This is to certify that the

dissertation entitled

The Plastome Mutator of Oenothera: A Molecular and Biochemical Examination of the Mutation Process

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany and Plant Pathology

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Date December 19, 1997

DATE DUE	DATE DUE	DATE DUE

THE PLASTOME MUTATOR OF OENOTHERA: A MOLECULAR AND BIOCHEMICAL EXAMINATION OF THE MUTATION PROCESS

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology
and
Genetics Program

1997

ABSTRACT

THE PLASTOME MUTATOR OF OENOTHERA: A MOLECULAR AND BIOCHEMICAL EXAMINATION OF THE MUTATION PROCESS

By

Tseh-Ling Chang

*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. Characterization of RFLP sites in a collection of mutants shows that insertion-deletion hot spots in the *pm* lines are defined by tandem direct repeats, implicating replication slippage or misalignment during recombination. To search for other DNA lesions that would not be visible as restriction fragment length polymorphisms, PCR-amplification products of the *psbB* gene were digested with a restriction endonuclease, denatured, and examined for single strand conformational polymorphisms (SSCP). Among 21 mutants, one 4-bp insertion and one point mutation were identified in *psbB*. The discovery that the *plastome mutator* can cause base substitutions as well as repeat-mediated insertions and deletions points to a likely defect in a component of the cpDNA replication machinery.

An *in vitro* system has been developed to investigate the chloroplast replication machinery of Oenothera. Few differences were observed between the *pm*- and wild-type chloroplast extracts provided with activated DNA. However, biochemical analyses show

that KCl is more essential for DNA syntheses by the *pm*-extracts than the wild-type extract. In a different assay, phagemids carrying a *pm* deletion hot spot were used to produce single stranded DNA as a template to examine DNA synthesis from a specific primer. Variation in extension of the primer were observed with the chloroplast replication extracts from the *pm*- and wild-type lines. The *in vitro* differences on the single stranded template allow topoisomerase and helicase to be ruled out as candidates for the genetic lesion which results in plastome mutator activities.

To Mu-Tsang,
My Beloved Husband

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Professor Barbara Sears for her encouragement and assistance throughout this research. It is certain that this work would not have been possible without her.

I would also like to thank my guidance committee members Professor Karen Friderici, Professor John Ohlrogge and Professor Kenneth Keegstra for their generous support and constructive suggestions during the period of this study.

Special thanks are given to late Professor James Asher for his help in establishing the SSCP technique, my college classmate Hao-Ping Chen who is a graduate student at Cambridge University for technical advice on chromatography, and Professors. Gordon Cannon and Sabine Heinhorst at University of Southern Mississippi for their support and fruitful discussion.

Kindly assistance and suggestions for my presentation from Monica Guhamajumdar and other colleagues are highly appreciated.

Finally, loving thanks is offered to my parents and my husband for their sacrifice and patience.

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Chapter 1

GENERAL INTRODUCTION

Organization of the plastid genome

Plastids are autonomously replicating organelles in photosynthetic eukaryotes. Since the discovery of the plastid genome (plastome) more than three decades ago, knowledge of the organization and structure of this molecule has accumulated dramatically (reviewed by Kirk and Tilney-Bassett, 1978; Palmer,1991; Shimada and Sugiura, 1991). Despite the fact that plastids with different shapes and functions exist in various tissues, the DNA composition is the same in all of them while differential expression of plastome genes distinguishes them from each other.

Plastids contain multiple copies of plastomes subdivided among nucleoids that are probably the units for segregation and division. The plastome molecules of higher plants are closed circular double stranded DNAs, with unit sizes that vary from 120- to 200- kbp (Palmer, 1991). Monomeric- or oligomeric units of plastomes have been observed in pea (Kolodner and Tewari, 1975), spinach (Deng et al. 1989), and watermelon (Bendich and Smith, 1990). Except for few species (e.g. legumes), most plastome molecules in higher plants contain two exact inverted repeats which are 20 to 30 kb in size and separated by a large and a small single copy region, as exemplified by the plastid genome map of *Oenothera* in Figure 1.1. The entire nucleotide sequences of the plastomes from the

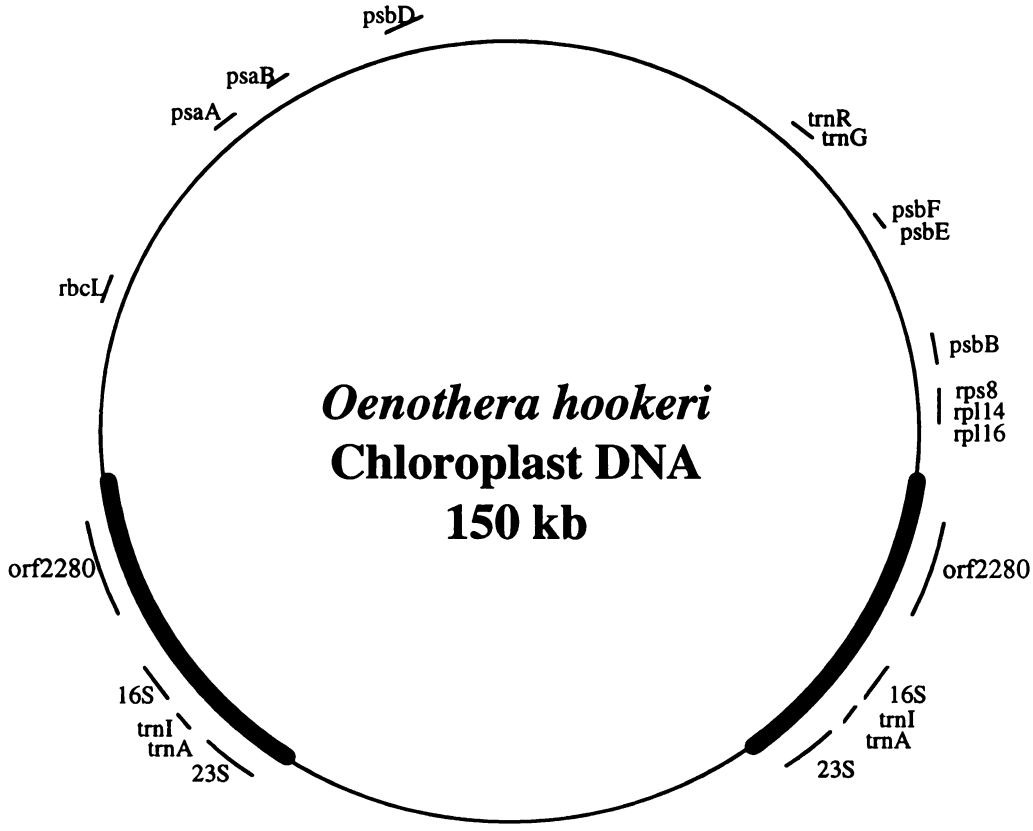


Figure 1.1 Circular map of *Oenothera* chloroplast DNA. Relative positions of genes used in this dissertation are as shown. Inverted repeat regions are represented by bold lines.

liverwort *Marchantia polymorpha* (Ohyama et al., 1986), tobacco (Shinozaki et al., 1986), rice (Hiratsuka et al., 1989), and maize (Maier et al., 1995) have been determined. Each plastome encodes 120 to 140 genes that are involved in photosynthetic electron transfer activities and its own transcription and translation. Often, a complex multi-subunit protein in organelles is encoded by genes from both nuclear and chloroplast genomes. The nuclear encoded precursors are transported into plastids through the guidance of transit peptides, they associate with proteins encoded in plastids, which then become functional. The best-known example is the ribulose bisphosphate carboxylase (rubisco) with its large subunit encoded by the plastome while the small subunit is encoded by the nuclear genome. Generally, plastomes are highly conserved in genome size and gene order. The main causes of length variation among plastomes include changes in the amount of repeated DNA, intron content, gene content, and other classes of deletion-insertion events. The majority of spontaneous length mutations are 1~10 bp in size (Palmer, 1991). In monocots, DNA rearrangement and amplification/diminishment of specific fragments were found to be induced in plastomes during callus induction and plant regeneration in cell/tissue culture (Day and Ellis, 1984; Fukuoka et al. 1994).

DNA replication in chloroplasts

Part of this section is based on reviews by Heinhorst and Cannon (1993) and Gillham (1993).

The most popular model of chloroplast DNA replication was proposed by Kolodner and Tewari (1975). Based on the electron microscopic (EM) examination of replicating cpDNA in pea and maize, they postulated that cpDNA replication in higher

plants involves the Cairns and the rolling circle models. It was interpreted that cpDNA replication begins with the Cairns mechanism in which DNA replication is initiated by two replication displacement loops (D-loops). The two origins expand towards each other to form the Cairns fork. Replication subsequently moves bidirectionally until the two ends meet each other at a site that is 180° around the DNA molecule from the initiation site. The DNA gaps left could either be sealed by DNA ligase or serve as the initiation site for rolling circle replication, through which new plastome molecules are synthesized through amplification. The mode of cpDNA replication in pea has been investigated *in vitro* (Reddy et al. 1994) and both types of replication intermediates (sigma structure of the rolling circle mechanism and theta structure of the Cairn mechanism) have been observed. Evidence obtained from other organisms seem to support this model to some degree. The replication intermediates of cpDNA from *Euglena*, *Chlamydomonas*, tobacco and *Oenothera* have been examined with electron microscopy and their replication D-loops were mapped (Koller and Delius, 1982; Ravel-Chapuis et al. 1982; Waddell et al. 1984; Chiu and Sears, 1992). In *Oenothera*, replication intermediates of the displacement loops from unidirectional synthesis of one strand of DNA were observed (Chiu and Sears, 1992), and in some cases, two D-loops were observed on the same molecule. The replication intermediates observed in *Euglena* (Koller and Delius, 1982; Ravel-Chapuis et al. 1982) and *Chlamydomonas* (Waddell et al. 1984) are mainly double-stranded arms resulting from bidirectional synthesis of two nascent strands, which suggested that discontinuous synthesis begins immediately after D-loop initiation (Heinhorst and Cannon, 1993), or the replication origins are too close to

discern from each other. After applying the technique of pulsed-field gel electrophoresis, Bendich and Smith (1990) suggested that the long linear molecules they observed may have resulted as the intermediates of rolling circle replication. The existence of plastome oligomers observed by Deng et al. (1989) might also seem to support the concept of rolling circle replication, except that no tail longer than 1.5 unit of the plastome length was discovered. Rather, frequent recombination events were proposed to explain the multimers observed in the chloroplasts of spinach.

Most of the proteins that are involved in cpDNA replication appear to be encoded in the nucleus. Evidence came from the observation that in white plastids of *iojap* maize, which lack chloroplast ribosomes and therefore do not contain any chloroplast-encoded proteins, the plastome DNA is nevertheless replicated (Walbot and Coe, 1979). Additionally, Rapp and Mullet (1991) showed that an inhibitor of chloroplast genome transcription (tagetitoxin) did not impair cpDNA synthesis during leaf development). Furthermore, in wheat, up to 80% of the chloroplast genome apparently can be deleted without affecting replication of the mutant plastomes (Day and Ellis, 1984). This line of evidence provides indirect proof that cpDNA replication is not largely dependent on plastome encoded proteins.

The abundance of plastomes and plastids in higher plant cells differ developmentally and tissue-specifically (Lamppa and Bendich, 1979; Possingham and Lawrence, 1983). Light seems to be an important factor stimulating synthesis of plastomes. Plastome numbers vary from 3300 to 12,000 copies per cell under dark and light growth conditions respectively in *Nicotiana tabacum* (Weissbach et al. 1985). It was estimated that in the leaf meristems of wheat, there are about 1000 plastome copies

per plastid before chloroplasts divide (Boffey and Leech, 1982), and the plastome number drops to ca. 60 per plastid in mature leaves. Conceivably, plastomes replicate rapidly at an early stage of leaf development, and then plastid division outpaces plastome replication resulting in reduction of plastome copy numbers per plastid. It has been suggested by Bendich (1987) that increases in the amount of cpDNA content occur in order to provide sufficient templates for expression of chloroplast encoded genes. Consequently, an adequate quantity of proteins will be made to meet the demand for elevated photosynthesis rates during leaf development. In older leaves of barley, the reduction of cpDNA copy numbers per plastid may also be caused by degradation of cpDNA in addition to redistribution of plastomes (Baumgartner et al. 1989). However, the copy numbers of plastome per plastid in epidermal cells of spinach does not change during leaf development (Lawerence and Possingham, 1986).

In vitro replication systems of chloroplast

Several *in vitro* replication systems have been constructed in different laboratories to study the process and regulation of DNA synthesis within the chloroplast. Thus far, the goal has been to establish a system that could support site-specific initiation of cpDNA replication.

In earlier work, crude extracts with DNA synthesis activity were obtained directly from isolated intact or disrupted chloroplasts of maize (Zimmermann and Weissbach, 1982), *Marchantia polymorpha* (Tanaka et al., 1984), and *Petunia hybrida* (de Haas et al., 1987), with no further purification. DNA synthesis in crude extracts from maize

depended on exogenously added plasmid containing plastome inserts, but no site specific initiation was observed. The same situation was observed in the crude chloroplast extracts from *Marchantia polymorpha*. In the crude extracts from *Petunia* chloroplasts, deoxynucleotide incorporation does not need exogenous DNA. In these systems, prevalent endogenous DNA and nuclease may have inhibited DNA replication and site specific initiation to some extent (Heinhorst and Cannon, 1993).

Recently, more refined *in vitro* replication systems of chloroplasts have been established following additional purification steps (Gold et al. 1987; Meeker et al. 1988; Carrilo and Bogorad, 1988; Heinhorst et al. 1989). Replication extracts having DNA polymerase activity are prepared by passing triton-disrupted chloroplasts through a diethyl-aminoethyl (DEAE) cellulose and a heparin column. Most endogenous cpDNA and DNA nucleases are removed at this step. Further chromatography steps may be performed in some cases for better purification.

A crude chloroplast replication system from *Chlamydomonas reinhardtii* was developed by Wu et al. (1986). A high salt extract of the thylakoid membrane fraction was isolated following the procedure developed by Orozoco et al (1985), to study site specific transcription of spinach extracts *in vitro*. A soluble protein fraction essential for cpDNA replication was added to the high salt extract to support this *in vitro* replication system. With increasing amount of inhibitors (ddCTP) of cpDNA replication, Wu et al. (1986) showed that cpDNA replication begins close to a site that was mapped by D-looping as a replication origin (ori A). Similarly, Meeker et al. (1988) showed that the partially purified pea chloroplast DNA polymerase initiated DNA replication at the vicinity of *Ori* regions mapped through EM studies. In maize, the *in vitro* replication

system (Carrillo and Bogorad, 1988) pointed to a site-specific initiation from a preferred template which did not match the D-loop sites observed by EM (Kolodner and Tewari, 1975 a & b). The inconsistency between the *in vitro* and *in vivo* studies was also observed in studies of tobacco in which a single replication origin was mapped by electron microscopy (Takeda et al. 1992) while an extra replication origin was mapped with both the *in vitro* and *in vivo* tools in another study (Lu et al. 1996).

***Plastome mutators* : an overview**

Plastome mutators are nuclear loci that greatly enhance the naturally low spontaneous rate of plastome mutation. According to the classification of Kirk and Tilney-Bassett (1978), these nuclear mutants can be grouped into two classes, based on the spectrum of mutant phenotypes induced. One example of the narrow-spectrum group is *iojap* of maize (Shumway and Weier 1967; Walbot and Coe 1979; Thompson et al. 1983, Han et al. 1992), in which affected leaves always have longitudinal white stripes of the same phenotype. The wide-spectrum group is exemplified by *chm* of *Arabidobsis* (Redei and Plurad, 1973; Mourad and White, 1992; Martinz-Zapater et al, 1992; Sakamoto et al. 1996), *cpm* of barley (Prina, 1992, 1996), and the *plastome mutator* (*pm*) of *Oenothera* (Epp 1973; Chiu et al. 1990; Johnson and Sears 1990a & 1990b; Johnson et al. 1991; Sears and Sokalski 1991; Chang et al. 1996). Mutant phenotypes vary in these *plastome mutators*, e.g. leaf deformation, and varying degree of pigmentation deficiencies of leaves. In all cases, the mutator activity is due to a recessive nuclear allele, capable of causing plastid defects only when it is homozygous. The mutations caused by the mutator are transmitted independently of the nuclear background. Variable features of the various

mutators suggest that they are likely to affect genes of different functions. The *iojap* locus has been cloned and sequenced (Han et al. 1992), but its function remains unknown. The *chm*-locus of *Arabidopsis* has three alleles, and two reports correlate mitochondrial DNA rearrangements with *chm* activity (Martinez-Zapeter et al. 1992; Sakamoto et al., 1996). Conceivably, the *chm* is a mutator of mitochondria conferring an impact on both mitochondria and plastids (Mourad and White 1992; Sakamoto et al. 1996). Analysis of mutants induced by the *cpm* mutator of barley implied that it may have been possibly involved in activation of transposable elements. However, no molecular evidence was provided in that report (Prina, 1996).

The *plastome mutator* of *Oenothera*

The *plastome mutator* of *Oenothera hookeri* strain *Johansen* was isolated by Epp (1973) through EMS mutagenesis. It has a lower penetrance than does *chm*, but it still causes variegated sectors to appear in about 35% of all *pm /pm* plants (Epp 1973; Sears 1983; Epp et al. 1987; Sears and Sokalski 1991). Genetic analysis showed that the *plastome mutator* is encoded by a recessive nuclear gene. This nuclear gene is capable of elevating the frequency of plastome mutations 200-1000 times higher than the spontaneous levels (Epp 1973; Sears and Sokalski 1991). Restriction fragment length polymorphism (RFLP) analyses revealed the association of the *plastome mutator* activity with cpDNA alterations in *pm* lines (Chiu et al. 1996). Sequence characterizations indicated that all of these variable sites are A-T rich (about 80%) and are surrounded by direct repeats (Chang et al. 1996). These *pm*-preferred targets are in all respects similar to

the sites where replication slippage events tend to occur frequently in prokaryotes (Levison and Gutman, 1988). A replication slippage model therefore has been proposed to explain the mode of action of the *plastome mutator* (Chang et al. 1996). Although most evidence collected in our laboratory points to a likely defect in a component of the cpDNA replication machinery, the real role of the *plastome mutator* in DNA metabolism remains elusive. In this research, molecular and biochemical approaches have been undertaken to study the *plastome mutator* of *Oenothera* from different angles.

As reported in Chapter 2, to search for other mutations that would not be visible as RFLPs, PCR-amplification products of several photosynthetic electron transfer related genes were digested with restriction endonucleases, denatured, and examined for single strand conformational polymorphisms (SSCPs). Variations thus identified were sequenced.

As discussed in Chapter 3, all of the previous variants recovered from the *plastome mutator* were in plants that carried plastome type I. To investigate whether the *plastome mutator* impacts different plastome types differentially, PCR amplification was performed with newly derived *pm/pm*-IV plants, to search for polymorphisms in previously identified mutation sites.

Chapter 4 describes an *in vitro* replication system that was adapted to assess DNA synthesis from segments of cloned *Oenothera* cpDNA. Additionally, a primed-template replication strategy was designed to investigate the cpDNA replication machinery from *pm*-plants. Plastome fragments containing previously identified *pm*-hot spots were cloned into phagemids to produce ssDNA as templates for replication experiments. Additionally, in order to observe *in vitro* site specific initiation in *Oenothera* chloroplasts, several

clones containing the putative origins of replication that were identified in cpDNA from *Oenothera* (Chiu and Sears, 1992) or other sources, (Meeker et al. 1988; Wu et al. 1986; Gold et al. 1987; Hedrick et al. 1993 and Lu et al. 1996) were examined with this newly developed *in vitro* system.

In Appendix A, a comparison of cpDNA content between the *pm*- and wild-type plants was conducted to study the impact of the *plastome mutator* on efficiency of cpDNA replication. The effect of the caylase treatment on the preparation of total DNA from *Oenothera* is also discussed in that context.

Chapter 2

CHARACTERIZATION OF DNA LESIONS CAUSED BY THE *PLASTOME MUTATOR* OF *OENOTHERA*

The main content of this chapter is from Chang et al. (1997)

Introduction

Initial studies of cpDNA in *pm*-induced mutants used restriction endonucleases that cut cpDNA infrequently, and no RFLPs were observed (Epp et al. 1987). In contrast, Chiu et al. (1990) used frequently cutting enzymes and showed that cpDNA polymorphisms were found at a high frequency in the *plastome mutator* lines of *Oenothera*. These RFLP sites consisted of deletions ranging from 50-500 bp. Examination of these *pm*-hot spots in natural lines revealed little variability relative to what was observed in *pm*-plants (Chiu et al. 1990). Sequence characterization of one RFLP site in a collection of *pm*-plants showed that different copies of numbers of 24-bp tandem repeats were deleted in two *pm* lines (Figure 2.1; Blasko et al. 1988), suggesting a repeat-mediated mutagenesis mechanism for the *plastome mutator*. Chang et al. (1996) examined the same fragment in another set of *pm* mutants, and all plants were found to harbor deletions relative to the progenitor cpDNA. No duplications were observed at this site. At another site where direct repeats exist in cpDNA, a deletion event eliminated one copy of a 29 bp tandem repeat in the non-coding spacer of *rps8-rpl14* in one member of the *pm* collection (Figure 2.2A; Chang et al. 1996). The plants also included a variant

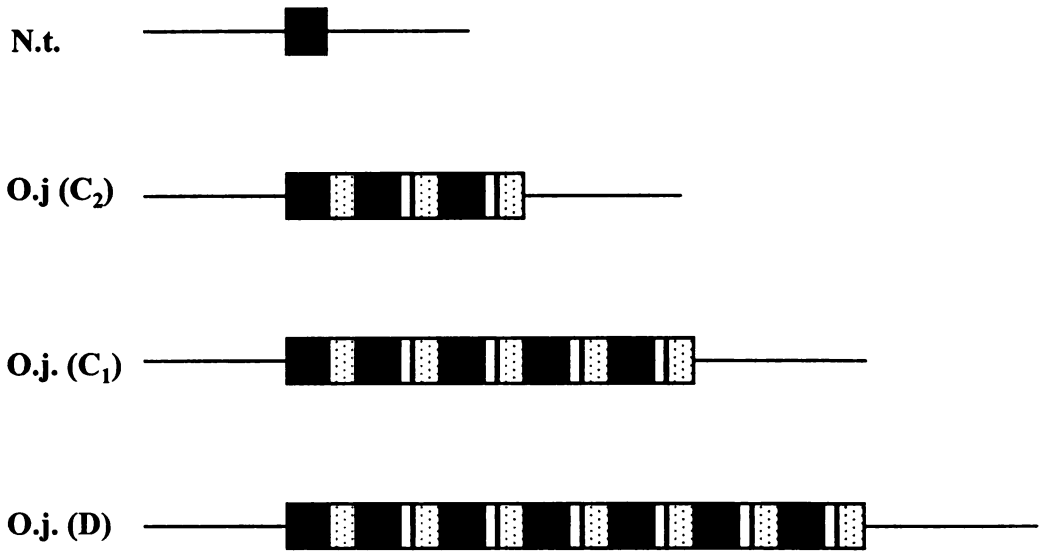


Figure 2.1. Simplified sequence comparison of the hypervariable region of orf2280 (Figure from Blasko et al. 1988) from the three *Oenothera* lines (C1, C2 and D), with the similar region from *Nicotiana tabacum* (*N.t.*). The first 16-bp of the 24-bp repeats are represented by black boxes, while the stippled box indicates the remaining 8-bp. The bars within the repeat units displays the only nucleotides difference between the near-repeat and the tandem repeats.

Figure 2.2. Sequence comparisons of sites responsible for RFLPs (Chang et al. 1996). In the regions of variability, sequences of the wild-type *Düsseldorf* line (wt) and mutant (*pm*) lines are aligned. Duplicated/deleted regions are framed by boxes; dashes indicate the absence of a comparable sequence. Direct and inverted repeats are indicated with arrows, dashed arrows indicate sequences of $G[A]_nT[A]_n$. (A) The *Cornell-2 pm*-line has a deletion of one copy of a 29-bp tandem repeat found in the wild-type between the *rps8* and *rpl14* genes (Wolfson et al. 1991). (B) The *Cornell-1 pm*-isolate has a 15-bp exact duplication relative to wild-type in the fourth largest *Bam*HI fragment located between *trnG* and *trnR* genes.

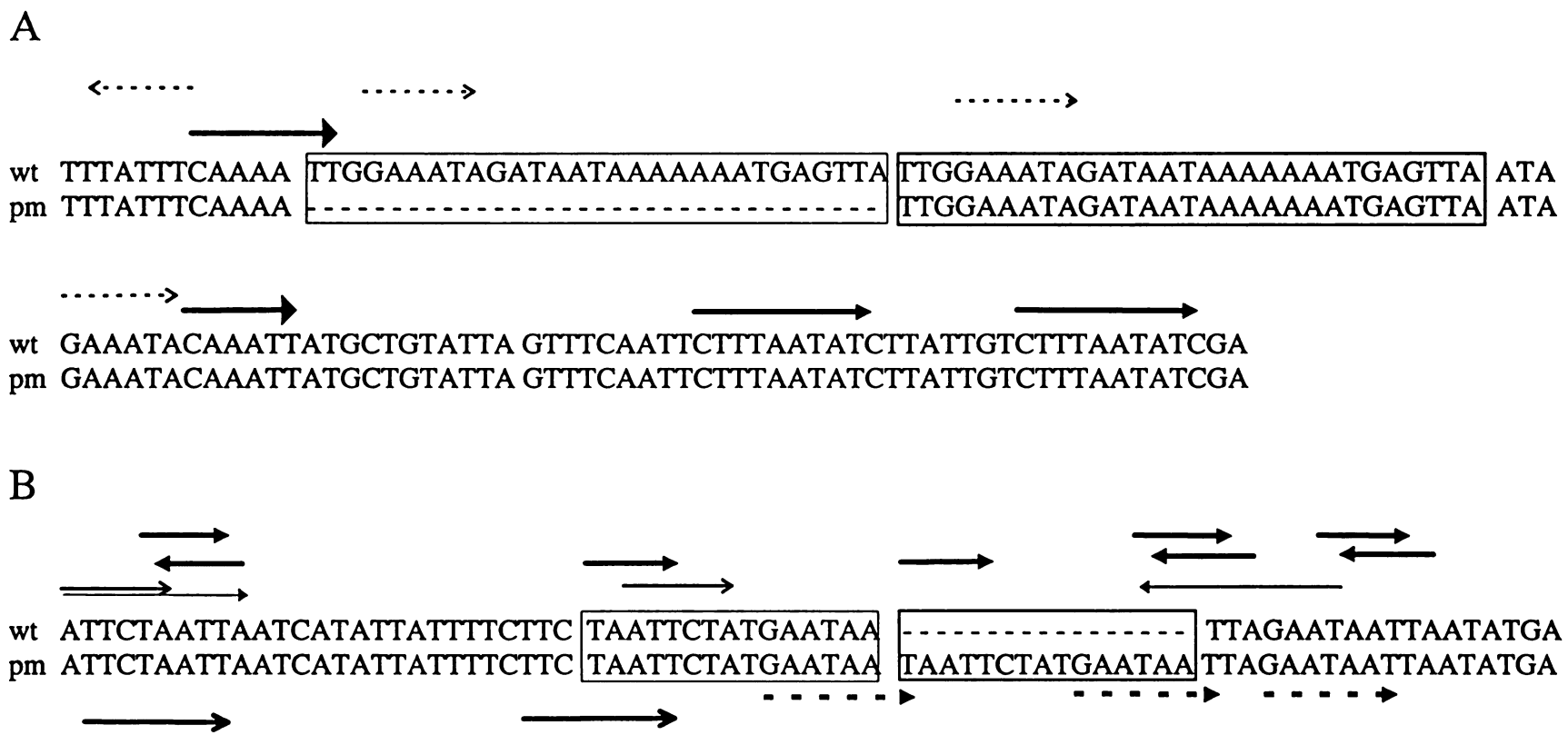

A
wt TTTATTTCAAAA TTGGAAATAGATAATAAAAAAATGAGTTA TTGGAAATAGATAATAAAAAAATGAGTTA ATA
pm TTTATTTCAAAA ---------- TTGGAAATAGATAATAAAAAAATGAGTTA ATA
wt GAAATACAAATTATGCTGTATTA GTTTCAATTCTTTAATATCTTATTGTCTTTAATATCGA
pm GAAATACAAATTATGCTGTATTA GTTTCAATTCTTTAATATCTTATTGTCTTTAATATCGA
B
wt ATTCTAATTAATCATATTATTTTCTTC TAATTCTATGAATAA ---------- TTAGAATAATTAATATGA
pm ATTCTAATTAATCATATTATTTTCTTC TAATTCTATGAATAA TAATTCTATGAATAA TTAGAATAATTAATATGA

Figure 2.2

with a unique 15-bp tandem duplication in another intergenic spacer (Figure 2.2B). Both inverted and direct repeats are found in the neighborhood of these indels, including 5- and 7- bp direct repeats at either end of the 15 base duplication. Larger deletions were identified in the 16S rRNA-trnI spacer, and these also occurred between direct repeats (Figure 2.3; Chang et al. 1996; Stoike 1997). Conceivably, these direct repeats mediated the duplication through replication slippage as explained below.

The replication slippage model was proposed by Albertini et al. (1982) to explain sites of deletion/duplication in prokaryotes (Figure 2.4). They pointed out that as DNA is unwound at the replication fork, the unreplicated and unpaired DNA could physically slip out of position. Slippage of the template strand during replication would result in deletion, while slippage of the daughter strand would yield duplications. Short direct repeats facilitate the mispairing of the template and daughter strands. Secondary structures were also likely to be involved in stabilizing the slipped strand. Preferred substrates for replication slippage include regions where DNA tends to be single stranded, sequences that are AT rich, and sites that have a simple alternating sequence (Albertini et al. 1982; Levinson and Gutman 1987).

If replication slippage is occurring in the *plastome mutator* lines at elevated frequencies, we predicted that we would see expansion/contraction of oligo-A tracks in cpDNA. Therefore, the mutability of two poly A tracks in *pm* plants was checked. Single adenine additions were observed in three of thirteen plants in a 13-bp track, but no variation was found in a nearby 11-base poly-A track (Figure 2.5; Chang et al. 1996). The differential susceptibility of the two oligo-A tracks to the *plastome mutator*-induced slippage could be due to the requirement of a substrate > 11bp or differences in the

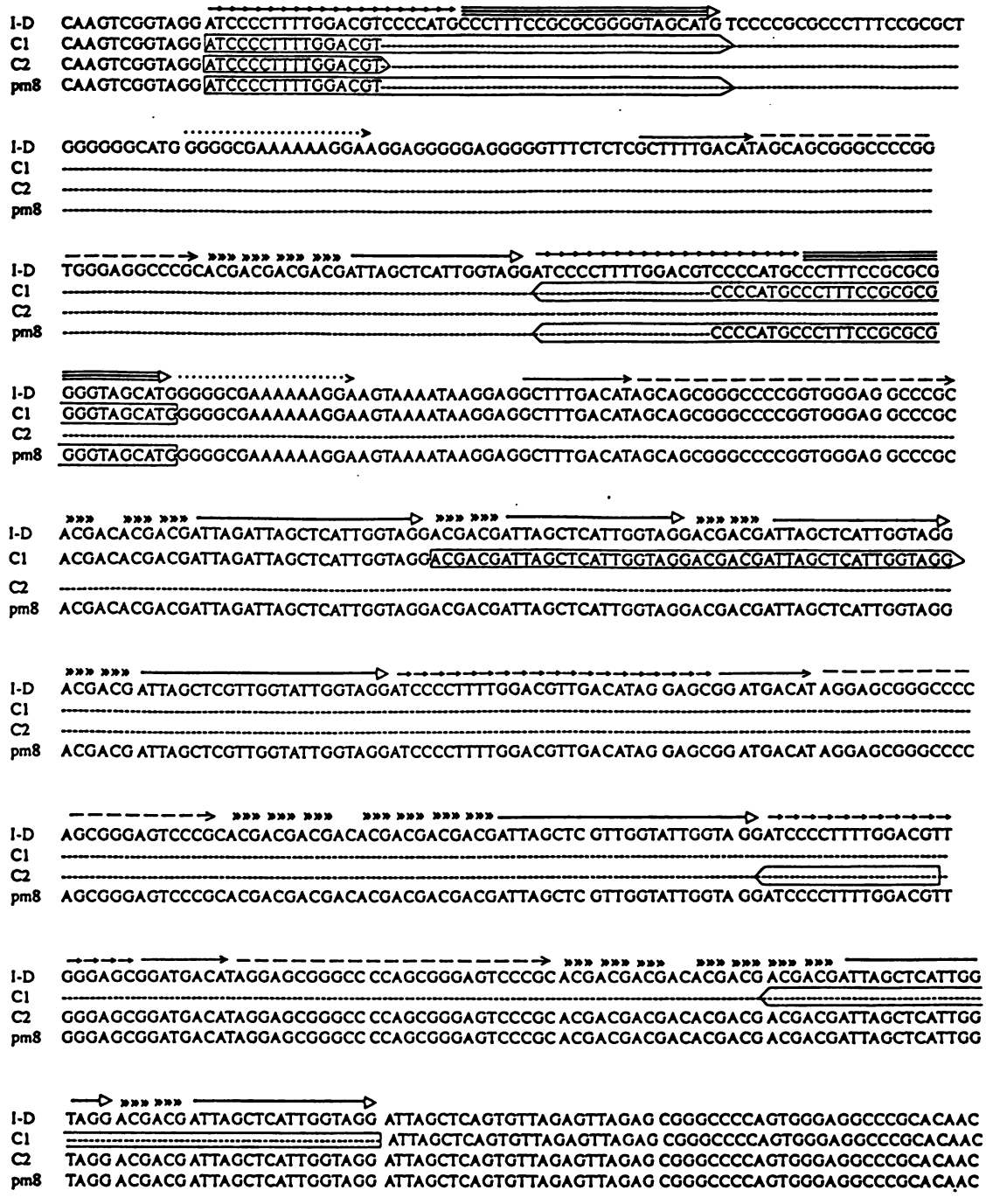


Figure 2.3 Sequence comparisons of the intergenic spacer of *16S rRNA-trnI* from *pm*-induced mutants (Stoike, 1998; Chang et al. 1996). *I-D*: wild type *Düsseldorf* line. *pm*-variants: *C1* (*Cornell-1*), *C2* (*Cornell-2*), *pm*8. Different families of repeat units are represented by various arrowheads. The borders of the deletions are framed by boxes.

Figure 2.4 Model of replication slippage. Boxes represent direct repeats and the direction of replication is indicated by arrowheads. A.) Slippage occurs during replication due to misalignment between repeat units of template and nascent DNA. B.) After another round of replication, deletion is recovered from slippage of template strand while duplication is obtained from slippage of nascent strand.

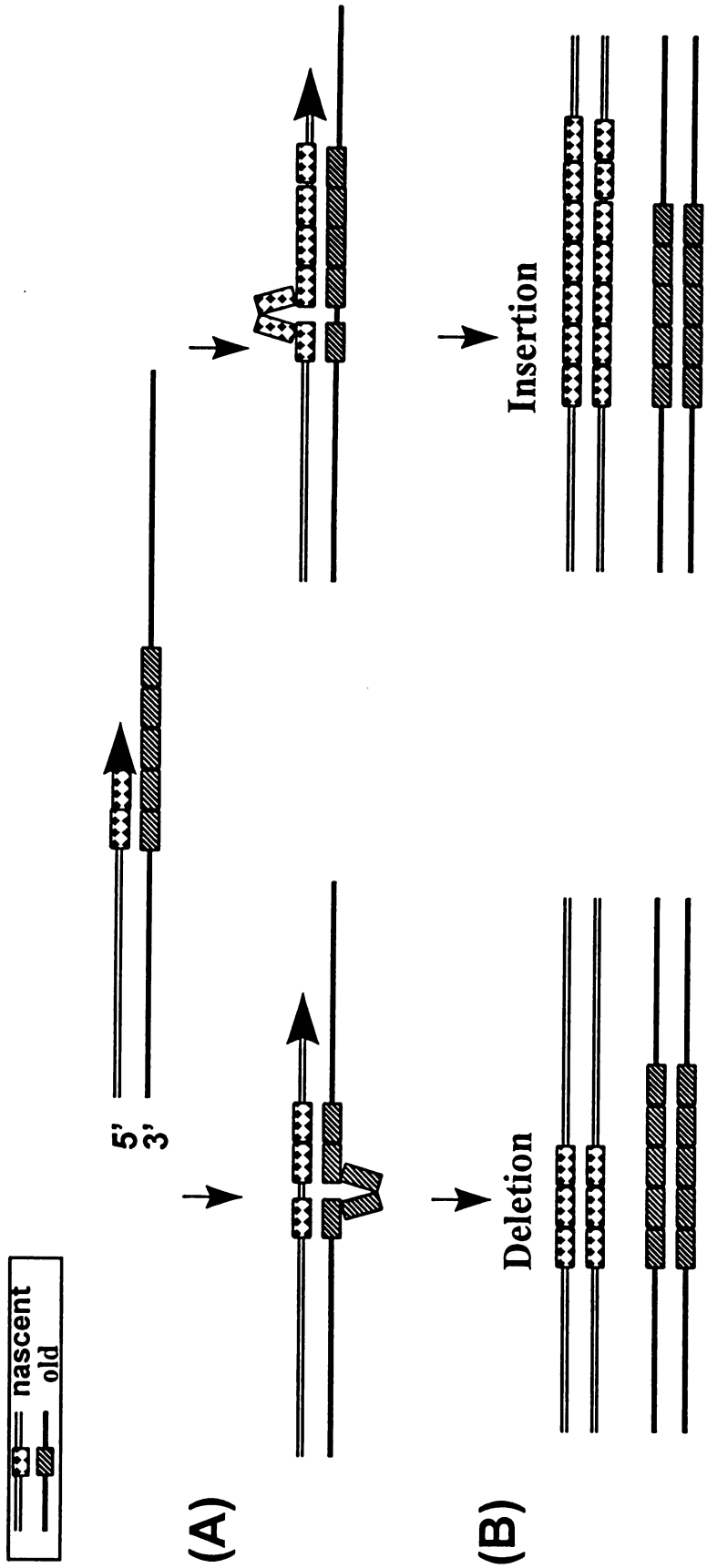


Fig. 2. 4

context of the oligo-A stretches. For example, in the analyses conducted thus far, one short repeat sequence $G[A]_nT[A]_n$, has been located in or near most of the *pm*-target sites (marked with dashed line and arrowhead in Figures 2.2 and 2.5).

Although we believe our evidence supports the interpretation that direct repeat-mediated replication slippage occurs at an elevated frequency in plants carrying the *Oenothera plastome mutator*, unequal inter- or intramolecular recombination could be an alternative explanation for the insertion and deletion events in *pm*-derived plants as postulated for some examples of repeat-mediated evolutionary change in cpDNA (Lin et al. 1984, Wallis et al. 1989, Palmer 1991).

During this analysis, we realized that it was possible that the *plastome mutator* causes more types of mutations than we had thus far recognized. Since our initial work focused on RFLPs, small indels or base substitution would have been overlooked. Analysis of SSCPs provides great sensitivity for revealing single base substitutions. Thus, it is the technique of use to screen for mutations in the present study. New variegated mutations were selected and a subset of plastome genes required for photosynthetic electron transfer were assayed for single strand conformational polymorphisms (SSCPs).

Materials and methods

Plant material. The *plastome mutator* seeds were obtained originally from Prof. W. Stubbe (University of Düsseldorf), who had perpetuated the line by self pollination of progeny descended directly from the original E-15-7 mutant of *Oenothera hookeri* strain Johansen (Epp 1973).

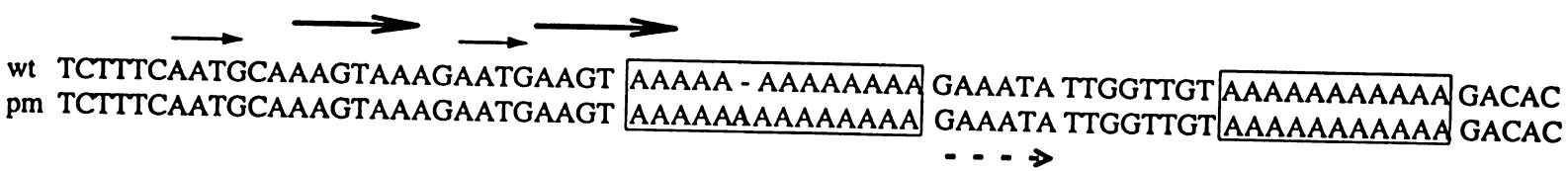


Figure 2.5. Sequence comparison of oligo-A stretches between the *rpl14* and *rpl16* genes (Chang et al. 1996). Sequences of two poly-A tracks between *rpl14* and *rpl16* genes from wild-type *Düsseldorf* line (Wolfson et al. 1991) and *pm* lines (*Cornell-2*, *pm8* and *pm32*) are aligned. Oligo-A regions are framed by boxes; other notations are as in Figure 2.2.

As explained by Chiu et al. (1990), plants of the strain Johansen are all descended from a single rosette collected in 1927 (Cleland 1935). The line that was mutagenized by Epp during his work at Cornell University, and from which Epp isolated the *pm* allele, is referred to as the *Cornell* line. Except for mutations caused by the EMS treatment, it should be identical to the wild-type *Düsseldorf* line of strain Johansen. The *Cornell-1* and *Cornell-2* lines contain photosynthetically-competent plastids, with a wild-type phenotype, although they contain cpDNA RFLPs relative to each other and relative to the *Düsseldorf* line (Chiu et al. 1990).

The *pm7* and *pm7ss* mutations were isolated from the same plant, and have been included in previous investigations (Johnson and Sears 1990a,b, Johnson et al. 1991; Chiu et al. 1990). These plastome I mutants have been maintained in leaftip culture in the Sears laboratory since 1983; they have an A/C, *pm/pm*$^+$ nuclear background. The *pm/pm7* line was obtained by self pollination of the original *pm7* plant; within this line, the *pm7* plastids have been kept for seven years in a *pm/pm* background, in vegatatively-propagated leaf tip cultures. These three lines were compared because the plastomes should be identical except for mutations caused by *pm*-activity. The 1993 field plants that were tested for new mutations were obtained by self-pollination of a single *pm/pm* plant from our 1992 field. These plants carried the *Cornell-1* type plastids, and are descended directly from sibs of the plants analyzed by Epp et al. (1987). New mutations induced by the *plastome mutator* were recognized as light green or white sectors in young *pm/pm* seedlings; these plants were selected and transplanted to the field. During the growing season, leaves were sampled for DNA extraction from 18 different plants containing pigment deficient sectors.

Preparation of nucleic acids. Plant total DNA extractions were performed as described by Fang et al. (1992).

Primer design, PCR amplification, restriction digestion, and SSCP analysis. Primers were designed based on sequence conservation of coding regions between tobacco and rice plastomes (Table 2.1). Oligonucleotides were synthesized at the Macromolecular Structure Facility at Michigan State University (East Lansing). PCR was performed with a Tempcycler II model 1105 (Coy Corporation, Grass Lake, Mich.) or a MiniCycler Model PTC-150-16 (MJ Research, Inc. Watertown, MA), using nucleotides from Perkin Elmer Cetus Corp. (Norwalk, CT), and Taq DNA polymerase from Boehringer Mannheim Biochemicals (Germany). PCR reactions were done in a 100ul total volume and overlaid with mineral oil. The amplification conditions consisted of 3 min at 94°C followed by 30 cycles of 1 min at 94°C and 1.5 min at 40°C and 2 min at 72°C, with a final extension for 10 min at 72°C, followed by prolonged incubation at 4°C. Amplification products were transferred to fresh tubes, precipitated and washed with ethanol, and dissolved in water. The amplification products were digested individually with restriction enzyme *Alu* I (*rbcL*), *Hae*III (*psaA*), *Taq* I (*psaB*), *Sau*3A (*psbB*), *Msp*I (*psbD*). The psbE+ F product is only 350 bp in size, and therefore it does not require restriction digestion. Visualization of single strand conformational polymorphisms (SSCPs) through MDE (JT Baker) gels and silver staining were performed as described by Morell et al (1993).

Table 2.1 Primers for PCR and sequencing

CpDNA region	Name*	Sequence
rbcL	rbcL**F**	5'-GTCACCACAAACAGA-3'
	rbcL**R**	5'-CTCCATTTGCAAGC-3'
psaA	psaA**F**	5'-CATTTCTCAAGAAC-3'
	psaA**R**	5'-CCTACTGCAATAATTC-3'
PsaB	psaB**F**	5'-GGTTTAGCCAAGGC-3'
	psaB**R**	5'-GAAAAGTGAGCTAA-3'
psbB	psbB**F**	5'-CCTTGGTATCGTGT-3'
	psbB**R**	5'-GTAGTTGGATCTCC-3'
	psbB3**F**	5'-GAATTAGATCGCGC-3'
psbD	psbD**F**	5'-GACTGGTTACG-3'
	psbD**R**	5'-CAAATCCTGCTGCA-3'
psbE+psbF	psbE+psbF**F**	5'-ATGTCTGGAAGCAC-3'
	psbE+psbF**R**	5-TTATCGTTGGATGAAC-3'
N.A.	universal**F**	5'-GTAAAACGACGGCCAGT-3'

*The direction noted for PCR primers **F**-forward (5'), **R**-reverse (3') corresponds to gene transcription for psbB gene and orf2280; all others correspond to the directionality of data entry from the tobacco chloroplast genome (Shinozaki et al. 1986). The direction of the universal primer was defined by the manufacturer (Gibco BRL).

Cloning and sequencing. The amplification product of the entire *psbB* gene, and the third largest fragment resulting from *Sau*3A digestion of the PCR product, were obtained from wild-type and mutant nucleic acids. The products were run on a 1.5% agarose gel to separate them from primers and other fragments, extracted from the gel with Qiaex (Qiagen Inc., Chatsworth, Calif.), cloned by conventional methods (Sambrook et al. 1989) in plasmid pUC18 (Norrander et al. 1983) in *E. coli* strain DH5α. Plasmids were purified using the Wizard mini prep kit (Promega Co., WI). Sequence data were obtained using ^{35}S-labeled dATP from New England Nuclear/Dupont (Wilmington, DE). The preparation of double strand DNA and dideoxy sequencing (Sanger et al. 1977) with Sequenase, were performed as outlined in the U. S. Biochemical (Clevand, Ohio) instruction manual. Primers are indicated in Table 2.1.

Results

The *psbB* gene is a site of *pm*-induced mutation in two albino isolates from 1993 field

DNAs from 18 new albino *pm*-mutants and the *pm7*, *pm7ss*, and *pmpm7* lines were amplified with primers specific for the *psbB*, *rbcL*, *psaA*, *psaB*, *psbD* and *psbE+ psbF* genes (Table 2.1). As indicated in the materials and methods section, all but the latter amplification products were digested with a restriction enzyme before denaturation and electrophoresis on an MDE gel. Most of the samples were a mixture of wild-type and mutant chloroplast DNA because nucleic acids were extracted from variegated leaves that

carried newly arisen mutations. In principle, any mutation in the segment of DNA being amplified, should be visualized as an extra band preceding or following the normal band (Morell et al. 1993). A mutant that has completely sorted out will give single rather than multiple bands, but the mobility will differ from the wild-type band. Since each band on the gel is composed of single stranded DNA, a mutational change will be reflected in two bands of DNA simultaneously. As shown in Figure 2.6, SSCPs were found at the *psbB* gene from an albino mutant "*93-b45*" and the leaftip culture line "*pmpm7*".

The PCR amplification products of the *psbB* gene were sequenced directly using internal primer psbB3F, but a frame-shift in mutant *93-b45* rendered the sequencing gel unreadable at the site of mutation because the sample contained a mixture of wild type and mutant DNA. Consequently, the PCR products from wild-type and mutant tissues were cloned and the plasmid insert was sequenced. As shown in Figure 2.7, a 4-bp insertion (TTTC) was found in mutant *93-b45* at position *84-87* of the *Oenothera* sequence. This mutation causes a frame-shift in *psbB* at a position equivalent to the 455th codon of the 501 codon tobacco gene, and thus is likely to be the reason for the albino phenotype. Through direct sequencing of the PCR product from *pm/pm7*, a single base transition (C/G → T/A) was found at position 150 of the *Oenothera* sequence in Figure 2.3. The observation that the mutation has sorted out and the wild-type sequence is lost is probably due to a founder's effect during subculturing and maintenance of the leaftip culture line.

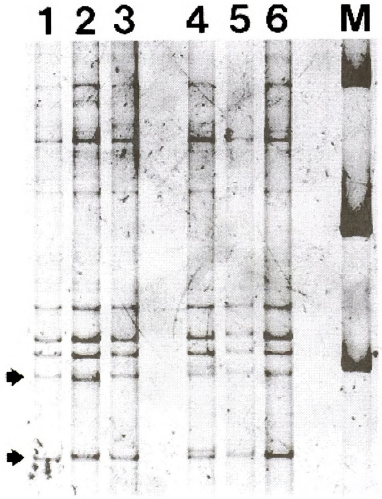


Figure 2.6. SSCP analysis of *pshB* gene (Chang et al. 1996). PCR products were digested with *Sau*3A, denatured and separated on an MDE gel. Lanes contain the following samples: 1. *pm7ss*, 2. *pmpm7*, 3. *pm7*, 4. *93-b45*, 5. *93-a31*, 6. *93-a28*, M: 1kb marker (Gibco). Arrowheads point to bands of altered mobility.

Figure 2.7. DNA sequence of *psbB* gene segments (Chang et al. 1996). Sequences from *Oenothera* wild-type (*OEN*), *93-b45* (*B45*) and *pm/pm7* (*pp7*) were aligned with the homologous region from cpDNA of *Nicotiana tabacum* (*NIT*). The numbering at the top left above the sequences refers to the location of the tobacco cpDNA sequence (Shinozaki et al. 1986); the wild-type *Oenothera* plastome I sequence numbers are shown underneath the sequence data. Dashes indicate the absence of a comparable sequence. In the tobacco sequence, bases that differ between the wild-type *Oenothera* sequence and tobacco are underlined. The 4-bp insertion and the base substitution are framed by boxes. The position of a stop codon created due to the frameshift in mutant *93-45b* is marked by the diamonds. Direct repeats are indicated with arrows.

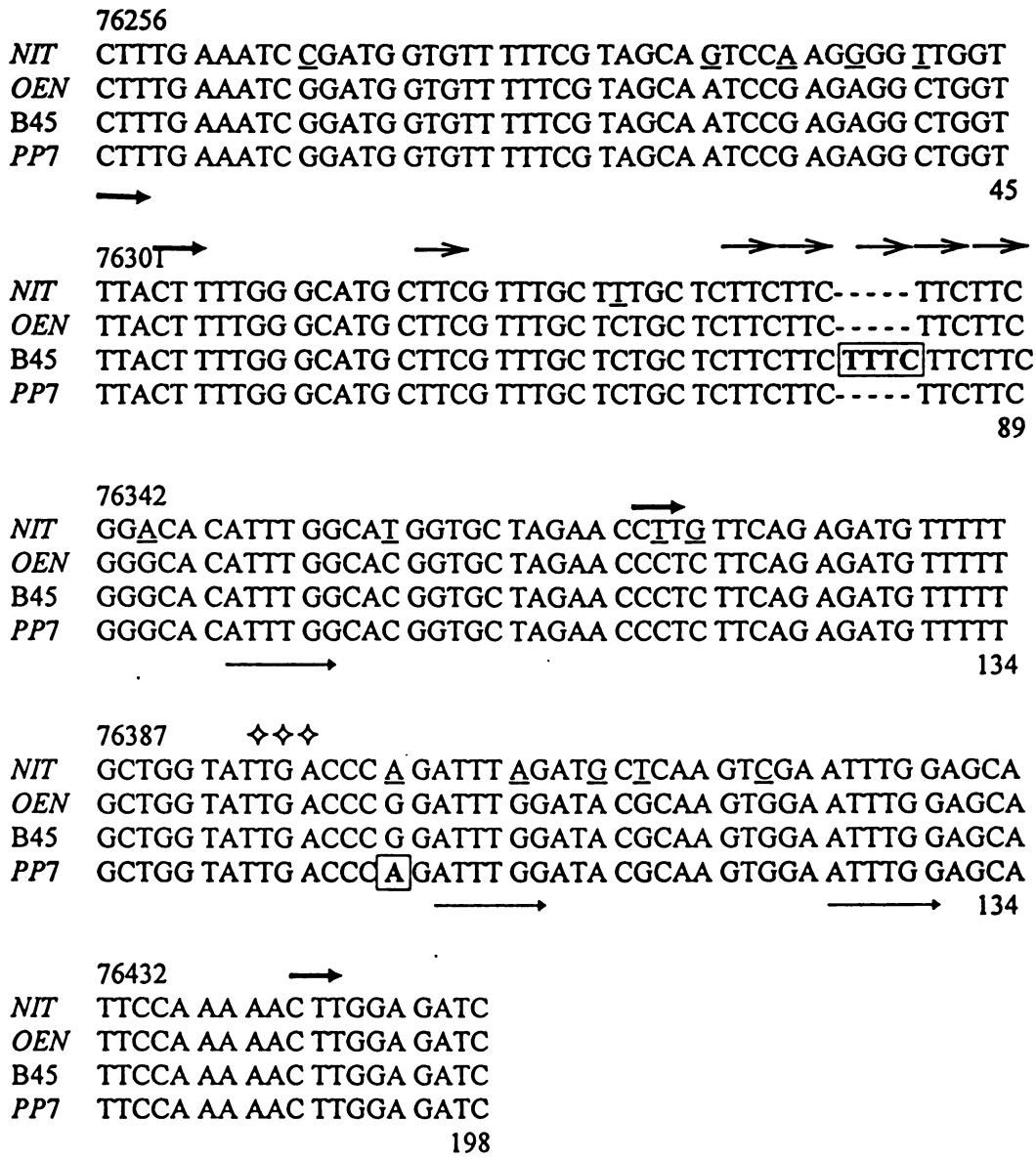

76256
NIT CTTTG AAATC CGATG GTGTT TTTCG TAGCA GTCCA AGGGG TTGGT
OEN CTTTG AAATC GGATG GTGTT TTTCG TAGCA ATCCG AGAGG CTGGT
B45 CTTTG AAATC GGATG GTGTT TTTCG TAGCA ATCCG AGAGG CTGGT
PP7 CTTTG AAATC GGATG GTGTT TTTCG TAGCA ATCCG AGAGG CTGGT
45
7630T
NIT TTACT TTTGG GCATG CTTCG TTTGC TTTGC TCTTCTTC-----TTCTTC
OEN TTACT TTTGG GCATG CTTCG TTTGC TCTGC TCTTCTTC-----TTCTTC
B45 TTACT TTTGG GCATG CTTCG TTTGC TCTGC TCTTCTTC TTTC TTCTTC
PP7 TTACT TTTGG GCATG CTTCG TTTGC TCTGC TCTTCTTC-----TTCTTC
89
76342
NIT GGACA CATTT GGCAT GGTGC TAGAA CCTTG TTCAG AGATG TTTTT
OEN GGGCA CATTT GGCAC GGTGC TAGAA CCCTC TTCAG AGATG TTTTT
B45 GGGCA CATTT GGCAC GGTGC TAGAA CCCTC TTCAG AGATG TTTTT
PP7 GGGCA CATTT GGCAC GGTGC TAGAA CCCTC TTCAG AGATG TTTTT
134
76387
NIT GCTGG TATTG ACCC A GATTT AGATG CTCAA GTCGA ATTTG GAGCA
OEN GCTGG TATTG ACCC G GATTT GGATA CGCAA GTGGA ATTTG GAGCA
B45 GCTGG TATTG ACCC G GATTT GGATA CGCAA GTGGA ATTTG GAGCA
PP7 GCTGG TATTG ACCC A GATTT GGATA CGCAA GTGGA ATTTG GAGCA
134
76432
NIT TTCCA AA AAC TTGGA GATC
OEN TTCCA AA AAC TTGGA GATC
B45 TTCCA AA AAC TTGGA GATC
PP7 TTCCA AA AAC TTGGA GATC
198

Fig. 2.7

Discussion

RFLP analysis has been used to detect mutations induced by the plastome mutator in previous studies (Chiu et al. 1990). Due to its limited resolution, only DNA alterations that range from 50-500 bp were detectable. In order to carefully assess the spectrum of mutations caused by the plastome mutator, I sought to utilize a method which is sensitive enough to reveal changes as subtle as a single base. To accomplish this, the SSCP technique was exploited to observe variation of PCR amplification products from photosynthetic electron transfer related genes. Restriction endonucleases digestion were used to digest the products of PCR reactions before gel electrophoresis, in order to increase the resolution of the SSCP analysis.

Using this procedure to assess PCR amplification products from 18 new albino mutants and three older lines that had all been derived from the same plant, one insertion mutation (*B45*) among a series of direct repeats, and a point mutation (*PP7*) were obtained in the *psbB* gene, as shown in Figure 2.7. The four-base insertion of TTTC occurred in the midst of a series of TTC tandem repeats. This 4-bp insertion causes a frameshift mutation, beginning with the 455th codon which is altered to specify Leu instead of Gly. Due to the frameshift, a truncated protein should result, which would be 19 amino acids shorter than the wild-type polypeptide, and which would also contain 28 missense amino acids at its carboxy end. Unfortunately, the *B45* plastome mutation was not recovered from the field, so this prediction cannot be tested. The TTTC insertion can be explained if slippage of the daughter strand occurred during replication, and resulted in an imprecise duplication. The second mutation identified in the *psbB* gene was

recovered in a *pm*-homozygous line, *pm/pm7*. This base substitution is not present in the original *pm7* mutant (lane 3 of Fig. 2.6), and would not alter the amino acid sequence. Thus, it is not responsible for the albino phenotype of the leaftip culture. Most likely, many other mutations have happened during the seven years of vegetative propagation of the *pmpm7* line. Although the base substitution in *pmpm7* did not involve any direct repeats, its recovery is consistent with our previous finding that NMU, a mutagen that causes point mutations, has a synergistic effect with the *plastome mutator* (Sears and Sokalski 1991). The recovery of this point mutation in the *plastome mutator* line indicates that *pm*-induced mutations are more variable than we had previously realized. At this time, the frequency of occurrence of point mutations relative to indels (insertion/deletion) is unclear, but further SSCP analysis of albino mutants should clarify the likelihood of occurrence of different types of mutations.

The involvement of the direct repeat motif in deletion/insertion mutations strongly points to replication slippage as being a major mutation mechanism in the *plastome mutator* line. In the plastome, likely examples of replication slippage have been documented in evolutionary comparisons (Treier et al. 1989, Wolfson et al. 1991, Madsen et al. 1993, and Sears et al. 1995), but in *pm/pm* plants, the frequency of replication-slippage must be greatly elevated.

Several lines of evidence have linked DNA arrangements among tandem repeats to replication errors. Frequencies of deletion formed between tandem direct repeats are increased to as much as 100 times in yeast with a temperature-sensitive DNA polymerase delta and the spectrum of mutations induced are also different than in the wild-type strain (Tran et al. 1995). The alpha-catalytic subunit of *E. coli* DNA polymerase III elevated

frameshift mutations dramatically when exonuclease was removed to eliminate proof-reading activities (Mo and Schaaper, 1996). Additionally, it has been shown that the mutation frequencies of regions containing short direct repeats depend on the orientation of replication (Rosche et al. 1995; Trinh and Sinden, 1991). In these studies, inserts containing asymmetric palindrome sequences with respect to the flanking direct repeats were designed, such that different misaligned intermediates could form on the leading and lagging strands during replication. Evidence obtained from these studies has suggested that deletions occur preferentially on the lagging strand during replication-associated events, although data from others led to a different conclusion (Westo-Hafer and Berg, 1991). The misaligned replication intermediate of the replication slippage model has been demonstrated in *E.coli* by Lovett and Feshenko (1996). A dysfunctional methyl-directed mismatch repair (MMR) pathway increases deletion frequency of 101-bp tandem repeats that differ from each other slightly (mismatches at four bases). Normally, such repeats would be removed by the MMR pathway during or soon after replication, because replication slippage would result in a misaligned-heterduplex intermediate that would be recognized by the MMR machinery.

Mismatch repair is known to repair mutations caused by replication slippage in both prokaryotic and eukaryotic systems (Radman and Wagner, 1986; Strand et al. 1993; Karren and Bignami, 1994; Kinzler and Vogelstein, 1996; Lovett and Feschenko, 1996). Conceivably, plastome mutator activity could be due to a defect in MMR. However, the MMR pathway rarely repairs mismatches longer than 8 bp (Radman and Wagner, 1986), and the repeat units of most of the *pm*-target sites are much longer. (Chiu et al. 1990; Chang et al. 1996) Thus, it is unlikely that the *plastome mutator* is deficient in mismatch

repair. Rather, it is likely that some protein involved in the replication/or recombination/ repair machinery is impaired.

A defect in cpDNA replication or repair has long been considered to be the likely cause of the high frequency of non-Mendelian mutation due to the *plastome mutator* (Epp 1973; Sears 1983; Chiu et al. 1990, Sears and Sokalski 1991). The completely recessive nature of the *pm*-allele indicates that it is probably a null allele, since a defective, but present polypeptide would probably increase the mutation level in the heterozygote. Thus, we deduce that the *pm*-homozygote probably lacks a polypeptide that participates in cpDNA metabolism. DNA helicase, which unwinds the helix, is a possible candidate for the *pm* gene product (Lahaye et al. 1991), as is the single-stranded DNA-binding protein (SSB), which stabilizes single stranded DNA during replication and repair (Van Dyck et al. 1992). Increased stalling of the replication complex due to absence of a helicase or SSB protein could increase the frequency of slippage and /or affect the proofreading function of the DNA polymerase. Absence of the 3'→ 5' exonuclease subunit of DNA polymerase would also result in many types of mutations since proofreading would be eliminated (Johnson et al. 1995).

Given the genetic traits and our deductions about possible functions of the *pm*-gene product, *in vitro* assays of cpDNA replication and other aspects of cpDNA metabolism are needed to discriminate among these possibilities.

Chapter 3

ANALYSIS OF THE *PLASTOME MUTATOR* PREFERRED TARGETS IN A SECOND TYPE OF PLASTOME

Introduction

According to Stubbe's genome categorization (1959), the Johansen strain from which the *pm* -allele was isolated contains genome type A and plastome type I. In addition to its native plastome type, the AA nuclear background is compatible to varying degrees with plastomes type II, III and IV (Kutzelnigg and Stubbe, 1974). Differences in inheritance patterns (Chiu et al. 1988) and photosynthetic rates (Glick and Sears, 1993) among various plastome types in a constant nuclear background were observed in our laboratory. It has been reported by Sears and Sokalski (1991) that albino sectors arose in *pm* -plants with plastome IV at a lower frequency than in *pm*-plants with plastome I. In order to study more carefully the impact of the *plastome mutator* on different plastome types, several previously identified *pm* -hot spots from plastome I were reexamined in our newly developed collections of *pm*-induced mutants from plastomes I and IV. The investigation of *pm* -I plants was performed by Lara Stoike, and is described elsewhere. The observation of the impact of the *plastome mutator* on plastome IV is presented and discussed below.

Materials and Methods

Plant Materials The wild type Johansen strain that carries plastome type IV was constructed by Prof. Stubbe who transferred the chloroplasts from *O. atrovirens* into the AA-Johansen nuclear background (Chiu et al. 1988). This line was maintained by self-pollination, and the source of seeds was field accession Nr. 95-4. To obtain seeds with a newly restored *pm/pm* genotype, plants with an *albicans/percurvans* genotype with plastome IV were used as the recipient for pollen from a Johansen line that was homozygous for the *pm* allele. Because the *albicans-percurvans* chromosomes form a circle of 14 in meiosis, only two types of meiotic product are produced (Cleland, 1972). Furthermore, because the *albicans* genome carries a pollen-lethal factor, and the *percurvans* genome carries an egg-lethal allele, the progeny of this cross are all of the genotype *albicans*-Johansen^{pm}, a heterozygote with plastome type IV. In the F1, a circle also forms at meiosis. Due to the pollen-lethal allele carried by the *albicans* strain and predominance of the Johansen genome during megaspore competition (Cleland, 1972), 99% of the F2 progeny are homozygous for the Johansen genome and hence the *pm* allele (accession Nr. 96-23). Nineteen *pm /pm* plants from the 96 field were identified as having mutant sectors, and were screened for DNA alterations.

Preparation of nucleic acids. It has been a problem to obtain good quality of templates for PCR amplification from *Oenothera* due to the excess amount of polysaccharide existing in the nucleic acid preparation (See Appendix B). Therefore, extraction of total nucleic acid was performed by the conventional protocol described by Fang et al. (1992), and was further purified by caylase digestion to remove the remaining

starch, as outlined by Rether et al.(1993). Nucleic acid samples were dissolved in a solution containing 50mM KoAc, 10mM EDTA, 5 g/ml RNAse and 0.5 mg/ml Caylase M3, then subjected to incubation at 37°C for overnight. After the caylase digestion, the pH of the samples was increased by adding 1M Tris-HCl (pH 8) to the final concentration of 50mM, followed by the conventional procedure of phenol/chloroform extraction and alcohol precipitation. Ethanol was replaced by ethoxyethanol to remove the digested polysaccharide more efficiently.

Other procedures. SSCP analysis, agarose gel electrophoresis, PCR amplification, programming and chemicals were described in detail in Chapter 2. Restriction digestions were performed following the manufacturer's instructions. Primers for PCR amplification are listed in Table 3.1.

Results:

No variation in the intergenic spacer region of 16S *rRNA-trnI* was observed

The intergenic spacer region of 16S*rRNA-trnI* genes was previously identified as a hypervariable region among the wild type plastomes (Sears et al. 1996). In the Sears *pm*-collection, all mutants had deletions at this site (Table 3.2; Chang et al. 1996). Due to inverted repeats in this region, strong secondary structures are capable of forming and appear to be highly susceptible to the *plastome mutator* in plastome I (Stoike, 1997): The mutability of this region in plastome IV to the *plastome mutator* was examined in the present study. The intergenic spacer region was amplified from *DNA isolated from plants*

TABLE 3.1. Primers for PCR and sequencing

CpDNA region	Name*	Sequence
psbB	psbBF	See Table 2.1
	psbBR	See Table 2.1
	psbB3F	5'-GAATTAGATCGCGC-3'
Bam3b	cpB3F	5'-GAATGGATTCAAAGAG-3'
	cpB3R	5'-AATTTGCGTCCAATAGG-3'
rpl14-rps8	cpRPL14F	5'-TTCTCGAGCCCCACT-3'
	cpRPL16R	5'-AAATGCCTATACGAATCAA-3'
	cpRPS8F	5'-TCTATTCATGTCAACATTTC-3'
orf2280	cpB12F	5'-CAACCTCTTTCAGAT-3'
	cpB12R	5'-ATTCCAGTTTGAGAG-3'
16S rRNA-trnI	Cpr16F'	5'-TCGTAACAAGGTAGCCGTAC-3'
	trnIA'	5'-CGACGCAATTATCAGGGGC-3'
N.A.	universalF	5'-GTAAAACGACGGCCAGT-3'

*The direction noted for PCR primers **F**-forward (5'), **R**-reverse (3') corresponds to gene transcription for *psbB* gene and orf2280; all others correspond to the directionality of data entry from the tobacco chloroplast genome (Shinozaki et al. 1986). The direction of the universal primer was defined by the manufacturor (Gibco BRL).

Table 3.2. Summary of pm-induced DNA alterations in *Oenothera pm*-stocks

Line	orf2280		Spacer of			
			trnG- trnR	*rpl16- rps8*	*16s rRNA-trnI*	*rpl14-rpl16*
	A	B				
D	large	large	1 x 15bp	2 x 29bp	789 bp	13bp
C1	med	med	2 x 15bp	2 x 29bp	Δ177 and Δ260 bp	13bp
C2	N.A.	small	1 x 15bp	1 x 29bp	Δ605 bp	14bp
pm7	small	small	1 x 15bp	2 x 29bp	Δ177 bp	13bp
pm7ss	small	small	1 x 15bp	2 x 29bp	Δ177 bp	13bp
pm8	small	small	1 x 15bp	2 x 29bp	Δ177 bp	14bp
pm12	small	small	1 x 15bp	2 x 29bp	Δ177 bp	13bp
pm11	med	med	1 x 15bp	2 x 29bp	Δ177 and Δ260 bp	13bp
pm32	med	med	1 x 15bp	2 x 29bp	Δ177 and Δ260 bp	14bp
pm35	med	med	1 x 15bp	2 x 29bp	Δ177 and Δ260 bp	13bp
pm38	med	med	1 x 15bp	2 x 29bp	Δ177 and Δ260 bp	13bp
pm43	med	med	1 x 15bp	2 x 29bp	Δ177 and Δ260 bp	13bp

*Alterations due to pm activity are boxed. The size of PCR amplification product of orf2280 DNA, and copy number of repeat sequences of Bam3b (trnG-trnR spacer), 29bp deletion (rpl16-rps8 spacer), and size of 13-, 14-bp oligo-A track (rpl14-rpl16 spacer) from three green lines, the Düsseldorf (D), Cornell-1 (C1), and Cornell-2 (C2) lines are compared to sizes and copy numbers from plastome mutator-induced mutants pm7, pm7ss, pm8, pm12, pm11, pm32, pm35, pm38, pm43.

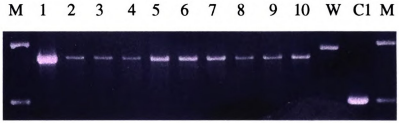
of the *pm /pm* IV-collection by primers Cpr16F'and trnIA' (Table 3.1), which flank the polymorphic region. The PCR amplification products are about 1 kb in size. Despite the many repeat units found in this area in plastome IV, no polymorphism was observed (Figure 3.1), which is in contrast to the highly variable phenotypes of the same region in plastome I.

orf2280 did not show variability in the *pm* -IV derived lines of *Oenothera*

Two variable regions in orf2280 of the *pm* -IV mutant collection were surveyed for variation. In one region (orf2280A) of other closely related *Oenothera* species, extensive stretches of 21 and 24 bp related repeats alternate with each other (Nimzyk et al. 1993). Region B is located downstream from region A. High frequencies of deletions have been observed in that region from our old *pm* -I stocks (Table 3.2), although an exact sequence composition remains to be determined (D. Jarrell, unpublished data). The PCR amplification products produced by primers OF3 and OR2 are about 600 bp in size for the wild type *Düsseldorf* line and 350 bp to 600 bp in our *pm* -I collection due to loss of several copies of repeat units. With the same pair of primers, region A of orf2280 was screened across the new *pm* -IV collection by PCR amplification. Their product sizes were estimated on a 2% agarose gel (Figure 3.2A). Region A of orf2280 in plastome IV is about 450 bp in size which is smaller than that of plastome I. However, no alterations were observed among all of the samples examined.

Region B of orf2280 contains a set of eight tandem 24-bp repeats in plastome I-D (Blasko et al. 1988). Deletion of three to five copies of the repeat units in this region had occurred in all members of the *pm*-I mutant collection (Table 3.2; Chang et al. 1996).

A)



B)

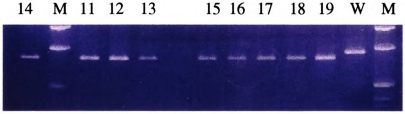
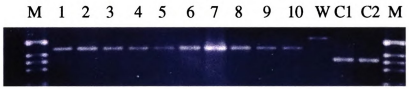


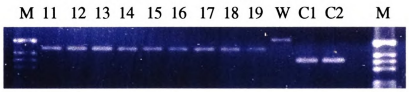
Figure 3. 1. A) and B) PCR analysis of the intergenic spacer region of *16S rRNA-trnI* on the 1.5% agarose gels. The primers cpr16F' and trnIA' amplified a segment of this hypervariable region from the following *O enothera* plastomes: A) *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10); Wild type *D üsseldorf* line (W), *Cornell-1* (C1). B) *pm*-IV mutant *95-14* (14), *95-11* (11), *95-12* (12), *95-13* (13),), *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), Wild type *Düsseldorf* line (D). The size markers (M) (From Gibco 1kb DNA ladder) in gel A are 1018 bp and 517 bp r espectively, in gel B are 1636-, 1018-, 517- and 396 bp respectively.

Figure 3. 2. A), B), C) and D) PCR analysis of orf2280 on the 1.5% agarose gels.
A) and B) The primers OF3 and OR2 amplified the variable region A of orf2280 (David Jarrell, unpublished data) from the following *Oenothera* plastomes: A) *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10); Wild type *Düsseldorf* line (W), *Cornell-1* (C1 from 93-field), *Cornell-2* (C2). B) *pm*-IV mutants: *95-11* (11), *95-12* (12), *95-13* (13),), *95-14* (14), *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), Wild type *Düsseldorf* line (D), *Cornell-1* (C1 from 93-field), *Cornell*-2 (C2). The size markers (M) (Gibco 1kb DNA ladder) are 517-, 396-, 344-, 298-bp respectively. C) and D) The primers cpB12F and cpB12R amplified the variable region B of orf2280 (Blasko et al. 1988) from the following *Oenothera* plastomes: C) *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10); Wild type *Düsseldorf* line (D), *Cornell-1* (C1 from 93-field), *Cornell-2* (C2). D) *pm*-IV mutants: *95-11* (11), *95-12* (12), *95-13* (13),), *95-14* (14), *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), Wild type *Düsseldorf* line (D), *Cornell-1* (C1 from 93-field), *Cornell-2* (C2). The size markers (M) (Gibco 1kb DNA ladder) are 517-, 396-, 344-, 298-bp respectively.

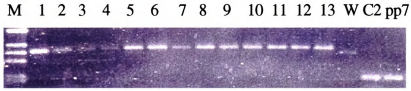
A)



B)



C)



D)

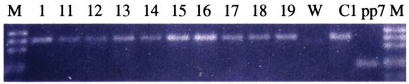


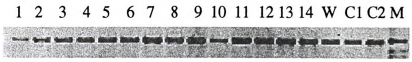
Figure 3.2

Using primers cpB12F and cpB12R listed in Table 3.1, the PCR amplification products of the wild type *Düsseldorf* line and *pm*-I plants were between 260 - 330 bp (Chang et al. 1996; Table 3.2). To assess the mutability of this region in *pm*-IV plants, PCR amplifications were performed with the same primers and products sizes were examined by agarose gel electrophoresis. Including the wild type control, all of the PCR products were about 350 bp, migrating slower than those of the wild-type *Düsseldorf* line (Figure 3.2B). This is consistent with previous observations that plastome IV contains more copies of the 24-bp repeats (Sears et al. 1995). No variability was observed across our *pm*-IV collection.

The *plastome mutator* did not induce polymorphisms in the variable *Hinf*I segment of the fourth largest *Bam*HI fragment

The fourth largest *Bam*HI fragment (*Bam* 3b) had been observed as an RFLP in comparisons of *pm* -lines (Sears 1983; Chiu et al. 1990), and had been found to have two polymorphic regions (Kaplan 1987). One of these was examined by subcloning and sequencing a variable *HinfI* fragment from *Bam* clones of the plastome I lines: *Düsseldorf*, *Cornell-1*, and *Cornell-2* lines. Subsequently, primers cpB3F and cpB3R were designed for amplification and direct sequencing of all of the members of our *pm*-I collection. One line (the original *Cornell-1* isolate) showed a 15 bp exact duplication in a non-coding region corresponding to the spacer between the *trnG* and *trnR* genes on the tobacco cpDNA map (Fig. 2.2B; Table 3.2; Chang et al. 1996). PCR amplification was performed to investigate variability of this region in the *pm*-IV mutant collection. As shown in Figure 3.3, the amplification products were about 500 bp in size. No mobility

A)



B)

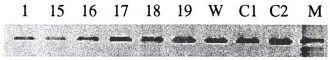


Figure 3.3. A) and B) PCR analysis of the *Hinf*I fragment of *Bam 3b* on the 1.5% agarose gels The primers cpB3F and cpB3R amplified this *Hinf*I variable segment (Chang et al. 1996) from the following *Oenothera* plastomes: A) *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10) *95-11* (11), *95-12* (12), *95-13* (13), *95-14* (14); Wild type *Düsseldorf* line (W), *Cornell-1* (C1), *Cornell-2* (C2). B) *pm*-IV mutants: *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), Wild type *Düsseldorf* line (W), *Cornell-1* (C1), *Cornell-2* (C2). The size markers (M) (From Gibco 1kb DNA ladder) are 517-, 396-, 344-bp, respectively.

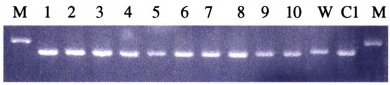
differences of PCR amplification products were found in this region in the *pm*-IV collection.

The intergenic spacer region of the *rpl16-rpl14-rps8* genes remains the same in *pm* lines

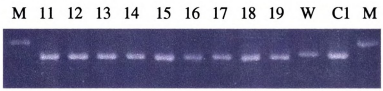
The *rpl16-rpl14-rps8* region was previously identified as a *pm* -target in *pm* -I plants (Chang et al. 1996): one of two 29-bp repeats was deleted (Fig. 2.2A; Table 3.2). Plastome types II, III, and IV contain only one copy of this 29-bp segment. Presumably, a 29-bp duplication occurred in the plastome I progenitor after its evolutionary divergence (Wolfson et al., 1991). Because the *plastome mutator* is able to induce both insertions and amplifications in *pm*-plants (Chang et al. 1996), I sought to determine if the one copy 29-bp fragment in plastome IV was amplified to two copies due to the effect of the *plastome mutator*. The whole intergenic spacer region was amplified with primers cpRPL16R and cpRPS8F (Table 3.1), and examined with agarose gel electrophoresis (Figure 3.4A). PCR amplification products from *pm*-IV plants were about 900 bp in size, migrating slightly faster than the products from the plastome-I plants due to their intrinsic difference in sequence (Wolfson et al. 1991). No other variation was observed across the *pm*-IV samples when they were compared with the wild type control. To better visualize the migration pattern of these products, samples were digested with the *BclI* restriction enzyme. The *BclI* enzyme restricted this intergenic spacer region into three fragments with approximate sizes of 450-, 300-, and 150- bp (See Figure 3.4B; Wolfson et al. 1991). The largest fragment of plastome IV is shorter than that of plastome I because it lacks the 29-bp duplication. The middle fragment from type IV samples ran slower than

Figure 3.4 PCR (A, B) and restriction digestion (C, D) analyses of the intergenic spacer region of the *rpl16-rpl14-rps8* genes on the 1.5% agarose gels The primers cpRPL16R and cpPRS8F amplified this variable intergenic region (Wolfson et al. 1991) from the following *Oenothera* plastomes: A) *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10), Wild type *Düsseldorf* line (W), *Cornell-1* (C1). B) *pm*-IV mutant: *95-11* (11), *95-12* (12), *95-13* (13), *95-14* (14), *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), Wild type *Düsseldorf* line (D), *Cornell-1* (C1). The size marker (M) (Gibco 1kb DNA ladder) is 1018-bp. In gels C and D, the products of PCR amplification from gels A and B were digested with endonuclease *Bcl*I. The sample order in gels C and D correspond to that in gels A and B respectively. The size markers (M) are 517-, 396-, 344-, 298-, 220-75- bp respectively. Fragments a, b, c are approximately 450, 300 and 150-bp in size respectively.

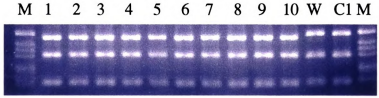
A)



B)



C)



D)

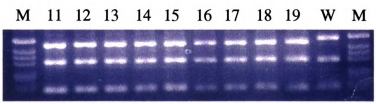


Figure 3.4

type I samples due to a 1-bp insertion in a 13-bp poly-A stretch and the expansion of an 11-bp poly-A track to 19 bp in type IV plastome. Other previously identified base substitutions in this region between the two plastome types would not have been resolved on an agarose gel.

The hypervariable region of the *psbB* gene remains intact in *pm*-IV isolates

SSCPs were found at the *psbB* gene of plastome I from an albino mutant "*93-b45*" and the leaftip culture line "*pmpm7*" (Figures 2.3; Chang et al. 1996). The polymorphic region of the *psbB* gene was then cloned and sequenced. Based on the sequence data, primer *psbB*4F was designed to be used with primer *psbB*R to amplify the surrounding 156 bp of the variable region (Table 3.1). PCR amplification was performed with these two primers to screen the *pm*-IV collection. The product sizes were assessed on an agarose gel and further examined by SSCP analysis. As presented in Figure 3.5A and 3.5B, no variation was observed among the samples.

Discussion

Several *pm* -hot spots were mapped previously by RFLP analysis (Chiu et al. 1990) and characterized by sequencing (Blasko et al. 1988; Chang et al. 1996). All of them occurred in regions surrounded by inverted or direct repeats. These *pm*-induced mutations probably resulted from misalignment of repeat units during recombination or replication. Several observations have led us to favor replication slippage as an explanation for the *pm*-mutations (See Chapter 2). Sequence analysis of one 13-bp oligo-A stretch revealed a one-bp insertion in several *pm*-I isolates, indicating that replication

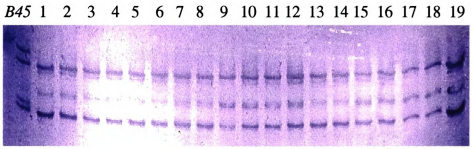


Figure 3. 5. SSCP analysis of the *psbB* gene on the MDE gel. This variable region (Chang et al. 1996) was amplified from plastomes of *pm*-IV plants with primers psbB4F and psbBR. The products of PCR amplification were denatured before sample loading. Samples order is as follow: *pm*-IV mutants: *95-1* (1), *95-2* (2), *95-3* (3), *95-4* (4), *95-5* (5), *95-6* (6), *95-7* (7), *95-8* (8), *95-9* (9), *95-10* (10) *95-11* (11), *95-12* (12), *95-13* (13), *95-14* (14), *95-15* (15), *95-16* (16), *95-17* (17), *95-18* (18), *95-19* (19), The sample *pm93-b45* (B45) in which a 4-bp insertion was recovered (Chang et al. 1996), was amplified as a size markers (156-160 bp).

slippage had probably occurred. In addition, two SSCPs were recovered in *pm*-I lines. One of them is an imprecise 4-bp duplication at a site containing stretches of 3-bp tandem repeats.

Prior to my study, all of the observations on the *pm*-induced mutations had been performed with its native plastome type I. Besides type I, the AA nuclear genotype of *pm*-isolates is also compatible with other plastomes (Stubbe, 1959). In the present study, *pm*-lines with plastome type IV were used to investigate the impact of *pm* on a non-native plastome. The data obtained by Sears and Sokalski (1991) indicated that the mutation frequencies of the *pm*-I, II and III plants are about the same, but pm-IV plants had much lower mutation frequencies. However, S. Baldwin (1995) found no statistically significant differences in the spontaneous appearance of white sectors in her comparison of *pm*-I and IV lines. Therefore, doubts had arisen about the genetic composition of the *pm* -line carrying plastome IV that was analyzed by Sears and Sokalski.

Because of the inconsistent results obtained from independent studies in our laboratory, we sought to carefully assess the impact of the *plastome mutator* of *Oenothera* on plastome types I and IV. Several RFLP sites recovered from *pm*-I plants were reexamined in our new *pm*-I and *pm*-IV collection. To be assured that all of the mutations observed are newly induced and not inherited from a previous generation, the plastome mutator genotype was newly restored as described in the materials and methods section. In the following discussion, the investigation of the *pm*-IV lines will be discussed. Parallel studies on the *plastome mutator* effect on plastome type I were performed by Lara Stoike in her thesis (1998).

Six previously identified RFLPs were examined in the *pm*-IV collection: the intergenic spacer of 16S *rRNA* and *trnI* genes, two polymorphic areas in orf2280, the *Bam* 3b fragment, the *rpl16-rpl14-rps8* intergenic spacer and the variable sequence of the *psbB* gene. None of them show polymorphisms in plastome IV. Similarly, in the examination of a new *pm* -I collection was performed by L. Stoike (Ph.D thesis, 1997), except for the intergenic spacer of 16S *rRNA-trnI*, no variation was observed. However, in the subsequent generation, variations could be discerned in both variable regions of the orf2280 region (Stoike, 1997). Whether the *plastome mutator* has the same effect on plastome IV remains unknown. Examination of the same kind of collection of second generation plants with plastome IV would answer this question.

The absence of variability in the 16S *rRNA* and *trnI* intergenic spacer in *pm*-IV plants is intriguing. Many direct and inverted repeats exist in this region in both plastome types I and IV (Sears et al. 1996), and it is a hotspot for evolutionary variation in the *Oenotheras* (Sears et al. 1996; Gordon et al. 1982). According to the analysis by the GCG program, a strong secondary structure is capable of forming at a site that suffers a high deletion rate in *pm*-I plants, (Stoike, 1998). The same region is also present in plastome IV, but no deletion was observed in my studies, contradicting the postulation that the potential secondary structure is associated with high frequencies of deletion. The discovery that no variation occur in the intergenic spacer of the 16S *rRNA* and *trnI* may implicate an important difference in the physiological function of this region in plastome IV. Alternatively, physiological differences between plastids carrying plastome IV and plastome I may render the 16S *rRNA-trnI* region differentially susceptible to the *plastome mutator*.

In prokaryotes, the deletion frequencies of the same secondary structure can differ by 1000 fold although they are just a single-bp apart in their positions on a plasmid (Das et al, 1987). A precisely controlled position effect on mutation frequencies was suggested, although the basis for this effect is still unclear (Leach, 1996). Perhaps the differing susceptibility of the spacer region between plastome I and IV is regulated similarly. Conceivably, the secondary structure may form less readily in plastome IV due to other adjacent sequences.

Data obtained by D. Jarrell (personal communication) and Chang et al. (1996), showed that in both the variable regions of orf2280, DNA alterations exist in every *pm*-isolate from our established *pm*-I mutant collection (Table 3.2). orf2280 is the largest open reading frame found in the plastome. Studies of tomato orf2280 showed that it might encode a protein expressed in the chromoplasts (Richards et al. 1991). Nevertheless, the real function of orf2280 remains elusive. Screening of the two variable region of orf2280 in the *pm*-IV collection revealed no variation, which is the same in the new *pm*-I stock. However, for the *pm*-I plants, polymorphisms start segregating out in the subsequent generation of heterozygous (*pm*/+). DNA deletions in this area occur in an "in frame" manner, which possibly would not impair the function of the orf2280 protein.

The two kinds of repeat units in orf2280, which are 21-24 bp respectively, could also serve as substrates for recombination (Blasko et al. 1988; Nimzyk et al. 1993). Therefore, both replication slippage and recombination mechanisms could be responsible for mutations in this area, and render it more susceptible to DNA alteration. It is interesting to note that the two most variable regions: "intergenic spacer of 16 *rRNA* and *trnI* genes" and "orf2280" are both located in the inverted regions of chloroplast where

presumably recombination occurs at high frequencies (Palmer, 1991). Introducing a dominant Rec$^-$ gene into plastomes of *pm*-plants would allow the contribution of recombination to be tested. The fact that in *pm*-I plants, deletions occurred at relatively high frequencies in orf 2280 and the rRNA operon, but not in most other intergenic spacers that contain repeats implies that gene expression may also be involved in mutagenesis of this open reading frame (Wierdle et al. 1996; Francino et al. 1996). For transcription, DNA must be unwound , and ssDNA can form hairpins. When confronted with secondary structures, the replication machinery of the plastome may "slip".

A 15-bp duplication exists in the *Bam* 3b region in one member of our historic *pm* -I stocks (Table 3.2; Chang et al. 1996). The amplification of this 15-bp segment from a single copy to a duplicate appears to have been mediated by replication slippage between a 5-bp repeat. The size of this repeat element would have provided inadequate homology to be involved in a recombinational misalignment. This varying segment was reexamined in both new *pm* -I and IV plants and no variation in this area was observed in the present study.

One copy of a 29-bp duplication in the intergenic spacer region of the *rpl16-rpl14-rps8* genes was eliminated in one of our old *pm* I-stocks (Table 3.2; Chang et al. 1996). This 29-bp region exists as only one copy in the wild-type plastome IV. Therefore, an amplification would have had to occur for a difference to be detected. However, no variation was observed in either of the new *pm* -collections.

An SSCP has been observed from our *pm* -I isolate as described in Chapter 2 and Chang et al. (1996). This polymorphic region of the *psbB* gene contains four copies of 3-bp direct repeats that offer a typical substrate for replication slippage in both prokaryotic

and eukaryotic systems (Radman and Wagner, 1986; Kinzler and Vogelstein, 1996). If chloroplast recombination resembles that in prokaryotes, the 12-bp would be small to mediate a misaligned recombination event. The *psbB* gene encodes the cp47, a chlorophyll binding protein of photosystem II. A severe mutation in this gene would be detrimental to the assembly of PSII (Richards et al. 1991), which would give it a selective disadvantage. In the plastome IV collection being analyzed, slippage events did not occur in the variable region of the *psbB* gene. Thus, despite its being a previously observed target of replication slippage, no variation was observed in this set of *pm* -IV plants.

The fact that no polymorphisms were observed in the newly developed generation of *pm*-isolates raises questions about the frequencies and segregation of *pm* -induced mutations. The genetic data (Epp, 1974; Sears and Sokalski, 1991) showed that white sectors start showing up soon after the *pm* -seedlings germinate, indicating mutations are induced and segregated out immediately after the *plastome mutator* genotype is regenerated. However, none of the specific spots investigated in this study were hit by it, or segregated out right away. We had hoped that examining the known mutations sites would provide information that could be used to analyze possible mechanisms involving in the *plastome mutator*. However, the investigation has shown that the different plastome types may have varying susceptibilities to the action of the plastome mutator.

Chapter 4

ESTABLISHMENT OF AN *IN VITRO* REPLICATION SYSTEM TO ASSESS DNA SYNTHESIS IN CHLOROPLASTS OF THE *OENOTHERA* PLASTOME MUTATOR

Introduction

In a few plants, chloroplast DNA replication has been studied using *in vitro* methods. In early studies, crude extracts with DNA synthesis activity were obtained from isolated intact or disrupted chloroplasts of maize (Zimmermann and Weissbach, 1982), *Marchantia polymorpha* (Tanaka et al., 1984), and *Petunia hybrida* (de Haas et al., 1987) with no further purification. In crude extracts from maize and *Marchantia polymorpha,* DNA synthesis occurred when exogenous plasmids containing chloroplast DNA inserts were added. However, no specific initiation site was detected. In the crude extracts from *Petunia* chloroplasts, the incorporation of deoxynucleotides into nucleic acids was observed, without the addition of exogenous DNA. In these systems, the assays were probably complicated by the presence of endogenous DNA and nucleases (Heinhorst and Cannon, 1993).

Recently, more refined systems of *in vitro* replication have been developed in *Chlamydomonas reinhardtii* (Wu et al., 1986), maize (Carrillo and Bogorad, 1988), pea (Meeker et al. 1988), soybean (Hedrick et al. 1993), and tobacco (Lu et al. 1996). In these systems, crude extracts were purified through chromatographic steps and fractions

containing DNA synthesis activity were collected for *in vitro* studies. Site specific initiations of replication were observed. In addition to DNA polymerase, RNA polymerase, helicase and topoisomerase were detected in these extracts (Hedrick et al. 1993; Reddy et al. 1994, and personal communication with Gordon Cannon).

As explained in Chapter Two, *plastome mutator* activity may result from defects in chloroplast DNA polymerase, topoismerase, helicase, SSB and proteins involved in an over-expressed S.O.S.-bypass pathway. Therefore the goals of the work described in this chapter were to develop an *in vitro* chloroplast replication system and to compare the DNA synthesis properties of extracts isolated from wild-type and *pm*-plants. In order to recover most of the relevant proteins in the chloroplast extracts while removing factors that inhibit or disturb the interpretation of results, isolated chloroplasts were disrupted and DNA polymerase and associated proteins were subsequently purified by DEAE cellulose chromatography.

In this study, procedures were adapted to allow me to study *in vitro* DNA synthesis using an *Oenothera* chloroplasts extract. In the following context, parallel comparisons of basic features of this system between the wild-type and *pm*-plants will be described. Plastome fragments containing previously identified *pm*-hot spots have been cloned into phagemids (Appendix C) to produce ssDNA as templates for the examination of DNA synthesis through primer extension.

Materials and methods

Plant materials. Both wild-type and the *pm/pm* plants are derived from *Oenothera hookeri* strain Johansen with plastome type I. The wild-type *Düsseldorf* line

of strain Johansen was described in Chapter Two. The *pm/pm* line was generated from self pollination of a variant of the *Düsseldorf* I line (field accession number: 96-40). Leaf tip cultures were maintained on the modified medium as described by Chiu et al. (1990). In order to obtain sufficient rapidly growing leaf materials, 7-10 day old leaftip cultures of *Oenothera* were harvested, providing a minimum of 80 g of plant material, from which chloroplasts were isolated. This stage of growth allowed leaves to be sampled before the developmental stage of expansion, in order to obtain strong replication activity in the chloroplast extracts.

Isolation of chloroplasts from *Oenothera*. (On the day that chloropalst were isolated, *Oenothera* leaf tip cultures were harvested, and homogenized for 7 seconds in 6-10 volumes of homogenization buffer containing 50mM Tris-HCl, pH 7.5, 6% sorbitol, 6 mM sodium ascorbic acid, 0.15% (w/v) polyvinyl pyrrolidone (pvp), 0.1% BSA, 3mM cysteine and 5 mM 2-mercaptoethanol. The debris was removed from the homogenate by pouring it through multiple layers of cheese cloth and 2 layers of Miracloth. Intact cells, starch, and nuclei were separated from the chloroplasts by differential centrifugation. Plastids were pelleted at 6000xg for 4 min, and subsequently washed with a solution containing: 50mM Tris-HCl, pH 7.5, 6% sorbitol, 6mM sodium ascorbic acid, 0.1% BSA, 3mM cysteine and 5mM 2-mercaptoethanol. The final chloroplast pellets were stored at -80°C for subsequent use.

Preparation of chloroplasts extracts. This protocol is adapted and modified from the Cannon-Heinhorst Laboratory (Hedrick et al. 1993). Purified

chloroplasts from *Oenothera* were resuspended in a minimal volume of buffer A containing 50mM Tris-HCl, pH 8.0, 100mM $(NH_4)_2SO_4$, 25% glycerol, 10 mM 2-mercaptoethanol, 1mM EDTA, 0.3 mM p-sulfonylfluoride (PTSF), 0.3 mM phenyl methyl sulfonyl fluoride (PMSF), and 1 mM ε-aminocaproic acid. Triton X-100 was added to a final concentration of 2.0 % and the solution was incubated and stirred on ice for 1 hour. The crude extracts were centrifuged at 9000 rpm in a DuPont Sorvall SS34 rotor for 30 min. The clear supernatant was loaded at a speed of 0.5ml/min onto a DE52 (Whatman Ltd. England) column pretreated with HCl and NaOH, equilibrated with buffer A in 0.5M $(NH_4)_2SO_4$. Before loading the sample, ten volumes of buffer A with 100 mM $(NH_4)_2SO_4$ were loaded at 3ml/min to wash and equilibrated the column. After the sample loading was finished, the column was washed with three volumes of buffer A with 100mM $(NH_4)_2SO_4$ at a rate of 2ml/min. The replication extracts were then eluted at 2ml/min by buffer A with 500 mM $(NH_4)_2SO_4$. Fractions showing DNA polymerase activity were collected together and dialyzed against 100 mM $(NH_4)_2SO_4$ buffer A with 0.1 % Triton X-100 and 0.1 mM EDTA. After dialysis, the replication extracts were concentrated to one tenth volume with centricon 10 concentration tubes (Amicon, Beverly, MA), and stored at -80°C for subsequent experiments.

DNA polymerase assays. The final volume of a standard DNA polymerase assay was 50 µl. It contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 125 mM KCl, 65 µg/ml activated DNA (DNAse treated calf thymus DNA; purchased from Sigma Biosciences, St. Louis), 33uM of each deoxynucleotide with ^{3}H-dTTP at 300-1000

cpm/pmole, 1 mM DTT and 140 μg/ml BSA (USB, Cleveland, Ohio). Aphidicolin (from Sigma Biosciences) was dissolved in DMSO at 20ug/ml. The final concentration of aphidicolin in a standard replication assay was 0.4 μg/ml. NTPs and dNTPs were purchased from Gibco BRL (Gaithersburg, MD). and ^{3}H-dTTP was obtained from Amersham (Arlington Heights, IL). The reaction began with the addition of 5 μl of the extract, followed by incubation at 37°C for 30 min. The quantity of radioactive incorporation into acid-insoluble materials was measured by transferring the entire reaction to a DEAE cellulose papers (Whatman, DE11) and washing once with 0.5 M Na_2HPO_4 for 10 min, H_2O/1 min and 95% ethanol/1min respectively. Liquid scintillation counting was performed to measure the precipitated radioactivity after the filters were dry.

Quantification of protein concentrations. Protein concentrations were quantified using the BioRad Protein Assay kit following the manufacturer's instructions.

Cloning of cpDNA substrates into phagemids and preparation of ssDNA.

Two clones containing the hypervariable region between the *16S rRNA* and *trnI* genes were obtained using the following procedures. The intergenic region was PCR amplified with primer cpr16F (5'-TCGTAACAAGGTAGCCGTAC-3') and trnIA' (5'-CGACGCAATTATCAGGGGC-3'). The amplification products were separated from the primers by agarose gel electrophoresis. The fragments of interest were then extracted from the gel with Qiaex (Qiagen Inc., Chatsworth, Calif.), followed by cloning with

conventional methods (Sambrook et al. 1989) into the *SmaI* site of pBluescript SK (Short et al. 1988). The pBluescript SK vectors are derived from pUC19, and contain a replication origin of f1 filamentous phage. Depending on the orientation of the f1 origin, pBluescript SKs are designated as (+) or (-). With the infection of helper phage, the (+) strain allows the recovery of the DNA sense strand of the *lacZ* gene while the (-) strain produces the antisense strand. *E.coli* strain XL1-Blue (Bullock et al. 1987) was used as the host for the constructions.

R408, an f1 helper phage (Russel et al. 1986) was used to infect transformed *E.coli* strain XL1-blue for propagation of ssDNA. The preparation of single strand phagemid DNA was performed as outlined in Sambrook et al. (1989). An overnight culture of the bacterial strain containing recombinant phagemid was infected with R408 at a final concentration of 2×10^{7} pfu/ml, incubated for 18 hours at 37°C with strong agitation (300 rpm/min). The overnight culture was transferred to a centrifuge tube and centrifuged at 12,000g for 5 min at 4°C. The supernatant was transferred to a fresh tube and precipitated in 4% (w/v) PEG 6000 and 3% NaCl for one hour, followed by resuspension in 10 mM Tris-HCl (pH 8). Three phenol/chloroform extractions were performed to remove proteins from the preparation. The upper, clean aqueous phase was transferred to a clean tube and precipitated with 1/10 volume of sodium acetate and two volumes of absolute ethanol at -20°C for one hour, followed by centrifugation at 12,000g for 20 min at 4°C for 10 min. The precipitate was washed with 70% ethanol and resuspended in TE buffer for subsequent use.

Analysis of products of DNA synthesis by alkaline gel electrophoresis.

Reaction mixtures (100 µl) contained 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 125mM KCl, 30uM dNTPs, 1mM DTT, 7.0 µg BSA, 0.5 µg single stranded recombinant phagemid DNA primed with 4 p mole SK primer labeled with γ-^{32}P (Andotek, Ca, 3000 Ci /mmole). The SK primer (5'-CGCTCTAGAACTAGTGGATC-3') complementary to the antisense strand of the lacZ gene on pBluescript SK (-) phagemid was synthesized at NBI Inc. (Plymouth, MN). The reaction mixtures were incubated at 30°C for 15 and 30 min. The reactions were terminated by the addition of EDTA and SDS to final concentrations of 10 mM and 0.1 % respectively, extracted twice with phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in 30mM NaOH and 1 mM EDTA for electrophoresis on a 1.5 % alkaline agarose gel (25cm x 15cm x 0.5cm) containing 30mM NaOH and 1mM EDTA at 40 V for overnight. After electrophoresis, the gel was washed in distilled water, dried under vacuum, exposed at -80°C to Kodak X-Omat AR X-ray film, or at room temperature to a storage phospho screen (Molecular Dynamics). Quantification of signals on the gel was performed by scanning the autoradiographs using the Image Quant Program (Molecular Dynamics), with data manipulation through Microsoft Excel. Background was measured in an adjacent lane, and was subtracted automatically.

Results

Properties of the replication extracts from *Oenothera* chloroplasts

Elution profile: Following the procedures described in the materials and methods section, chloroplasts of *Oenothera* were isolated by differential centrifugation and disrupted with triton. The crude extracts of chloroplasts were loaded onto a DEAE cellulose column, DNA binding proteins were step-eluted with the high salt buffer containing 0.5M $(NH_4)_2SO_4$. Fractions containing DNA polymerase were selected based on their ability to incorporate ^{3}H-dTTP into acid insoluble materials when DNase treated calf thymus DNA was supplied as substrate.

The elution profile of fractions displaying DNA polymerase activity from the *Oenothera* chloroplasts of wild-type and *pm/pm* plants is shown in Figure 4.1. With the step elution procedure, chloroplast DNA polymerase was recovered as a single peak from both the wild-type and *pm*-plants. The maximum level of ^{3}H-dTTP incorporation is significantly higher (5-10 times) than the background level (Figure 4.1 fraction 4 vs. fraction 1) even before the concentration step. The heights of peaks varied in different preparations, possibly as a result of slightly differing tissue culture conditions or minor variations in the purification process for each preparation. However, the peak activity showed up in approximately the same fractions in every preparation, regardless of the batch or the lines of plant materials used. As shown in the representative data in Table 4.1, the DEAE cellulose column afforded a five to seven fold purification of the DNA synthesis activity. The enhanced specific activity of DNA polymerase after chromatographic purification steps (Table 4.1 and Figure 4.1) may be due to higher concentration of proteins involved in replication activity relative to other proteins, or a

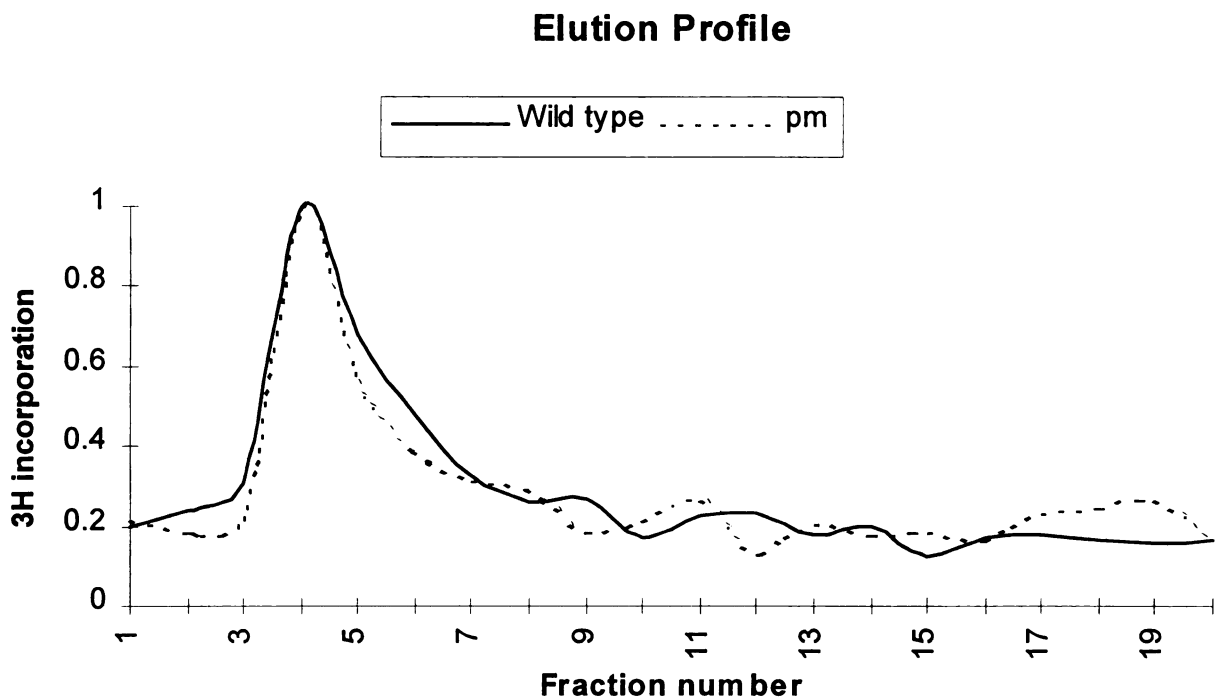


Fig. 4.1 Elution profile of DNA synthesis activity. Ractions containing nucleotide incorporation activity were collected. DNA polymerase assays were carried out as described in materials and methods section

Table 4.1 Purification of the chloroplast DNA polymerase

	Protein (ug)	Activity (*units)	specific activity units/ug	purification
A. Wild type				
Crude extract	202.8	42.5	0.2	1
DEAE cellulose	10.4	15	1.4	7
B *pm*				
Crude extract	169.6	72.8	0.4	1
DEAE cellulose	14.8	33.8	2.2	5

Due to differing culture conditions and the amount of plant materials, DEAE resins used, the extent of purification of extracts for each batch is not exactly the same as each other.
*A unit of activity is defined as 1 nmol of deoxynucleotide incorporated into acid-insoluble material per hour.

reduced amount of replication inhibitors present in the crude extracts, e.g., nuclease and endogenous nucleic acids (Heinhorst and Cannon, 1993). The extent of purification in each preparation differs due to slight variations in experimental conditions. No important differences were observed between wild-type and *pm*-plants in terms of elution profile and the extent of purification of replication-active proteins.

Purity : The purity of the replication extract of *Oenothera* chloroplasts was tested using aphidicolin, which is known to be a strong inhibitor of the nuclear DNA polymerase α (Sala et al. 1980; Reviewed by Heinhorst et al. 1989) and other nuclear polymerases to varying extents, while not inhibiting the γ type DNA polymerases of organelles. Therefore, it is commonly used to test for nuclear contamination in organelle replication extracts. The diagnostic concentration of aphidicolin used in this study (20 μg/ml) does not allow synthesis of either leading or lagging strands by the nuclear enzyme. As shown in Table 4.2, after the addition of aphidicolin, the incorporation rate of dNTP remains at 90-95 % of the original value. Thus, DNA synthesis activity in our system is predominantly resistant to aphidicolin. This line of evidence indicates that the DNA polymerase from chloroplasts is a γ type and is responsible for almost all of the DNA synthesis activity observed.

Other features: As summarized in Table 4.2, this system is completely dependent on $MgCl_2$ and exogenous DNA. The addition of KCl has a minor effect on the replication activity on the wild type plants while the *pm*-plants are more affected by the

Table 4.2 Requirement for *in vitro* DNA synthesis

Reaction conditions	DNA synthesis activity, % Wild type	*pm*-plants
Complete	100	100
Plus aphidicolin	95	90
Minus replication extracts	2	2
Minus calf thymus DNA, activated	4	3
Minus Mg++	2	3
Minus KCl	73	37
Minus dNTPs	9	9
Plus rNTPs	86	87
Plus ATP	76	83
-40 primer	<1	N.A.
M13 template	16	N.A.
-40 primer+ M13 template	117	N.A.

concentration of KCl. The replication extract is dependent on deoxynucleotides, but does not need ribonucleotides or ATP alone for the incorporation of dTTP into nucleic acid. In fact, the ribonucleotides seem to slightly inhibit deoxynucleotide incorporation. These basic features are very similar to systems characterized from other organisms, including *Chlamydomonas* (Wu et al. 1986), soybean (Heinhorst et al. 1989), and maize (Carrillo et al. 1988). There are no differences observed between the wild-type and *pm*-plants for these basic features, except for the KCl requirement.

Kinetics of [^{3}H]-TTP incorporation: The incorporation activity of *in vitro* DNA synthesis was quantified during a time course. Because of the slight differences in each preparation, the incorporation activity is presented as a percentage of the highest activity in each set of experiments. The comparison of the incorporation activity between the wild type and *pm*-plants is shown in Figure 4.2. No significant differences exist between the two types of samples in their overall incorporation rate during the entire two hour incubation. At the end of the time course experiments, both of them were still at the peak of the incorporation activity, which is consistent with observations from other systems (Zimmermann and Weissbach, 1982; Tanaka et al. 1984; Wu et al. 1986; de Hass et al. 1987) where the incorporation activities last longer than one hour.

Substrate concentration: Initially, the concentration of activated DNA used was 65 μg/ml which is the same amount used in the Cannon-Heinhorst Laboratory for the DNA replication extract assay. However, later observations indicated that reduction in the amount of activated DNA led to higher activity. As shown in Figure 4.3, the

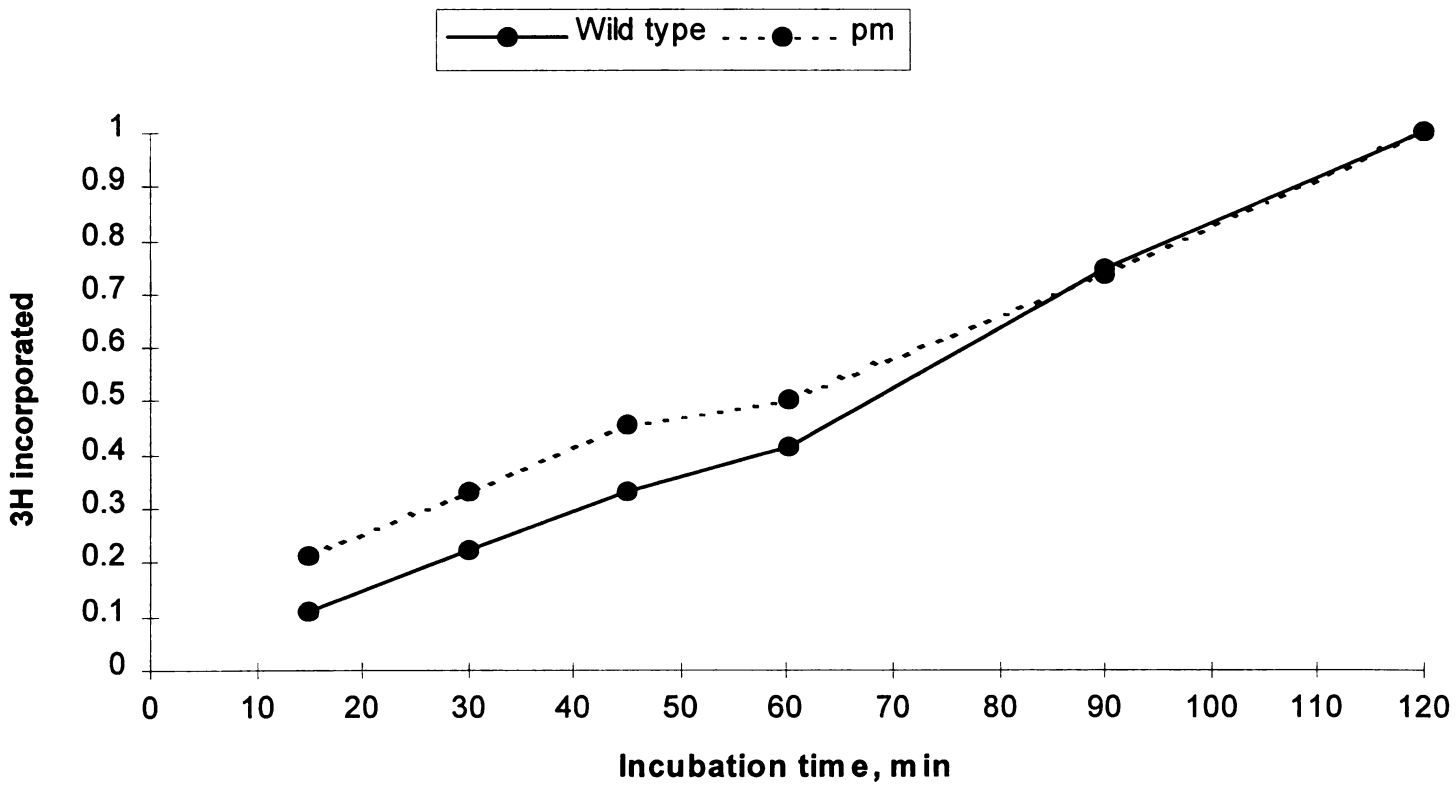


Fig. 4.2 Duration of DNA synthesis. Time course of nucleotides incorporation. Assays were carried out as outlined in materials and methods section.

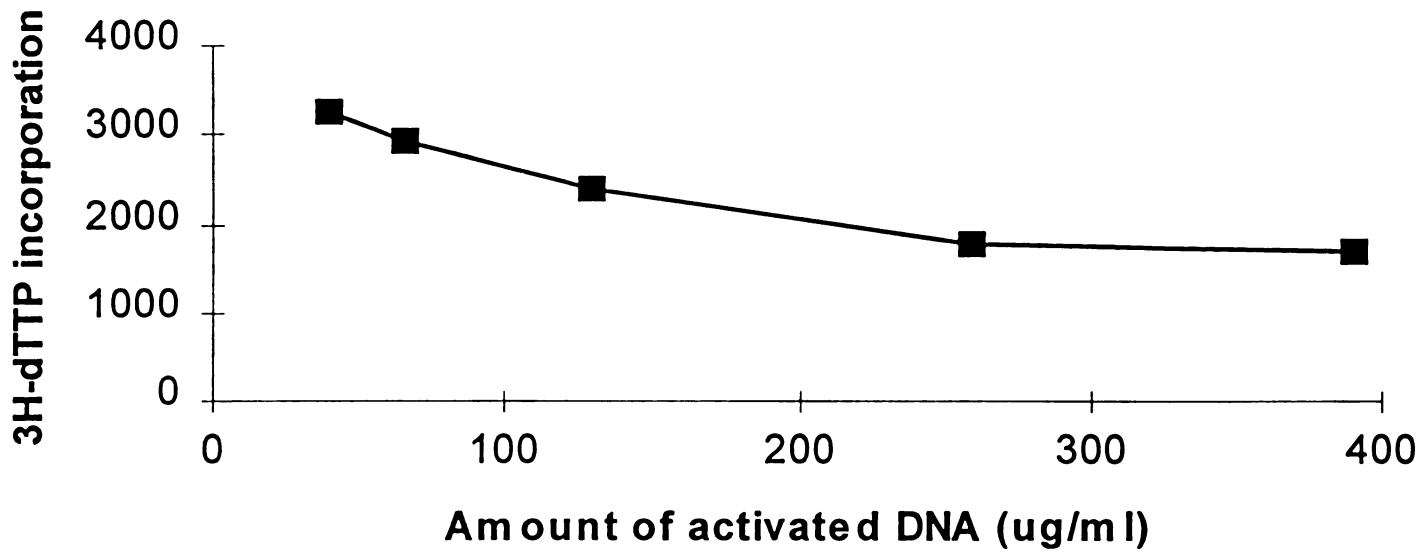


Fig. 4.3 Activated DNA requirement. Effect of substrate concentration on DNA synthesis was measured as outlined in materials and methods section.

incorporation of dTTP continued to climb when the amount of activated DNA was decreased to 39 μg/ml. The amount of activated DNA required to reach the peak activity was not followed further because of the slow increment in incorporation activity with decreasing DNA content. Final concentration of activated DNA of 39 μg/ml or less was used for later experiments.

$MgCl_2$ optimum: As described in the previous section, $MgCl_2$ is essential for *in vitro* DNA synthesis by the chloroplast replication extracts. The influence of Mg^{++} concentration on [^{3}H]-dTTP incorporation in replication extracts from both the wild type and *pm*-plants has been investigated in this study. As shown in Fig. 4.4, an optimum concentration of Mg^{++} at 0.5 mM was observed for extracts from both the wild type and *pm*-plants. As explained before, due to the slight differences of the replication extracts from each preparation, the incorporation of [^{3}H]-dTTP is expressed as percentage of the peak activity.

Variations in primer extension were observed with the replication extracts from wild-type and *pm*-plants

To observe the products of primer extension using the *Oenothera* chloroplast replication extracts from wild-type and *pm*-plants, I used an end-labeled primed-ssDNA that would enable me to observe both quantitative and qualitative aspects of DNA synthesis. Although the *in vitro* replication system is purified through one DEAE cellulose column to remove endogenous nucleic acids and it is dependent on exogenous substrates for replication, extensive contaminating DNA and RNA appear to be present

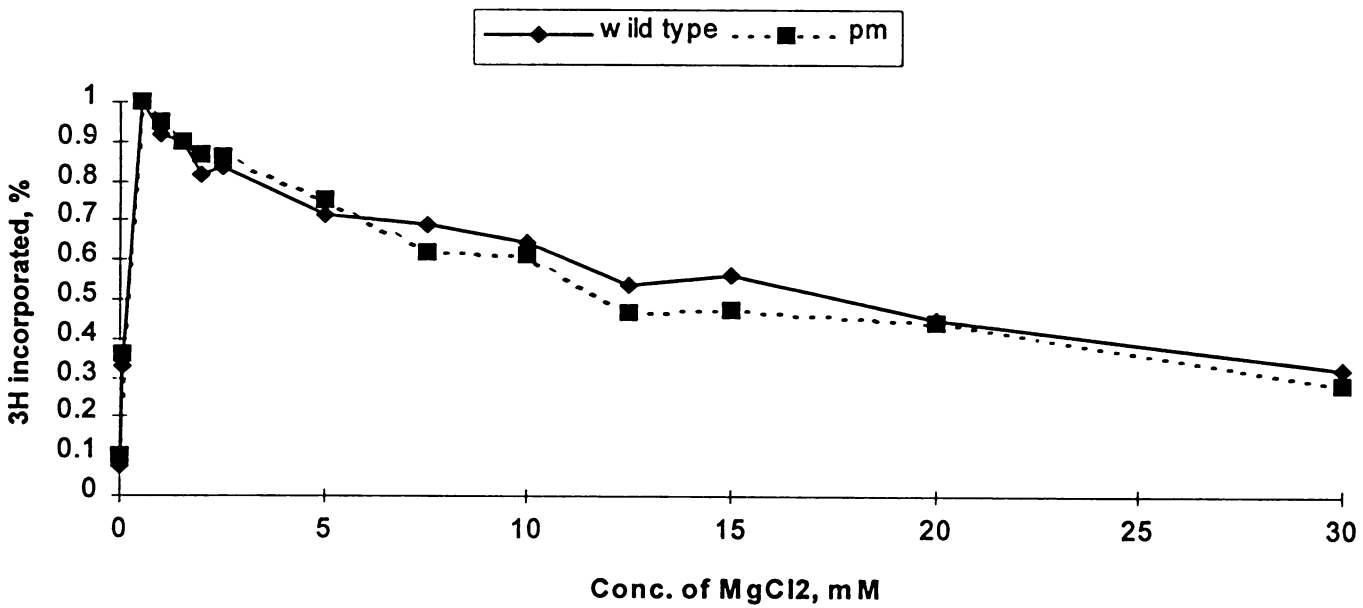


Fig. 4.4 Dependence of DNA synthesis on $MgCl_2$. Impact of $MgCl_2$ concentration on nucleotide incoporation was measured as outlined in materials and methods section.

based on ethidium bromide fluorescence in agarose gel electrophoresis (data not shown). Thus, to avoid competition with the contaminating nucleic acids, an end-labeled primer was chosen which would be complementary to the vector but not to the chloroplast genome. The *ori*B segment was chosen for this assay because it is a deletion hot spot of the *plastome mutator* and contains numerous secondary structures was chosen for this assay. It was cloned into phagemid to produce ssDNA as described in the materials and methods section and appendix C1. DNA polymerase has been reported to be very sensitive to secondary structures. It will stall at secondary structures that have formed in the template when no SSB is available to provide assistance (Williams and Kaguni, 1995). Thus, if SSB is limiting or non functional in the extracts, we expect to see DNA synthesis pausing at those secondary structures.

Fig. 4.5 shows the products synthesized by the primed-chloroplast extracts after 15 and 30 min incubation. Three main pausing sites (bands 1, 2 and 3) are apparent: (approximately 530-, 300-, 150-bp). For each extract, signals obtained from both time points (15 and 30 min) have approximately the same pattern of distribution.

For the wild type, the most intense signal is in a band of about 530 bp in size (Fig. 4.5, bands 1 and 2), indicating that the strongest pause site is at this position. The relative intensity of the bands was then quantified using a Phospho Imager, and these values are depicted in Fig. 4.6. Less radioactivity accumulates in the smaller bands, which also represent pause sites due to secondary structure.

For the extract from the *pm*-line, the results are also consistent between the two time points. Although discrete bands are observed for DNA segments of similar size between wild type and *pm*-extracts, the 530 bp band is a less abundant product in the *pm*-

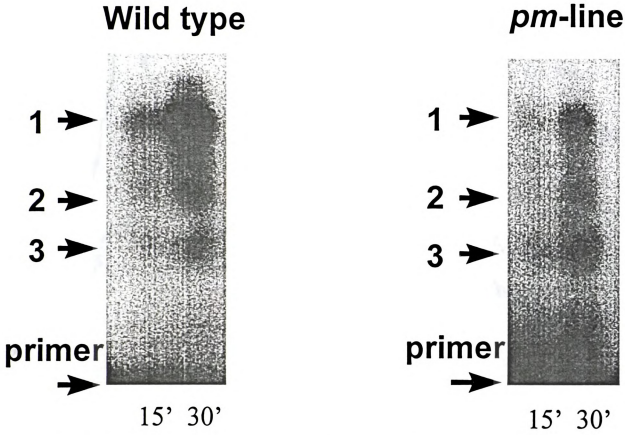


Figure 4.5 DNA synthesis on a primed-single-stranded template. The wild-type and *pm*-extracts were added to the reaction mixes and incubated for 15 or 30 min. Products were denatured and resolved on an alkaline agarose gel, as described in the materials and methods.

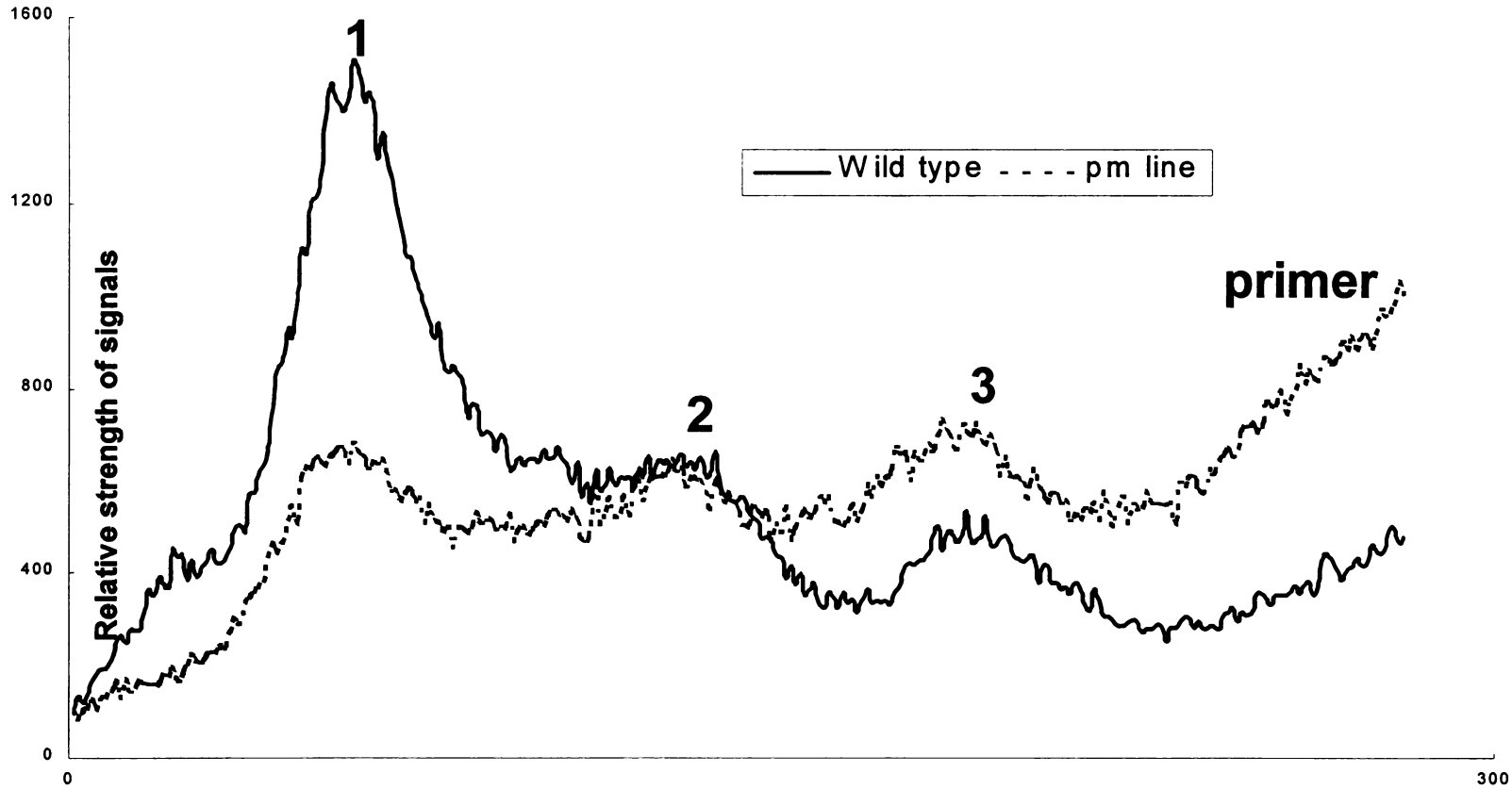


Fig. 4.6 Quantification of DNA synthesis through secondary structures. Signals obtained from primer extension at 30 min were integrated. Four peaks corresponding to pause sites 1, 2, 3 and primers shown in Fig. 4.5 are indicated.

sample (band1; Fig. 4.5). More radioactivity is distributed among smaller products. The quantification is also depicted in Fig. 4.6.

Discussion

Previous studies have indicated that the *plastome mutator* of *Oenothera* causes elevated mutation frequencies in the chloroplast genome (Epp 73; Sears and Solkalski, 1991). Sequence characterization of mutations recovered from *pm*-plants have led us to the hypothesis that replication slippage is the consequence of the defective product in the *plastome mutator* phenotype. As discussed in Chapter Two, possible candidates for the defective product in the *plastome mutator* include DNA polymerase, SSB, helicase, topoisomerase as well as proteins involved in an over-expressed S.O.S. system or the mismatch repair mechanism. In the present report, the *in vitro* replication systems from *Oenothera* chloroplasts of both wild-type and *pm*-plants were compared and characterized, in search of differences in the replication machinery of chloroplasts in *pm*-plants.

In vitro DNA synthesis is not inhibited by aphidicolin at 20 μg/ml. Since aphidicolin is a strong inhibitor of nuclear DNA polymerase α from both plants and animals (Sala et al. 1980; Misumi and Weissbach, 1982), the DNA synthesis we observed should be due to organelle DNA polymerase γ. In addition to chloroplasts, the other source for γ type DNA polymerase is mitochondria. However, contamination by the mitochondrial enzyme is unlikely, due to the differential centrifugation used to initially isolate the chloroplasts.

The incorporation of [^{3}H]-dTTP into acid insoluble materials with our system is highly dependent on the addition of exogenous DNA, $MgCl_2$, and dNTPs, which are similar to other systems. rNTPs and ATP had slight inhibitory effects on the *in vitro* DNA synthesis, consistent with the data from soybean (Hedrick et al. 1993) and maize (Zimmermann and Weissbach, 1982). In some other systems, both rNTP and ATP have very little stimulating effect on DNA replication (Tanaka et al. 1984; Wu et al. 1986; de Haas et al. 1987; Carrillo and Bogorad, 1988). These observation indicate that RNA primers are not necessary to stimulate DNA synthesis when activated DNA is provided as the template.

Despite the finding a few minor variations of the chloroplast replication extracts from *Oenothera* and other plants, no major differences were observed between the wild-type and *pm*-plants regarding the aforementioned features of the replication extracts. One exception is the KCl requirement. As indicated in Table 4.2, the presence of KCl in the reaction mixtures is more critical for the extract from the *pm*-plants than for the wild type extract. In chloroplast replication extracts from pea and spinach (Sala et al. 1980; McKown and Tewari, 1984), KCl is necessary for high levels of DNA synthesis. In extracts from liverwort (Tanka et al. 1984), soybean (Hedrick et al. 1993), and the wild type *Oenothera*, KCl is relatively unimportant. Observations from other *in vitro* replication systems maybe helpful in interpreting these results. Williams and Kaguni (1995) found that the addition of *E.coli* SSB lowers the KCl optimum of Drosophila pol γ. Both KCl and SSB contribute to the destabilization of secondary structures, thus improving the processivity of pol γ. If similar interactions occur for chloroplast

replication proteins and DNA, the lower nucleotide incorporation efficiency of *pm*-extracts in the absence of KCl may indicate that a defect in SSB exists in *pm*-chloroplasts. Although the specific components in the *Oenothera* chloroplast extracts were not analyzed in this work, data obtained from similar chloroplast systems, e.g., pea (Reddy et al., 1994) and soybean (Hedrick et al. 1993; Gordon Cannon and Sabine Heinhorst, personal communication) have indicated that in addition to DNA polymerase γ, primase, topoisomerase, helicase, and RNA polymerase and many DNA binding proteins are also present in the replication extracts after chromatography. Therefore, it is reasonable to suspect that a protein with SSB traits may exist in the replication extracts, a deficiency in the *pm*-extracts may make the KCl requirements for DNA synthesis different from the wild type.

The distribution of radioactive DNA products (Fig. 4.5) was quantified to analyze DNA synthesis. Most of the products of wild-type extracts results from pausing at the top band while the products of primer extension from *pm*-extracts were more broadly distributed. Thus, DNA synthesis in the wild type-system appears to be more processive than that with *pm*-system. This tendency was even more clear when the data were integrated and quantified. As shown in Fig. 4.6, most of the signals from the wild-type accumulate at the top band (peaks 1) while in the parallel experiments with *pm*-material, signals were evenly distributed along the entire lane. IN repeating this experiments, I tested equal amount of *pm* and wild-type specific activities, and still observed the disparity in the banding patterns. When the amount of replication extract were doubled, no change was observed in the bands, indicating that the substrates are not limiting, and

that the larger bands are not a consequence of reinitiation of synthesis. These data indicate that the replication extracts from the *pm*-line have difficulty extending the primers. This is observed despite the fact that the *pm*-extracts added to the reaction mixtures were twice as active in deoxynucleotide incorporation in the initial assay with calf thymus DNA than were the wild-type extracts. The two differences in the products of DNA synthesis indicate that either DNA polymerase or SSB may be involved in mutation activities of the plastome mutator since those two components of the replication machinery are sufficient for replication of ssDNA phage.

Several lines of evidence in bacteria have linked DNA rearrangements among tandem repeats to the DNA polymerase. Frequencies of deletion formed between tandem direct repeats are increased to as much as 100 times in yeast having a temperature-sensitive DNA polymerase delta and the spectrum of mutations induced is also different than in the wild-type strain (Tran et al. 1995). The alpha-catalytic subunit of *E. coli* DNA polymerase III elevates frameshift mutations dramatically when the exonuclease subunit is absent and therefore eliminate proof-reading activities (Mo and Schaaper, 1996).

The *in vitro* chloroplast replication system developed here could be utilized further to investigate the roles of DNA polymerase and SSB in the mutagenesis process of the *plastome mutator*. Data obtained in the present study implicated the chloroplast DNA polymerase or SSB as potential candidates for the *pm* defect. However, the involvement of other accessory proteins in the replication complex could not be completely excluded since the replication extracts were only partially purified. A closer

scrutiny of these individual protein through experiments designed specifically for each of them is recommended.

Chapter 5

AN *IN VITRO* REPLICATION SYSTEM OF *OENOTHERA* CHLOROPLASTS DOES NOT SUPPORT INITIATION OF REPLICATION AT THE PREVIOUSLY IDENTIFIED "*ORI*" REGIONS

Introduction

Electron microscopy has been used to observe replicative intermediates of the plastome from pea (Kolodner and Tewari, 1975), *Chlamydomonas* (Waddle et al. 1984), *Oenothera* (Chiu and Sears, 1992) and tobacco (Takeda et al. 1992). Based on their observations of DNA spreads from pea and maize, Kolodner and Tewari (1975) proposed that the replication of the plastome initiates from two displacement (D) loops located about 7kb apart on complementary strands. As replication proceeds, the two D-loops move toward each other in a unidirectional manner. After the two D-loops pass each other, bidirectional semicontinuous DNA synthesis begins. DNA replication terminates when the two Cairns forks meet at a site approximately 180° from the starting point. Based on their observation of lariat structures, Kolodner and Tewari proposed that rolling circle replication takes place subsequently to further amplify the plastome. Recently, both the Cairns type- and rolling circle- replications have been observed with an *in vitro* replication system from tobacco. However, no other electron microscopic reports of DNA spreads have indicated the presence of lariats. Two pairs of putative replication origins

(*ori*A and *ori*B, Figure 5.1) were mapped in plastomes I and IV of *Oenothera* by electron microscopy (Chiu and Sears, 1992). Each pair of origins is located in the inverted repeat region flanking the *16S rRNA* gene. The relatively imprecise localization has not yet been refined, since no *in vitro* replication system has been developed for *Oenothera* chloroplasts.

As an alternative to electron microscopy, partially purified chloroplast replication extracts from pea (McKnown and Tewari, 1984), *Chlamydomonas reinhardtii* (Wu et al. 1986; Waddell et al. 1984), maize (Gold et al. 1987), soybean (Hedrick et al. 1993) and tobacco (Takeda et al. 1992; Lu et al. 1996) have been used to identify chloroplast DNA replication origins through observation of site specific initiation. Presumably, these replication extracts contain proteins essential for site specific initiation of cpDNA replication, including DNA polymerase and other accessory proteins (Hedrick et al. 1993; Reddy et al. 1994). In my experiments, I sought to use the newly developed *Oenothera* chloroplast *in vitro* replication system to examine several sites that had been identified as putative replication origins of the plastome. (Figure 5.1). Two of the putative origins tested (Figure 5.1A) are located in or near the rRNA operon in *Oenothera* (Chiu and Sears, 1992), pea (Meeker et al. 1988 and Reddy et al. 1994), and tobacco (1996). The other potential origin (Figure 5.1B) maps to the vicinity of the *rpl16* gene region in *Chlamydomonas reinhardtii* (Wu et al. 1986), soybean (Hedrick et al. 1993) and maize (Gold et al. 1987), but not in *Oenothera.*

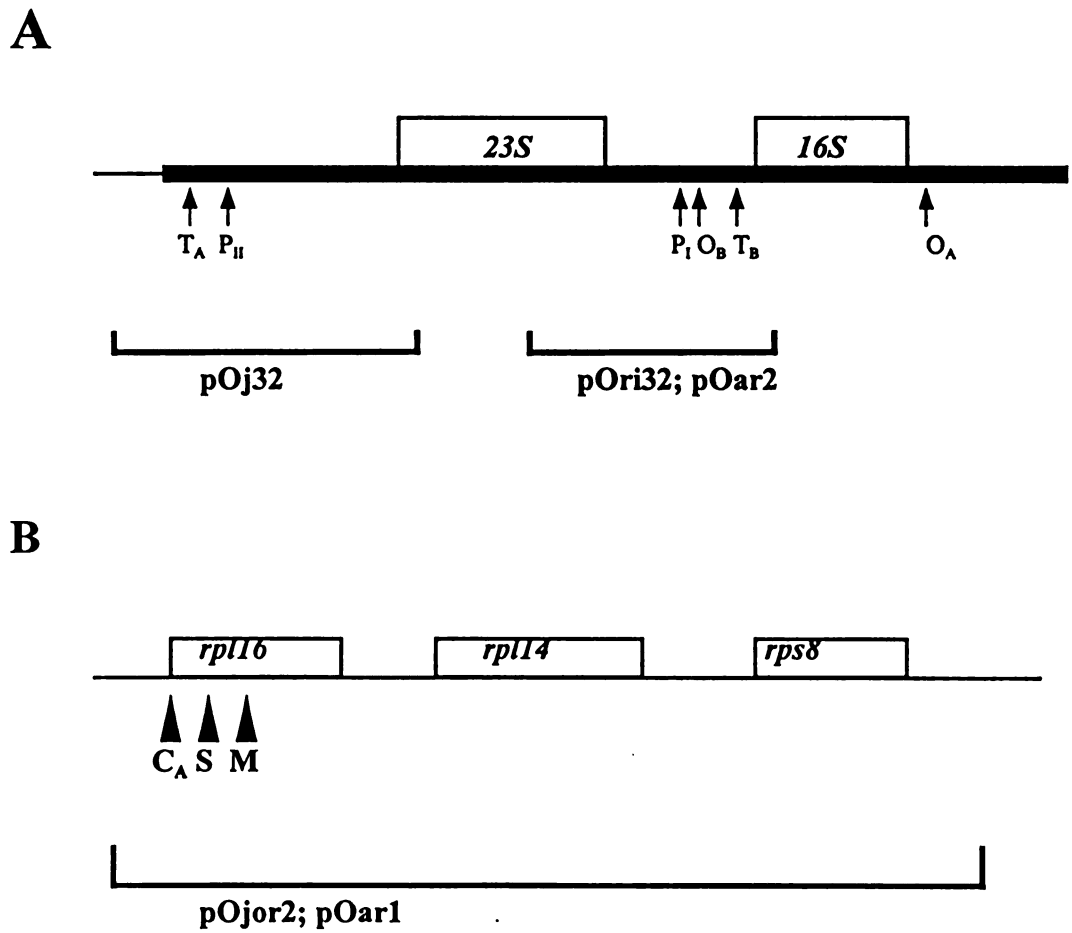


Figure 5.1 **A)** Putative origins of plastome replication mapped in the vicinity of the 23S rRNA and 16S rRNA genes in pea (P_I, P_{II}; Meeker et al. 1988), *Oenothera* (O_A, O_B; Chiu and Sears, 1992), and tobacco (T_A and T_B Liu et al. 1993). Bold black line represents the inverted repeats of plastome in tobacco and *Oenothera* . Thin line extended to the left end represents the small single copy region for tobacco and *Oenothera* . Despite the fact that the inverted repeat does not exist in pea, the relative locations of origins are very similar among the three species in question. T_n and P_n represent of plastome replication from tobacco and pea, respectively. The location of the 5 kb insert containing *ori*B in clones pOri32, pOar2 and a 5.5 kb insert countaining partial 23S rRNA gene in clone pOj32, respectively, are indicated below the *ori* map. **B.)** Approximate locations of putative replication origins relative to *rpl16* genes for *Chlamydomonas* (Wu et al. 1988), soybean (Hedrick et al. 1993) and maize (Carrillo and Bogorad. 1988) are indicated by arrowhead. The region covered by the insert of plasmid pOjor2, pOar1 is shown below with a bold line.

Materials and methods

Plant materials Both wild-type and the *pm/pm* plants are *Oenothera hookeri* strain Johansen with plastome type I. The wild-type *Düsseldorf* line of strain Johansen is described in Chapter Two. The *pm/pm* line is generated from self pollination of a variant of the *Düsseldorf* line of plastome I (field accession number: 96-40). Leaf tip cultures were maintained on a medium described by Chiu et al (1990).

Plasmid construction and preparation Plasmids used in this study are listed in Table 5.1. The 5 kb *EcoRI* fragment of the *16S rRNA-trnI* spacer from the *Düsseldorf* line was constructed in pBR328 (Sears et al. 1996) and transferred into pBluscript⁻, where it was renamed as pOri32 in the present study. This region contains a putative origin of replication (*ori* B) identified by electron microscopy (Chiu and Sears, 1990). Plasmid pOar2 contains the same region from plastome IV in pBR328 (Sears et al. 1996). Plasmid pOj32 was constructed in pBR322. It covers the 5' end of the *23S rRNA* region where one of the replication origins from pea and tobacco were observed (unpublished data, Figure 5.1A). Plasmid pOj108 is constructed in pBR322. It contains the fifth largest BamHI fragment from *the O. johansen* plastome which is similar in size to the insert in pOj32 (unpublished). The 2.5 Kb *EcoRI* fragment of the *rpl16-rpl14-rps8* region from *Cornell*-1 and plastome IV were obtained as clones in pUC18 as strains pOjor2, pOar1 respectively (Wolfson et al. 1991). The region containing the *rpl16* gene has been mapped as one of the D-loops (*ori* A) in the plastome of *Chlamydomonas reinhardtii* (Waddle et al. 1984), it also supports *in vitro* dNTP incorporation into acid insoluble material in *Chlamydomonas* (Lou et al. 1987; Wu et al. 1986) maize (Gold et al. 1987; Carrillo and

Table 5.1 Plasmids used in site specific initiation experiments

Plasmid	Features
A. Intergenic region of *16S rRNA-trnI* genes	
(Putative origin in Oenothera, tobacco and pea)	
pOri32	Plastome I
pBluescript⁻	vector of 3-2
pOar2	Plastome IV
pBR328	vector of B57
B. Intergenic region of *23S rRNA-16S rRNA* genes	
(Putative origin in tobacco, pea)	
pOj32	Plastome I
pOj108	a random insert of the same size as pOj32
pBR322	vector of pOj32and pOj108
C. The vicinity of rpl16-rpl14-rps8 genes	
(Putative origins in chalymydomonas, maize, soybean)	
pOjor2	Plastome C1
pOar1	Plastome IV
pUC18	vector of pOjor2 and pOar1

Bogorad 1988) and soybean (Hedrick et al. 1993). Plasmid DNA was prepared following the standard mini prep procedure described by Sambrook et al (1989).

Isolation of chloroplasts from *Oenothera* Using a procedure described by Chiu et al. (1990) *Oenothera* leaf tip cultures (ca. 80 g) were harvested 7-10 days after being transferred into fresh medium, and homogenized in 6-10 volumes of homogenization buffer containing: 50mM Tris-HCl, pH 7.5, 6% sorbitol, 6 mM sodium ascorbic acid, 0.15% (w/v) polyvinyl pyrrolidone (pvp), 0.1% BSA, 3mM cysteine and 5 mM 2-mercaptoethanol. The debris from the homogenate was removed by pouring through multiple layers of cheese cloth and two layers of Miracloth. Intact cells, starch, lipids and nuclei were removed from the chloroplasts by differential centrifugation. Plastids were pelleted at 6000xg for 4 min and subsequently washed with the same basic medium lacking PVP. The final chloroplast pellets were stored at -80°C for subsequent use. When the plastid pellet was observed with phase microscopy, <20% of the plastids were brightly luminescent (i.e. intact). However, 80% of the organelles that were pelleted were extremely small and not at all luminescent. These are most likely to be composed of rapidly-dividing proplastids.

Preparation of chloroplast extracts This protocol has been adapted and modified from the Cannon-Heinhorst Laboratory at the University of Mississippi (Hedrick et al. 1993). Purified chloroplasts from *Oenothera* were resuspended in a minimal volume of buffer A containing: 50mM Tris-HCl, pH 8.0, 100mM $(NH_4)_2SO_4$, 25% glycerol, 10 mM 2-mercaptoethanol, 1mM EDTA, 0.3 mM p-sulfonylfluoride

(PTSF), 0.3 mM phenyl methyl sulfonyl fluoride (PMSF), and 1 mM ε-aminocaproic acid. Triton X-100 was added to a final concentration of 2.0 % and the solution was incubated and stirred on ice for 1 hour. The crude extracts were centrifuged at 9000 rpm in a Sorvall SS34 rotor for 30 min. The clear supernatant was loaded onto a 10 ml DE52 (Whatman Ltd. England) column pretreated with HCl and NaOH and equilibrated with buffer A in 0.5M $(NH_4)_2SO_4$. Before loading the sample, ten volumes of buffer A with 100 mM $(NH_4)_2SO_4$ was loaded to wash and equilibrate the column. After sample loading was finished, three volumes of buffer A with 100mM $(NH_4)_2SO_4$ was loaded to wash the column. The replication extracts were then eluted by buffer A with 500 mM $(NH_4)_2SO_4$. Fractions were tested for activity using the system described in chapter 4 and activated calf thymus DNA. Fractions showing DNA polymerase activity were pooled together and dialyzed against 100 mM $(NH_4)_2SO_4$ buffer A with 0.1 % Triton X-100 and 0.1 mM EDTA. After dialysis, the replication extracts were concentrated to one tenth volume with Centricon 10 concentration tubes at 5000 x g (Amicon, Beverly, MA) and stored at -80°C for subsequent experiments.

DNA polymerase assays. Each reaction for a standard DNA polymerase assay was performed in a 50 ul volume, containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 125 mM KCl, 39-65 ug/ml activated DNA (DNAse treated calf thymus DNA), 30uM of each of the four deoxynucleotides with ^{3}H-dTTP at 300-1000 cpm/pmole, 1 mM DTT and 140 ug/ml BSA. Aphidicolin was dissolved in DMSO at 20ug/ml. The final concentration of aphidicolin in a standard replication assay is 0.4 ug/ml. The reaction began with the

addition of 5 ul of the enzyme sample, followed by the incubation at 37 °C for 30 min. The quantity of radioactivity incorporated into acid-insoluble material was measured by transferring the entire reaction to a DEAE cellulose paper (Whatman, DE11), and washing once with 0.5 M Na_2HPO_4 for 10 min, H_2O for 1 min and 95% ethanol for 1min. Liquid scintillation counting was performed to measure the precipitated radioactivity after the filters were dry.

Quantification of protein concentrations Protein concentrations were quantified by the protein assay kit (BioRad) following the manufacturer's instructions.

DNA replication initiation assay The standard replication initiation assay was adapted from that of Hedrick et al. (1993). Each 50 ul assay contained 50 mM Tris-HCl pH 8.0, 12 mM $MgCl_2$, 100uM each GTP, CTP, UTP, 2mM ATP, 20 uM each dATP, dCTP, dGTP, 2uM ^{3}H-TTP (1000-3000 cpm/pmole), 0.1 mg/ml acetylated BSA and 20 ng/ul substrate DNA. After the addition of chloroplast extract (1-10ug protein/ul reaction), the initiation experiments were incubated at 25°C for 1 hour. The quantity of radioactivity incorporated into acid-insoluble material was measured as outlined in the DNA polymerase assay.

Results

The replication extracts of *Oenothera* chloroplasts do not support dNTP incorporation within either the *23S rRNA-16S rRNA-trnI* or the *rpl16-rpl14-rps8* regions.

Plastome regions examined in this study are shown in Figure 5.1. As explained in the materials and methods section, two regions were investigated: the vicinity of the *23S rRNA* and *16S rRNA*, and the intergenic region of the *rpl16-rpl14-rps8* genes. In addition to these areas, the appropriate vector DNA was also used as a control substrate for each initiation experiment. The replication extracts from both wild type and *pm*-plants were assessed for site specific initiation, as described in the materials and methods section.

The *ori* B site was chosen for the preliminary assays because this region appears to be one of the replication origins in pea and tobacco, and its complete sequence has been determined and analyzed (Sears et al, 1996, Hornung et al. 1996; Stoike and Sears, 1997). In my assay, the *ori*B regions from both plastome types I and IV were examined, and neither of them stimulate the DNA synthesis activity of the replication extracts (Figure 5.2; pOri32 and pOar32).

The 5'-end flanking the *23S rRNA* gene was mapped as one of the replication origins in both tobacco and pea, but not in *Oenothera* (Figure 5.1). Preliminary observations of Lara Stoike (personal communication) suggested that this area might be a putative replication origin for the *Oenothera* plastome. Therefore, plasmid pOj32 containing this region was included in the present study. Additionally, plasmid pOj108 with a plastome insert similar in size to that of pOj32 was used as a control substrate. No significant level of DNA synthesis was observed in this set of experiments. However, the

vector pBR322 control seemed to promote a high level of DNA synthesis in extracts from both the wild type and *pm*-plants (Figure 5.2).

The intergenic region of the *rpl16*-ribosomal protein operon is thought to contain the replication origins in maize (Carillo and Bogorad, 1988), *Chlamydomonas* (Wu et al. 1988) and soybean (Hedrick et al. 1993), as determined by *in vitro* analysis. Therefore, I was interested to test whether this region may function as an origin in the *Oenothera* plastome. For this purpose, plasmids were used that carry this portion of the *rpl16* operon from the Cornell-1 plastome (clone pOjor2) and wild type-plastome IV (clone pOar1). Although the Cornell-1 line originated from the *pm* line, our studies have indicated its cpDNA is identical to the wild-type in this region. Thus, this clone was used instead of a much larger clone containing this fragment from the wild-type-I (*Düsseldorf* line), where a risk would have existed that a higher background would have been obtained. Additionally, pUC18, in which the pOjor2 and pOar1 were constructed, was used as a control to observe the background incorporation level. Although the overall DNA synthesis in these clones seemed higher than all other parallel experiments, no significant levels of ^{3}H-dTTP incorporation is observed when these results are compared with the control vector.

Discussion

A partially purified *in vitro* replication system of *Oenothera* chloroplasts was described in Chapter IV. With similar procedures, *in vitro* replication systems have been developed from pea (McKnown and Tewari, 1984), *Chlamydomonas reinhardtii* (Wu et al. 1986),maize (Gold et al. 1987), soybean (Hedrick et al. 1993) and tobacco (Lu et al.

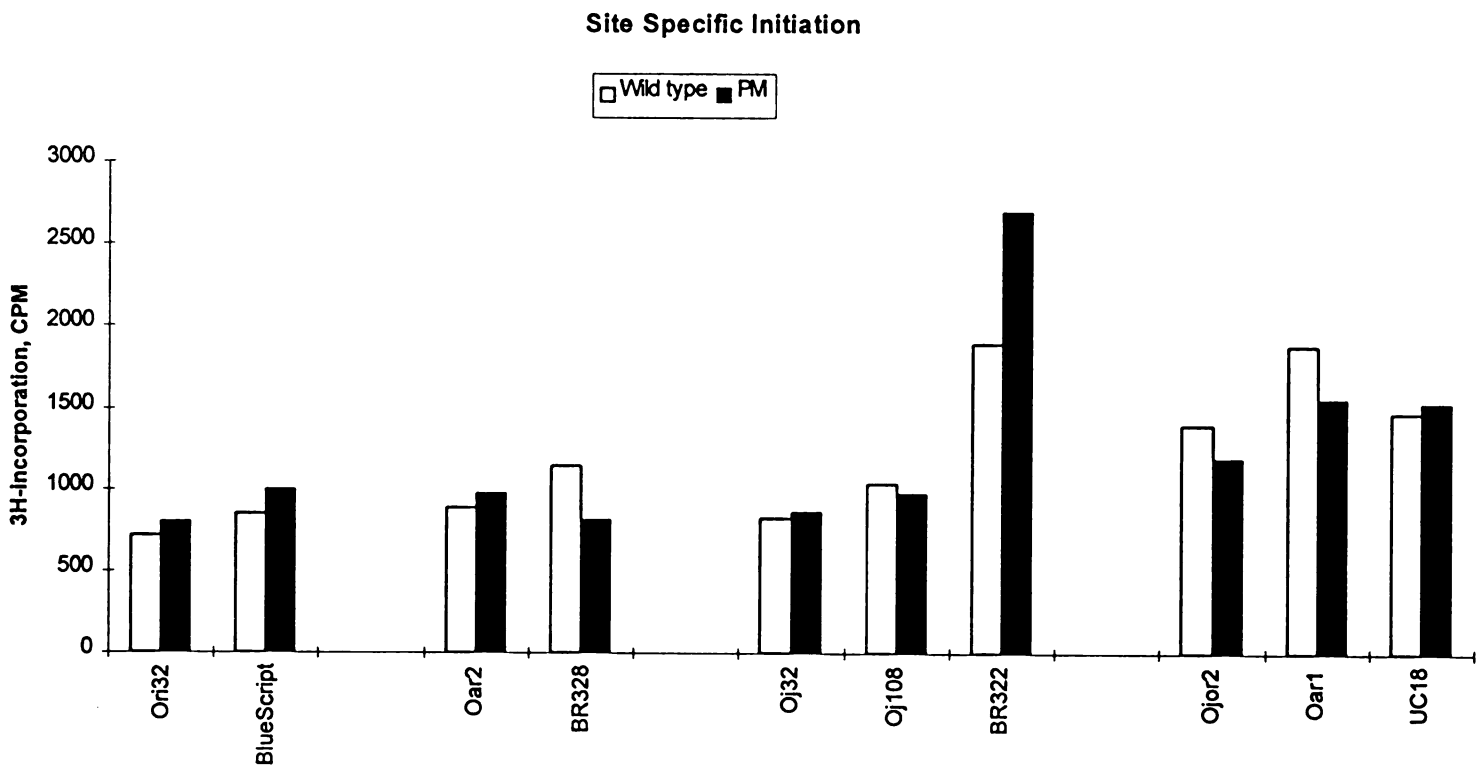


Figure 5.2 Template preference of the Oenothera replication extracts. Different plasmid templates containing *Oenothera* cpDNA restriction fragments (Table 5.1) were tested with the replication extracts from Oenothera chloroplasts. Experiments were carried out as described in the materials and methods section.

1996) for the observation of site specific initiation of replication. In these systems, the presence of DNA polymerase was used as the standard to define the replication activity in the chromatographic purification steps. However, other replication related proteins are also present in the extracts. For example, in the replication extracts of soybean chloroplasts, activities of gyrase, topoisomerase, helicase, and RNA polymerase were also detected in addition to the DNA polymerase activity (Hedrick et al, 1993). Similarly, in the high salt extracts of pea chloroplasts, the fractions containing activities of primase, helicase and topoisomerase overlap with the ones that have the DNA polymerase activity (Reddy et al. 1994). Since a similar preparation procedure was followed, the replication extracts obtained from *Oenothera* chloroplasts should contain most of the essential components for site specific initiation. In this chapter, this newly developed *in vitro* replication system from *Oenothera* was used to investigate the *in vitro* function of putative origins of *Oenothera* previously identified using the electron microscope (Chiu and Sears, 1992), and some potential *ori* sites that had been recognized in other organisms (McKnown and Tewari, 1984; Wu et al. 1986; Gold et al. 1987; Hedrick et al. 1993; Lu et al. 1996).

Despite the fact that the *in vitro* replication system from *Oenothera* chloroplasts actively incorporated ^{3}H-dTTP into acid insoluble materials from primed, single stranded templates (Table 4.2), it did not support site specific initiation of any of the potential replication origins (Figure 5.2). Although *in vitro* replication data does not always coincide with the *in vivo* observation (Carrillo and Bogorad, 1988; Hedrick et al. 1993), it is surprising that no incorporation activity was observed in the *ori*B region of the *Oenothera* plastome (Chiu, 1990). The *ori*B of *Oenothera* was mapped to the intergenic

spacer of 16S-23S rRNA, which is consistent with the *ori*A of tobacco (Lu et al. 1996) and *ori*I of pea (Meeker et al. 1988) (Figure 5.1). Evidence from *Chlamydomonas* (Wu et al. 1986) and maize (Gold et al. 1987) showed that a putative origin of replication supporting *in vitro* replication could be delimited to a fragment that is as little as 450 bp. However, the replication activity was not detected if the essential cis-elements of initiation were missing. For the *ori*A fragment of tobacco. both sides of the *Bam*HI restriction site within the *EcoRI* fragment containing *ori*A were needed for significant replication activity *in vitro* (Lu et al. 1996). The two clones flanking each side of the *Bam*HI site did not support *in vitro* replication alone (Nielsen et al. 1993). Thus, the lack of incorporation of nucleotides with clones pOri32 and pOar1 of the *Oenothera* plastome could be due either to the absence of the essential cis-elements capable of supporting initiation of replication, or alternatively, the trans-acting factors for site specific initiation may not have been copurified with fractions containing active DNA polymerase. Another possibility is that the accuracy of measurement of D-loop sites in *Oenothera* plastomes may have been affected by the use of a small circular size marker (φ174) in EM work to estimate the long fragments resulting from restriction digestions (Chiu and Sears, 1992).

The *rpl16-rpl14-rps8* region also did not support *in vitro* replication. This observation is consistent with the failure to identify this region as a D-loop in the EM studies (Chiu and Sears, 1992).

Both the *in vivo* and *in vitro* data from pea (Kolodner and Tewari, 1975; Meeker et al. 1988) and tobacco (Lu et al. 1996) indicated that the two putative origins flank the *23S rRNA* (Figure 5.1) while in *Oenothera*, the two D-loops are located on each side of

the *16S rRNA* gene. Perhaps using more clones overlapping the *rRNA* operon can provide a complete answer for the *ori*B puzzle of *Oenothera*. Additionally, a thorough examination of the kinds of proteins present in the replication extracts is recommended to assure the reliability of the data obtained from the *in vitro* replication system of *Oenothera* chloroplasts.

Chapter 6

SUMMARY AND CONCLUSIONS

The *plastome mutator* of *Oenothera* is capable of causing elevated frequencies of non-Mendelian mutations that are 200-1000 times higher than spontaneous levels. Several *pm*-hot spots have been mapped, and sequence characterization of these *pm*-induced RFLP sites showed that all of them occurred in regions that contain direct/ inverted repeats. Subsequently, replication slippage was suggested as being responsible for the mutations caused by the *plastome mutator*. Evidence obtained from the insertion mutations recovered from a poly-A track of several *pm*-isolates provided further support for this model. The objective of this dissertation was to examine the mutagenesis process of the *plastome mutator* in several ways. One goal was to accomplish more thorough examination of the types of mutation induced by the *plastome mutator* and their frequencies of occurrence in different plastome types. A second goal was the development of an *in vitro* replication system to investigate the replication enzymes of *pm*-chloroplasts biochemically. The effect of the *plastome mutator* on plastome copy numbers was examined using Southern blotting experiments.

Due to the limited resolution of RFLPs, only mutations ranging from 50-500 bp were recovered from *pm*-plants in the initial studies (Chiu et al. 1990). It was unknown whether the *plastome mutator* causes a broader spectrum of mutations. Therefore, a more

sensitive screening technique was needed for a thorough examination of the types of mutations induced by the *plastome mutator*. The single stranded conformational polymorphism (SSCP) technique served this role. Combined with PCR amplification and restriction endonuclease digestion, the SSCP technique was used to screen six photosynthetic electron transfer-related genes from 37 *pm*-plants having chlorophyll deficiencies. Two mutations were recovered from the *psbB* gene. One of these was a 4-bp frameshift insertion, that looked like a typical product of replication slippage. A truncated protein would be generated due to shifting of the coding frame of the *psbB* gene, and hence could have been responsible for the albino phenotype. Another mutation recovered by SSCP analysis was also located in the *psbB* gene. It is not surrounded by repeats sequences, nor by stretches of a single oligonucleotide. Sequence characterization of this area revealed no features of preferred substrates for replication slippage. Nevertheless, this line of evidence is consistent with our previous finding that the *plastome mutator* has a synergistic effect with NMU, a mutagen that predominantly induces point mutation. It also pointed to the likelihood that the *plastome mutator* may have caused more varieties of mutations than those which we had previously observed. The discovery that the *plastome mutator* can cause base substitutions as well as repeat-mediated insertions and deletions points to a likely defect in a component of the cpDNA replication machinery.

The *plastome mutator* of *Oenothera* carries genotype A and plastome type I. It is compatible with plastome type II, III and IV to differing degrees. Evidence obtained by Sears and Sokalski (1991) suggested that the *plastome mutator* causes a lower frequency of chlorophyll deficient mutants in plastome IV, but subsequent work in the Sears laboratory showed no significant differences between plastome type I and IV (Baldwin,

1995). In order to carefully assess the impact of the *plastome mutator* on different plastome types, previously identified RFLPs were examined among newly-derived *pm*-IV mutants. A parallel experiment with plastome I was performed by Lara Stoike, who found that the oriB region is targeted almost immediately, but deletions at other sites can be observed only in a second plant generation. With plastome IV, no activity was observed at the DNA level, although an equivalent frequency of albino sectors was observed. The finding that the super hot spot "oriB" in plastome I is not affected by the *plastome mutator* in the plastome IV background is intriguing. The very strong secondary structure corresponding to frequent deletions in *pm*-I is also present in *pm*-IV. This line of evidence indicates that factors other than the secondary structures are also involved in mutagenesis of the *plastome mutator*. To make the analyses perfectly parallel, the plastome IV plants need to be carried through a second generation.

Before my project was initiated, all of the *pm*-induced mutations had been examined long after they were induced. Because the results pointed to the involvement of replication slippage, questions arose about the integrity of the chloroplast replication in the *plastome mutator*. Therefore, in order to initiate an investigation of the replication enzymes of *pm* and to observe the induced events *in vitro*, the establishment of an *in vitro* replication system was necessary.

With slight modification of the system from the Cannon and Heinhorst Laboratory (Hedrick et al. 1993) and some technical improvements specific to *Oenothera* plant materials, an *in vitro* replication system was developed from *Oenothera* chloroplasts. For the first time, the activity of such extracts from *Oenothera* reached a workable activity 5-10 times higher than the background level. This system is able to incorporate [^{3}H]-dTTP

into acid insoluble materials and the incorporation activity was stable for longer than two hours. In this system, DNA synthesis is dependent on exogeneously added substrates and nucleotides. $MgCl_2$ is essential for DNA synthesis, but KCl is apparently not critical for DNA synthesis of an extract prepared from wild-type chloroplasts. However, it is interesting to note that the presence of KCl seemed important for DNA synthesis in the *pm*-system. It has been reported that the addition of SSB to DNA polymerase lowered the requirement of Drosophila mitochondrial DNA polymerase for KCl. Further examinations designed specifically for assessment of SSB composition in *Oenothera* chloroplasts will indicate whether a defective SSB gene is the *plastome mutator* we have sought for.

A primed-template replication strategy was designed to observe processive DNA replication. Plastome fragments containing previously identified *pm*-hot spots have been cloned into phagemids to produce ssDNA as templates for replication experiments. The processivity of *pm*-replication extracts is apparently lower than that of the wild-type extracts. In the experiment with wild-type extracts, DNA synthesis stalled at a strong secondary structure while the *pm* extracts stall earlier, at the numerous weaker secondary structures present in the template. As a consequence, *pm*-DNA polymerase appears to have difficulty processing DNA replication. A primed-template replication system needs two main proteins: DNA polymerase and SSB. Since DNA polymerase tends to stall at the secondary structures if SSB is not present at the replication fork (William and Kaguni, 1995), the discovery that *pm*-replication extracts is less processive indicates that either the DNA polymerase or the SSB is not functioning well in the *pm*-extract.

To investigate the role of the *plastome mutator* on copy number control, copy numbers of plastomes were compared between the wild-type and *pm*-plants. As shown in appendix B, my preliminary data revealed insignificant differences of plastome copy numbers between the wild-type and *pm*-plants. Evidence obtained from this experiment indicated that copy number of the plastome in *pm*-plants might not be affected by the *plastome mutator*. In prokaryotes, the prevalent mechanism controlling copy numbers is through the initiator protein or less often, through other replication related proteins, as discussed in appendix A. Since plastome copy numbers are normal in *pm*-plants, we suggest that the initiator protein of plastome replication is not a candidate of the *plastome mutator* .

Studying the mechanism of mutagenesis of the *plastome mutator* of *Oenothera* has been a long term project in our laboratory. My thesis research has enhanced our understanding of the *plastome mutator* in several ways. From a molecular aspect, the spectrum of the types of mutations induced by the *plastome mutator* was found to be broader than what was previously known. Other data obtained from biochemical analyses indicated that the *plastome mutator* is likely to involve a defect in a chloroplast DNA polymerase or SSB. These observations suggest a future direction for the study of the *plastome mutator*. Experiments should focus on assessing the functions of these two proteins in *pm*-plants.

APPENDIX A

EFFICIENCY OF *IN VITRO* REPLICATION USING DOUBLE-STRANDED AND SINGLE STRANDED TEMPLATES.

In order to start DNA replication, an initiator recognizes the replicator (origins) (Huberman 1995). With help from other accessory proteins at the replication fork, a primer or nick is made, then replication proceeds. A defect in any of these proteins would render the replication mechanism malfunctional. As explained in Chapter 2, evidence obtained in our laboratory has suggested that the *plastome mutator* is involved in DNA replication in *pm*-chloroplasts. To be able to characterize the *plastome mutator* defect biochemically, I hoped to assay the frequency of errors using an *in vitro* replication system. Defects in a helicase or topoisomerase would be visualized with a ds-template, while defects in a single stranded binding protein would be observed using a ss-template. Thus, a comparison of DNA replication with ds- and ss-templates would facilitate a better understanding of the accessory proteins involving DNA replication.

In this section, a heterologous primed-template replication system consisting of the replication extracts from soybean chloroplasts and the substrates from clones containing fragments of the *Oenothera* plastome are described. The goal was to use the abundant soybean material to prepare a chloroplast replication extract to test the potential substrates for the *in vitro* replication system that I was going to develop. The intention was to test both dsDNA and ssDNA templates for their utility in assessing properties of the chloroplast replication extracts. Several attempts have been made to overcome the thermodynamic barrier of double stranded DNA lacking an origin of replication.

Materials and methods

The details of the preparation of the soybean extracts and the conditions of DNA polymerase assays described in Hedrick et al. (1993).

Isolation of chloroplasts from soybean cells During my three-month visit to Dr. Gordon Cannon's Laboratory at the University of Southern Mississippi, the SB-1 cell line of *Glycine max* (L.) Mer. V. Corsoy was incubated photomixotrophically on KT medium (Horn et al. et al. 1983), and harvested for chloroplasts (Hedrick et al. 1993)

Results:

Impact of varying ratios of primer/ds-template on replication activities were tested at room temperature with undenatured dsDNA

To start testing the possibility of using a primed-dsDNA replication system, undenatured dsDNA was used as a substrate. The template used in this test contains the 2.5 kb *EcoRI* fragment of the *rpl16-rpl14-rps8* gene region from plastome IV in pUC18 (Wolfson et al. 1991). The primer BS27 is complementary to the 3'end of the *rpl16* gene. 0.5 ug of the template was used in each assay, with the molecular ratios of primer/template adjusted to 10:1 and 100:1 accordingly. A summary of the results from this test is listed in Table A.1, including controls. Even a 10-100 fold excess of primers to template molecules does not enable priming for the replication of this dsDNA template.

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Table A.1 DNA synthesis activity of different primer/template ratio at room temperature

Relative concentrations		DNA synthesis activity (%)
primer	template	
10	1	1
100	1	1
100	H_2O	1
H_2O	1	1
H_2O	H_2O (blank control)	1
activated DNA (DNase digested calf thymus DNA, 3ug)		100

Impact of template denaturation on replication activities

In ssDNA sequencing reactions, samples are heated up to 65°C, followed by slow cooling to anneal the primer and the template. To apply this approach to my assay, samples were denatured at 70°C for 10 minutes in the presence of primers, and slowly cooled to room temperature for 30 min before the replication experiments. In addition to the primer/template amounts used in the first set of experiments, a ratio of 1000/1 was also used. The results of replication activity for each experiment are summarized in Table A.2.

The replication activity was slightly increased in the experiments in which the ratio of primer/template was 100/1. However, it is insignificant compared to the level of replication when activated DNA was the substrate of replication (Table A.2). The decrease of template concentration did not affect the replication efficiency. Heating samples to 70°C before replication did not seem helpful for initiation of replication.

Table A.2 DNA synthesis activity of different primer/template ratios at 70°C

Relative concentrations		DNA synthesis activity (%)
primer	template	
10	1	1
100	1	2
10	0.1	1
10	0.01	2
10	0.001	1
100	H_2O	1
H_2O	1	1
H_2O	H_2O (blank)	1
activated DNA (3 ug)		100

Impact of boiling and alkali treatments of double stranded template on replication activity

Two clones and two primers were used for the following experiments. Both clones pOar1 (Wolfson et al. 1991) and pOjr2 (Sears et al. 1996) were described in Chapter 5. Primer BS49 is complementary to the 3' end of the *16S rRNA* gene in the clone B54. The universal forward primer is at the 5' end of the multiple cloning sites of pUC18 (Yanisch et al. 1985). This primer is used for annealing to clone pOar1. The boiling and alkali treatments that are both known to denature dsDNA (Sambrook et al. 1989), were tested.

A) 100°C treatment

Samples of template and primer were denatured at 100°C for 10 min, slowly cooled to room temperature for another 30 minutes, then left on ice before being added to

Table A.3 DNA synthesis activity of different primer/template ratios after 100°C treatment

Relative concentrations			DNA synthesis activity (%)
	primer	template	
pOar1	10	1	2
	100	1	2
	100	H_2O	2
	H_2O	1	2
pOar2	10	1	2
	100	1	2
	100	H_2O	2
	H_2O	1	2
	H_2O	H_2O (blank control)	1
	activated DNA (3ug)		100

the replication reaction. The results of replication activity obtained for each experiment are summarized in Table A.3. This 100°C boiling treatment did not boost replication activity.

B. The alkali treatment and the primed-ssDNA replication system

The alkali treatment renders dsDNA susceptible to DNA polymerase in sequencing reactions (Lim and Pene, 1988). Therefore, it was chosen to test the materials used in the present study. Templates were denatured in 0.2M NaOH and 0.2mM EDTA at 37°C for 30 minutes, precipitated by NaOAc and ethanol, then resuspended for use in the replication reaction. Because the double stranded templates are denatured into two

interlocked ssDNAs after the treatment, the ratios of primer/template were reduced to 1/1 and 10/1 accordingly. The levels of nucleotide incorporation in each experiment are summarized in Table A.4. The replication levels were slightly increased in the 10/1 (primer/template) reaction for both samples, indicating the initiation of replication after annealing of synthetic primers on alkali-denatured dsDNA.

In the last set of preliminary tests of this replication extract, the M13 ssDNA and the -40 primer adjacent to the cloning site (USB) were used for the replication study. The level of replication of this primed-ssDNA system was 10-15 times higher than all other treatments tested (Table A.4).

Table A.4 DNA synthesis activity of different primer/template ratios with alkali treatment

Relative concentrations			DNA synthesis activity (%)
	primer	template	
pOar1	1	1	3
	10	1	4
	10	H_2O	2
	H_2O	1	2
pOar2	1	1	3
	10	1	6
	10	H_2O	2
	H_2O	1	2
M13	1	1	29
	1	0	3
	0	1	3
	H_2O	H_2O (blank)	1
	activated DNA (3ug)		100

Conclusion

Double stranded DNA is an inert substrate for replication unless it contains the origin of replication (Kornberg, 1991). Therefore, single stranded DNA is usually the template of choice for biochemical assays of DNA replication when the initiation at the origins is not the focus of study or when DNA synthesis on the lagging strand is under study (O'Donnell and Kornberg, 1985; Nielsen et al. 1991; Willams and Kaguni, 1995; Tuteja et al. 1996). In this report, several attempts have been made to break the thermodynamic barrier of native dsDNA. Nevertheless, data obtained from the tests still suggested that ssDNA is a better substrate for annealing of an *in vitro*-synthesized oligonucleotide to prime DNA replication. Thus, the primed-ssDNA replication system has been the system of choice for the research performed in Chapter 4.

Conclusion

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APPENDIX B

THE *PLASTOME MUTATOR* OF *OENOTHERA* DOES NOT AFFECT PLASTOME COPY NUMBERS

Rationale

In wheat (Day and Ellis, 1984), spinach (Lawrence and Possingham 1986), barley (Baumgartner et al. 1988) and rice (Cuzzoni et al. 1995), plastome copy number changes in response to different physiological conditions and developmental stages of the plant (Lamppa and Bendich, 1979; Lawerence and Possingham, 1986). It has been postulated that templates for replication are randomly chosen, and new cpDNAs are partitioned into new chloroplasts in a random fashion, regardless of the genotype (Berky, 1994).

Several lines of genetic and physical evidence indicate that plastome copy number control is "relaxed" rather than "strict" (reviewed by Birky, 1994), similar to the maintenance of high copy number plasmids in prokaryotes. In prokaryotes, the prevailing mechanism for control of plasmid copy number is through inhibition of initiator protein binding to the replication origin (reviewed by Khan, 1996). However, other studies point out that mutations in the replication complex also can impact copy number control. For example, a DNA polymerase I mutation in *Rhizobium leguminosarum* reduces the levels of resident plasmids (Crank et al. 1994).

As discussed in previous chapters, replication slippage has been postulated as the mechanism responsible for the *plastome mutator* activity (Sears et al. 1995; Chang et al. 1996; Stoike and Sears 1997). Several proteins that are involved in movement of the

replication fork have been considered as potential candidates for the *plastome mutator* product, e.g. DNA polymerase, helicase, topoisomerase, and SSB (Chang et al. 1996). Investigations with other organisms have indicated that defects in these proteins may not only affect replication fidelity, but may also impact replication efficiency (Meyer et al. 1979; Nowicka et al. 1994; Vincent et al. 1996). Therefore, I sought to investigate the impact of the *plastome mutator* on cpDNA replication, by comparing the relative amount of cpDNA in wild-type and *pm*-plants.

Materials and Methods

Plant materials The origin of the *plastome mutator* and plant materials used in this study have been described in Chapter II. The *pm/pm* plants were obtained by self pollination of *albicans*-Johansen^{pm} heterozygote carrying plastome type IV. Due to the pollen-lethal allele carried by the *albicans* strain and the megaspore competition between the Johansen and *albicans* strains, 99% of the progeny are homozygous for the *pm* allele. The *pm*/+ heterozygote background was obtained by crossing the *pm/pm* plants from the 94 field season (field accession 94-23) to the +/+ Johansen strain with plastome type IV (field accession 94-4). Because the *plastome mutator* of *Oenothera* creates some pigmentation deficiencies, it was conceivable that these phenotypes (rather than the nuclear genotype) could have a secondary effect of altering the cpDNA content in the chloroplasts. Thus, leaf samples were carefully chosen, to enable all possible comparisons. In addition, because plastome copy numbers change in different developmental stages, leaves were harvested at the same size (10 cm) and age (4 weeks old). The one exception is the sample *pm50-2*, which was from a middle size (5cm)green

plant, purposely sampled to observe the developmental effect. In total eight samples were harvested, each representing a unique combination of nuclear background and phenotype. These include one wild-type plant 95-4 (1), two *pm/pm* plants: *pp93* (green) (2), *pp92* (variegated) (3), and five *pm*/+ plants: *pm*50-4 (green) (4), *pm*50-3 (light green) (5), *pm*50-2 (green) (6), *pm*50-1 (variegated) (7), *pm*69 (variegated) (8).

Preparation of nucleic acids. Extractions of total plant DNA were performed as described by Fang et al. (1992). Half of the total nucleic acids from each sample as then subjected to further purification by caylase digestion as follows (Rether et al.1993): DNA samples were dissolved in a solution (500ul) containing 50mM KoAc, 10mM EDTA, 5 g/ml RNAse and 0.5 mg/ml Caylase M3, then subjected to overnight incubation at 37°C. After the caylase digestion, the pH of the samples was increased by adding 1M Tris-HCl (pH 8) to a final concentration of 50mM, followed by the conventional procedure of phenol/chloroform extraction and alcohol precipitation. However, ethanol was replaced by ethoxyethanol to remove digested polysaccharides more efficiently.

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Southern blotting and quantification of chloroplast DNA content. Southern blotting was performed following the conventional procedure described by Maniatis et al. (1982), with ^{32}P-labeled dATP from New England Nuclear/Dupont. Two clones containing DNA fragments from *Oenothera* were used as probes for blotting. The plastome probe contains a 2.4 kb fragment of the *petA* gene, (Johnson and Sears, 1990) and the nuclear probe has a random nuclear genomic insert of 8 kb (Sears et al. 1997).

Approximately the same amount of sample (1ug/each sample) was loaded twice onto each of two separate gels, and hybridized with the nuclear and chloroplast probes (0.5 ug/each probe) respectively. After hybridization, the blots were exposed to the storage phosphor screen (Molecular Dynamics) at room temperature. Signals on the gel were quantified using Image Quant Program (Molecular Dynamics), with data manipulation through Microsoft Excel.

Results

Southern blotting results are shown in Figure B1 (no caylase treatment) and Figure B2 (caylase-treatment). Signals obtained from these two gels were calculated and drawn using Excel software as displayed in Table 1 and Figure 3.

Measurements of plastome content in the "no-caylase treatment" experiments. Southern blotting results are shown in Figure 1A (gel A) and Figure 1B (gel B). Eight samples were loaded twice on each gel, providing two replicates. Gel A was hybridized to the chloroplast probe while the gel B was hybridized to the nuclear probe. As displayed in Table 1, signals from the two replicates (set 1 and set 2) on each gel were listed and averaged. The averages obtained from the chloroplast blot (C) were divided by the averages obtained from the nuclear-blot (N) to normalize the quantity of hybridization on a per-cell basis (C/N). No pronounced variation in plastome contents was observed among the samples, except that sample 3 (variegated plant *pp92*) has a lower C/N ratio (3.75) compared to all other samples that together have an average of 5.35.

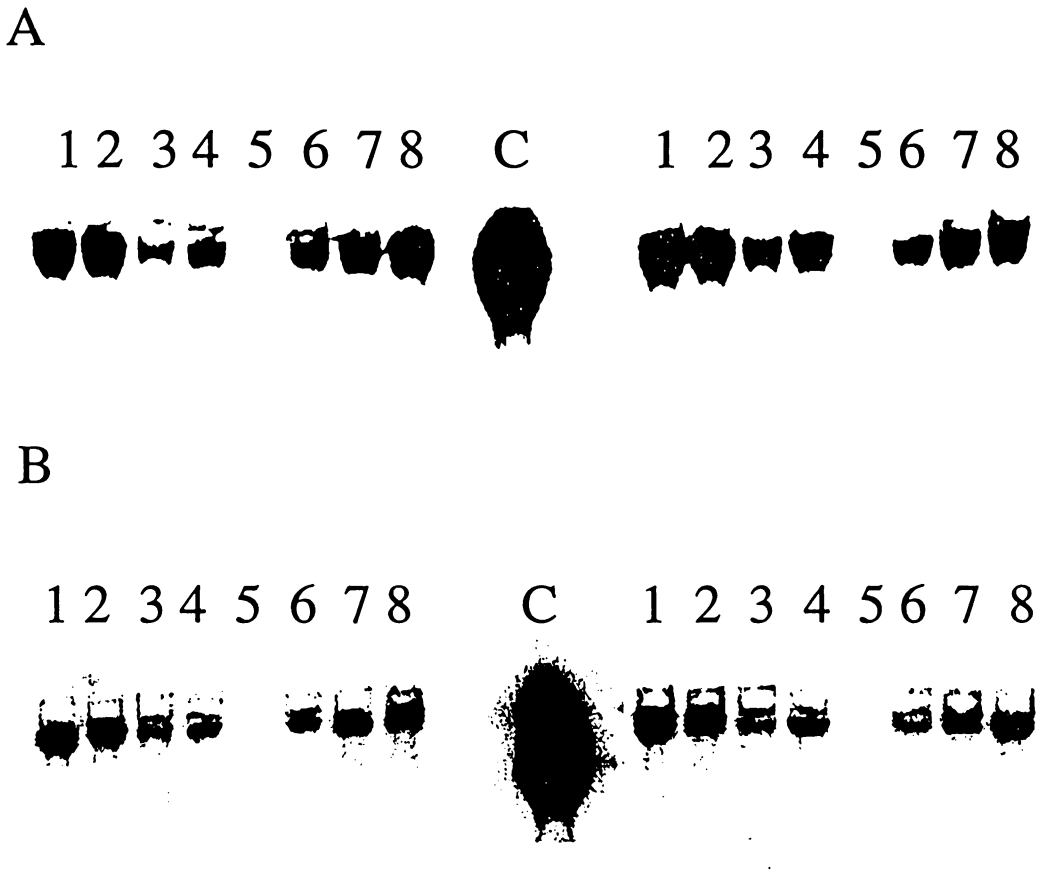

Figure B.1 Southern blot of undigested DNA hybridized with (A) cpDNA probe and (B) nuclear probe. Total DNA was extracted by the conventional method, and no caylase treatment was performed. Duplicate samples of total DNA were loaded into two separate gels, then hybridized with (A) a plastid probe and (B) a nuclear probe respectively, as described in materials and methods. Lanes contain the following samples: one wild-type plant (1) 95-4; two *pm/pm* plants: (2) *pp93* (green), (3) *pp92* (variegated); five *pm/+* plants: (4) *pm50-4* (green), (5) *pm50-3* (light green), (6) *pm50-2* (green and middle size), (7) *pm50-1* (variegated) and (8) *pm69* (variegated). C (control): the same probes used in each experiment were loaded into the agarose gels to use as a control for hybridization.

Table B.1 DNA content of plastids in *pm*-plants. Panel (A) and (B): DNA content of the plastids (C) and nucleus (N) as measured by Southern blotting. Signals obtained from the two replicates of the same gel were listed and averaged. The normalized values of plastid DNA levels were calculated by dividing the plastid signals (C) with nuclear signals (C/N). Panel (A) lists values of (C), (N) and (C/N) determined from the blot with no caylase treatment. Panel (B) shows numbers counted from the caylase-treated experiments. Sample numbers in Panels (A) and (B) represent *pm*-plants as follow: one wild type plant (1) 95-4; two *pm*/*pm* plants: (2) *pp93* (green), (3) *pp92* (variegated); five *pm*/+ plants: (4) *pm50-4* (green), (5) *pm50-3* (light green), (6) *pm50-2* (green and middle size), (7) *pm50-1* (variegated), (8) *pm69* (variegated). All plants carried plastome type IV.

Table B.1 DNA content of plastids in pm-plants

A

		Chloroplast (C)			Nucleus (N)			(C/N)
	Name, genotype	set 1	set 2	Average	set 1	set 2	Average	
1	95-4(g), +/+	3764658	5600738	4682698	770586	1087105	928846	5.04
2	pp93(g), pm/pm	3482925	4596755	4039840	688249	848925	768587	5.26
3	pp92(v), pm/pm	1106787	1595946	1351367	329220	392375	360798	3.75
4	pm50-4(g), pm/	1711274	2813284	2262279	330052	458006	394029	5.74
5	pm50-3(lg), pm/	174663	219253	196958	N.A.	34636	34636	5.69
6	pm50-2(gm), pm	1868102	1577969	1723036	345077	276162	310620	5.55
7	pm50-1(v),pm/+	2277153	2612470	2444812	489718	433530	461624	5.30
8	pm69(v), pm/+	3693718	3067619	3380669	769616	619736	694676	4.87

B

		Chloroplast (C)			Nucleus (N)			(C/N)
	Name, genotype	set 1	set 2	Average	set 1	set 2	Average	
1	95-4(g), +/+	5844162	6392345	6118254	944149	871045	907597	6.74
2	pp93(g), pm/pm	4248394	5197539	4722967	723399	844526	783963	6.02
3	pp92(v), pm/pm	2551661	3457046	3004354	398254	385320	391787	7.67
4	pm50-4(g), pm/	1453891	1597227	1525559	286028	349907	317968	4.80
5	pm50-3(lg), pm/	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
6	pm50-2(gm), pm	2472538	2458411	2465475	381941	365686	373814	6.60
7	pm50-1(v),pm/+	4225103	4820357	4522730	551733	495452	523593	8.64
8	pm69(v), pm/+	3507574	3872326	3689950	476114	392733	434424	8.49

Measurements of plastome content in caylase-treated samples The Southern blots displayed in Figure B. 2 were handled exactly the same as described above except that the DNA had been treated with caylase. The signals obtained from both gels were integrated and are listed in Table 1. The green tissue samples have similar plastome contents in all nuclear backgrounds. Except that the green plant *pm50-4* shows a lower plastome content (C/N: 4.8) relative to all other green samples (average C/N: 7.36). Overall, plastome content seems higher in variegated plants, (*pm50*-1, *pm69* and *pp92*) compared to the green plants (*pp93*, *pm50-4* and *pm50-2*). In other words, the main differences among them depend more on their phenotypes (variegated vs. green), rather than the nuclear backgrounds (+/+ vs. *pm*/+ vs. *pm/pm*) from which these materials are sampled. The relative differences among samples are more obvious in the caylase-treated samples than in these that were not treated with caylase,

Discussion

A defect in the replication complex has been proposed to cause elevated frequencies of mutations in *pm*-plants (Sears et al. 1995; Chang et al. 1996). Thus, the impact of the *plastome mutator* on plastome replication was investigated through comparing the copy numbers of plastomes between the wild-type and *pm*-plants.

In this experiment, one set of samples followed a standard procedure for DNA extraction in the Sears Laboratory, while a replicate set was treated with caylase in addition to the conventional method of DNA extraction. Without the additional caylase treatment, no obvious differences of cpDNA levels were observed in various nuclear

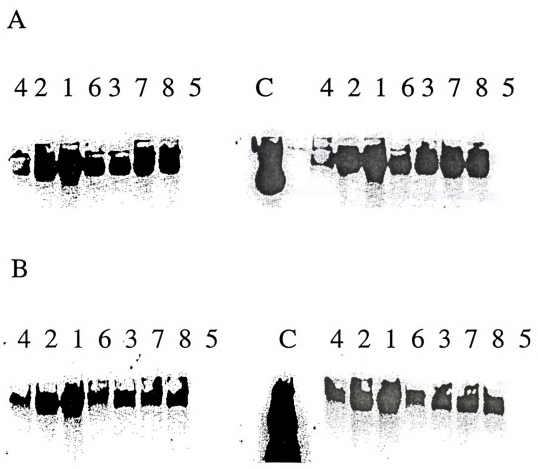

Figure B.2 Southern blot of undigested DNA with (A) cpDNA probe and (B)nuclear probe. Total DNA was extracted by the traditional method, followed by caylase treatment. Duplicate samples of total DNA were loaded into two separate gels, then hybridized with (A) a plastid probe and (B) a nuclear probe respectively. Lanes contain the following samples: one wild type plant (1) 95-4; two *pm/pm* plants: (2) *pp93* (green), (3) *pp92* (variegated); five *pm/+* plants: (4) *pm50-4* (green), (5) *pm50-3* (light green), (6) *pm50-2* (green and middle size), (7) *pm50-1* (variegated), (8) *pm69* (variegated), and C (control). The same probes used in each experiment were loaded into the agarose gels to use as a control (C) for hybridization.

backgrounds, except for one variegated plant *pp92*, in which lower plastome copy number was obtained. In the caylase-treated samples (Figure B.3), the cpDNA copy numbers in variegated plants tended to be higher than in the green lines, regardless of the nuclear background. The lowest cpDNA content was in a green *pm*/+ plant *pm50-4*, which is inconsistent with the data from the set that was not treated with caylase. The different results between samples that were not treated with caylase that were otherwise replicates indicates that the variations were probably not due to the unusual physiological condition of the plant line in use, e.g. altered plastid numbers per cell (Epp and Parthasarathy, 1987). Rather, the effect of caylase treatment or handling errors maybe responsible for the differences.

Carbohydrates that are isolated along with the nucleic acid preparations from *Oenothera* pose an obstacle for accurate pippeting and quantification of DNA. Digestion of the carbohydrates with caylase (Rether et al. 1993) appeared to be a procedure that would improve our purification of DNA. The pippeting of DNA samples becomes much easier due to the reduced viscosity of samples after caylase treatment. In this report, the effect of caylase treatment was tested with the Southern blotting experiments. The relative strengths of signals among samples were stronger in the caylase-treated samples than in the non-treated samples. The divergent results obtained from the different treatments suggest that caylase treatment may have unmasked the interference caused by large amount of carbohydrates present in the DNA preparation. However the possibilities that the observed effects of the caylase treatment originated from pippeting errors or other handling mistakes during sample preparation, can not be excluded.

The evidence displayed here is still preliminary. Despite the variable results gained from the two sets of Southern blots, neither of them reveal any pronounced distinction of cpDNA copy numbers among different nuclear backgrounds. Therefore, it seems unlikely that the *plastome mutator* of *Oenothera* plays a direct role in copy-number control of plastomes.

APPENDIX C

CLONING OF *PM*-HOT SPOTS INTO THE PHAGEMID PBLUESCRIPT SK

Introduction

Due to the uncertainty of the precise location of the replication origins, a primed-replication reaction was designed to study the replication machinery of *Oenothera* chloroplasts. In *E.coli*, double stranded DNA is an inert substrate for primed-replication (Kornberg, 1991), and similar results were observed in my chloroplast replication extracts (Appendix A). Therefore, a primed-ssDNA replication was developed as a system to study DNA replication of an extract prepared from *Oenothera* chloroplasts. The ultimate goal for this cloning project was to observe replication slippage events *in vitro*.

Several fragments containing previously identified *pm*-hot spots were cloned into the phagemid pBlueScript SK for further usage. Stretches of tandem repeats that were designed based on the preferred targets of the replication slippage mechanism, were synthesized *in vitro* and inserted into the 3'end of the *rbcL* gene of *Chlamydomonas* (Sears, unpublished data). The 1kb fragment containing this *rbcL* end and part of the 5'-end of *atpB* gene, was also cloned into the phagemids for further manipulation.

Materials and Methods

Vectors used are pBluescript SK (+/-) (Short et al. 1988) derived from pUC19. The pBluescript SKs contain a replication origin of f1 filamentous phage. Depending on

the orientation of the f1 origin, pBluescript SKs are designated as (+) or (-). With the infection of helper phage, the (+) strain allows the recovery of the sense strand of the *lacZ* gene while the (-) strain produces the antisense strand. *E.coli* strain XL1-Blue (Bullock et al. 1987) was used as the host for constructions. In all cases, the inserts were transferred from other constructed clones containing replication slippage targets. Based on the known physical maps of these previously constructed clones, restriction enzymes were selected and the digested fragments were separated by electrophoresis on agarose gels. The fragments of interest were then extracted from the gel with Qiaex (Qiagen Inc., Chatsworth, Calif.), followed by cloning using conventional methods (Sambrook et al. 1989).

Results

Cloning results are summarized in Table C.1. Ten clones were obtained. All of the insert fragments contained previously recognized *pm*-hot spots (Chang et al. 1996), or *in vitro* designed replication slippage targets (Sears, unpublished data). The phagemids pBlueScript SK (+) and (-) were used as the cloning vectors. ssDNA produced from phagemid has the advantage of being stable, and can be easily maintained within bacteria (Sambrook et al. 1989). Additionally, the two transcription promoters- T3 and T7 flanking both sides of the multiple cloning sites of the phagemid pBluescript SK may be of use to produce RNAs from one strand or the other for future research. Therefore, that vector was chosen for this cloning project. As a record of all of these clones, their features and origins are described in this appendix.

Table C.1 Phagemids constructed for propagation of ssDNA

Name[#]	Insert (origin, size, flanking restriction sites) & vector [(+) or (-)] of use		feature of interest
pORIF pORIR	rRNAs spacer; 5kb; EcoRI	+ +	hypervariable region in the intergenic region of *16S rRNA-trnI*
pRPF pRPR	rpl16-rpl14-rps8 spacer; 2.5 kb; EcoRI	+ +	2 copies of a 29-bp repeat oligo-A stretches
p2280F p2280R	orf2280 coding region; 450 bp; RsaI-HincII	+ -	one set of 8 tandem 24-bp repeats
pRB3F pRB4F pRB4R pRB6F	interruppted coding region of rbcL gene; 1kb; EcoRI-EcoRV	+ + - -	*in vitro* synthsized multiple copies of CTTT (preferred-targets of replication slippage)

[#] The direction designated for clones. **F**-forward, **R**-reverse corresponds to gene transcription for 16S rRNA (Sears et al. 1996), rpl16 (Wolfson et al. 1991), and orf2280 (Blasko et al. 1988) genes of Oenothera plastome and rbcL gene of Chlamydomonas plastome (Goldschmidt-Clermont and Rahire, 1986) respectively.

pORIF+ and pORIR+ were both constructed in pBlueScript SK (+), with the same inserts arranged in opposite directions. The insert fragment was from the hypervariable region between the 16S *rRNA* and *trnI* genes from the Düsseldorf strain containing plastome I (Sears et al. 1996). When the host *E.coli* is infected with the helper phage R408, the sense strand of *16S RNA* gene could be generated from pORIF+ while the antisense strand could be produced from pORIR+.

pRPF+ and pRPR+ were both cloned in pBlueScript SK (+). They have the same inserts but in different directions. The insert DNA was from the intergenic spacer region of *rpl16-rpl14-rps8* of the *Cornell-1* variant of plastome I (Figure 5.1B; Wolfson et al. 1991). The ssDNA propagated from pRPF is the sense strand of the *rpl16* gene while the ssDNA from pRPR is the antisense strand.

p2280F+ and p2280F- were cloned in pBlueScript SK (-), (+) respectively, with the insert containing one set of 8 tandem repeats in orf2280 from plastome I (Blasko et al. 1988). With the infection of the helper phage R408 to its host *E.coli*, the p2280F+ produces the sense strand of the orf2280 while the p2280R- produces the antisense strand.

The inserts in **pRB3F+, pRB4F+, pRB4F-** and **pRB6F-** are 1kb fragments which contain part of the 5'end of the *rbcL* gene and part of the 3' end of the *atpB* gene from plastome of *Chlamydomonas*. The reading frame of the *rbcL* gene was interrupted by several stretches of tandem repeats, that had been synthesized *in vitro* and inserted at a BspMI cut site (Sears, unpublished data).

pRB3F+ is cloned in pBlueScript SK (+), it contains a monomer insert of 5'-gtttCTTTCTTTCTTTCTTTCTTT-3', at the 5'end of the *rbcL* gene. Propagation of pRB3R would generate the sense strand of the *rbcL* gene.

pRB4R-, pRB4F+ were cloned in pBlue Script SK (-), (+) respectively. They contain two units of the same insert in pRB3F+ at the 5'end of the *rbcL* gene. The sense strand of the *rbcL* gene can be produced when the host strain of pRB4F+ is infected with the helper phage R408 while the antisense strand would be produced from pRB4F-.

pRB6F- was cloned in pBlueScript SK(-). It contains a similar repeat sequence at the 5'end of the *rbcL* gene. The repeat is 5'-GTTTCTTTCTTTCTTTCTTTCTTTCTTT-

3'. With the help from the helper phage R408, the antisense strand of *rbcL* gene could be generated from pRB6F-.

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