymes are the same for plastome types I and IV (Gordon et al. 1982).

Digestion with restriction enzymes greatly reduced the frequency with which D-loops were observed. A total of 54 SalI fragments bearing D-loops (Figure 2.3) were characterized: 29 from plastome I and 25 from plastome IV. Nine out of these 54 fragments contained two adjacent D-loops (Figure 2.3C). The sizes and numbers of the D-loop containing fragments are presented in Figure 2.4A. According to the observations with intact cpDNA, D-loops should be expected in at least two seperate regions of Oenothera cpDNA (Figure 2.1B and C). As shown in Figiure 4A, D-loops were most frequently found in SalI fragments between 15 and 20 kb, but they were also present in some larger SalI fragments (30-40 kb). However, since the largest expected SalI fragment is around 30 kb (Gordon et al. 1982), fragments much larger than 30 kb are most likely products of incomplete SalI digestion. To calculate the observed sizes

Figure 2.1. Closed circular molecules of Oenothera chloroplast DNA (plastome type IV) with D-loops. Arrows indicate the displaced strand. ØX174 DNA and its RF form (M) are used as internal standards for single stranded and double stranded DNA, respectively. (A) Part of an intact cpDNA molecule that contains two adjacent D-loops. Bar, 1kb. (B) An intact cpDNA molecule with two distant D-loops. Bar, 5kb. (C) Part of an intact cpDNA molecule containing two D-loops approximately 30 kb apart. The D-loop on the left has a short tail resulting from strand migration. Bar, 5 kb.

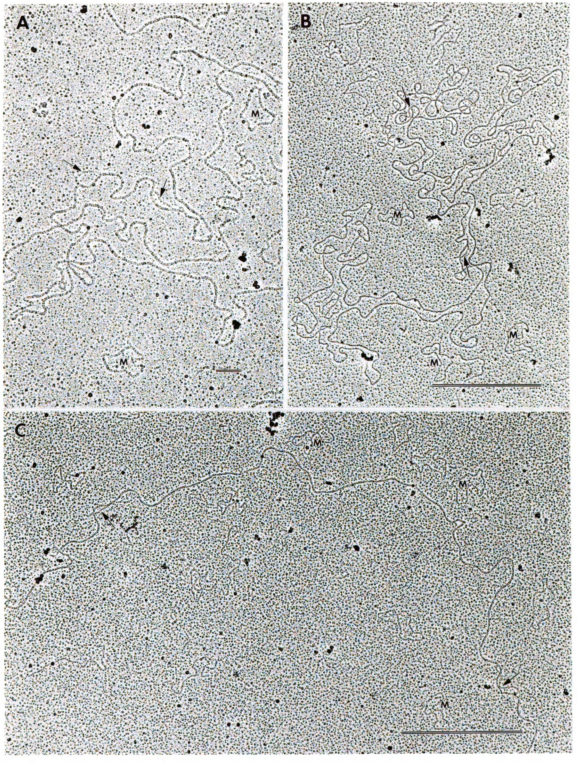
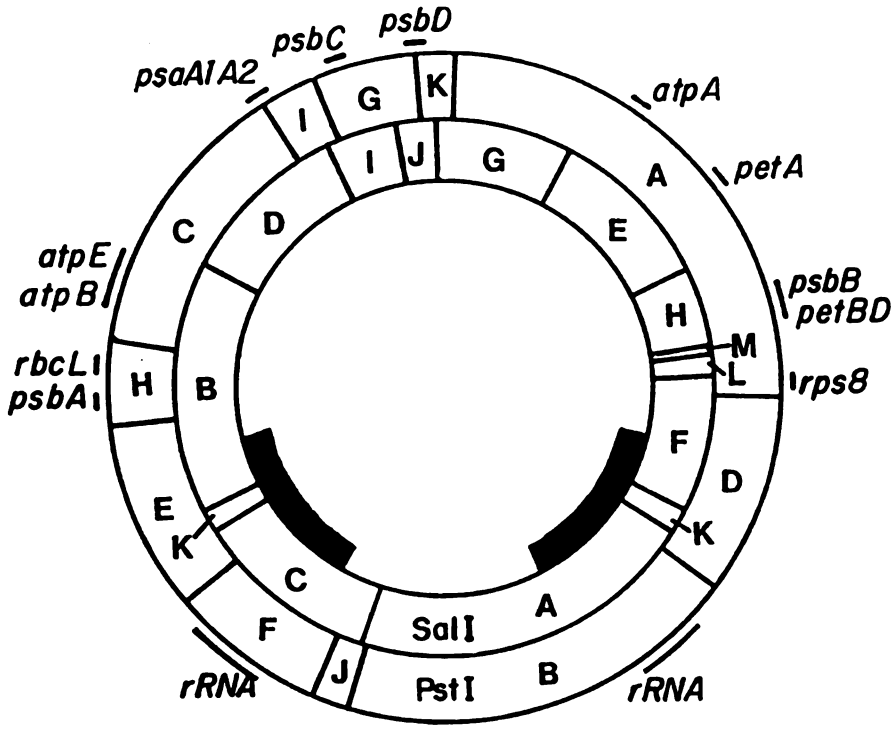

A
M
M
B
M
M
M
M
C
M
M
M

Figure 2.2. Restriction and partial gene map of Oenothera cpDNA. From Gordon et al. 1982 and Herrmann et al. 1983. The large inverted repeats, characteristic of DNA from most higher plant chloroplast genomes, are outlined by black arcs. SalI and Pst I fragments are labeled, with the largest fragment in each case designated as A.


psbC
psbD
psaA1A2
atpA
petA
atpE
atpB
psbB
petBD
rbcL1
psbA1
rps8
rRNA
rRNA
SalI
PstI
A
B
C
D
E
F
G
H
I
J
K
L
M

Figu

with

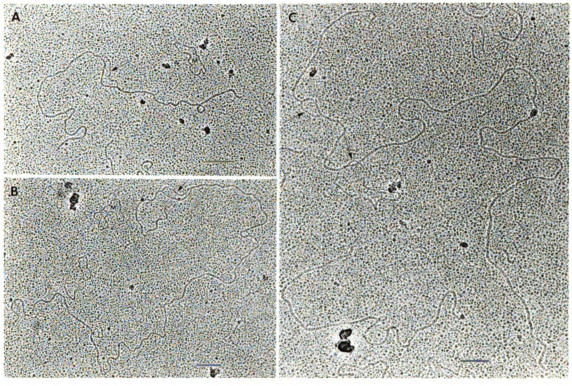
Arro

with

loop

Figure 2.3. Electron micrographs of cpDNA (plastome type I) digested with SalI.

Arrows point to the displaced strand of each D-loop. (A) 18 kb fragment with one D-loop, (B) 30 kb fragment with one D-loop, (C) Two adjacent D-loops on the same fragment. Bar, 1 kb.


A
B
C

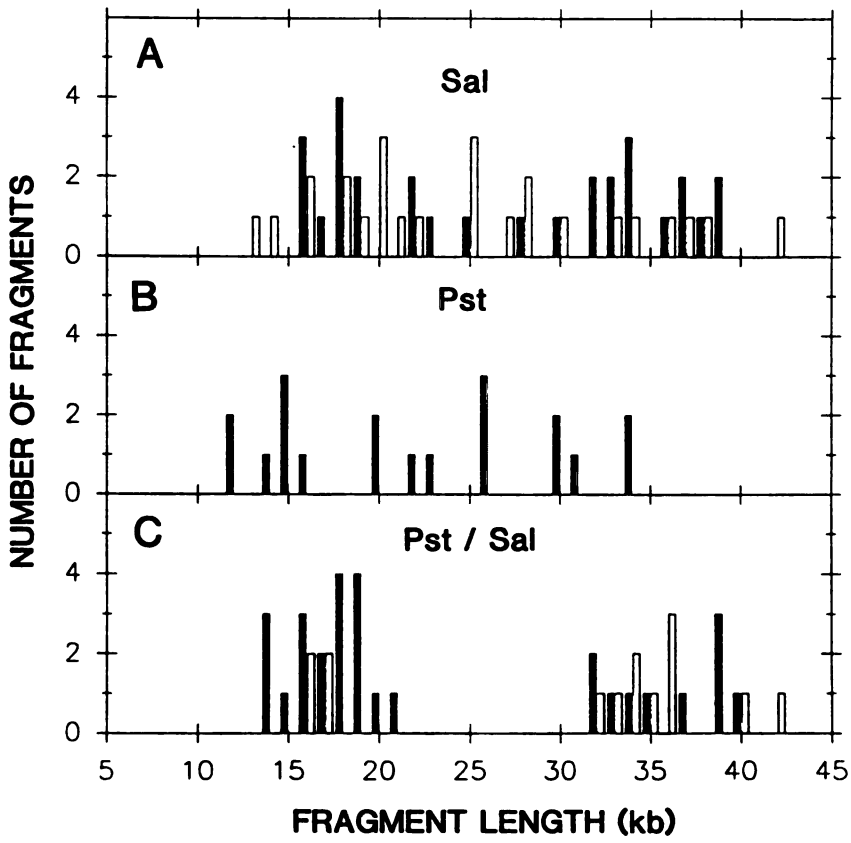
for D-loop-bearing SalI fragments, those SalI fragments smaller than 22 kb were grouped into one class and those larger than 22 kb the other. For both plastome types I and IV (Table 2.1), the smaller class of D-loop-containing fragments had an average size of 18 kb (n=23), and the larger class had an average size around 32 kb (n=31). These sizes are comparable to the third largest (Sal C, 18 kb) and the largest (Sal A, 30 kb) SalI fragments, respectively. Sal A and Sal C are two neighboring fragments spanning most of the inverted repeats and the entire small single copy region of the Oenothera plastome (Figure 2.2).

When plastome type I DNA was digested with PstI, D-loops were observed on fragments ranging from 12 to 34 kb (Figure 2.4B). Those fragments smaller than 20 kb have an average size of 14 kb (n=7), which is the expected size of the major PstI fragment that overlap Sal C (Figure 2.2). The size distribution of those fragments larger than 20 kb are quite scattered. However, judging from the position of the D-loops on these fragments (see below), most of these fragments should represent the PstI fragments (Pst B) that overlap Sal A (Figure 2.2).

If D-loops are located within the regions spanned by Sal A and Sal C, double digestion of cpDNA with PstI and SalI should also yield two fragments that carry D-loops (Figure 2.2). For both plastome types I and IV, the double digestions of PstI and SalI resulted in two size groups of D-loop-containing fragments (Figure 2.4C) with average sizes around 17 kb and 36 kb respectively (Table 2.1). Although the same sample gave the expected patterns for PstI and SalI double digestion when visualized on an agarose gel, the average sizes of these D-loop-containing

Figure 2.4. Length distribution of cpDNA fragments containing D-loops from plastome types I and IV.

Fragments generated by digestion with (A) SalI (B) PstI (C) PstI + SalI. CpDNA fragments of plastome type I are represented by filled bars, while those of plastome type IV are represented by empty bars. The vertical axis indicates the number of fragments in each size class observed to have D-loops.


A
Sal
B
Pst
C
Pst / Sal
NUMBER OF FRAGMENTS
0
2
4
5
10
15
20
25
30
35
40
45
FRAGMENT LENGTH (kb)

fragments are larger than the lengths of the expected fragments (Table 2.1). Conceivably, complete SalI and PstI double digestions bring D-loops closer to the end of the fragments and that might have rendered the D-loops less stable due to strand migration.

To orient the positions of the D-loops on the restricted cpDNA fragments, the location of D-loops on each fragment was measured. A length, d, was defined as the shortest distance from an end of the fragment to a terminus of the D-loop. It is clear that D-loops can occur at two adjacent sites on the same region of the cpDNA (Figure 2.1A). The proximal terminus was chosen as the point of measure for the D-loops closer to the end of the fragment and the distal terminus was chosen for the more distant D-loops. Each terminus of the D-loops defined in this way probably coincides with the replication origin for one daughter strand, according to the model for the initiation of cpDNA replication in land plants (Kolodner and Tewari 1975b). In fact, since most of the D-loops are relatively small in size, the placement of a D-loop was not affected greatly by the choice of terminus. Two classes of d were observed and the measurement in these classes were grouped as d_1 and d_2 (Table 2.1).

If the D-loops on fragments Sal A and Sal C lie within the inverted repeats, then D-loops on both PstI fragments should be 2 kb closer to the end of the fragments than D-loops on the SalI fragments (Figure 2.4). As shown in Table 2.1, this prediction is fulfilled since d_1 and d_2 from PstI fragments are about 2-3 kb shorter than measurements derived from the related SalI fragments. The shortening of arm length

Table 2.1. Distribution of D-loops in cpDNA of Oenothera plastomes I and IV

Enzyme	Plastome	d_1[a] (n)	d_2[a] (n)	Obs. length (n)	Exp. length[b]
SalI	I	3.6±0.6 (6)	8.1±0.3 (5)	18.0±0.5 (11)	Sal C 17.9
	IV	4.2±0.5 (7)	9.1±0.5 (5)	18.0±0.8 (12)	Sal C 17.8
	I	6.1±0.7 (13)	11.3±0.8 (10)	32.5±1.2 (18)	Sal A 30.2
	IV	6.3±0.5 (8)	10.9±0.4 (8)	31.3±1.6 (13)	Sal A 30.2
PstI	I	1.9±0.4 (6)	5.6±0.3 (3)	14.1±0.6 (7)	Pst F 14.2
	I	3.0±0.8 (2)	8.2±0.9 (9)	26.8±1.4 (12)	Pst B 28.4
PstI	I	3.0±0.3 (9)	7.0±0.5 (12)	17.5±0.5 (19)	Pst F 14.2
+	IV	3.0±0.4 (3)	7.2 (1)	16.6±0.3 (4)	Pst F 14.2
SalI	I	4.2±0.4 (6)	10.9±0.7 (6)	36.0±1.0 (10)	P+S A 28.2
	IV	4.3±0.6 (5)	9.9±0.5 (7)	35.8±1.0 (10)	P+S A 28.2

[a] Average distance ± standard error (in kb) between the terminus (as defined in text) of D-loop structure and the nearer end of the fragment.

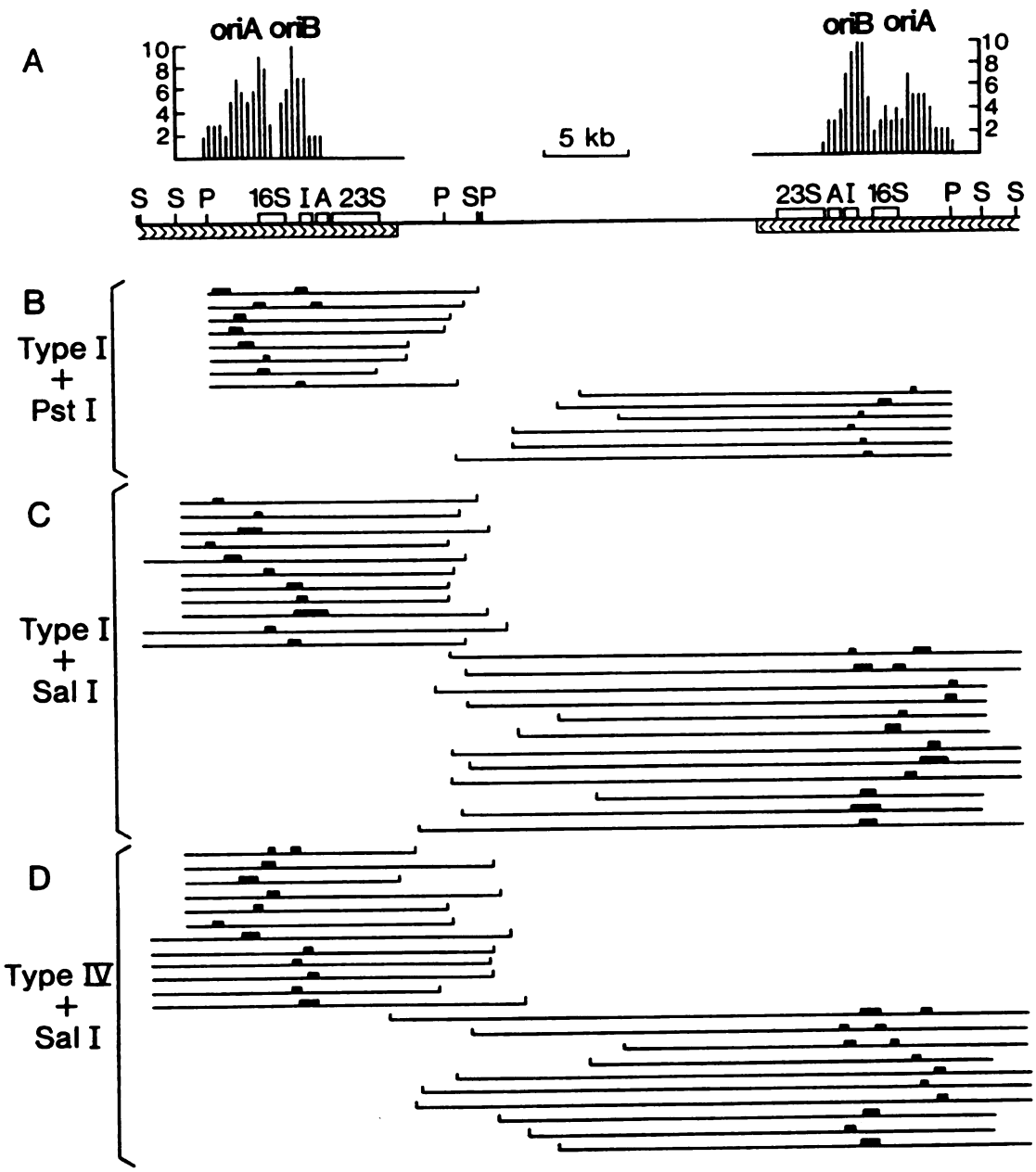
[b] Length expected from physical map of Gordon et al. (1982). Sizes expressed in megadaltons in the original paper have been converted to kilobase pairs.

coincides well with the position of PstI sites on Sal A and Sal C (Figure 2.4). This placement of D-loops would further predict that the shorter distance from a D-loop to the end of a SalI fragment should be the same for both Sal A or Sal C. However, this distance is about 2 kb larger in the case of Sal A than SalC (Table 2.1). This discrepancy can be explained if many of the larger SalI fragments that contain D-loops are the products of partial digestion: Sal A joined to the adjacent Sal K. Consistent with this interpretation, the average length of the larger SalI fragment is 2 kb greater than the expected length (Table 2.1). Furthermore, two distant D-loops are observed to be at least 30 kb apart on intact cpDNA molecules (Figure 2.1B, C). This distance can be explained only if the D-loop initiation sites are located toward the distant ends of the two neighboring SalI fragments within the inverted repeat.

Each pair of D-loop initiation sites is in the close vicinity of rRNA genes.

In order to see the positions of D-loop initiation sites relative to genes on the plastome, most of the SalI or PstI fragments containing D-loops were aligned to the end of their short arms (Figure 2.5B, C and D). Fragments that are much longer than the predicted sizes due presumably to incomplete digestions were not included in this analysis. Either Sal A or Sal C fragments that were slightly longer and may have included the neighboring Sal K were aligned in Figure 2.5 to the appropriate SalI site 2 kb away. In some cases, the original alignment placed the D-loop initiation sites within the 16S or 23S rRNA genes.

Figure 2.5. Proposed alignment of D-loop-containing molecules relative to the rRNA operon and the small single copy region of Oenothera cpDNA. (A) Frequency distribution of the D-loop structures, summing data from plastomes I and IV. Physical map of the Sal A (right) and Sal C (left) region. The positions of genes within the rRNA operon are indicated with open boxes, with 16S and 23S indicating the rRNA genes, I designating trnI and A designating trnA. The most likely location for the D-loop initiation sites are indicated with oriA and oriB. (B-D) Scale of lines indicates length of fragments containing D-loops (indicated as black boxes): (B) plastome type I DNA digested by PstI, (C) plastome type I DNA digested by SalI, and (D) plastome type IV DNA digested by SalI.


A
oriA oriB
10
8
6
4
2
5 kb
oriB oriA
10
8
6
4
2
S S P 16S I A 23S P SP
23S A I 16S P S S
B
Type I
+
Pst I
C
Type I
+
Sal I
D
Type IV
+
Sal I

Since the rRNKA genes are highly concerved, and have never been cited to function as origins of DNA replication, We realigned these fragments to alternative restriction sites. Restriction analysis of the cloned 14 kb Pst F fragment from both plastome types revealed only a 100 bp deletion in plastome type IV relative to type I (Chapter 3), and therefore, data from both plastome types were pooled to establish a more precise localization of D-loop initiation sites. As shown in the D-loop distribution frequency histogram (Figure 2.5A), two regions appear to be preferential sites for D-loop initiation on both copies of the inverted repeat: One (oriA) is 1 - 2 kb upstream from the 16S rRNA gene and the other (oriB) is in the spacer of the rRNA operon near the tRNA-Ile gene. The distance between the two spacer regions is about 30 kb, which coincides with the distance between two distant D-loops in Figure 2.1C.

Discussion

Both the transmission efficiency of Oenothera plastids and their competitive multiplication rates are influenced by the plastome type (Schötz 1974, Chiu et al. 1988). Since plastids can multiply even in the absence of protein synthesis within the plastid (reviewed by Boffey 1985), the different multiplication efficiencies of Oenothera plastids may be attributable to differences in the non-coding components of the plastomes, such as the origins of cpDNA replication. This possibility inspired the present study.

CpDNA replication in Oenothera was found to be initiated through the

formation of D-loops (Figure 2.1). One pair of D-loop initiation sites was mapped to each copy of the large inverted repeat (Figure 2.2) of the plastome: one lies 1 - 2 kb upstream from the 16S rRNA gene, while the other lies in the spacer between the two rRNA genes (Figure 2.5). In these respects, plastome type I, having the highest transmission efficiency, is indistinguishable from plastome type IV, having the lowest efficiency in transmission. Hence, differential plastid multiplication in Oenothera cannot be explained simply by differences in the number or placement of functional cpDNA replication origins present in the plastome. However, this does not preclude the possibility that sequence differences at the replication origin could affect the efficiency of replication initiation.

In order to locate the replication origins, care was taken to preserve unstable D-loop replication intermediates in the purified DNA. All the restriction digestions in this study were performed at room temperature for a limited amount of time to minimize strand migration. This may have contributed to the frequent appearance of partial digestion products in the DNA spreads. Despite the measures taken to minimize strand migration, D-loops were observed far less frequently in digested cpDNA than in uncut samples. It is conceivable that D-loops lying near the ends of fragments or on small fragments may have been lost. However, the relative locations of D-loops on uncut DNA are consistent with the four D-loop initiation sites determined on the restricted fragments.

In no other organism besides Oenothera has cpDNA replication been shown to proceed by two pairs of D-loops. In maize, cpDNA fragments that can

initiate replication *in vitro* have been identified (Gold *et al.* 1987). Whether these fragments can initiate cpDNA replication *in vivo* have not been confirmed by electron microscopy. Pea, the only other land plant in which the D-loop initiation sites of cpDNA have been localized, has only one pair of cpDNA replication origins near the rRNA operon: one in the spacer region within the rRNA operon, as in *Oenothera*, and the other several kb downstream from the 23S rRNA (Meeker *et al.* 1988). However, the plastome of pea differs from most other land plants in that it has only one copy of the rRNA operon (Palmer and Thompson 1981). Since the rRNA operon generally is found within the large inverted repeat that is common to the cpDNA of most land plants (reviewed by Palmer 1985) including *Oenothera*, it is likely that the number and the placement of replication origins in the *Oenothera* plastome is typical of those in plastomes of most other land plants. The placement of replication origins near the rRNA operon may have some functional importance since several algae also have their cpDNA replication origins near the rRNA operon although the exact position differs with respect to rRNA genes (Koller and Delius 1982, Ravel-Chapuis *et al.* 1982, Waddell *et al.* 1984, preliminary results in Yamada *et al.* 1986).

The two adjacent D-loop initiation sites in the *Oenothera* plastome may represent separate origins for the replication of each strand, as suggested in studies on cpDNA replication in pea and maize (Kolodner and Tewari 1975a, b) and mitochondrial DNA replication in animals (reviewed by Clayton 1982). It has been proposed that unidirectional synthesis of a daughter strand from each neighboring origin results in a replication eye (Cairns structure, Cairns 1963) with two double stranded arms, from

which bidirectional DNA synthesis proceeds (Kolodner and Tewari 1975b). Although no such structures were observed in this study, replication forks with two double stranded arms were observed in some fragments. In cpDNA of the algae Chlamydomonas (Waddell et al. 1984), Euglena (Koller and Delius 1982; Ravel-Chapuis et al. 1982) and Acetabularia (Santulli et al. 1983), loops with two double stranded arms are the major or only replication intermediates that have been observed. Apparently, in these organisms, either the same origin is used for the replication of both strands or the replication origins for each strand are very close to each other. It will be necessary to analyze cpDNA replication in vitro (Wu et al. 1986, Meeker et al. 1988) to locate the exact sites of replication initiation in plastids of Oenothera and to reveal further features of the cpDNA replication origins that may be common to land plants and algae.

CHAPTER 3

PROLIFERATION OF DIRECT REPEATS NEAR THE OENOTHERA CHLOROPLAST DNA ORIGIN OF REPLICATION

Introduction

Plastids can be transmitted from both parents in crosses of Oenothera, the evening primrose (reviewed by Kirk and Tilney Bassett, 1978), resulting in progeny in which two different plastid genomes (plastomes) may coexist in the same cell. This biparental plastid transmission facilitates the study of plastome-plastome as well as plastome-genome interactions. Five major types of plastome are found among species of the subsection Euoenothera (reviewed by Stubbe, 1989), and the relative efficiency of plastid transmission is strongly influenced by the plastome types involved in the crosses (Schötz, 1974, 1975, Chiu et al. 1988).

As a first step towards understanding molecular factors contributed by the plastome that may affect the efficiency of chloroplast multiplication, origins of Oenothera chloroplast DNA (cpDNA) replication were localized through electron microscopic analysis of restriction fragments carrying replication intermediates (Chapter 2). CpDNA replication in Oenothera is initiated through the formation of displacement loops (D-loops). One pair of replication initiation sites was found within each copy of the large inverted repeat of cpDNA from both plastome type I,

which has the highest transmission efficiency and plastome type IV, which has the lowest. One D-loop initiation site (oriA) lies about 1-2 kb upstream from the rRNA operon, and the other (oriB) lies in the spacer between the 16S and 23S rRNA genes.

In this chapter, restriction fragment length polymorphisms in regions surrounding the D-loop initiation sites were analyzed to search for local sequence variations that might affect the efficiency of replication initiation. A highly variable region was identified in the 16S rRNA-trnI spacer near a D-loop initiation site (oriB) of Oenothera plastome. Analysis of this intergenic region revealed multiple duplications of related sequence motifs. Mechanisms that might be responsible for the hypervariability in this region and the possible impact of these length mutations on DNA replication will be discussed.

Material and Methods

Plant material. Wild-type Oenothera plastomes from the following strains were used in this study: Oenothera hookeri str. Johansen carrying the Düsseldorf line of plastome type I (I^D) (Chiu et al. 1990), or plastome type II, III or IV (Chiu et al. 1988); and Oenothera hookeri standard with its native plastome type I (I^{HK}). The plastome from Cornell-1 (I^{C1}), a derivative of I^D, was obtained from O. hookeri str. Johansen homozygous for the plastome mutator allele (Chiu et al. 1990). Chloroplast DNA was isolated according to Chiu et al. (1990).

Restriction analysis and sequencing. Enzymes for restriction analysis and cloning were obtained from GIBCO-BRL. SalI fragments that were 18 kb in size (Sal C, Figure 3.1A) from six Oenothera plastomes were purified by phenol/chloroform extraction from 0.8% low melting agarose following gel electrophoresis in TAE buffer (Maniatis et al. 1982), and cloned into the SalI site of pRL498 (Elhai and Wolk 1988). The largest EcoRI fragments (R2, the second largest EcoRI fragment of Oenothera cpDNA, approximately 5 kb) in Sal C from six Oenothera plastomes were subcloned into the EcoRI site of pBR328. Subsequent electrophoresis of restricted DNA was performed in TBE buffer (Maniatis et al. 1982).

EcoRI-BssHII subfragments of R2 (Figure 3.1A) from the two type I plastomes, I^{D} and I^{C1}, were obtained by cutting the two R2 clones (pOjD-R2 and pOjC1-R2) with BssHII, filling in the ends with Klenow fragment of DNA Polymerase I, and then cutting with EcoRI. The fragments thus obtained were ligated with pIC20H (Marsh et al. 1985) that had been digested with EcoRI and SmaI. Plasmids containing the smaller EcoRI-BssHII subfragment from R2 of I^{D} and I^{C1} were identified according to the sizes of their inserts and were named pOjD-BE1 and pOjC1-BE1 respectively. Deletion derivatives of pOjD-BE1 were obtained using exonuclease III (Henikoff 1984). First, pOjD-BE1 was digested with SstI and EcoRI, cutting in the polylinker region derived from pIC20H. The DNA was then treated with exonuclease III for different lengths of time, resulting in unidirectional digestion starting from the EcoRI end, and the enzyme was inactivated at 70°C for 15 minutes. The ends of the resulting fragments were made blunt with mungbean nuclease and rejoined. Two derivatives were obtained by this method: pOjD-BE2 and pOjD-BE3,

carrying 600 bp and 400 bp inserts, respectively. Other deletion derivatives of pOjD-BE1 and pOjC1-BE1 were obtained by cutting the original plasmids with either HincII or BamHI and religating.

The DNA sequences of the inserts in pOjD-BE1 and pOjC1-BE1 were obtained using Sequenase (United States Biochemical Corporation) and methods for double stranded DNA sequencing (Zhang et al. 1989). To obtain sequences from both strands, all the deletion clones of pOjD-BE1 and pOjC1-BE1 mentioned above were also used.

Computer programs used for sequence analysis. A computer program, EDITBASE, for two dimensional comparison of nucleic acid sequences provided by Niels Nielsen (Purdue University) was used to search for repeated sequences. Regions capable of forming secondary structures were found using PC-FOLD (Zuker 1989).

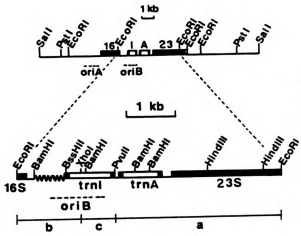
Results

One D-loop region is proximal to a highly variable region of the *Oenothera* plastome.

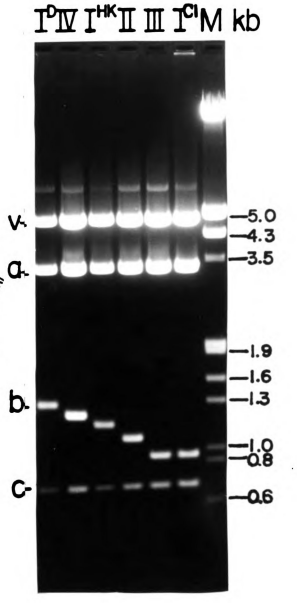
The third largest SalI fragment (SalC) from *Oenothera* plastome types I and IV contains two D-loop initiation sites (Ch. 2). This fragment was cloned from six *Oenothera* plastomes and analyzed after restriction with EcoRI (Figure 3.1A and data not shown). No length polymorphism was

Figure 3.1. Localization of the hypervariable region in the rRNA operon of Oenothera cpDNA. (A) Physical map of SalC (top) and R2 (bottom) fragments of the Oenothera plastome. The genes for 16S rRNA and 23S rRNA, trnI (I) and trnA (A), are represented with filled boxes. The region of variable size is represented with a wavy line. BamHI sites within this region are not shown. The two preferential sites for D-loop initiation (oriA and oriB) are indicated with dashed lines since their precise locations are still unknown. (B) Restriction digestion patterns of the R2 fragments from six Oenothera plastomes. Plasmids, each containing an R2 fragment inserted into the EcoRI site of pBR328, were digested with EcoRI + PvuII + XhoI. Lanes contained digested R2 clones from plastomes I^{D}, IV, I^{HK}, II, III and I^{C1}. Molecular weight markers (M) consisted of lambda DNA digested with EcoRI and HindIII. Fragments marked a, b, and c are indicated in the diagram in panel A, and fragment V is from the pBR328 vector.

A


1 kb
SalI
PstI
EcoRI
16S
I A
23S
oriA
oriB
1 kb
EcoRI
BamHI
BspHII
NciI
PvuII
HindIII
16S
trnI
trnA
23S
oriB
b
c
a

B


I^D Ⅳ I^HK Ⅱ Ⅲ I^Cl M kb
5.0
4.3
3.5
1.9
1.6
1.3
1.0
0.8
0.6
a
b
c

detected for the 4 kb subfragment that contains one D-loop initiation site (oriA) located upstream from the rRNA operon. However, the size of the EcoRI fragment that lies internal to the rRNA operon varied among the Oenothera plastomes that were examined. This approximately 5 kb fragment (R2) contains oriB, a second D-loop initiation site (Figure 3.1A). R2 fragments derived from these Oenothera plastomes were subcloned into pBR328 and analyzed further.

A conserved PvuII site at the end of the gene for tRNA-Ile (trnI) and an XhoI site in the trnI intron are present in the rRNA operons from chloroplasts of several land plants (Massenet et al. 1987). Restriction digests showed that these two sites are also present in Oenothera (Figure 3.1A). Restriction analysis of the cloned R2 fragments showed that the length variation among six Oenothera plastomes is contained within the smaller EcoRI/XhoI fragment (fragment b in Figure 3.1). Further restriction analysis confined the variable region to the sequences bounded by EcoRI and BssHII (Figure 3.1A and data not shown). This region covers sequences separating the 16S rRNA gene from trnI and is close to oriB. The fragment from five wild-type Oenothera plastomes ranged in size from 400 bp to 900 bp. The variable fragment in plastome I^{C1}, a variant of I^{D}, is much smaller than that of its wild-type predecessor and has a size similar to that of plastome type III (Figure 3.1B).

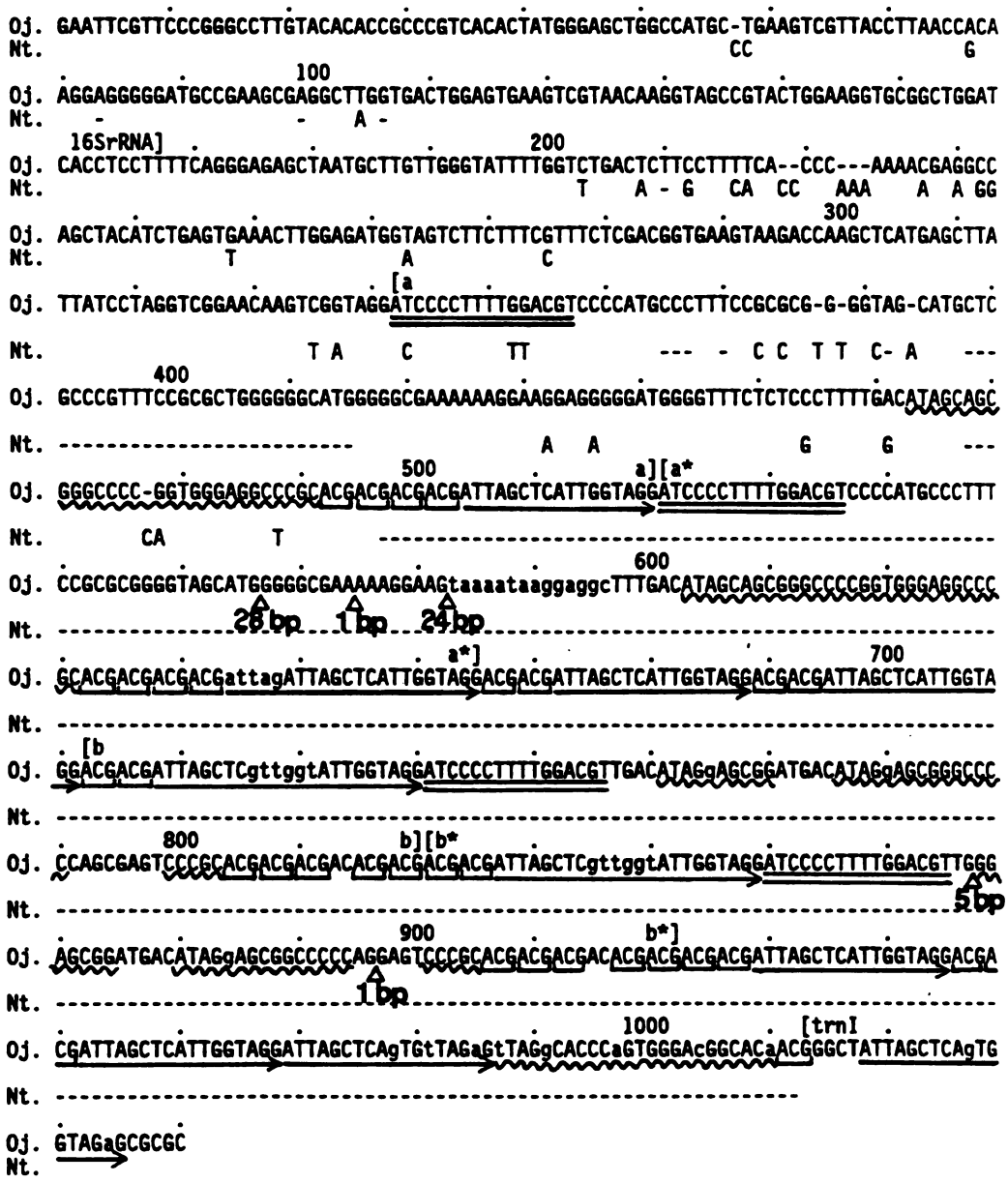
Sequence comparison of the 16S rRNA-trnI spacer.

The DNA sequences of the variable EcoRI-BssHII fragments (Figure 3.1A)

from I^D and I^{C1} were determined. The 1040 bp DNA sequence from I^D is shown in Figure 3.2 and is compared with the same region from the tobacco plastome. Overall homology between the two sequences is quite high (90% sequence identity), excluding two insertions in Oenothera cpDNA relative to tobacco: one of 28 bp (positions 388 to 415) and the other of 516 bp (positions 497-1012). The larger insertion occurs immediately 5' to trnI.

Analysis of the 516 bp insertion revealed multiple repeated sequences. For example, positions 521 to 665 (segment a* in Figure 3.2) is a 125 bp duplication of positions 342 to 520 (segment a in Figure 3.2) with two deletions of 28 and 24 bp, and two small insertions (lower case letters in a*, Figure 3.2). The 28 bp deletion in a* makes this region more similar than a to the equivalent region in the tobacco plastome. The smaller duplication, b (109 bp) and b* (102 bp), consist of DNA sequence that can also be found at the junction between a and a* (positions 498 to 536) and three segments within a* (positions 599 to 612, 599 to 617 and 627 to 643). Interspersed between these two major repeated segments are several 17 bp direct repeats (symbolized by a long arrow) first appearing at the end of a. This 17 bp segment is almost identical to part of the 5' trnI exon (15/17 match). The 5' end of the 17 bp repeats is usually accompanied by tracks of $(ACG)_n$, which also appear at the 5' junction between trnI and the spacer in tobacco plastome.

Figure 3.2. Sequence comparison of the EcoRI-BssHII fragment from Oenothera hookeri str. Johansen plastome I^D (Oj.) and N. tobaccum (Nt.). Tobacco sequences identical to that of Oenothera are not shown. Base substitutions in tobacco relative to Oenothera are indicated with capital letters while missing bases in both sequences are represented by dashes. Two families of large repeated segments in Oenothera are indicated as a, a*, b and b*. The positions and sizes of two deletions in a* relative to a are indicated by Δ, with the number of base pairs deleted indicated underneath. Long arrows represent a repeating 17 bp unit. Double lines indicate a repeating 16 bp unit. Wavy lines indicate the sequences equivalent to the region immediately upstream of trnI in tobacco plastome. Horizontal brackets indicate the repeating ACG element. Lower case letters represent inserted or substituted bases in the repeated units.


Oj. GAATTCGTTCCCGGGCCTTGTACACACCGCCCGTCACACTATGGGAGCTGGCCATGC-TGAAGTCGTTACCTTAACCACA
Nt. CC G
100
Oj. AGGAGGGGGATGCCGAAGCGAGGCTTGGTGACTGGAGTGAAGTCGTAACAAGGTAGCCGTACTGGAAGGTGCGGCTGGAT
Nt. - - A -
16SrRNA]
200
Oj. CACCTCCTTTTCAGGGAGAGCTAATGCTTGTTGGGTATTTTGGTCTGACTCTTCCTTTTCA--CCC---AAAACGAGGCC
Nt. T A - G CA CC AAA A A GG
300
Oj. AGCTACATCTGAGTGAAACTTGGAGATGGTAGTCTTCTTTCGTTTCTCGACGGTGAAGTAAGACCAAGCTCATGAGCTTA
Nt. T A C
[a
Oj. TTATCCTAGGTCGGAACAAGTCGGTAGGATCCCCTTTTGGACGTCCCCATGCCCTTTCCGCGCG-G-GGTAG-CATGCTC
Nt. T A C TT --- - C C T T C- A ---
400
Oj. GCCCGTTTCCGCGCTGGGGGGCATGGGGGCGAAAAAAGGAAGGAGGGGGATGGGGTTTCTCTCCCTTTTGACATAGCAGC
Nt. ---------- A A G G ---
500
a][a*
Oj. GGGCCCC-GGTGGGAGGCCCGCACGACGACGACGATTAGCTCATTGGTAGGATCCCCTTTTGGACGTCCCCATGCCCTTT
Nt. CA T ----------
600
Oj. CCGCGCGGGGTAGCATGGGGGCGAAAAAGGAAGtaaaataaggaggcTTTGACATAGCAGCGGGCCCCGGTGGGAGGCCC
Nt. ---------- 26 bp 1 bp 24 bp ----------
a*]
700
Oj. GCACGACGACGACGattagATTAGCTCATTGGTAGGACGACGATTAGCTCATTGGTAGGACGACGATTAGCTCATTGGTA
Nt. ----------
[b
Oj. GGACGACGATTAGCTCgttggtATTGGTAGGATCCCCTTTTGGACGTTGACATAGgAGCGGATGACATAGgAGCGGGCCC
Nt. ----------
800
b][b*
Oj. CCAGCGAGTCCCGCACGACGACGACACGACGACGACGATTAGCTCgttggtATTGGTAGGATCCCCTTTTGGACGTTGGG
Nt. ---------- 5 bp
900
b*]
Oj. AGCGGATGACATAGgAGCGGCCCCCAGGAGTCCCGCACGACGACGACACGACGACGACGATTAGCTCATTGGTAGGACGA
Nt. ---------- 1 bp ----------
1000
[trnI
Oj. CGATTAGCTCATTGGTAGGATTAGCTCAgTGtTAGaGtTAGgCACCCaGTGGGAcGGCACaACGGGCTATTAGCTCAgTG
Nt. ----------
Oj. GTAGaGCGCGC
Nt.

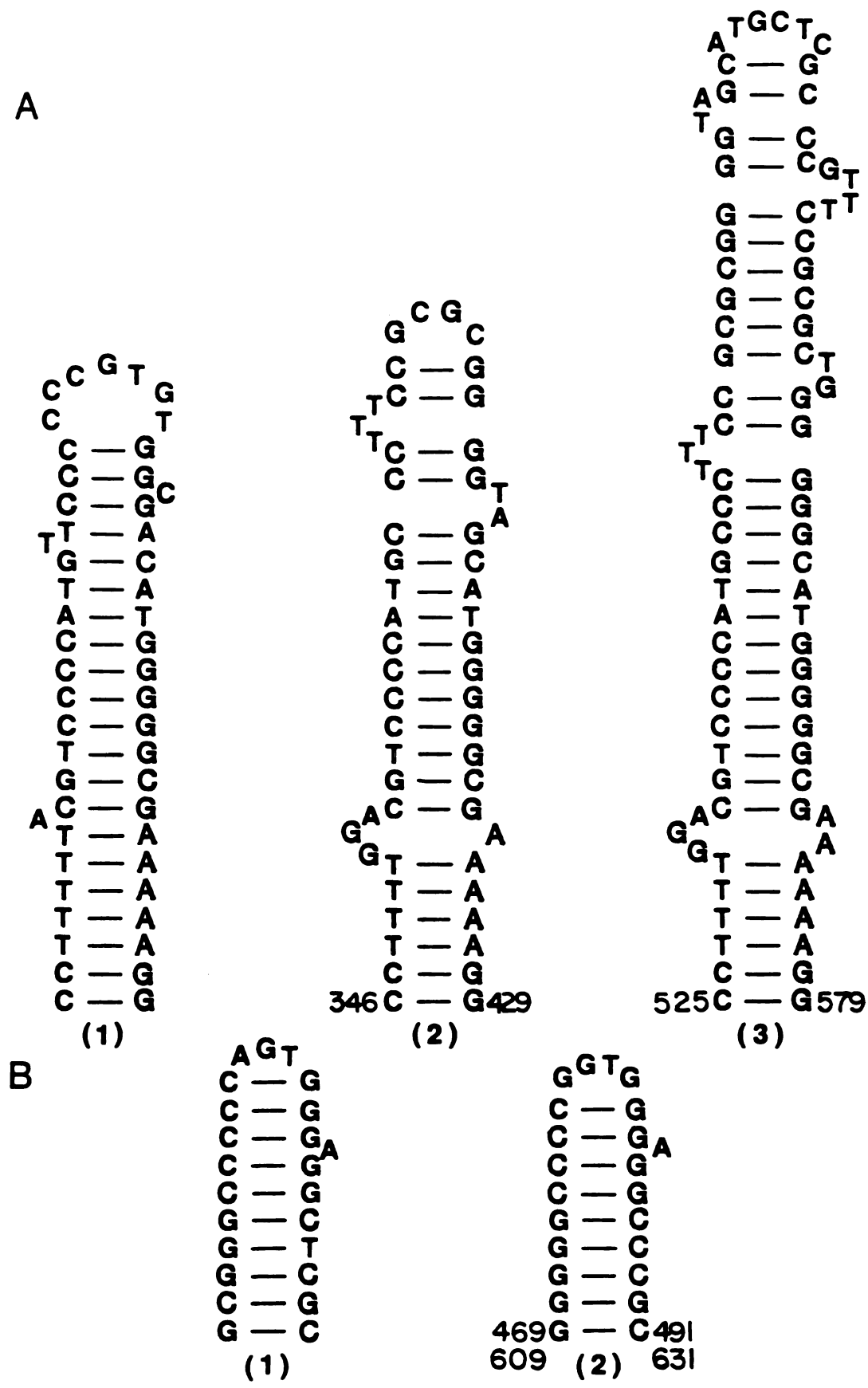
The sequence of the duplicated region in Oenothera has the potential to form stable hairpin structures.

The duplicated segments a and a* (Figure 3.2) of Oenothera plastome I^D contain two regions that can form very stable hairpin structures (Figure 3.3). The stem-loop structure in segment a* (base pairs 526 to 578) has a similar structure in the tobacco plastome (stem 104 in de Lanservin et al. 1987). The stem-loop structure in the analogous position of segment a (base pairs 346 to 429) is larger by 28 bp, owing to an insertion, relative to a*, that extends the palindrome (Figure 3.2). The smaller hairpin structure (Figure 3.3B) can form in both a (positions 469 to 491) and a* (positions 609 to 631), and is very similar to a structure in tobacco (stem 107 in de Lanservin et al. 1987). Besides these two stable hairpin structures, another 25 bp segment in a (positions 433 to 457) also has the potential to from a stem-loop structure. This segment is absent in a* due to a 24 bp deletion.

Most of duplicated sequences in the spacer have been deleted in the cpDNA of a strain derived from the plastome mutator line.

Plastome I^{C1} arose from I^D under the influence of the plastome mutator gene (Chiu et al. 1990). The 604 bp EcoRI-BssHII fragment from I^{C1} has the identical nucleotide sequence as that of I^D except for two large deletions (Figure 3.4). The first deletion removes the 179 bp segment a completely, while the second deletion removes 257 bp (positions 712 to 968 including segment b, b* and two units of the 17 bp direct repeat with accompaning ACG tracks. No base substitutions were found.

Figure 3.3. Secondary structures in the repeated segment a and a* of Oenothera plastome I^D and the equivalent region in tobacco cpDNA. A. The first stem-loop in (1) tobacco (ΔG = -41.9 Kcal/mole), in (2) Oenothera segment a* (ΔG = -43.2 Kcal/mole and in (3) Oenothera segment a (ΔG = -76.5 Kcal/mole). B. The second stem-loop in (1) tobacco (ΔG = -22.2 Kcal/mole) and in (2) Oenothera segment a and a* (ΔG = -26.9 Kcal/mole). The numbers at both ends of the stem-loop structures correspond to the positions of the bases in Figure 2.


A
B
(1)
(2)
(3)
346
429
525
579
469
491
609
631

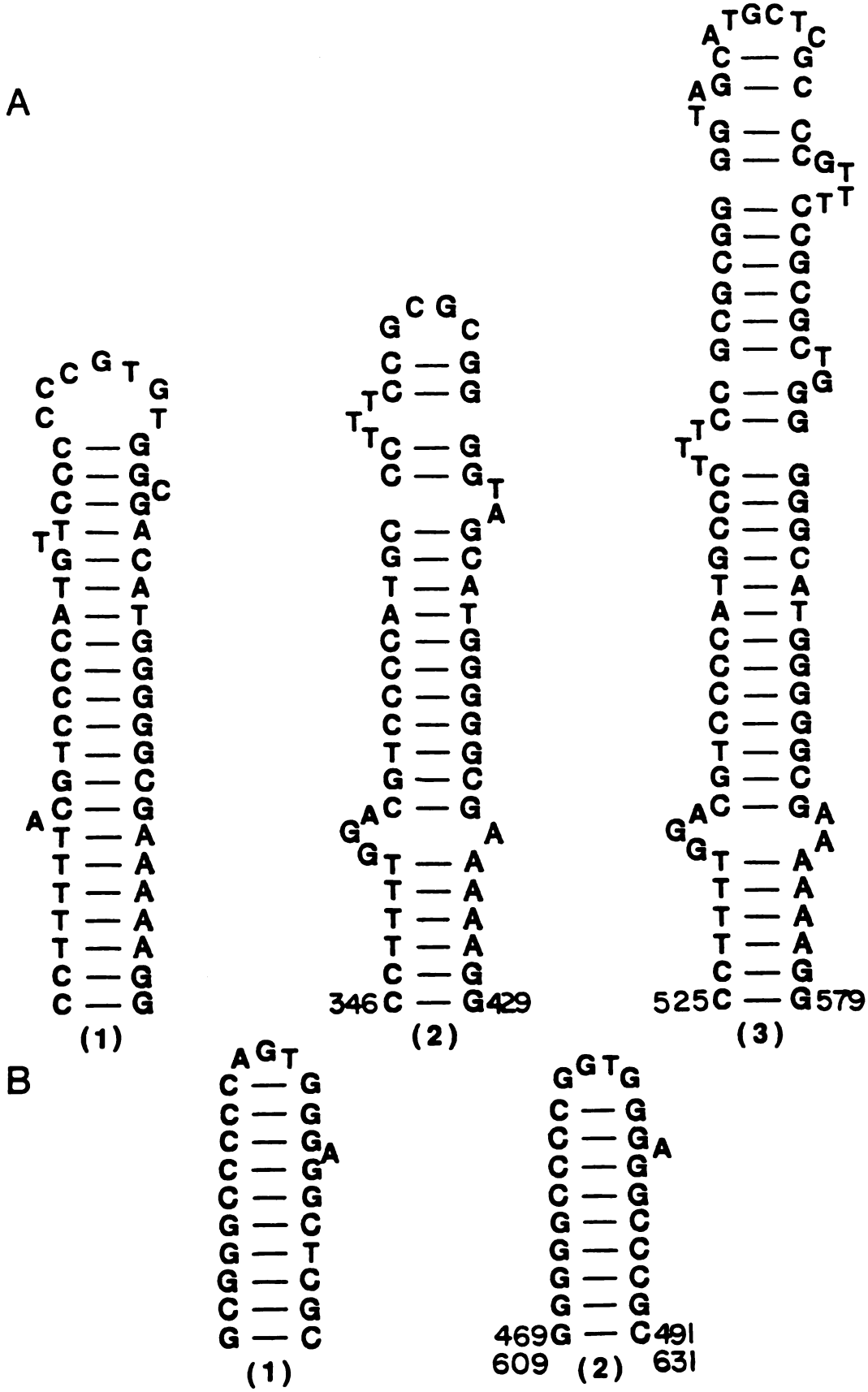
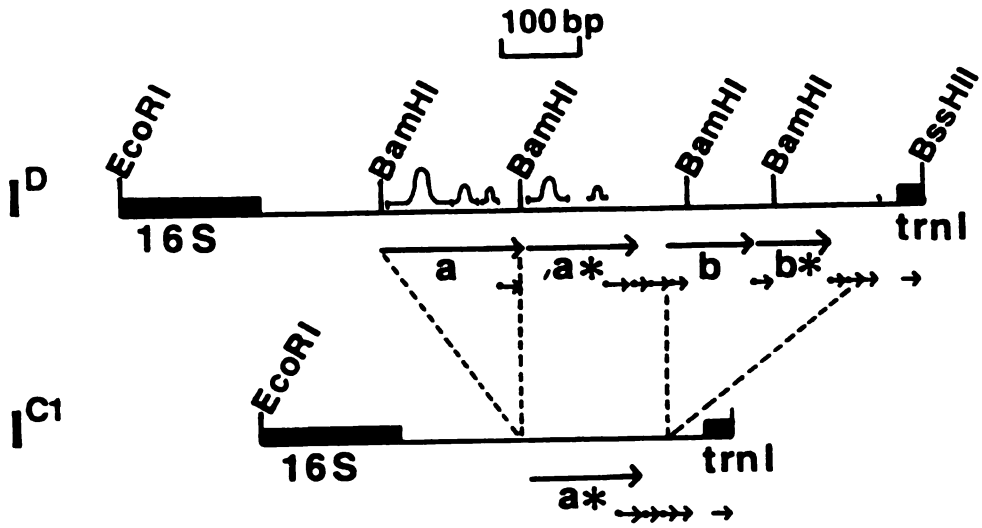

A
B
(1)
(2)
(3)
346
429
525
579
469
491
609
631

Figure 3.4. Restriction map of the variable EcoRI-BssHII fragment in Oenothera plastomes I^{D} and I^{C1}. Two major families of repeated segments are indicated as a and a*, b and b*. The symbol indicates the positions where stem-loop structures may form. Small arrows represent the 17 bp direct repeat and each dot represents pairs of the 3-bp ACG motif shown in Figure 2. The 16-bp repeat in Figure 2 is not indicated seperately, because each copy lies in each of the major repeats, a, a*, b and b*.


100 bp
EcoRI
BamHI
BamHI
BamHI
BamHI
BssHII
I^D
16S
trnI
a
a*
b
b*
EcoRI
I^C1
16S
trnI
a*

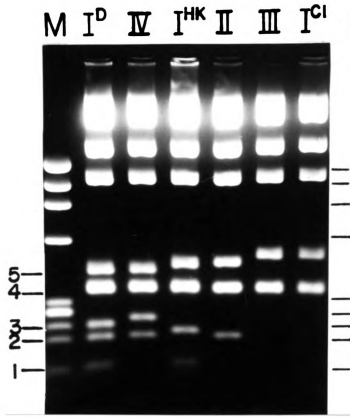
Multiple repeats are not stable in the evolution of Oenothera plastomes.

Since there is one BamHI site (5'GGATCC3') at the junction between the 17 bp and the 16 bp repeats (symbolized by an arrow and a double line respectively in Figure 3.2) in each major repeat unit of plastome I^D (Figure 3.2 and 3.4), the presence or absence of the major repeated segments in other Oenothera plastomes can be deduced by comparing the number and position of BamHI sites with that of plastome I^D and I^{C1} (Figure 3.5A). The results of the restriction analysis are summarized in Figure 3.5B. The five fragments of I^D in Figure 3.5A and B are numbered according to their increasing sizes. Plastome type IV has a novel fragment in place of fragments 1 and 3, hence it probably does not contain segment b. Plastome I^{HK} from Oenothera hookeri std. has a novel fragment instead of fragments 2 and 3, thus it probably does not carry segment a*. Plastome type II probably lacks segment a* and b since a novel fragment replaces fragments 1, 3 and 5. Plastome III has exactly the same number and sizes of restriction fragments as that of plastome I^{C1}, hence, it probably has only segment a*. Subsequent sequencing of plastomes I^{HK} and II (Sears, unpublished results) using primers deduced from the I^D sequence have confirmed the predictions for those two plastomes.

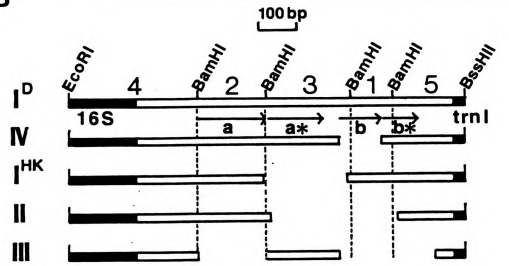
Figure 3.5. Comparison of the variable 16S-trnI spacer among Oenothera plastomes.

A. Ethidium bromide-stained gel of 1.8% agarose with DNA from R2 clones of Oenothera cpDNA digested with EcoRI and BamHI. The five small fragments from plastome I^D are numbered according to their sizes. B. Deduced restriction maps of the variable region in R2 fragment. Gaps indicate regions that are absent in each plastome. Fragment 5 is bracketed by two BamHI sites with one of them outside of the map (see Figure 1A).

A


M
I^D
IV
I^HK
II
III
I^Cl
bp
1353
1078
872
603
310
270
234
194
118
5
4
3
2
1

B


100 bp
EcoRI
BamHI
BamHI
BamHI
BamHI
BssHII
I^D
4
2
3
1
5
16S
a
a*
b
b*
trnI
IV
I^HK
II
III

Discussion

A highly variable region of Oenothera cpDNA was found to be located near one of the D-loop initiation sites (oriB) in the intergenic region Legend to Figure 3.5 here
separating 16S rRNA and trnI genes. This variability stands in contrast to the conservation of this region in other land plant plastomes (de Lanversin et al. 1987, Massenet et al. 1987, Markowicz et al. 1988). The size of this intergenic region ranges from approximately 410 to 845 bp in six Oenothera plastomes, compared to a range of 273 to 310 bp in the six plastomes of land plants that have been sequenced (Koch et al. 1981, Ohyama et al. 1986, Shinozaki et al. 1986, de Lanversin et al. 19871, Massenet et al. 1987, Hiratsuka et al. 1989). The longest of the 16S rRNA-trnI spacer region examined (from plastome I^D) is very similar in sequence to the same region in the tobacco plastome, except for two additional stretches of DNA. Restriction analysis and DNA sequencing of this region from five wild-type Oenothera plastomes indicates that it is rich in repeated sequence motifs and sequences capable of forming stem-loop structures (Figures 3.2, 3.3, 3.5). It is likely that this region of the Oenothera plastome evolved from an ancestral sequence similar to that of tobacco and the initial expansion of this intergenic region in Oenothera probably occurred before the divergence of plastome types.

The major repeat units (a, a*, b and b* in Figure 3.2) in the 16S rRNA-trnI spacer of plastome I^D are flanked by numerous short direct repeats. Slipped-strand mispairing, also known as replication slippage (Moore 1983, Levinson and Gutman 1987, Allgood and Silhavy 1988), has been put

forward as a mechanism to explain the occurrence of DNA length mutations mediated by short direct repeats in bacterial as well as eukaryotic DNA. The slipped-strand mispairing model proposes that the resumption of DNA replication after slippage and mispairing of the daughter strand with a preceding short homologous sequence duplicates the region between two short direct repeats (Figure 3.6A). On the other hand, mispairing between one short direct repeat on the daughter strand with the other repeat unit ahead of the replication fork would cause the loss of one of the repeated units and the region between the two (Figure 3.6B).

In bacterial DNA, slipped-strand mispairing is facilitated by conditions that stabilize or create single-stranded DNA, such as the presence of palindromes or the mutation of single-stranded DNA binding protein (reviewed by Allgood and Silhavy 1988). The hypervariable region in the rRNA operon is between the two neighboring D-loop initiation sites (oriA and oriB in Figure 3.1A) of the Oenothera plastomes. Initiation at either origin would create a displaced strand of parental DNA between the two replication origins (Kolodner and Tewari 1975). Indeed, single-stranded DNA up to 3 kb has been observed in the Oenothera plastome (Chapter 2). Furthermore, several stem-loop structures that could be formed in the major repeat units of plastome I^D (Figure 3.3) may serve to bring distant short direct repeats into close proximity (Glickman and Ripley 1984). Overall, this intergenic region presents itself as an ideal site for slipped-strand mispairing.

Figure 3.7 shows a plausible sequence of events using slipped-strand mispairing that may have led to the present structure of the 16S rRNA-

Figure 3.6. The generation of duplications (A) or deletions (B) by slipped-strand mispairing of direct repeats during replication of DNA. Parallel lines represent complimentary strands of DNA, while shaded boxes represent short, direct repeats (SDR). In (A), DNA replication continues after slippage and mispairing of the daughter strand with a preceding SDR on the parental strand. This duplicates the region between two SDRs. In (B), DNA replication continues after mispairing between one SDR on the daughter strand with the other SDR ahead of the replication fork. This causes the loss of one of the SDRs and the region between the two.

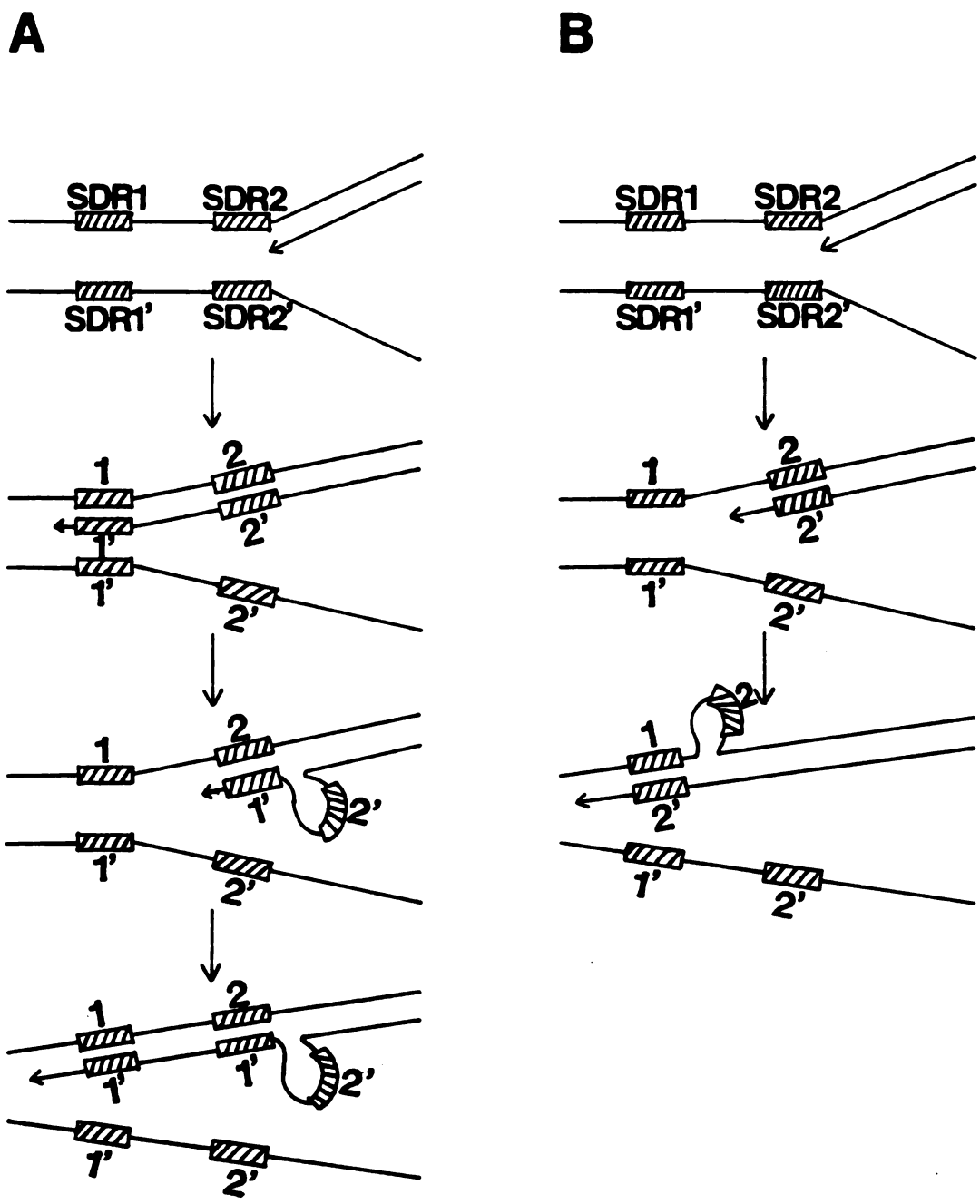
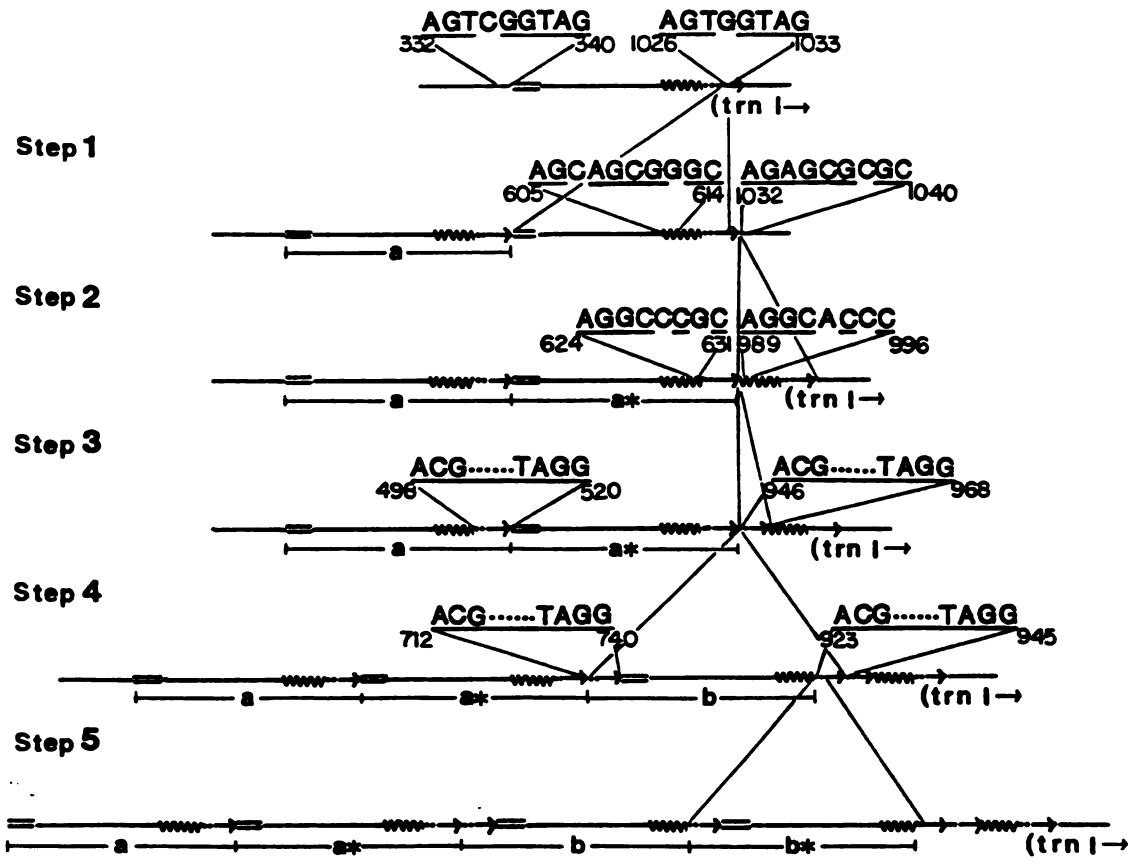

A
SDR1
SDR2
SDR1'
SDR2'
1
2
1'
2'
B
SDR1
SDR2
SDR1'
SDR2'
1
2
1'
2'

Figure 3.7. Major steps in the expansion of the 16S rRNA-trnI spacer of Oenothera plastome I^D starting from sequences similar to other land plant plastomes. Symbols used to represent major direct repeats are the same as those in Figure 2 except for the ACG element, which is represented by a dot. Each step represents a duplication event mediated by slipped strand mispairing during DNA replication (Figure 6A). Sequences of the short direct repeats proposed to have mediated the mispairing are shown above the horizontal lines with the matched bases underlined. The numbers indicate the position of these sequences in Figure 2. Minor addition/ insertion events and base substitutions required to produce the ultimate sequence of plastome I^D are not shown.


AGTCGGTAG
AGTGGTAG
332
340
1026
1033
(trn I→
Step 1
AGCAGCGGGC
AGAGCGCGC
605
614
1032
1040
a
Step 2
AGGCCCGC
AGGCACCC
624
631
989
996
a
a*
(trn I→
Step 3
ACG.....TAGG
ACG.....TAGG
496
520
946
968
a
a*
(trn I→
Step 4
ACG.....TAGG
ACG.....TAGG
712
740
923
945
a
a*
b
(trn I→
Step 5
a
a*
b
b*
(trn I→

trnI region of Oenothera plastome I^D. It is presumed that the ancestral sequence of this region was similar to that of the tobacco plastome. As illustrated in Figure 3.7, the first major step is probably the duplication of the region bracketed by two short direct repeats, one (5'AGTCGGTAG3')immediately upstream from segment a and the other (5'AGTGGTAG3') within trnI, due to mispairing of one short direct repeat on the daughter strand with the preceeding short direct repeat on the parental strand (Figure 3.6A). In the subsequent step, the region between 5'AGCAGCGGGC3' in a* and a similar segment 5'AGAGCGCGC3' in trnI is duplicated. These two duplication events form the precursor of a*. The duplication of the 17 bp motif (symbolized with an arrow) and the accompanying ACG elements (symbolized as dots) upstream to trnI (step 3) may be facilitated by the mispairing of two flanking segments, 5'AGGCCCGC3' and 5'AGGCACCC3'. This duplication must occur prior to the formation of segment b, which is accomplished through the mispairing of two 17 bp motifs (step 4). Subsequently, segment b itself is duplicated through a similar process (step 5). Replication slippage may also be responsible for the deletion of a 24 bp segment in a* (Figure 3.2), since the equivalent region in a can form a stem-loop structure, which is known to facilitate replication slippage (Glickman and Ripley 1984).

Perhaps because long stretches of repeated sequences tend to be unstable in the chloroplast genome (reviewed by Palmer, 1985), at least some of the major repeat units are absent in other wild-type Oenothera plastomes examined (Figure 3.5). In plastome I^{C1}, a recent derivative of I^D, most of the repeated sequences including segments a, b and b* have been deleted. The changes in I^{C1} are most simply explained by the occurence

of two events, each of which resulted in a sizable deletion (Figure 3.4). The 179 bp deletion appears to have resulted from recombination or slipped-strand mispairing between 46 bp of sequence identity: the region of segment a and a* before the 28 bp deletion. The 257 bp deletion appears to have resulted from a similar event between 46 bp of sequence identity: two iterations of the 17 bp motif preceded by two ACG elements.

Can the observed cpDNA length variation between the two D-loop initiation sites affect the efficiency of cpDNA replication? The duplicated segment a in Oenothera plastome I^D has the potential of forming two stable stem-loop structures (Figure 3.3). These secondary structures are very likely since the formation of D-loops would render this region single stranded. It is known that replication forks tend to pause at sequences that can form stem-loop structures (Huang and Hearst 1980, Weaver and DePamphilis 1982, Bedinger et al. 1989). One of the deletions in I^{C1} removed segment a and hence two potential stem-loop structures that could contribute to pausing. In comparing the transmission abilities of plastomes I^D and I^{C1}, genetic data hinted that these differences could have some influence (Appendix C). Crosses in which I^D or I^{C1} competed against maternal plastomes type I to III did not reveal a significant difference, however, crosses in which I^D or I^{C1} competed against maternal plastome type IV, I^{C1} was detected in a higher percentage of the progeny than was I^D (65.8% biparental, n = 144 vs. 36.6% biparental, n = 314). It should also be noted that in the genomic background of O. hookeri str. Johansen, the relative transmission efficiency of the three foreign plastome types (III > II > IV, Chiu et

al. 1988) is in reverse to the number of the repeat units present in their 16S rRNA-trnI spacer (III < II < IV, Figure 3.5). The effiency of plastid transmission is influenced by many different factors (Chapter 1), however, and the development of an in vitro cpDNA replication system is necessary to test the predicted pausing sites near the cpDNA replication origin and their potential influence on the efficiency of plastid multiplication.

CONCLUSION

The object of this dissertation was to understand the roles of the nuclear and plastid genomes in controlling plastid transmission, using Oenothera as the experimental system. Prior to this work, it was generally held that the pattern of plastid inheritance in Oenothera is determined primarily by the plastome, due to different intrinsic rates of plastid multiplication associated with each plastome type (Schötz 1954, 1974, 1975; Chiu et al. 1988). Data presented in Chapter 1 modified this concept and stressed the important role of plastome-genome interactions in determining the outcome of plastid transmission. In general, there is a positive correlation between the relative success of the plastid in transmission and the relative compatibility of the plastome with the nuclear genome, suggesting that the interactions between plastid and nuclear genomes can influence plastid transmission by affecting the efficiency of plastid multiplication. These studies also found low frequencies of plastid transmission from the paternal side when the pollen had poor vigor due to an incompatible plastome-genome combination, indicating that plastome-genome interactions may affect the input of plastids at fertilization.

Although Schötz (1954, 1974, 1975) concluded that the plastome had a predominant effect on plastid transmission, he also noticed that the

competitive ability of a plastome was highest in its native nuclear

background. In this dissertation, the ability to compare results from reciprocal crosses and to compare the four Oenothera plastomes simultaneously in several different nuclear backgrounds made it possible to obtain a more complete view of the role of both plastid and nuclear genomes in plastid transmission.

Besides plastome-genome interactions, traits determined mainly by the parental nuclear genomes, such as the size of the flower and the the growth capacity of the pollen tube, were also observed to have an effect on plastid transmission, independent of plastome type. For example, a much higher frequency of paternal plastids was detected in the progeny when strains with smaller flowers were used as the maternal parent. Pollen from small-flowered strains were observed to have low growth capacity when germinated in vitro (Chapter 1). These pollen also showed a lower efficiency in transmitting plastids in crosses. These findings suggest that if the time between pollination and fertilization is lengthened, degeneration of plastids may occur in the pollen generative cells. This hypothesis is supported by the fact that to some extent a higher frequency of paternal plastids was detected in plantlets derived from seeds collected from the upper half of the capsule than in seeds from the lower half (Appendix B). A direct test for the connection between the frequency of plastid transmission and the degeneration of plastids would be to examine the number of cpDNA aggregates (Corriveau and Coleman 1988) in the pollen generative cells at different times after pollen germination in culture.

If generative cell plastids degenerate during pollen tube growth, then

Oenothera may differ only slightly from other plant species that exhibit uniparental maternal plastid inheritance due to plastid degeneration in the generative cell (Hagemann and Schröder 1989). Hence, the knowledge we gain from the study of plastid transmission in Oenothera may be pertinent to plastid transmission in a much larger group of land plants. Theoretically, those plants that carry plastids in the pollen generative cells should be able to transmit plastids from the male parent if favorable conditions for the delivery and the multiplication of paternal plastids are met. Supporting this view are the findings that even in tobacco and Epilobium, plants known to exhibit solely maternal plastid inheritance, DNA from the paternal plastids can be detected occasionally in the progeny (Medgyesy et al. 1986, Schmitz and Kowallik 1986).

As mentioned previously, the results of Schötz (1974, 1975) that the plastome determines the success of plastid transmission led to the the belief that each Oenothera plastome type has its own intrinsic rate of multiplication. This concept is in apparent contradiction to the finding that plastids of barley and maize seem to be able to multiply in the absence of plastid protein synthesis. These is also evidence that plastids of tobacco multiply in the presence of an inhibitor of plastid protein synthesis (reviewed by Boffey 1985). On the other hand, one should recognize that while plastome-encoded proteins may not be essential, they nontheless may regulate or increase the rate of plastid multiplication through their interaction with nuclear gene products.

This dissertation sought to examine whether the plastome-dependent

efficiency of plastid multiplication could be modulated through a non-coding function of the plastome. If one accepts Schötz' hypothesis or the modified view of plastid transmission suggested in Chapter 1, then the origin of cpDNA replication is an outstanding candidate for the plastid component that modulates the efficiency of plastid multiplication. Since initiation of DNA replication is the first step in plastid multiplication, differences in the replication origins among the plastome types may influence the efficiency of cpDNA replication and plastid multiplication. Chapter 2 mapped the cpDNA replication origins from Oenothera as a first step towards examining this possibility.

According to electron microscopic data, initiation of cpDNA replication in Oenothera proceeds through the formation of D-loops at four separate sites. These initiation sites are located within the large inverted repeat region, on both sides of the 16S rRNA gene. An attempt to use two-dimensional gel electrophoresis to more precisely localize the D-loop initiation sites was not successful (Appendix D). According to the available data, there is no difference in the number and the positions of cpDNA replications between plastome types I and IV, the two plastomes with the most distinct transmission properties. Hence, if the origins are the sites in Oenothera plastomes that vary with respect to DNA replication, they must differ from each other in a more subtle way.

In mapping the cpDNA replication origins, a hypervariable region that differentiated the Oenothera plastomes was noted between the 16S rRNA and the trnI genes. This hypervariable region was investigated further (Chapter 3) because of its proximity to two of the four D-loop

initiation sites and, hence, the possibility that differences in this region could account partially for differences in plastid transmission. Sequencing of this hypervariable region revealed multiple direct repeats that most likely arose through duplications of part of trnI and the sequences immediately upstream from trnI. Some of the repeated regions contain segments of DNA that are capable of forming stable stem-loop structures. These secondary structures could potentially block replication fork movement and hence affect the efficiency of cpDNA replication and plastid multiplication. In order to test this hypothesis, the transmission efficiencies of a wild-type plastome I (I^D) and plastome I^{C1} (Appendix A), a derivative of I^D with a deletion of most of the direct repeats were compared (Appendix C). In the process of gathering these data, my other crossing experiments (Chapter 1) indicated that significant variation in plastid transmission can occur from year to year and from flower to flower, at different times in the flowering season. Thus, it is not valid to compare most of the data from the I^D and I^{C1} crosses of Appendix C. Subtle differences may exist between the two plastomes, but side by side comparisons are necessary to avoid variations in other factors that may also affect the apparent efficiency of plastid transmission.

This dissertation began with a simple hypothesis to explain the molecular mechanisms behind the differential plastid transmission in Oenothera, but ended by confronting the complex nature that governs plastid inheritance in higher plants. Basically, the genetic results suggest that the outcome of plastid transmission depends not only on the number of plastids in the gametes but also on events that take place

before and after fertilization. Furthermore, all of these events rely heavily on the interactions between the plastid and the nuclear genomes. Due to the large size of the nuclear genome and the number of genes involved, it will be easier to investigate the genetic basis for these crucial interactions from the side of the plastome. Thanks to the recent development of plastid transformation techniques (Svab et al. 1991), it is now possible to replace genes within the plastid. Experiments using cpDNA from one Oenothera plastome type to replace the homologous region of a different plastome type may be used to localize plastid genes that are responsible for plastome-genome compatibility. Oenothera, a classical favorite of cytogeneticists, may play an important role in helping us to understand the complicated nature of plastome-genome interactions.

APPENDICES

APPENDIX A. *OENOTHERA* CHLOROPLAST DNA POLYMORPHISMS ASSOCIATED WITH PLASTOME MUTATOR ACTIVITY

----From Mol. Gen. Genet. (1990) 221:59-64

Mol Gen Genet (1990) 221: 59–64

MGG

Oenothera chloroplast DNA polymorphisms associated with plastome mutator activity

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Summary. *Oenothera* plants homozygous for a recessive allele at the plastome mutator (*pm*) locus show non-Mendelian mutation frequencies that are 1000-fold higher than spontaneous levels. Chloroplast DNA (cpDNA) was isolated from nine mutants and two green isolates of the plastome mutator line. cpDNA restriction patterns were compared to cpDNA from a representative of the progenitor Johansen strain, and cpDNAs from all eleven plastome mutator lines show changes of fragment mobility due to deletion events at five discrete regions of the plastome. Most of the mutants have cpDNA restriction patterns identical to that of one of the green isolates from the plastome mutator line, and therefore, most of the differences in fragment length are probably not responsible for the mutant phenotypes. In contrast to the plastome mutator line, cpDNA from several populations of a closely related wild-type *Oenothera* species have few restriction fragment length polymorphisms. This suggests that both mutation frequencies and site-specific cpDNA deletions are elevated in the plastome mutator line, and implicates a defect in the cpDNA repair or replication machinery.

Key words: Plastome mutator – *Oenothera* – Chloroplast DNA polymorphisms

Introduction

Chloroplast DNA (cpDNA) coding sequences are highly conserved (Curtis and Clegg 1984; Zurawski and Clegg 1987), which may be due in part to the low frequency of spontaneous mutation (Michaelis 1969; Kutzelnigg and Stubbe 1974). However, comparisons of the cpDNA restriction patterns and physical maps from closely related plant species have revealed the relatively frequent occurrence of insertion/deletion events (Gordon et al. 1982; Salts et al. 1984; Schmitz and Kowallik 1986; Doebley et al. 1987) or rearrangements (reviewed by Palmer 1985).

A number of higher plant isolates have elevated rates of plastid genome (plastome) mutations, including *Arabidopsis thaliana* (Redei and Plurad 1973), maize (Shumway and Weier 1967; Walbot and Coe 1979; Thompson et al. 1983), *Petunia* (Potrykus 1970), and *Oenothera* (Epp 1973). The plastome mutator activity in the Johansen strain of *O. hookeri* is the result of a nuclear gene that, when homozygous for the mutant *pm* allele (*pm pm*), induces mutations in the plastome (Epp 1973). A large collection of plastome mutator-induced chloroplast mutants has been generated by several laboratories (Epp 1973; Sears 1983; Epp et al. 1987). All mutations isolated thus far were recognized initially as chlorotic sectors that appeared in *pm/pm* plants. In confirmation of the initial findings of Epp (1973), such plastome mutations are inherited in a non-Mendelian fashion and display an array of phenotypes; independent mutants show varying degrees of chlorosis (Epp 1973) and differ in the extent of plastid development (Epp and Parthasarthy 1987) and in the accumulation of particular proteins (Johnson 1988). Epp et al. (1987) have recently shown that the cpDNAs isolated from different plastome mutator-induced chlorotic sectors are identical in restriction pattern to cpDNA of the wild-type, when cut with enzymes that digest cpDNA infrequently. In contrast, our experiments utilized more frequently cutting enzymes applied to a different group of plastome mutator-induced mutants and two green isolates, and we have found restriction fragment length polymorphisms (RFLPs). Comparisons of the cpDNA restriction patterns from isolates of the plastome mutator (*pm*) line and from the wild-type *O. hookeri* str. Johansen suggest that the cpDNA alterations in the *pm* line result from deletions at several sites that were previously identified as "hot spots" in the evolution of cpDNA (Gordon et al. 1982; Stein and Hachtel 1986).

Materials and methods

Plant material. Seeds from *O. elata* were generously provided by Prof. W. Stubbe and Dr. W. Dietrich from plants collected from the Mexican states of Puebla, Mexico (near the city of Toluca), and Hidalgo (Steiner 1951), catalogued in the University of Düsseldorf collection as Puebla, Toluca, and Hidalgo, respectively. The Johansen strain of *O. hookeri* was established from a single rosette collected in 1927 by D.A. Johansen (Cleland 1935). From 1930 it was maintained in R. Cleland's collection by self-pollinations, and in the 1950s seeds were provided to H. Stinson (Cornell University, Ithaca, NY), E. Steiner (University of Michigan, Ann Arbor), and W. Stubbe (University of Düsseldorf, FRG), where the strain was perpetuated by further self-pollinations (Fig. 1). We refer to the plant lines established at these institutes as the *Cornell*, *Ann Arbor*, and *Düsseldorf* lines of strain Johansen. The only representatives of the *Cornell* line that have been preserved are descended from an isolate (E-15-7) derived from ethyl methane sulfonate (EMS) mutagenesis, and exhibiting the original plastome mutator activity (Epp 1973).

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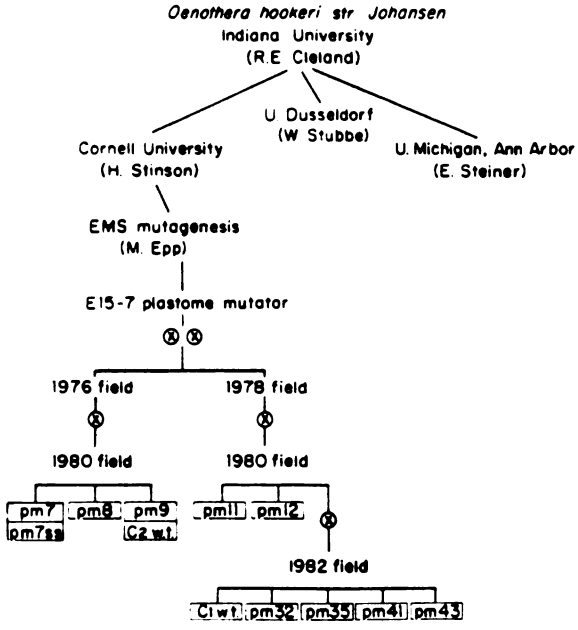

Fig. 1. Pedigree of mutant and wild-type lines descended from the original Johansen isolate of *Oenothera hookeri*. An unknown number of self-pollinations were performed in the propagation of the Johansen strain at all four institutions, where the stocks were maintained. Ethyl methane sulfonate (EMS) mutagenesis was used to produce the plastome mutator isolate (E-15-7) at Cornell (Epp 1973). Several self-pollinations (indicated by an *encircled x*) were performed with progeny of this line to generate more seed prior to the 1976 and 1978 field plantings indicated in the pedigree. In the plastome mutator line, mutants are indicated by *pm* designations, while green isolates are indicated as C_1 and C_2. Different lines obtained from the same plant have adjoining *boxes*

Both *O. elata* and *O. hookeri* have an A/A nuclear background and a plastome type I, according to the classification scheme of Stubbe (1959), and according to Raven et al. (1979) they should be reclassified as a single species. The original *pm/pm* line was inbred for several generations to produce the plastome mutator seed stocks, which were maintained by Prof. W. Stubbe after 1973. Figure 1 indicates the pedigree for the plastome mutator-induced mutants (*pm7, pm7ss, pm8, pm9, pm11, pm12, pm32, pm35, pm41,* and *pm43*) that were isolated from progeny of further self-pollinations. All of these mutants have pigment alterations; their phenotypes range from white to relatively green, but none are photosynthetically competent. To produce the plant lines used here, flowers from these plants were emasculated and crossed as the female parent to a pm^+/pm^+ line as described by Kutzelnigg and Stubbe (1974). These crosses accomplished two objectives: (i) seeds were produced, which could be stored, or used immediately for the establishment of leaf-tip cultures and (ii) the plastid mutations were stabilized by placement in a heterozygous nuclear background.

In order to produce a pm^+ *pm* line containing the same plastome type in a heterozygous nuclear background for use as a control, green siblings of plants containing the *pm32, pm35, pm41,* and *pm43* mutations in the 1982 field plot were crossed as the female parent with pollen from *O. hookeri* strain Johansen carrying plastome type IV, because plastome IV transmits its plastids to fewer than 1% of the progeny (Chiu et al. 1988). We refer henceforth to this green line of *O. hookeri* strain Johansen as "*Cornell-1*". To obtain leaf tissue for plastid isolations, *Cornell-1* plants were grown in a glasshouse in sandy soil.

Another green isolate (*Cornell-2*) carrying a second cpDNA variant was recovered from the 1980 field season by crossing the same plant that carried the *pm9* mutation to a plant line with the *albicans/percurvans* genotype (Cleland 1972) and plastome type IV; the resulting progeny have an A/C nuclear background. Plants having plastome type I in combination with the A/C nuclear background are pale green due to nuclear-plastome incompatibility (Kutzelnigg and Stubbe 1974) and therefore, it was necessary to grow them as leaf-tip cultures as described by Johnson and Sears (1990). To establish pure mutant cultures, seeds were surface-sterilized with 40% commercial bleach and germinated on agar medium lacking plant hormones (Stubbe and Herrmann 1982). The resulting seedlings were propagated as leaf-tip cultures to allow the plastids to sort out during vegetative growth. Cultures of pure mutant tissue were maintained by transferring them to fresh medium every 4 weeks.

Isolation of chloroplast DNA. Chloroplast DNA was prepared as described by Chiu and Sears (1985). Bisbenzimide (Hoechst 33258, Sigma) was used for the CsCl gradients at a final concentration of 0.01 mg/ml (after Hudspeth et al. 1980). After buoyant density equilibrium centrifugation, the upper (cpDNA) band in the gradient was collected. If the lower band of nuclear DNA was so broad that it contaminated the upper cpDNA band during its removal from the gradient, the DNA band was further purified on a second gradient under the same conditions. Bisbenzimide was removed by three extractions with NaCl-saturated isopropanol, the remaining cpDNA-CsCl mixture was diluted twofold with water, and precipitated by the addition of two volumes of ethanol or one volume of isopropanol. The resulting cpDNA pellet was washed with 70% ethanol and dissolved in 10 mM TRIS, 0.1 mM EDTA, pH 8.0.

Restriction analysis and Southern blotting. Restriction endonuclease digestions were performed according to the manufacturers' specifications (Bethesda Research Laboratories and Boehringer Mannheim Biochemicals). Fragments were separated by electrophoresis in 0.8% agarose gels in TAE buffer (40 mM TRIS, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) and visualized with ethidium bromide staining according to Maniatis et al. (1982), using *Hin*dIII-digested lambda phage DNA as size markers. Standard procedures were used for Southern blotting and nick-translation (Maniatis et al. 1982), using ^{32}P-labeled dATP from New England Nuclear Dupont. All probes were made from plasmids having cloned cpDNA from the *Düsseldorf* line.

Results

Comparisons of cpDNA from plastome mutator lines and the wild-type O. hookeri *str. Johansen*

CpDNA was isolated from nine mutants and two green isolates (*Cornell-1* and *Cornell-2*) from the plastome muta-

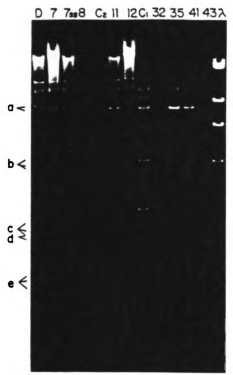

Fig. 2. *Cla*I restriction patterns of cpDNA from plastome mutants and green isolates. CpDNA from three green isolates, the *Düsseldorf* (D), *Cornell-1* (C_1), and *Cornell-2* (C_2) lines are compared to cpDNA from plastome mutator-induced mutants *pm7, pm7ss, pm8, pm11, pm12, pm32, pm35, pm41,* and *pm43*. Lanes with cpDNAs from the mutants are given the numerical *pm*-designation. DNA size standards at the *right* were obtained by *Hin*dIII digestion of lambda DNA. The letters *a–e* to the *left* of the gel indicate the positions of fragments that vary in mobility

tor line and from the *Düsseldorf* and *Ann Arbor* lines of strain Johansen. No differences were observed in the comparison of restriction patterns of the *Düsseldorf* and *Ann Arbor* lines (data not shown), and therefore the *Düsseldorf* line has been used to represent the progenitor cpDNA. In the comparisons of the plastome mutator lines, restriction fragment length polymorphisms (RFLPs) were observed when the DNAs were digested with *Eco*RI, *Bam*HI, and *Cla*I. Since the *Cla*I digestions revealed the most variability (5 RFLPs), this enzyme was used for the subsequent analyses. In Fig. 2, five variable *Cla*I fragments are apparent in the ethidium bromide-stained agarose gel, although the size differences of the larger fragments are more difficult to discern in this 0.8% agarose gel than in a gel with a lower percentage of agarose. Comparing the cpDNAs from the green isolates, the *Cornell-2* line differs from both the *Cornell-1* and *Düsseldorf* lines in all five variable fragments. Four of these five fragments also differ in mobility between the *Cornell-1* and *Düsseldorf* lines. In all cases, the mobility differences represent variations in fragment size of 50–500 bp.

The cpDNAs of *pm35, pm41,* and *pm43* have the same restriction pattern as line *Cornell-1*, as does *pm32*, although the digest shown in Fig. 2 contains some partial digestion products for this line. The cpDNAs of *pm7, pm7ss,* and *pm12* are identical to the cpDNA of the *Cornell-2* line. *Pm7* and *pm7ss* are two different mutants with quite different pigmentation phenotypes that were isolated from a single plant. The restriction pattern of mutant *pm8* is the same as that of the *Cornell-2* line, except for variable fragment "c", which is about 300 bp larger than the equivalent fragment in *Cornell-2* and has the same mobility as that of the wild-type *Düsseldorf* line. Similarly, the restriction patterns of the *pm11* mutant resembled that of the *Cornell-1* class except that the variable fragment "b" was approximately 300 bp smaller than the analogous fragment in *Cornell-1*. Among all five variable fragments detected, fragments from the *Düsseldorf* line are always the largest.

Localization of the five variable regions on the physical map of the Oenothera *plastome*

The five variable *Cla*I fragments were located on the physical map of *Oenothera* cpDNA (Gordon et al. 1982) by Southern hybridization (Figs. 3, 4; additional data not shown). Fragment "a" hybridized to a cloned 5.1 kb *Eco*RI fragment from the wild-type *Düsseldorf* line, which contains the entire spacer region between the 16S and 23S rRNA genes and most of the 23S rRNA (Gordon et al. 1982). This segment of the inverted repeat (see Fig. 3) is found on the largest and third largest *Sal*I fragments of *Oenothera* cpDNA (*Sal* fragments 1 and 3 in Fig. 3). The variable region within fragment *a* was further determined to be in the spacer near the 16S rRNA (data not shown). The corresponding fragments in lines *Cornell-1* and *Cornell-2* are 500 bp and 300 bp smaller, respectively, than that of the *Düsseldorf* line.

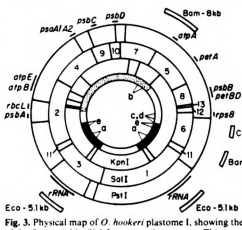

Fig. 3. Physical map of *O. hookeri* plastome I, showing the location of the five variable *Cla*I fragments (letters a–e). This map contains the *Pst*I, *Kpn*I and *Sal*I sites and gene loci determined previously (Gordon et al. 1982; Herrmann et al. 1983), and shows the position and sizes of fragments produced by *Bam*HI, *Cla*I, and *Eco*RI digestion, which were used as probes for southern hybridization. The relative sizes of the major *Sal*I fragments are included, with 1 being the largest. The 2.5 kb *Cla*I fragment is equivalent to fragment c, but its precise location within the *Sal*I fragment has not been determined. In the innermost circle, the inverted repeat regions are indicated as *black arcs*, and the position of a large inversion in the physical map, relative to spinach, is indicated by the *stippled arc*

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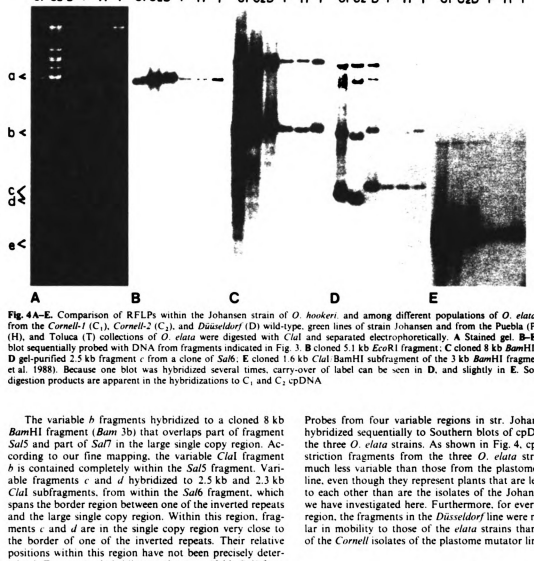

Fig. 4A–E. Comparison of RFLPs within the Johansen strain of *O. hookeri*, and among different populations of *O. elata*. CpDNA from the *Cornell-1* (C_1), *Cornell-2* (C_2), and *Düsseldorf* (D) wild-type, green lines of strain Johansen and from the Puebla (P), Hidalgo (H), and Toluca (T) collections of *O. elata* were digested with *Cla*I and separated electrophoretically. **A** Stained gel. **B–E** Southern blot sequentially probed with DNA from fragments indicated in Fig. 3. **B** cloned 5.1 kb *Eco*RI fragment; **C** cloned 8 kb *Bam*HI fragment; **D** gel-purified 2.5 kb fragment *c* from a clone of *Sal6*; **E** cloned 1.6 kb *Cla*I·BamHI subfragment of the 3 kb *Bam*HI fragment (Blasko et al. 1988). Because one blot was hybridized several times, carry-over of label can be seen in **D**, and slightly in **E**. Some partial digestion products are apparent in the hybridizations to C_1 and C_2 cpDNA

The variable *b* fragments hybridized to a cloned 8 kb *Bam*HI fragment (*Bam* 3b) that overlaps part of fragment *Sal5* and part of *Sal7* in the large single copy region. According to our fine mapping, the variable *Cla*I fragment *b* is contained completely within the *Sal5* fragment. Variable fragments *c* and *d* hybridized to 2.5 kb and 2.3 kb *Cla*I subfragments, from within the *Sal6* fragment, which spans the border region between one of the inverted repeats and the large single copy region. Within this region, fragments *c* and *d* are in the single copy region very close to the border of one of the inverted repeats. Their relative positions within this region have not been precisely determined. Fragment *e* hybridizes to the same 13 kb *Sal6* fragment as well as to the *Sal2* fragment, indicating that it lies within the inverted repeat of the cpDNA. The variable region *e* has been further defined by hybridization to a 3 kb *Bam*HI fragment described by Blasko et al. (1988), and mapped to the edge of the inverted repeats.

Comparison of RFLPs of O. hookeri *str. Johansen and* O. elata

We hoped to determine whether the RFLPs observed in the plastome mutator line represent a normal level of variability, or indicate that rapid changes were occurring in the cpDNA of this line. Thus, we extended our comparisons to the closely related *O. elata*. CpDNAs were isolated from plants representing three independent collections of *O. elata* from Toluca, Hidalgo, and Puebla, Mexico (Steiner 1951). Probes from four variable regions in str. Johansen were hybridized sequentially to Southern blots of cpDNA from the three *O. elata* strains. As shown in Fig. 4, cpDNA restriction fragments from the three *O. elata* strains were much less variable than those from the plastome mutator line, even though they represent plants that are less related to each other than are the isolates of the Johansen strain we have investigated here. Furthermore, for the variable region, the fragments in the *Düsseldorf* line were more similar in mobility to those of the *elata* strains than to those of the *Cornell* isolates of the plastome mutator line.

Discussion

A great number of plastome mutator-induced plastid mutations have been collected in the past 10 years (Epp et al. 1987), and yet the nature of the *pm* gene function is still unknown. The goal of the current study was to investigate the nature of plastome mutator-induced mutations in order to elucidate the function of the *pm* gene.

We have compared RFLPs of cpDNA from descendants (two green and nine mutant isolates) of the original *pm pm* plant and from two representatives of wild-type *O. hookeri* strain Johansen, from which the plastome mutator line was derived. Five *Cla*I fragments of the cpDNA were variable among these isolates. Among the isolates from the plastome mutator line, the RFLPs can be classified into two major types, represented by lines *Cornell-1* and *Cornell-2* and by

Table 1. Sizes of variable fragments *a–e* in *Oenothera hookeri* strain Johansen

Plant line[a]	*D*	C_1	C_2	*pm7*	*pm7ss*	*pm8*	*pm11*	*pm12*	*pm32*	*pm35*	*pm41*	*pm43*
Fragment[b]												
a	9.0	8.5	8.7	8.7	8.7	8.7	8.5	8.7	8.5	8.5	8.5	8.5
b	4.6	4.5	4.3	4.3	4.3	4.3	4.2	4.3	4.5	4.5	4.5	4.5
c	2.5	2.35	2.2	2.2	2.2	2.5	2.35	2.2	2.35	2.35	2.35	2.35
d	2.25	2.25	2.15	2.15	2.15	2.15	2.25	2.15	2.25	2.25	2.25	2.25
e	1.55	1.50	1.40	1.40	1.40	1.40	1.50	1.40	1.50	1.50	1.50	1.50

[a] *D, Düsseldorf*; C_1, *Cornell-1*; C_2, *Cornell-2*
[b] Fragment sizes are given in kb

two minor classes, consisting of the mutants *pm8* and *pm11*, each of which have cpDNAs differing from that of the *Cornell-2* or *Cornell-1* line, respectively, by only one fragment. The comparisons with two wild-type lines descended from the original Johansen isolate and three collections of *O. elata* from the wild (Fig. 4) indicate that the cpDNA variability correlates with the plastome mutator ancestry of the Cornell strains. Since the isolates from the *pm* line invariably have fragments that are smaller than the analogous fragments in the *Düsseldorf* and *Ann Arbor* lines, we conclude that cpDNA deletions have occurred recently within the plastome mutator line.

The five variable regions have been mapped on the plastome (Fig. 3): two of them (*a, e*) are inside the inverted repeat, while the other three (*b, c, d*) are in the large single copy region. As indicated in Table 1, the sizes of fragments *a–e* are highly variable, with more than two size classes being observed for each fragment. It is probably not coincidental that all five variable regions overlap with the evolutionary "hot spots" previously identified on the *Oenothera* plastome (Gordon et al. 1982; Stein and Hachtel 1986).

Length mutations smaller than 50 bp would not have been detected in this study due to the limitation of resolution imposed by agarose gel electrophoresis, and thus we cannot address the frequency of occurrence of smaller length alterations. Among the nine mutants analyzed, only one contained cpDNA in which a specific deletion (fragment b of *pm11*) clearly distinguished the mobility of a fragment from the homologous fragment in any of the green plant lines. Thus, with the exception of this one fragment, we conclude that the deletions that result in the RFLPs are not likely to be responsible for the mutant phenotypes. Presumably, undetected small insertions, deletions, or base substitutions are responsible for the chlorotic phenotypes of the other mutants and, possibly, also of *pm11*.

A number of mechanisms have been proposed to explain the elevated mutation rates in *pm* plants (Redei and Plurad 1973; Epp 1973; Sears 1983). These include defects in the replication or repair machinery and the suggestion that *pm* encodes a repressor of a chloroplast transposable element. The evidence presented here argues against the involvement of a transposable element because changes in fragment size seem to be unidirectional, favoring deletions; also Southern hybridizations show that no sequences are shared among the variable fragments and hence no common element could be involved in the deletions. Furthermore, sequencing of variable region e (Blasko et al. 1988) showed that it has none of the features of known transposable elements (reviewed by Charlebois and Doolittle 1989). Rather, the RFLPs are due to the presence of a variable number of 24 bp direct repeats. Other short repeats in *Oenothera* cpDNA have been identified and are probably involved in deletion/insertion events on an evolutionary scale (Stein and Hachtel 1988a, b). We predict that similar short direct or inverted repeats will be implicated in the other four cpDNA hypervariable regions in the plastome mutator line. Such repeats could be the substrate for plastome mutator activity, if the *pm* defect involves a recombination/repair system or the replication apparatus. Thus, a single mechanism is hypothesized to be responsible for the high frequency of both the chlorotic mutations and the cpDNA deletions. Determination of cpDNA sequence changes responsible for the mutant phenotypes may more clearly implicate one of the above mechanisms.

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APPENDIX B. RELATIONSHIP BETWEEN SEED POSITION AND THE EXTENT OF BIPARENTAL PLASTID TRANSMISSION

Rationale

For all Oenothera strains examined by DAPI-staining, over 90% of the pollen generative cells contain one or more plastid DNA aggregates (Corriveau and Coleman 1990; personal communication). One might expect that these pollen should be able to transmit their plastids to the progeny. However, results in Chapter 1 indicate that paternal plastids are not generally transmitted with such a high efficiency. It was noted that when the parental plastome types are held constant, biprental transmission of plastids occurs more frequently when the maternal parent is a small-flowered strain or when the paternal parent produces pollen with higher growth capacity. These observations indicate that the timing between pollination and fertilization may be an important factor in Oenothera plastid transmission. Conceivably, plastids in the pollen generative cell degenerate as the time increases between pollination and fertilization.

The purpose of this investigation was to test the hypothesis that there is loss of plastids in Oenothera during growth of the pollen tube by examining the relationship between the outcome of plastid transmission and the position of seeds in the capsule. This experimental approach is

based on the assumption that pollen tubes have to grow longer in order to fertilize ovules in the lower half of the ovary. If there is degeneration of plastids during pollen tube growth, pollen reaching the lower part of the capsule may carry fewer viable plastids, resulting in a lower frequency of plastid transmission from the paternal parent in that region. To examine this possibility, for a number of crosses, the frequency of biparental plastid transmission in seeds obtained from the upper half of the capsule was compared with that in seeds from the lower half.

Results and Conclusion

All the Oenothera strains, except for $A^{j}A^{j}$-I^{c1}, used in the crosses described below are listed in Ch. 1, Table 1. $A^{j}A^{j}$-I^{c1} is a strain of O. hookeri str. Johansen carrying plastome I^{c1}, which is a derivative of plastome I^{D} (Chiu et al. 1990). Mutant plastids used in the crosses were maintained in O. hookeri str. Johansen (genotype $A^{j}A^{j}$), and all the crosses were made in the 1989 field season. Dried, mature seed capsules were broken in half and seeds from the upper and the lower halves were collected separately.

Results from "Green x White" and "White x Green" crosses are presented in Table B.1, section A and section B, respectively. When strains $A^{a}C^{p}$-II, $B^{p}C^{f}$-IV and $A^{r}C^{p}$-IV were used as paternal parents, seed development was limited to the top portion of the capsule, most likely due to

Table B.1. Frequency of biparental plastid transmission (BP) obtained from seeds in upper (U) or lower (L) half of the capsule[a]

A. Green x White crosses

		Maternal					
		A^aC^p-IV	A^aC^p-II	B^pC^f-IV	A^rC^p-IV	A^jA^j-I^D	
		% BP (n)	% BP (n)	% BP (n)	% BP (n)	% BP (n)	t[b]
Paternal							
I-b	U	—	45.3 (53)	73.8(160)	59.4(133)	19.0(142)	0.48
	L	—	55.6 (54)	61.7(170)	64.8 (88)	20.3(133)	
I-z	U	86.9 (61)	69.5 (82)	86.8(159)	85.0(127)	46.4(112)	0.57
	L	78.8 (66)	80.5 (82)	81.1 (37)	94.4(108)	46.3(123)	
II-e	U	72.4 (87)	—	78.6(145)	80.2 (91)	2.9(104)	2.61*
	L	66.0 (97)	—	72.1(140)	72.9 (96)	0 (131)	
III-g	U	80.8 (73)	—	90.1(151)	83.3(126)	19.6(107)	2.10*
	L	88.3(128)	—	91.6(142)	89.1 (83)	24.7 (93)	
IV-a	U	47.6 (63)	4.9(102)	—	38.7(111)	—	2.70**
	L	29.8 (57)	7.5 (93)	—	22.4 (85)	—	

B. White x Green crosses

		Maternal					
		I-b	I-z	II-e	III-g	IV-a	
		% BP (n)	% BP (n)	% BP (n)	% BP (n)	% BP (n)	t
Paternal							
$A^jA^jI^{C1}$	U	25.8(128)	22.4 (98)	50.0(104)	37.8(172)	74.7(150)	2.14*
	L	17.6(108)	23.1(117)	39.4(127)	21.7 (23)	55.0(229)	
A^aC^pIV	U	21.7 (46)	9.1 (33)	4.4 (45)	12.7 (63)	29.9(104)	0.91
	L	21.9 (32)	11.2(143)	3.7 (54)	5.3 (38)	11.8 (17)	

[a] All the strains are as listed in Ch. 1, Table 1. The nuclear background of the male parents in section A and the female parents in section B is A^jA^j.

[b] $t = (p_U - p_L)/s$ where $s = p_U(1 - p_U)/n_U + p_L(1 - p_L)/n_L$. p_U and p_L are the %BP from the upper and lower halves of the capsule respectively. n_U and n_L are the number of the seedlings in each sample. The degrees of freedom for t is taken to be infinity (Steel and Torrie 1980).

* $P < 0.05$.

** $P < 0.01$.

limited pollen tube growth (see discussion in Chapter 1). Hence, no meaningful comparisons can be made for these crosses and only data from crosses using $A^{j}A^{j}$-I^{c1} and $A^{a}C^{p}$-IV as the paternal parents are presented in section B.

As shown in Table B.1, the frequency of biparental plastid transmission does not differ a great deal between seeds situated in one half of the capsule compared to the other half. In slightly more than 50% of the crosses, a higher frequency of biparental plastid transmission was obtained from the upper half of the capsule. For the purpose of testing whether there is a relationship between the differences obtained from two halves of the capsules and the plastome types of the paternal parents, the data obtained from all crosses with the same paternal parent were pooled and analyzed by t-tests. According to this analysis, the frequencies of biparental plastid transmission obtained from the two halves of the capsule are significantly different in four out of the seven sets of crosses. In three sets of crosses involving paternal plastomes II-epsilon, IV-alpha and I^{c1}, a higher frequency of biparental plastid transmission was obtained from the upper half of the capsule. However, the reverse was true in crosses that have plastome III-gamma from the paternal parent.

At first glance, the differences in %BP between the two halves of the capsule are not as dramatic as one would expect if there is an extensive loss of plastids during pollen tube development. However, limitations in the method used for this analysis have to be considered. The lengths of the styles of Oenothera flowers range from 4 to 10 cm, depending on the

species. The ovary, situated at the end of the style, is only 1 cm in length. Thus, the full length of the ovary comprises only a relatively small fraction of the total distance a pollen tube must extend prior to fertilization. If, as I postulate, a greater distance implies a greater time required for growth of the pollen tube, then it follows that the time required for a pollen tube to grow from one end of the ovary to the other comprises a small fraction of the total time between pollination and fertilization. The experiment presented here presumes that appreciably more degradation of paternal plastids takes place within the second half of the small duration as compared to the first half. It may be beyond the power of the experiment to detect such small differences against biological variability. Furthermore, very rapidly growing pollen tubes may experience no degradation during their passage through the style. Conversely, very slowly growing pollen tubes may seldom reach the base of the ovary at all, which may explain the observation that viable seeds could be obtained only from the upper part of the capsule in some crosses (Chapter 1).

Taking into consideration the limitations mentioned above, the results obtained are consistent with the hypothesis that there is degeneration of plastids in the generative cell as a pollen tube extends towards the egg. Furthermore, the extent of plastid degeneration may be greater in plastomes having lower transmission efficiencies (II-epsilon and IV-alpha) in the given nuclear background (A^JA^J).

APPENDIX C. COMPARISON OF TRANSMISSION OF PLASTOMES I^{D} AND I^{C1}

Rationale

The transmission of plastids in Oenothera can be influenced by multiple factors contributed by either the nuclear or the plastid genomes (Chapter 1). One major focus of the research described in this dissertation was to examine the relationship between the efficiency of cpDNA replication and the efficiency of plastid transmission. Electron microscopic examination of cpDNA replication intermediates did not point to any difference in the number and the location of the replication origins between two plastomes with distinct transmission properties (Chapter 2). However, subsequent analysis indicated that a highly polymorphic region of cpDNA lay close to one of the replication origins (Chapter 3). Conceivably, differences in this region could affect the functioning of the origin if the sequence contains the recognition sites for replication proteins or regulatory sequences flanking these sites. In order to test whether differences in this region could affect the transmission abilities of plastids, crosses were conducted to compare two closely related plastomes that differ in this region.

Plastome I^{C1} is derived from plastome I^{D} under the influence of the plastome mutator activity (Chiu et al. 1990). Four differences in

restriction fragment length have been detected between these two type I plastomes. One of the length mutations lies within the 16S rRNA-trnI spacer, close to one of the members of each pair of replication origin (Chapter 3). Analysis of the nucleotide sequences show that some repeated segments capable of forming stable stem-loop structure in the 16S rRNA-trnI region of plastome I^{D} are absent from plastome I^{C1}. Since stem-loop structures are known to affect the movement of replication forks, it was of interest to determine if the transmission of plastome I^{C1} is more efficient than that of I^{D}.

Results and Discussion

In order to compare the transmission efficiencies of plastome I^{D} and plastome I^{C1}, crosses were made using the constant nuclear background of Oenothera hookeri str. Johansen ($A^{J}A^{J}$), with various mutant plastids (Chapter 1) in the maternal parent and either plastome I^{D} or I^{C1} in the paternal parent. Seeds from these crosses were sterilized and germinated as described in Chapter 1.

The frequencies of biparental plastid transmission (%BP) from crosses made in different years are listed separately in Table C.1, due to my observation that significant differences may occur in plastid transmission from the same crosses performed in different years. As discussed in Chapter 1, seasonal variation in transmission frequency may be related to variation in the sizes of the maternal flowers. As a

Table C.1. Frequency of biparental plastid transmission in crosses with mutant plastids transmitted by the female parent and plastome I^{C1} or I^{D} transmitted by the male parent.

	Paternal Plastome				
	I^{C1}		I^{D}		
Maternal	1988	1989	1984/1985[b]	1988	1989
Plastome	% BP[a] (n)	% BP (n)	% BP (n)	% BP (n)	% BP (n)
I-b	23.0±3.2 (174)	22.0±2.7 (236)	40.9±5.2 (88)	—	—
I-z	—	22.8±2.9[+] (215)	9.7±2.9 (103)	—	30.3±4.2[+] (122)
I-e	21.9±3.0 (192)	—	21.1±3.9 (109)	—	—
I-e[c]	32.3±4.8[+] (93)	—	—	24.1±4.6[+] (87)	—
II-e	—	44.2±3.3 (231)	26.7±4.0 (120)	—	—
III-g	24.4±4.6 (86)	35.9±3.4 (195)	21.7±2.9 (203)	—	—
IV-a	73.0±4.1 (115)	62.8±2.5[+] (379)	40.0±4.1 (140)	—	36.6±2.7[+] (314)

[a] see Table 1.2.

[b] These data are taken from Chiu et al. (1988).

[c] The nuclear background of this strain is albicans/[h]Johansen ($A^{a}A^{j}$).

[+] The transmission efficiencies of plastomes I^{C1} and I^{D} can be compared in these crosses.

consequence, meaningful comparisons between plastomes I^{C1} and I^{D} can be made only using data obtained from the same year. Three pairs of crosses meet this criterion. Data from these crosses are marked with a plus in Table C.1. The transmission efficiency of plastome I^{D} was higher than I^{C1} when the maternal parent contributed plastome I-zeta. The reverse was true when the maternal parent was an albicans/^{h}Johansen hybrid strain carrying mutant plastome I-eta. However, the difference between plastomes I^{C1} and I^{D} in these two pairs of crosses was not dramatic. A larger difference between I^{C1} and I^{D} was observed when the maternal parent contributed mutant plastome IV-alpha. In these crosses, plastome I^{C1} from the paternal parent was detected in 63% of the progeny while plastome I^{D} only appeared in 37% of the progeny. Overall, it appears that the transmssion efficiencies of plastomes I^{C1} and I^{D} are very similar. The difference between the two may be visible only when the frequency of biparental transmission is very high.

APPENDIX D: ATTEMPTS TO LOCATE THE ORIGIN OF DNA REPLICATION IN THE CHLOROPLAST OF *OENOTHERA* USING TWO-DIMENSIONAL GEL ELECTROPHORESIS

Rationale

Replication of chloroplast DNA is an important step in the multiplication of chloroplasts. This dissertation examines the hypothesis that differences in the structure of origins of cpDNA replication among various *Oenothera* plastome types may contribute to the observed differences in the efficiency of plastid transmission. In Chapter 2, two pairs of origins of cpDNA replication were located using electron microscopy. However, the electron microscopic data can only localize the D-loop initiation sites within a 1-2 kb region. In order to study the initiation of cpDNA replication, it is necessary to identify the D-loop initiation sites more precisely.

Among alternative methods of mapping DNA replication origins, the two-dimensional gel electrophoresis technique developed by Brewer and Fangman (1987) has been used most frequently. In this method, restricted DNA fragments are seperated according to their mass in the first dimension on a low percentage agarose gel at low voltage. In the second

dimension of gel electrophoresis, DNA fragments are seperated according to both mass and shape on a high percentage gel at high voltage. Deviations in mobility occur with restriction fragments derived from DNA at different stages of replication, and these can be visualized by the use of radiolabeled probes. Restriction fragments that carry replication forks will form an arc ("fork arc"), which rises above and then returns to the line of simple linear fragmnets. Fragments containing replication bubbles (i.e. origins of replication) will migrate even slower than those with a simple replication fork and form an additional arc ("bubble arc") above the "fork arc". The position of the origin of replication on the restriction fragment can be determined according to the relative positions at which the bubble arc ends.

The two-dimensional gel method has been used successfully to localize origins of DNA replication in the highly amplified region of yeast (Linskenns and Huberman 1988), Drosophila (Delidakis and Katatos 1989), and mammalian chromosomes (Vaughn et al. 1990). These origins of replication all form replication eyes with double stranded arms. It is not known if the two-dimensional gel technique can be used to characterize origins of replication that form displacement loops, such as those in the genomes of mitochondria and chloroplasts. This section describes an initial effort to employ the two-dimensional gel technique to localize origins of cpDNA replication in Oenothera.

Results and Discussions

Chloroplast DNA from Oenothera hookeri str. Johansen carrying either plastome I or IV was used for this project. Methods used for the isolation, purification, and storage of cpDNA are as described in Chapter 2. CpDNA was digested with restriction enzymes at room temperature for two hours immediately before electrophoresis. A protocol provided by Dr. M. Linskens (Roswell Park Memorial Institute, New York) was used for two-dimensional gel electrophoresis. In a typical experiment, the first dimension of electrophoresis was run overnight on a 0.4% agarose gel (prepared in TAE buffer) at 0.75 to 1.0 V/cm. Both the gel and the running buffer contained low concentrations of ethidium bromide (0.1 ug/ml). At the end of the first dimension, the sample lane was excised, rotated by 90 degrees, and cast on top of a 1.2% agarose gel (prepared in TBE buffer supplemented with 0.5 ug/ml ethidium bromide). The second dimension was run at 4 V/cm for 8-10 hours at 4°C. At the end of the electrophoresis, the DNA was depurinated in 0.25 M HCl for 10 min and was transferred onto a GeneScreen (NEN/DuPont) membrane using buffer containing 0.4 M NaOH and 1 M NaCl. In order to establish the conditions for detecting origins of cpDNA replication, the 5 kb EcoRI fragment (R2) that carries one of the D-loop initiation sites identified by electron microscopy (Chapter 2) was used as a probe for Southern hybridization.

Three restriction enzymes, EcoRI, ClaI and SalI, were used to digest Oenothera cpDNA. These enzymes generate fragments with a wide range of sizes (5 kb, 8kb and 18 kb respectively) that can be recognized by the

R2 probe. Since the retardation of replication intermediates on the second dimension is more obvious with a smaller fragment, one might expect EcoRI and ClaI to give better results. However, for all three enzymes tested, only spots representing non-replicating linear fragments were detected either by X-ray autoradiography or by a beta-scaning device.

Since the two-dimensional gel technique has never been used for mapping origins that initiate replication through formation of D-loops, it is possible that the technique has to be modified to suit the D-loop type of replication initiation. For example, the mobility of fragments containing D-loops on the gel may not be as low as the mobility of those carrying double-stranded replication eyes. However, the fact that even fork arcs were not detected indicates the quality of cpDNA used in the experiments was not optimal. There are several ways to improve the quality of cpDNA. First of all, since replication intermediates tend to be unstable, it may be necessary to preserve these structures before restriction digestion. In their electron microscopic study, Meaker et al. (1988) reported that no D-loops could be found on fragments smaller than 5 kb unless cpDNA was cross-linked by Trioxalen before digestion with restriction enzymes. Cross-linking is also necessary to see arcs formed by double stranded replicating bubbles on two-dimensional gels (Vaughn et al. 1990). Secondly, the frequency of replicating DNA in the sample should be increased. Methods such as BND-cellulose chromatography (Levine et al. 1970) have been used to enrich for replicating DNA before electrophoresis. However, the most efficient way would be to isolate cpDNA from cells in which cpDNA is actively replicating. This could be

achieved by using suspension culture cells that can be partially synchronized for cpDNA synthesis (Yasuda et al. 1988).

In conclusion, based on the preliminary work described above, a significant amount of work will be required before the two-dimensional gel technique can be adapted to study DNA replication in chloroplasts. Nevertheless, the procedure would provide valuable information by allowing a more precise localization of replication origins. Furthermore, since this method is based on different shapes of the DNA fragments at different stages of replication (Brewer and Fangman 1987), it can also be used to locate the terminus of cpDNA replication (Kolodner and Tewari 1975b).

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