This is to certify that the

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Characterization of Chloroplast DNA
From Wild-type and Mutant Plastids
of Oenothera hookeri str. johansen

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Sara Anne Kaplan

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Barbara B. Sears

Major professor

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CHARACTERIZATION OF CHLOROPLAST DNA FROM WILD-TYPE AND MUTANT PLASTIDS OF *OENOTHERA HOOKERI* STR. JOHANSEN.

By

Sara Anne Kaplan

A THESIS

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ABSTRACT

CHARACTERIZATION OF CHLOROPLAST DNA FROM WILD-TYPE AND MUTANT PLASTIDS OF *OENOTHERA HOOKERI* STR. JOHANSEN

By

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Chloroplast DNA (cpDNA) from two plastome mutants and three wild-type plastome I lines of *Oenothera johansen* were analyzed by restriction endonuclease mapping and Southern hybridization. Although characterizations indicated that no major DNA changes could be correlated to the mutation events, the restriction fragment analyses did suggest that two BamHI DNA fragments were variable.

Fragments were localized by Southern hybridization experiments on the physical map of the chloroplast genome. The larger BamHI fragment is located in the large single copy region; the other is located within the inverted repeat at or near the junction of the large single copy region. Restriction endonuclease mapping analysis of the two variable BamHI fragments from cpDNA revealed small differences between the cpDNAs in the form of discrete insertions and deletions. Previously, insertions and deletions in cpDNA had been observed only between more distantly related groups of plants.

This thesis is dedicated to:

Dr. and Mrs. William D. Kaplan

David H. Kaplan

Professor Frederick I. Kaplan

and the memory of Mr. Kenneth Aberle

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Oenothera:

"A hopelessly confused and freely hybridizing group, early introduced into Europe and there cultivated, and, like other plants of the garden, intermixed; then spreading to waste or open ground."

Fernald (1950) Grays Manual of Botany

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LIST OF ABBREVIATIONS

bp	base pair(s)
Ci	Curie(s)
d	day(s)
EMS	ethylmethanesulfonate
g	gram(s)
h	hour(s)
k	kilo (1 X 10^3)
kbp	kilo base pair(s)
l	liter(s)
ml	mililiter(s)
M	Molar
mM	mili Molar
min	minute(s)
n	nano (1 X 10^{-9})
rpm	revolutions/minute
rt	room temperature
s	second(s)
μ	micro (1 X 10^{-6})
UV	ultra violet
w/v	weight/volume

CHAPTER 1

INTRODUCTION AND

LITERATURE REVIEW

1.1. INTRODUCTION

The characterization of the chloroplast as being the part of the plant responsible for photosynthesis, and for its other functions, was a long process which took place over several hundreds of years by some of the great early scientists including Hales, Priestley, Ingen-Housz, and deSassure. (Reviewed by Hoober 1984, Arnon 1955, and Rabinowitch 1945). The chloroplast is perhaps the most conspicuous and, because of its importance to the plant cell, certainly the most extensively characterized type of plastid. In addition to photosynthesis, chloroplasts are responsible for amino acid and fatty acid biosynthesis, nitrogen assimilation and partial synthesis of chlorophyll, carotenoids, and plastoquinone (Kirk and Tilney-Bassett 1978).

Many aspects of chloroplast genetics have been investigated, including the organization, expression, and the interaction of chloroplast genes with the nuclear genome of the plant. Because the chloroplast genome, on the whole, is greatly conserved throughout the plant kingdom (Palmer 1987a, Stein et al. 1986, Gillham et al. 1985, and Palmer 1985a, 1985b), it is also the subject of evolutionary studies. The study of the subgenus *Euoenothera* including the genus *Oenothera* has added a wealth of information on the study of chloroplast inheritance. Species within this plant group have an unusual arrangement of chromosomes, three well

characterized nuclear genome types and five genetically distinct plastid types (reviewed by Kutzelnigg and Stubbe 1974, Kirk and Tilney-Bassett 1978, Cleland 1972, and Burnham 1962). Genetic studies of the chloroplast also include the induction and characterization of mutants. Plastid genome (plastome) mutations have been characterized in many plants including *Oenothera* (reviewed by Sears and Boerner 1986). In one case, a variety of chloroplast mutations in *Oenothera* chloroplast DNA have been induced by a nuclear gene (*Plastome mutator*) (Epp 1972, 1973).

1.2. INHERITANCE OF THE CHLOROPLAST.

In 1900, the Mendelian laws of inheritance were rediscovered. A few years later, investigators found exceptions to these laws which showed the manner in which chloroplast traits were inherited. Work by Correns in 1909 and Baur in 1909 (reviewed by Gillham 1978, Kirk and Tilney-Bassett 1978, and Cleland 1972) revealed two types of chloroplast inheritance: uniparental and biparental. Correns' work on *Mirabilis jalapa* showed that only the female parent contributed plastid traits to the offspring. This mode of inheritance has been designated uniparental-maternal. Baur performed reciprocal crosses on *Pelargonium* between green and variegated plants, and found green, white and variegated progeny in the F_1 generation. This mode of

inheritance has been designated as biparental. *Oenothera* displays biparental inheritance of plastids. Even in cases of biparental inheritance, plastids from the maternal parent are generally favored (Sears 1983, Gillham 1978, and Cleland 1972). Since the work of Baur and Correns, chloroplast inheritance in many plants has been examined. In most of the algae and ferns examined to date, the predominant type of inheritance is uniparental. Conifers display biparental inheritance although it is skewed towards paternal transmission of plastids (Ohba 1971, reviewed by Sears 1980 and Kirk and Tilney-Bassett 1978). Among the angiosperms, uniparental-maternal inheritance appears to be the most common pattern, as it has been observed in 80% of angiosperms studied so far. However, biparental inheritance may be more widespread than was previously thought. Through the use of mutant plastids, Schmitz and Kowallik (1986) have shown that *Epilobium*, a species previously believed to have uniparental-maternal chloroplast inheritance, does display some degree of biparental chloroplast inheritance.

1.3. CHARACTERIZATION OF CHLOROPLAST DNA.

Evidence indicating the presence of nucleic acids in the chloroplast first came from a report by Chiba (1951), using Feulgen staining techniques. In the cytological studies which followed, including autoradiography, electron

microscopy, high speed density centrifugation, and reassociation kinetics, the presence of nucleic acids (DNA and RNA) in the chloroplast was confirmed. (Kowallik and Herrmann 1972, Gunning 1965, and Ris and Plaut 1962). Chloroplast DNA in broad bean was demonstrated as being distinct from nuclear DNA due to a higher ratio of adenine to guanine (Kirk 1963). Wells and Birnsteil (1969) distinguished chloroplast DNA from mitochondrial DNA by the chloroplast DNA's lack of 5-methylcytosine. The circular nature and location of the chloroplast DNA molecule within the chloroplast was established through the work of Manning et al. (1971). Subsequent work by investigators demonstrated that chloroplast DNA ocurred naturally in supercoiled form (Gunning 1965, and Ris and Plaut 1962), was contained within discrete areas called nucleoids and that there were 20 - 50 copies of the DNA per chloroplast with an average density of around 1.697 g / cm^3, and a d(G + C) content of 38% for most higher plants (Herrmann et al. 1975, Kolodner and Tewari 1972, Herrmann and Possingham 1980, and Kowallik and Herrmann 1972).

Chloroplast DNA has been characterized from lower plants including the algae, bryophytes, and ferns. Some algae which have been investigated include: *Xanthophyceae* (Yellow-green: *Vaucheria sessilis* (Herrmann and Possingham 1980, Behn and Herrmann 1977), *Chrysophyceae* (golden algae): *Olisthidiscus leteus* (Aldrich and Catallico 1981),

Bacillariophyceae (diatoms): (Palmer 1985b), *Phaeophyceae* (brown algae): *Dictyota dicoma* (Kushel and Kowallik 1985) and *Phylaiella littoralis* and *Sphacelaria sp.* (Dalmon and Loiseaux 1983), *Chlorophycophyta* (green algae): *Chlamydomonas* (Rochaix 1978), *Acetabularia* (Green and Burton 1970), and from *Euglena gracilis* (Gray and Hallick 1978). Chloroplast DNAs have also been characterized in the bryophyte, *Marchantia polymorpha* (Ohyama et al. 1986) and some ferns from the genus *Osmunda* (Stein et al. 1986).

Chloroplast DNA from many higher plants has been examined. Research on cpDNA of gymnosperms is not as extensive as the research on angiosperms cpDNA with only one gymnosperm, *Ginkgo biloba,* (Palmer and Stein 1986) characterized thus far. Restriction mapping and sequencing of cpDNA has occurred in some 200 species representing 33 families of angiosperms including both monocotyledonous and dicotyledonous plants (Palmer 1987a, Palmer and Stein 1986, and Palmer 1985). Some representative angiosperm families which have been extensively studied are the *Leguminosae* (Palmer 1987a), *Solanaceae* (Shinozaki et al. 1986, Sugiura et al. 1986) and the *Onagraceae* (Gordon et al. 1982, Gordon et al. 1981).

1.4. MAPPING THE CHLOROPLAST GENOME.

During the past ten years, the circularity of chloroplast DNAs has been observed as a general feature of many plant groups from the primitive to the advanced. Their sizes have been calculated either by contour length measurement of electron micrographs or by summing up the molecular weights of restriction endonuclease cut DNA fragments.

By far the most powerful technique used in the characterization of cpDNA is the use of restriction fragment length polymorphisms (RFLPs). The first use of RFLPs in the comparison of cpDNAs was by Atchison, Whitfeld and Bottomley (1976). This group used the restriction endonuclease EcoRI in digests of cpDNA from different plant species as well as on plants within the same genus. They found that although the cpDNAs are similar in gross base composition, they show distinctly different restriction digestion patterns. Subsequently, RFLPs have been used as the primary method in determining the gene order in cpDNA (Whitfeld and Bottomley, 1983). Restriction mapping has generated the preliminary data necessary for the sequencing of the entire sequences of cpDNAs from tobacco (Shinozaki et al. 1986) and a liverwort (Ohyama et al. 1986).

Studies involving restriction fragment cloning in conjunction with cell free translation and immunological techniques allowed the location of many genes on cpDNA

including those for thylakoid proteins (Herrmann et al. 1983). This study also reported a 50 kilobase (kb) inversion between spinach and *Oenothera* in the large single copy region of the cpDNA. This inversion is located between the ATPase and the cytochrome f genes and between the large sub-unit of RuBISCO and psbA genes (figure 4.1). Inversions have also been found in the large single copy region of wheat and mung bean cpDNA when compared with spinach (Palmer et al. 1982).

All chloroplast DNAs isolated are circular molecules ranging from 120 kbp in pea to 217 kbp in geranium (Palmer et al. 1987b). Most chloroplast DNAs are represented by only one size class with the exceptions of *Acetabularia* (Green and Burton 1970), *Euglena gracilis* (Jenni et al. 1981), *Phylaiella littoralis* and *Sphacelaria sp.* (Dalmon et al. 1983), green and brown algae. In higher plants, rice was reported by Moon et al. (1987) to consist of heterogeneous cpDNA molecules. With respect to plastome size, the green algae are the most divergent group, with cpDNA molecules ranging from 85 kbp to 292 kbp (Palmer 1985a, 1985b).

In general, the chloroplast DNA molecule is divided into four parts: two areas of single copy DNA designated as the large and small single copy regions, respectively, separated by two large inverted repeats ranging from 10 - 11 kbp to as large as 76 kbp (Palmer et al. 1987a, Stein and Palmer 1986

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and Palmer 1985a, 1985b). The single copy regions are relatively d(A + T) rich, the repeated regions are relatively d(G + C) rich in their base compositions (Gillham et al. 1985). The unique regions of the chloroplast DNA encode the majority of chloroplast proteins including the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBISCO), proteins associated with of photosystems I and II and proteins of the ATPase complex. The inverted repeats contain genes for ribosomal proteins and some tRNAs. The ribosomal genes are similar to prokaryotic ribosomal genes and are arranged in operons (Shinozaki 1986, reviewed by Gillham et al. 1985, Palmer 1985a). The plastome contains spacer regions between genes. Compared to plant nuclear and mitochondrial genomes these are much smaller and fewer in quantity (Shinozaki et al. 1986, Gillham 1985, Palmer 1985b, Gordon et al. 1981, 1982).

The inverted repeat regions in the chloroplast genome allow reciprocal recombinations to occur. This results in chloroplast DNA molecules in isomeric form differing only in the relative orientation of their single copy sequences (Palmer 1983). The inverted repeats are considered to be primitive (Palmer et al. 1987a) and are thought to confer stability to the molecule since a strong correlation has been shown between the presence of the inverted repeat and the rarity of gross rearrangements in the chloroplast DNA

molecules which contain them (Palmer 1985a, Mubumbila et al. 1984, Palmer and Thompson 1981, 1982).

Small rearrangements occur within cpDNA (Palmer et al. 1987a) and are less common within the chloroplast genome containing inverted repeats, but they do occur (Palmer 1985a, Gordon et al. 1982, Palmer and Thompson 1982, Palmer et al 1987a). In the case of *Oenothera*, its five genetically- classified plastome types can be distinguished by a series of small insertions and deletions within the spacer regions of the chloroplast DNA, however, these differences do not alter the overall restriction endonuclease DNA fragment order (Gordon et al. 1982).

With the exception of the geranium chloroplast genome (Palmer et al. 1987b), most of the chloroplast genome does not contain large areas of repeated sequences outside the inverted repeats (Palmer et al. 1987a). Small repeated sequences have been reported in the chloroplast DNA of subclover (Palmer et al. 1987a), wheat (Bowman and Dyer 1986) and *Chlamydomonas* (Palmer 1985b).

1.5. *OENOTHERA*, AN IMPORTANT GENETIC SYSTEM.

The genus *Oenothera* (evening primrose), sub-genus *Euoenothera*, is an important genetic system but it is also a complicated one. The subgenus *Euoenothera* has three basic haploid genome types designated A, B, and C, and may give

six combinations. The group also contains five genetically distinct plastid types. The plastid types vary in their restriction endonuclease digestion patterns as well as their ability to green in certain nuclear backgrounds. The five plastome types also differ in their DNA replication rates. Plastome type I is the fastest, and plastome type IV is the slowest. *Oenothera* has been the focus of many experiments in genetics, cytogenetics and cytoplasmic inheritance ever since deVries' first publication in 1901. deVries' work was continued by Renner and his students and is extensively reviewed by Burnham (1962), Tilney-Bassett (1978), and Cleland (1972). The work performed by Renner, his students and Cleland established *Oenothera* as having a unique characteristic in its organization of chromosomes. Most *Oenothera* species contain 7 pairs of chromosomes. The chromosomes may be linked together in a chain or ring at the first meiotic division (Cleland 1972). These rings are the result of a series of equal subterminal reciprocal translocations between chromosome ends. Such translocations are deleterious in most plants because exchanges are ordinarily unequally spaced. Unequal translocations, in most cases, result in a high level of non-disjunction and consequent sterility. In *Oenothera*, however, the translocations that have survived are the result of equal exchanges so that the centromeres are evenly spaced in the circles and little sterility ensues. Crossing-over between

these homologous regions serves to stabilize the chromosomes in the ring or chain configuration. In the ring arrangements, independent assortment and crossing over of ring chromosomes are effectively suppressed during meiosis. *Oenothera* has other characteristics which make it the plant of choice to study cytoplasmic inheritance including biparental inheritance of plastids, and the ability to perform crosses between species.

1.6. MUTATIONS OF THE CHLOROPLAST.

Studies of the chloroplast genome including its role in the physiology and genetics of the plant have been greatly advanced through the investigation of mutations in the plastome (Boerner and Sears 1986, and Gordon et al. 1980). Plastome mutants affecting many physiological and genetical processes have been isolated and studied in a large number of plants (reviewed by Boerner and Sears 1986).

1.7. THE *PLASTOME MUTATOR* GENE.

A mutator gene has been defined as a gene which controls the frequency of occurrence of mutation events in a particular system (Cox 1976). The mutator genes found in prokaryotic systems may serve as useful models for those found in eukaryotic organelles, since mitochondria and chloroplasts are considered to be prokaryotic in nature

(Palmer 1985b and Bohnert et al. 1982). Mutator genes have been shown to affect many cellular processes in bacteria including the DNA polymerases and DNA repair (Cox 1976). They may also affect removal and replacement of specific nitrogenous bases (Coulandre et al. 1978), nucleotide removal, replacement, photomonomerization of uv-induced thymine dimers, and recombination (Kimball 1979), or the induction of a transposable element (Kleckner 1981). Mutator genes which cause a variety of non-Mendelian mutations in mitochondria of yeast (Backer and Floury 1985), and chloroplasts of *Arabidopsis* (Redei 1973, Redei and Plurad 1973) and of *Oenothera* (Epp 1972, 1973) have been identified. The mutator genes of higher plants have been called "*plastome mutator*" (Epp 1972, 1973) or "*chloroplast mutator*" (Redei 1973). Prior to the isolation of a plastome mutator strain in *Oenothera*, the number and type of plastome mutants was limited primarily to spontaneous mutations most of which were described by Kutzelnigg and Stubbe (1974). These spontaneous mutations arise at a frequency of 0.01-1.3% in a population (Michaelis 1969). The frequency of mutations induced by *plastome mutator* is 30%. Since then, investigators have had some success in creating plastome mutants in higher plants by the use of chemical mutagens (reviewed by Boerner and Sears 1986).

Two types of plastome mutator systems in plants have been demonstrated to date. One type always results in the expression of the same mutant phenotype as seen in

albostrians of barley (Boerner et al. 1976), and *iojap* of maize (Shumway and Weier 1967, Thompson et al. 1983). These mutators are possibly the cause of a loss of chloroplast ribosomes during a critical stage in the development of the plant. The second type of plastome mutator is found in *Arabidopsis* (Redei 1973, Redei and Plurad 1973), *Petunia* (Potrykus 1970), and *Oenothera* (Epp 1972, 1973). These mutators induce a variety of chloroplast mutations.

It is the *plastome mutator (pm)* gene of *Oenothera* which is the focus of chapter 3 of this thesis. Chapter 4 deals with the characterization of small discrete insertions or deletions found of the chloroplast DNA during comparisons of wild-type plants of *Oenothera hookeri* str. johansen.

CHAPTER 2

MATERIALS AND METHODS

2.1. PLANT MATERIAL.

Three wild-type cultivars and two *plastome mutator* induced mutants of *Oenothera johansen* were used in the studies. Table 2.1 lists the plant lines their genotypes and their phenotypes. The pedigree of these lines is shown in Figure 2.1.

Wild-type lines D and C_1 were grown in the greenhouse in sandy soil. Wild-type line C_2 was maintained as shoot meristem cultures since it was available only in an A/C nuclear background (plastome I plants are not as vigorous in the A/C background as they are in their natural A/A nuclear background (Kutzelnigg and Stubbe 1974), and therefore need to be grown on nutrient medium). The mutant plants *pm8* and *pm11* along with wild-type line C_2 were maintained as shoot meristem cultures, in accordance with Gamborg (1975) and Stubbe and Herrmann (1982) on a modified N.T. medium (Nakata and Takabe 1971) containing a 1:6 ratio of BAP (benzylamino purine) to NAA (napthylacetic acid). Mutant *pm11* grew better and had more leaf expansion when grown on M/S medium (Murashige and Skoog 1962) with a 1:2 ratio of auxin to cytokinin (Table 2.3). The plants were maintained as shoot meristem cultures and were transferred to fresh plates every three to four weeks as needed.

Table 2.1 Plant lines and their origins. Mutant lines arose as sectors on plants homozygous for the *pm* gene. Capital letters denote the nuclear genome type. Roman numerals denote the plastome type. The genetic composition of wild-type *O. johansen* is AAI. Five digit numbers represent the year on the field and field designation.

Line	Female parent	Male parent	Genotype	Phenotype
D	*Joh* AAI	*Joh* AAI	AA	wild-type
C_1	81/124 AAI	*Joh* AAIV	^{pm}AA	wild-type
C_2	80/960 AAI	*Joh* ACIV	^{pm}AC	wild-type
pm11	80/960 *pm11* AAI	*Joh* ACIV	^{pm}AC	*pm11* white
pm8	80/959 *pm8* AAI	*Joh* ACIV	^{pm}AC	*pm8* pale green

Figure 2.1. Pedigree of Plant Lines. Pedigree of plant lines from Epp's Cornell *Oenothera johansen pm pm* plastome I line. The five digit numbers following the arrows represent the year in which the seed was planted (first two digits) and the field plot number (last three digits). Roman numerals refer to plastome type and capital letters refer to the genome type. Line D is a different *O. johansen* isolate from the Duesseldorf collection and is not included in this pedigree.

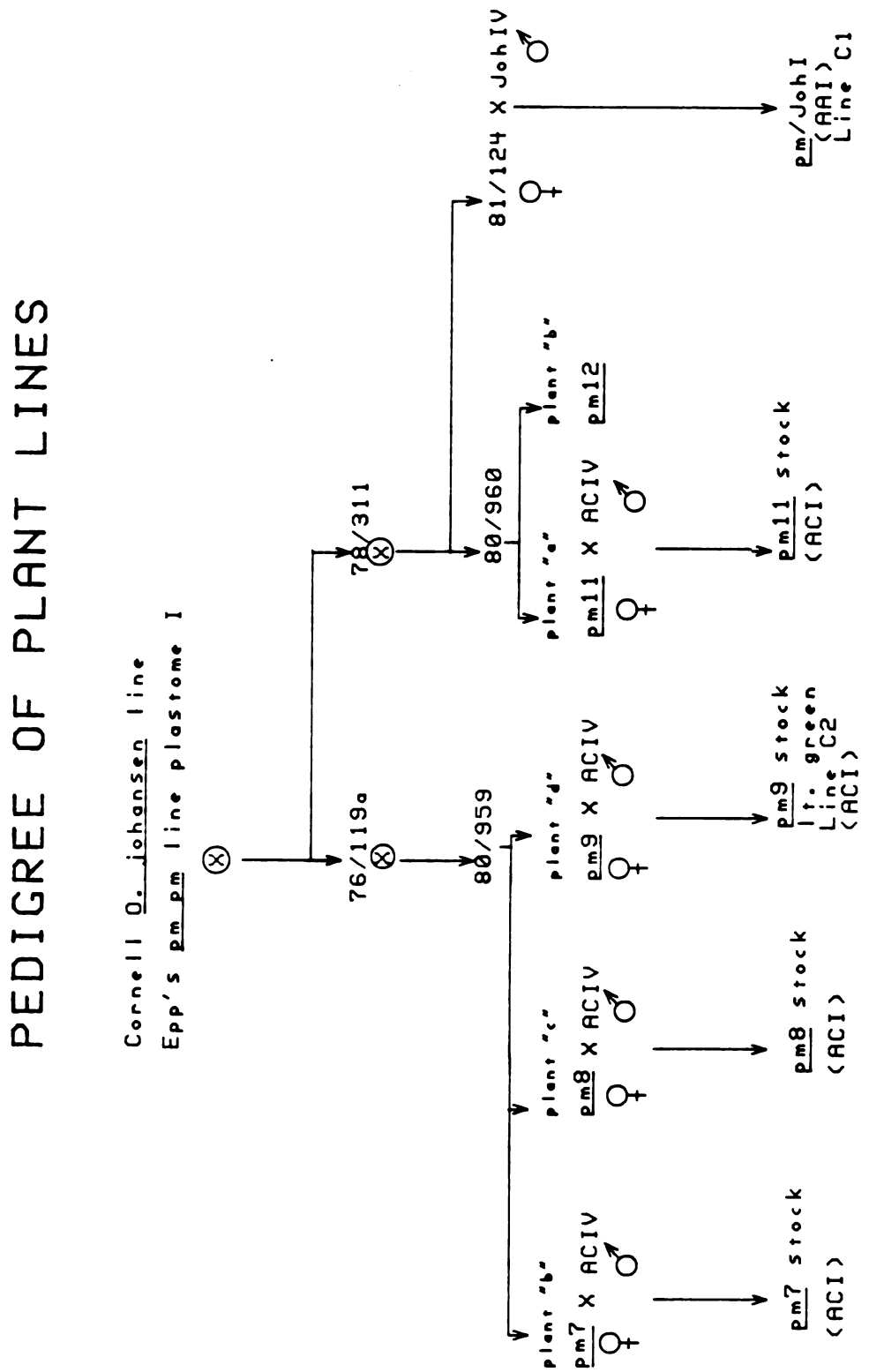

PEDIGREE OF PLANT LINES
Cornell O. johansen line
Epp's pm pm line plastome I
76/119a
78/311
80/959
80/960
plant "b"
plant "c"
plant "d"
plant "e"
pm7 X ACIV
pm8 X ACIV
pm9 X ACIV
pm11 X ACIV
81/124 X JohIV
pm12
pm7 stock
(ACI)
pm8 stock
(ACI)
pm9 stock
lt. green
Line C2
(ACI)
pm11 stock
(ACI)
pm/JohI
(AAI)
Line C1

Figure 2.1

Table 2.2. Modified N.T. Medium for the maintenance of shoot meristem cultures.

Component	N.T. Medium Amount per liter
N.T.-major elements stock	125 ml
N.T.-minor elements stock	1 ml
N.T. vitamin solution	10 ml
NAA solution	5 ml
BAP solution	10 ml
Sucrose	60 g
Agar	8 g

The pH was adjusted to 5.8 with KOH prior to addition of agar and then autoclaved.

N.T. stock solutions:

N.T.Major elements Component	Amount per liter, pH5.8
NH_4NO_3	6.6 g
KNO_3	7.6 g
$CaCl_2 \cdot 2H_2O$	1.76 g
$MgSO_4 \cdot 7H_2O$	9.86 g
KH_2PO_4	5.44 g
Na_2EDTA	0.30 g
Fe-citrate	0.22 g
H_2BO_3	0.05 g
$MnSO_4 \cdot H_2O$	0.18 g
$ZnSO_4 \cdot 7H_2O$	0.07 g

N.T.Minor Elements	Amount per 100 ml
KI	83.0 mg
$Na_2 MoO_4 \cdot 2H_2O$	25.0 mg
$CuSO_4 \cdot 5H_2O$	2.5 mg
$CoSO_4 \cdot 7H_2O$	3.0 mg

N.T.Vitamin Solution	Amount per 100 ml
Meso (Myo-)-Inositol	1.000 g
Thiamine-HCl	0.010 g

NAA Stock
Napthylacetic acid 30 mg/100 ml

BAP Stock Solution
Benzylamino purine 10 mg/ 100 ml

Table 2.3. M.S. Medium

Component	M/S medium Amount per liter
M/S stock solution 1	20 ml
M/S stock solution 2	10 ml
M/S stock solution 3	10 ml
M/S stock solution 4	10 ml
$Na_2FeEDTA$	10 ml
thiamine	2 ml (2.5 mg/ml)
Pyridoxine	1 ml (0.5 mg/ml)
nicotinic acid	1 ml (0.5 mg/ml)
BAP	2 ml (1 mg/ml)
NAA	1 ml (1 mg/ml)
myo-inositol	100 mg
sucrose	60 g
(2[N-Morpholino]-ethanesulfonic Acid)(MES)	0.5 g
agar	8.0 g

The pH was adjusted to 5.8 with 1 N KOH prior to the addition of agar, and then autoclaved.

M/S stock solutions Component	Amount per liter
M/S 1	
NH_4NO_3	82.5 g
KNO_3	95 g
M/S 2 (Sulfate)	
$MgSO_4 \cdot 7H_2O$	37 g
$MnSO_4 \cdot H_2O$	1.69 g
$ZnSO_4 \cdot 7H_2O$	1.058 g
$CuSO_4 \cdot 5H_2O$	0.0025 g
M/S 3 (Halide)	
$CaCl_2 \cdot 2H_2O$	44.0 g
KI	0.083g
$CoCl_2 \cdot 6H_2O$	0.0025 g
M/S 4 (PBMo)	
KH_2PO_4	17.0 g
H_3BO_3	0.62 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g

2.2. BACTERIAL STRAINS AND PLASMIDS.

The bacteria *Escherichia coli (E. coli)* strains ED8654: [*gal K, gal T, trp R, met B, hsd R, sup E, lac Y]* (Bork et al 1976) and HB101: [*hsd S* (r_B-,m_B-), *rec A, ara, pro A, lac Y, gal K, rps L, xyl, mtl, sup E,*] (Boyer and Roulland-Dussoix 1969) were used in this study. They were maintained on Luria Broth (L.B.) plates for working cultures and as frozen cultures for long term storage. Luria Broth consists of : 5 g NaCl, 10 g Bacto tryptone (Difco), 5 g yeast extract (Difco), 1 g glucose [(15 g Agar(Difco or Sigma)] per liter (Maniatis et al. 1982). *E. coli* storage medium consists of: 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.05 g Na-Citrate, and 0.01 g $MgSO_4$, 50 % Glycerine per liter. Frozen cultures were maintained at -20°C.

Bacterial strains containing the plasmid pBR322 were maintained on L.B. plates containing either 80 μg/ml ampicillin (amp) or 12.5 μg/ml tetracycline (tet). Recombinant plasmids containing inserts cloned into the tet resistance gene were maintained on L.B. + amp plates, or as frozen cultures lacking antibiotics in the storage medium described above.

2.3. DNA PREPARATION.

2.3.1. Chloroplast DNA Isolation.

2.3.1.1. Chloroplast isolation from abundant amounts of plant material.

For plants which were grown in the greenhouse, leaf material was abundant. Chloroplasts were first purified as described below. DNA was then extracted from the isolated organelles.

The leaves of *Oenothera* contain compounds, such as phenolics, which make the isolation of chloroplasts difficult. Because young leaves of *Oenothera* plants contain lower concentrations of phenolic compounds and other secondary metabolites than do older ones, most of the leaves were trimmed from the plants about three weeks before the scheduled harvest in order to promote new growth. These plants were then placed in the dark one to four days before harvest in order to deplete their starch reserves. 200 to 250 g of leaves were then harvested and washed in distilled H_2O, damp dried and weighed. The leaves were homogenized in a buffer containing 6% sorbitol, 6 mM EDTA, 1 mM ascorbic acid, 3 mM cysteine, 0.15% (w/v) polyvinyl pyrrolidone (PVP), 0.1% bovine serum albumin (BSA), 50 mM Tris pH 7.5. This buffer is based on the one described by Herrmann (1982) with modifications adapted from Somerville et al. (1981), Loomis (1974), and Galliard (1974). The homogenate was

filtered through one layer of 100 micron mesh gauze followed by filtration through two layers of miracloth (Calbiochem). The chloroplasts were pelleted at 6,000 rpm at 4°C and were washed in the same buffer described above, but lacking PVP. The chloroplasts were then purified over a 10% - 80% sucrose step gradient buffered with 10mM Tris pH8.0, 1mM EDTA. The band containing the chloroplasts was separated and diluted with 50 mM Tris, 20 mM EDTA pH 7.5 and pelleted at 10,000 rpm 4°C for 10 min. The chloroplast pellet was resuspended in an equal volume of 50 mM Tris, 100 mM EDTA, 15 mM NaCl pH 8.5 for subsequent lysis.

2.3.1.2. Isolation of cpDNA from limited amounts of plant material.

Plant material from tissue culture was generally in a much more limited supply than material from greenhouse grown plants.

2.3.1.2a. Protoplast method.

Protoplasts were isolated from 10 to 50 g of tissue. The tissue was first chopped with a razor blade and placed in glass petri plates (5 g per plate) in a solution of 2% Cellulysin, 1% Macerase, 1% Driselase, 13% Mannitol in a buffer containing 27.2 mg KH_2PO_4, 101.0 mg KNO_3, 246.0 mg $MgSO_4 \cdot 7H_2O$, 0.16 mg KI, 0.025 mg $CuSO_4 \cdot 5H_2O$, 1.48 g $CaCl_2 \cdot 2H_2O$ per liter at pH 5.8. The plant material was incubated overnight at room temperature in the dark. Undigested plant material was separated from the protoplasts by filtration

through an 80 micron stainless steel mesh screen. The filtered protoplasts were pelleted by centrifugation at 2,000 rpm at room temperature for 15 min. The pellet was resuspended in a buffer containing 400 mM Sorbitol, 5 mM EDTA, 100 mM Tris pH 8.0, 0.1% BSA, and 0.3% Mercaptoethanol and allowed to equilibrate on ice for 20 min.

The protoplasts were lysed by forcing the suspension through an 18 gauge needle three times. The lysate was pelleted by centrifugation in a swinging bucket (Sorvall HB4) rotor at 4°C at 4,500 rpm for 5 min. The pellet was resuspended in an equal volume of 50 mM Tris pH 8.0, 20 mM EDTA for organelle lysis and cpDNA isolation.

2.3.1.2b. Modified whole cell DNA method.

The whole cell DNA method was modified from DeBonte and Matthews (1984). 10 to 50 g of plant material was homogenized in the chloroplast isolation buffer described above and filtered through one layer of 100 micron mesh and one layer of miracloth. The homogenate was then pelleted by centrifugation at 9,000 rpm in a Sorvall GSA rotor at 4°C for 20 min. The pellet was resuspended in chloroplast wash buffer and pelleted as before. The pellet was then resuspended in 5 ml 50mM Tris, 20mM EDTA pH 8.5 for lysis.

2.3.2. PURIFICATION OF cpDNA.

The chloroplasts were lysed and the cpDNA was liberated from the membranes to which they associate by the addition of 1% Sarkosyl and 1 mg/ml Pronase, followed by gentle mixing at 4°C for 4 h. Chloroplast DNA was separated from nuclear DNA by CsCl buoyant density equilibrium centrifugation in the presence of bisbenzamide (Hoechst 33258) (Mueller and Gautier 1975, and Preisler 1978). 20 µg bisbenzimide and 1.1 g/ml CsCl were added per ml of lysate and the refractive index was adjusted to 1.3960. CsCl gradients were run in a Sorvall OTD-B6 ultracentrifuge with vertical rotors TV865 for small volume samples or a TV850 rotor for samples greater than 30 ml. Gradients were run at 40,000 rpm to 42,000 rpm at 19°C for 12 to 15 h.

The upper band which contained cpDNA was removed from the gradient by fractionation. Bisbenzimide was removed by at least three extractions using CsCl- or NaCl-saturated isopropanol. The salt was removed by dialysis at 4°C with three changes of 10 mM Tris pH 8.0, 1mM EDTA (TE).

2.3.3. PRECIPITATION OF DNA.

DNA was precipitated by the addition of either 2/3 volume 5M NH_4OAC or 1/20 volume 3M NaOAC, 1 µl 20 mg/ml glycogen and 2.5 volumes absolute ethanol at -20°C for at least 4 h.

Large volumes greater than 3 ml were centrifuged at 8,000 rpm at 4°C for 1 h in a Sorvall HB4 rotor. Small volumes were centrifuged in a microfuge at 4°C for 15 min. The purified cpDNA was then resuspended in the appropriate amount of 10 mM Tris pH 8.0, 0.1 mM EDTA (T 1/10 E).

2.3.4. PURIFICATION OF PLASMID DNA.

2.3.4.1. Large Scale isolation of Plasmid DNA.

For purification of large amounts of plasmid DNA, two methods were used depending on the size of the plasmid.

2.3.4.1a. Triton Lysis method.

This method has been modified from Maniatis et al. (1982). It was used for plasmids larger than 15,000 bp. A 1 liter culture of *E. coli* containing the plasmid was grown in L.B. + amp in a shaker incubator at 37°C until the cell culture reached an optical density (O.D.) $_{650}$ of 0.4. A final concentration of 170 μg/ml Chloramphenicol was added for amplification of the plasmid. Incubation of the cultures continued at 37°C for at least 12 h or overnight.

The cells were pelleted at 3,000 rpm at 4°C for 30 min., washed with 20 ml 25 mM Tris pH 8.0, 10 mM EDTA,and pelleted at 6,000 rpm at 4°C for 10 min. The pellet was carefully resuspended in 15 ml 50 mM Tris pH 8.0, 25% sucrose and transferred to a 40 ml centrifuge tube. 3 ml of a freshly prepared solution of 5 mg/ml lysozyme (Sigma) was added and

the cells were incubated on ice for 15 min. 15 ml of Triton lytic solution (3 ml 10% w/v Triton X-100, 75 ml 0.2M EDTA, 15 ml 1.0M Tris pH 8.0, 7 ml H_2O per 100ml) was added and the cells were incubated on ice for another 30 min. The lysate was then centrifuged in a Ti 60 rotor (Beckman) at 35,000 rpm at 4°C for 30 min in a Sorvall OTD-B6 ultracentrifuge. The top of the supernatant containing the plasmid DNA was carefully removed with a siliconized large bore Pipette. 100 µl (10 mg/ml)of a RNase A stock solution was added to the plasmid DNA, followed by incubation at 37°C for 45 min. 5M NaCl was added to a final concentration of 0.5M and the nucleic acids were precipitated with an equal volume of isopropanol at -20°C for at least 2 h. The nucleic acids were then pelleted in a Sorvall HB4 rotor at 6,000 rpm at 4°C for 1 h. The pellet was dried and reconstituted in 6 ml TE. For each ml, 180 µl of a 10 mg/ml Ethidium bromide (EtBr) stock solution and 1.1 g CsCl was added. The refractive index was adjusted to 1.3915 followed by ultracentrifugation at 42,000 rpm at 19°C for 12 to 15 h.

2.3.4.1b. Physical Shearing Method.

The physical shearing method was obtained as a personal communication from M. Gurevitz (developed by John Williams, DuPont), and was used to purify plasmid DNA from small plasmids of less than 15,000 bp. A 200 ml culture of cells was grown, amplified and pelleted as described previously.

The cells were resuspended in 5 ml 10% sucrose in TES buffer (50 mM Tris pH 8.5, 50 mM NaCl, 5 mM EDTA). 100 µl lysozyme (35 mg/ml) and 10 µl RNase A (10 mg/ml) were added followed by incubation at 37°C for 10 min. 5 ml 2% sarkosyl in TES buffer was added, the lysate resumed incubation at 37°C for another 10 min. The DNA was then sheared through a 10 ml glass pipette until the solution was less viscous and would fall dropwise from the pipette. The lysate was then transferred to a tared 125 ml erlenmeyer flask, 1.5 ml EtBr (10 mg/ml) was added and the weight was adjusted to 23 g with TES buffer. To this solution, 21 g solid CsCl was added and the refractive index adjusted to 1.3915, followed by ultracentrifugation in a Sorvall TV850 rotor at 40,000 rpm at 19°C for at least 18 h.

2.3.4.2. Isolation and purification of Plasmid DNA following ultracentrifugation.

Following ultracentrifugation, the gradient was fractionated to recover the lower band containing supercoiled plasmid DNA. The EtBr was removed by extraction with sec-butanol. CsCl was removed by dialysis in ddH_2O at room temperature for 1.5 h. The volume was reduced to 1 ml by several extractions of sec-butanol, followed by phenol and $CHCl_3$ ($CHCl_3$: isoamylalcohol 24 : 1) extractions. The DNA was precipitated as previously described.

2.3.4.3. Rapid small scale isolation and purification of plasmid DNA (mini preps).

The mini prep method is a modification of a procedure described by Maniatis et al. (1982). Mini preps were used to isolate small quantities (1 to 5 μg) of plasmid DNA in order to screen for recombinants in cloning experiments or any time small quantities of DNA were sufficient.

Bacterial cultures were grown in 5 ml L.B. + amp in a shaker incubator at 37°C for 8 h or overnight. Cells were pelleted at 3,000 rpm at 4°C for 5 min. if from liquid culture, transferred to 1.5 ml microfuge tubes and pelleted by a brief spin in a microfuge. Occasionally, cells were taken directly from an L.B. + amp plate. The pellet was resuspended in 350 μl STET buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris pH 8.0). 25 μl Lysozyme (1 mg/ml) was added, the samples then placed in a boiling water bath for 40 s. followed by centrifugation in a microfuge for 10 min. The top 200 μl of the supernatant was removed and to it 3 μl of RNase A (1 mg/ml) was added and the samples incubated at 37°C for 30 min. The samples were then extracted with phenol/$CHCl_3$, and precipitated. Plasmid DNA was then pelleted as described above, resuspended in T 1/10E and checked for purity on a small agarose gel.

2.4. RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

Restriction endonucleases were purchased from the following companies: International Biotechnologies, Inc., Boehringer Mannheim Biochemicals, Bethesda Research Laboratories, and New England Biolabs. Reactions were carried out according to company specifications using 1 to 3 units of enzyme per μg of DNA in a reaction volume of 20 μl per 1 μg DNA for 2 to 4 h. Enzyme digestion reactions were stopped by the addition of EDTA to a final concentration of 10 mM. If the restricted DNA needed to be reacted with another enzyme under different conditions, or needed to be concentrated to a smaller volume, the DNA was precipitated.

2.5. GEL ELECTROPHORESIS.

2.5.1. Agarose gel electrophoresis.

Agarose gel electrophoresis was performed as described by Maniatis et al. (1982). The concentration of the agarose gels depended upon the sizes of the DNA fragments which needed to be resolved from restriction endonuclease digestions. Electrophoresis buffer TAE (.004M Tris, 0.001M EDTA pH 8.0 with acetic acid) was used for resolution of large molecular weight fragments of 10 kbp or more, and TBE (0.089M Tris, 0.089 boric acid, 0.002M EDTA pH 8.0 with HCl) for the resolution of DNA fragments less than 10 kbp. In

order to be able to monitor the progress of the electrophoresis, a running dye of 0.1% bromophenol blue (BPB) in 30% glycerol was added to the samples. Ethidium bromide (EtBr) was added to the gel and the running buffers at a final concentration of 0.5 μg/ml. It was then possible to monitor the progress of the electrophoresis by watching the dye and by looking directly at the DNA using a hand held UV light. Gels were run at room temperature at a current of 25 to 50 mAmps with constant voltage.

2.5.2. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was set up according to Maniatis et al. (1982). The concentration of the polyacrylamide gels varied from 5% to 20% depending on the sizes of the DNA fragments. The running-buffer for PAGE was exclusively TBE lacking EtBr. PAGE was run at room temperature at 10 to 15 mAmps (50 volts).

In order to obtain the best resolution, samples were loaded in volumes not exceeding 5 μl and run with 1/10 volume of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll (type 400) in H_2O). Following electrophoresis, the gels were stained in a buffer of 1 μg/liter EtBr in TBE for 15 to 30 min, examined on a UV transilluminator, and photographed.

2.5.3. Calculation of molecular size of bands separated by electrophoresis.

The approximate sizes of DNA bands on electrophoresis gels were calculated as described by Sealey and Southern (1982) by extrapolation from a plot of log molecular weight versus distance traveled of marker DNAs listed in table 2.3. The entire ranges of fragment sizes are listed in the appendix.

Table 2.4. Marker DNAs used in gel electrophoresis.

Marker	DNA	Fragment Size Range
1	phage λ digested with HindIII + EcoRI	25kb - 0.5kb
2	BRL 123 bp ladder	4000bp - 123bp
3	pBR322 DNA digested with HinfI	1600bp - 50bp
4	pBR322 DNA digested with MspI	620bp - 50bp

2.6. PURIFICATION OF DNA FROM GELS.

2.6.1. Purification of DNA fragments from agarose gels.

2.6.1.1. Crush and extract method.

This method was used for the purification of DNA fragments from agarose gels containing many bands in close proximity. DNA digestions were run on the lowest possible

concentration of agarose which would provide resolution of the band(s) of interest. The band(s) were then located under UV light and removed from the gel with a sterile scalpel. The gel piece was then placed in a microfuge tube and frozen at -70°C for 10 min. The blunt end of a small spatula was then used to crush the frozen piece of gel while still in the microfuge tube. An equal volume of phenol was added, mixed gently and frozen at -70°C for 10 min, followed by centrifugation in a microfuge for 5 min. The aqueous layer containing the DNA was removed and saved on ice while the organic layer was back extracted with an equal volume of TE as before. The aqueous layers were pooled, extracted two more times with phenol, and twice with $CHCl_3$: isoamylalcohol (24:1). The DNA was then precipitated.

2.6.1.2. Electroelution onto DEAE Cellulose Membrane.

Schleicher and Schuell (S&S) NA-45 DEAE cellulose membrane was used when isolating DNA fragments which were easily separated by electrophoresis. Restricted DNA was run on a 0.6% agarose gel until a good separation was achieved. A block was cut from the gel just below the band of interest. A piece of S&S membrane was cut to fit and was inserted into the gap. The gel block was replaced behind the membrane and the current set at 75 mAmps until the band of DNA moved onto the membrane (about 20 min). The progress was monitored by a hand held UV lamp. The membrane was then removed from the gel, washed briefly in sterile ddH_2O and

placed into a microfuge tube containing 400 µl 1.5M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA. The membrane holding the DNA was then incubated at 65°C for 4 h. Following incubation, the membrane was removed and discarded, the DNA was then precipitated.

2.6.2. Purification of DNA from polyacrylamide gels: Crush and Soak method.

The EtBr stained gel was placed on a UV transilluminator; the band(s) of interest were located and carefully removed with a sterile scalpel. Each gel fragment was then placed into a 5 ml disposable syringe fitted with an 18 gauge needle which had been cut to 1/4 of its original length. The syringe was placed above a microfuge tube, the whole system was then placed in a 30 ml corex centrifuge tube and was spun at 5,000 rpm at room temperature in a desk top centrifuge for 10 min. Elution buffer (0.5M NH_4OAC, 1mM EDTA pH 8.0) was added to the crushed acrylamide, and the slurry was incubated with shaking at 37°C for at least 8 h or for overnight. The acrylamide was separated from the DNA by centrifugation through a quick-sep (Isolab) tube. 5M NH_4OAC was added to a final concentration of 2M, and the DNA was precipitated.

2.7. SOUTHERN TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE PAPER.

Southern transfer of DNA to nitrocellulose paper was done as described by Maniatis et al. (1982).

2.8. HYBRIDIZATION OF SOUTHERN FILTERS.

A slight modification of the technique described by Southern (1975) and Maniatis et al. (1982) was used. Nitrocellulose filters were prehybridized at 68°C for 8 h or overnight in 6X SSC (1X SSC is 0.15M NaCl, 0.015M NaCitrate, pH 7.0), 0.5% SDS, 5X Denhardts solution (0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g), 100 μg/ml denatured salmon sperm DNA, Hybridizations with radioactive DNA probes were performed in 6X SSC, 0.01M EDTA, 5% SDS, 100 μg/ml denatured salmon sperm DNA. Hybridizations were done at 68°C when homologous probes were used. The filters were washed in 2X SSC, 0.5% SDS at room temperature for 5 min., 2X SSC, 0.1% SDS at room temperature for 15 min. and 2 washes in 0.1X SSC, 0.5% SDS at 68°C for 30 min. each. For heterologous probes, the final washes were at less stringent temperatures. The filters were allowed to air dry followed by autoradiography.

2.9. NICK TRANSLATION.

Radioactive probes were prepared by nick translation with ^{32}P labeled nucleotides (NEN-DuPont). The procedure used was a modification of that of Rigby et al. (1977). 0.5 to 1 µg DNA was labeled in a reaction volume of 50 µl containing 50mM Tris pH 7.8, 7.5mM $MgCl_2$, 10mM mercaptoethanol, 0.05 mg/ml BSA, and one or more ^{32}P labeled dNTPs (20 to 40 µCi specific activity greater than 600 µCi). Cold dNTPs were added at a concentration of 12.5 nM, omitting the ones used to label the DNA. The DNA substrate was then digested by adding 4 µl fresh DNase I (BMB)(100 ng/ml) for 1 min. at room temperature immediately followed by the addition of 1 to 5 units of DNA polymerase I (BRL, BMB, or NE Biolabs) and incubation at 14°C for 1 to 2 h. The unincorporated ^{32}P nucleotides were separated from the labeled DNA over a Sephacryl S-200 (Pharmacia) column. The incorporation of ^{32}P into the DNA was quantified with DE-81 (Whatman) ion-exchange paper as described by Maniatis et al. (1982) and by measurements in a Beckman LS-133 scintillation counter.

The nick-translated DNA (probe) was denatured by heating at 100°C for 3 min. before adding to the hybridization mix.

2.10. CLONING.

Comparison of the cpDNAs required large amounts of material. In order to obtain sufficient quantities of these cpDNAs, the cpDNA fragments of interest were isolated and cloned into plasmid pBR322 using *E. coli* HB101.

2.10.1. Preparation of Vector DNA.

For cloning of BamHI cpDNA fragments, plasmid pBR322 was cut with the restriction endonuclease BamHI. In order to prevent recircularization of non-recombinant plasmids, BamHI digested pBR322 was treated with alkaline phosphatase (Calbiochem). For this reaction, 5 units of phosphatase for each μg pBR322 DNA was added to the restriction endonuclease digestion. Following incubation at 37°C for 30 min, the phosphatase was inactivated by incubation at 70°C for 30 min followed by 3 extractions with phenol and 3 extractions with $CHCl_3$. The phosphatased pBR322 DNA was then precipitated.

Cloning DNA fragments which had been digested with BamHI + EcoRI did not require phosphatase treatment of the vector. A BamHI/EcoRI digestion of the pBR322 vector yields two DNA fragments of 3986 bp and 376 bp which are easily separable by gel electrophoresis. The larger band was isolated from the gel with S&S membrane as described above, and ligated to the cpDNA to be cloned.

2.10.2. Ligation.

Ligation of DNA was carried out with an excess of insert DNA at a final concentration of 0.1 μg/μl in a reaction containing 66 mM Tris pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM DTT, 0.1 mM ATP and ligase. The amount of ligase added ranged from 1 to 5 units, depending on the age of the enzyme. The ligation reaction was incubated at 4°C for 12 to 18 h. The reaction was monitored by gel electrophoresis.

2.10.3. Transformation.

2.10.3.1. Transformation by the Calcium Chloride Procedure.

Transformation using the calcium chloride procedure was done as described by Maniatis et al. (1982) with the following modifications:

I. Transformation competent cells: 100 ml L.B. was inoculated with 1 ml of an overnight culture of *E. coli* strain HB101. The cells were grown with shaking at 37°C until the $O.D._{650}$ reached 0.4, or approximately 3 x 10^8 cells/ml. The cells were transferred to a sterile centrifuge jar and chilled on ice for 1 h. The suspension was centrifuged at 8,000 rpm at 4°C for 5 min. and gently resuspended in ice cold sterile 50 mM $CaCl_2$. The cells were incubated on ice for 20 min. and pelleted again. The cells were resuspended in 8 ml ice-cold 50 mM $CaCl_2$ and 15% glycerol and allowed to sit on ice overnight or were frozen immediately in liquid nitrogen in 100 μl alliquots in pre-

chilled microfuge tubes. The competent cells were stored at -80°C. The efficiency of transformation ranged from 5 x 10^5 to 1 x 10^7 per μg intact pBR322 DNA; the cells remain competent for up to 3 months.

II. Transformation: Approximately 50 to 200 ng of DNA in ligation buffer was added directly to 100 μl of *E. coli* HB101 transformation competent cells. The cells were incubated on ice for 1 h, followed by a heat pulse at 42°C for 2 1/2 min., placed on ice, and transferred into 1 ml L.B., and incubated at 37°C without shaking for 2 h. The cells were then plated on L.B. + amp plates and incubated at 37°C for no more than 12 h.

2.10.3.2. Transformation using a modification of the Hanahan procedure.

The protocol for this modification of the Hanahan procedure was obtained through Ellen Johnson (personal communication).

I. Preparation of transformation competent cells: A modification of the procedure developed by Hanahan (1983) was used when a high transformation efficiency was required. This procedure was used to clone the BamHI 3b fragments. 100 ml SOB medium (20 g Bacto tryptone, 5 g yeast extract, 5 g NaCl, 5 ml (0.5M) KCl, 10 ml (10 mM) $MgSO_4$ per liter) was inoculated with 1 ml cells from a 2.5 ml overnight culture in SOB medium. The cells were grown to an $O.D._{650}$ of 0.5 to 0.6 with shaking at 37°C. Cells were collected in a

centrifuge tube which had been prerinsed with SOB and chilled. The cells were then incubated on ice for 5 min. The cells were pelleted in an HB4 rotor at 2,500 rpm at 4°C for 15 min. The pellet was gently resuspended in 33.3 ml of ice cold FSB (10 mM KAc, 100 mM KCl, 45 mM $MnCl_2$ 4 H_2O, 10 mM $CaCl_2$), and incubated on ice for 5 min, and pelleted once again. The cells were resuspended in 8 ml ice-cold FSB and 280 µl DMSO was added, with gentle mixing. The cells were incubated on ice for 5 min, followed by the addition of another 280 µl DMSO. The cells were then aliquotted into chilled microfuge tubes (200 µl /tube) and immediately frozen in liquid nitrogen. The transformation competent cells were stored at -80°C.

Transformation efficiencies with this method yielded 1 x 10^6 colonies per µg intact pBR322 DNA for HB101 cells and 1 x 10^7 colonies per µg DNA for ED8654 cells. Cells remained competent for up to 3 months.

II. Transformation: 10 ng of DNA was added to thawed competent cells, and incubated on ice for 30 min. The cells were heat-pulsed at 40°C for 2 min followed by the addition of L.B. broth. The cells were incubated at 37°C with shaking for 1 h. and spread on L.B. + amp selection plates and incubated at 37°C for no more than 12 h.

2.10.4. Screening for recombinant clones.

Screening for recombinant colonies of bacteria was done as described by Gergen et al. (1979) and Grunstein and

Hogness (1975) with the following modifications. After transformation, amp^r colonies were transferred to L.B. + amp and L.B. + tet grid plates and grown overnight at 37°C. Colonies displaying an amp^r tet^s phenotype were transferred to L.B. + amp grid plates. This second grid contained both positive and negative controls. The positive controls were *O. johansen* cpDNA clones carrying Bam 3a, Bam 3b, or Bam 12 fragments. The negative control contained the plasmid pBR322.

The colonies were grown on the screening grids for no more than 12 h at 37°C. A sterile Whatman 541 circle was placed on top of the bacterial colonies. After a few minutes, the filters holding the bacterial colonies were removed from the plates.

In order to lyse the cells in the colonies and denature the DNA, the filters were washed twice with agitation for 5 min. in each of the following solutions: 0.5M NaOH, 0.5M Tris pH 7.5, and 2X SSC (1X SSC is 0.15M NaCl, 0.015M NaCitrate, pH 7.0). The filters were rinsed briefly in 95% ethanol and air dried.

The filters were prehybridized in sealed plastic bags for at least 5 h. at 55°C to 68°C in 0.5% N P 40 (Sigma), 100 μg/ml denatured salmon sperm DNA, 6X SET (0.9M NaCl, 180mM Tris pH 8.0, 6 mM EDTA). Hybridization was done at 0° - 3°C below the T_d (temperature of denaturation) which, under the most stringent conditions in the study was 68°C. The

hybridization buffer was the same as the prehybridization buffer but included 1 μg/filter denatured pBR322 DNA and ^{32}P nick translated probe. The hybridization was done overnight (at least 8 h).

Following hybridization, the filters were removed from the plastic bags and washed three times for 5 min. each in 6X SSC at just below the T_d (for the most stringent condition). The filters were allowed to air dry, followed by autoradiography.

CHAPTER 3

PLASTOME MUTATOR

3.1 INTRODUCTION

Plastome mutants are a useful resource for the study of the genetic content and the contribution of plastid genes to chloroplast development (reviewed by Boerner and Sears 1986). Initially, such mutations were obtained mainly through the isolation of spontaneous mutants (Kutzelnigg and Stubbe 1974, Stubbe and Herrmann 1982). To obtain additional plastid mutants, Epp (1972, 1973) tried to induce chloroplast mutations in *Oenothera hookeri* strain johansen using irradiation and chemical mutagens including ethylmethanesulfonate (EMS). Although, in immediate terms, Epp was unsuccessful at obtaining chloroplast mutations, his chemical mutagenesis experiments with EMS resulted in an M_2 plant line with a high frequency of chloroplast mutations. In this line, Epp was able to identify a recessive nuclear gene which, when homozygous, induces a variety of non-Mendelian mutations differing in phenotypic expression (Epp 1972, 1973). He called the nuclear gene *plastome mutator (pm)*. Once induced, the plastid mutations are not dependent upon the presence of the nuclear *pm* gene. They are inherited in a non-Mendelian fashion.

Although Epp discontinued his work with the plastome mutator for about ten years, he sent seeds to Professor W. Stubbe at the University of Duesseldorf who continued to

propagate the line. The *pm* plant lines used in Sears' lab were derived from Stubbe's stocks.

Flowers on shoots which carried newly-arisen mutant sectors were emasculated and used as the female parent in crosses with wild-type plants. Such a cross places the mutant plastids into a nuclear background which is heterozygous for the *pm* gene. Heterozygous *pm*/+ progeny lack the plastome mutator activity and thus provide a stable nuclear background for the maintenance of the mutant plastids. These seeds were then surface sterilized and germinated on MS medium. From the resulting seedlings, plant meristem cultures were initiated and mutant sectors were selected and maintained on N.T.-agar medium. Initial characterizations by Sears demonstrated that two mutants, *pm8* and *pm11* appeared to have an altered BamHI restriction endonuclease digestion pattern when compared to a wild-type sibling (line C_2). Two chloroplast DNA fragments, Bam 3b and 12 were identified as being different in size from the wild-type fragments by approximately 200 bp (Sears 1983, Sears and Kaplan 1984). These observations led to the proposal that the *pm* gene could have caused a major rearrangement or transposition within chloroplast DNA. To test this possibility, my initial project was to isolate the chloroplast DNA fragments which differed between wild-type and mutant plastids and to characterize them using restriction endonuclease mapping.

3.2. RESULTS

3.2.1. CLONING.

The twelfth largest BamHI fragment (Bam 12) was one of the fragments which was thought to have a different mobility in mutant *pmll*. In order to amplify this DNA fragment it was cloned. cpDNAs from the plant lines designated *pmll* and C_1, shown in Figure 2.1, using the protoplast method.

The Bam 12 fragment was purified from gels using the crush and extract method described in chapter 2 and was inserted into pBR322. *E. coli* strain HB101 host cells were transformed using the calcium chloride procedure. Following selection for the amp^r, tet^s phenotype, recombinant plasmids containing the Bam 12 fragment were identified through Southern hybridization as described in section 2.2.7.3. The clone designations are shown in Table 3.1 and include a clone from the cpDNA clone library which had been constructed in the Sears laboratory from a wild-type *O. johansen* strain from the Duesseldorf collection. This line shall be referred to as "Line D" in this thesis. The insert from plasmid pOj119 from line D was nick-translated and was used as the hybridization probe.

Table 3.1. Recombinant plasmids of BamHI fragments 3b and 12 and their origins.

Plant Line	Plasmid Bam 12 Clone Name	Plasmid Bam 3b Clone Name
D	pOj119	pOj118
C_1	pOjo4	pOjo6
pm11	pPm11	

Recombinant plasmid DNAs containing Bam 12 were isolated using the physical shearing method.

3.2.2. COMPARISON OF RESTRICTION ENDONUCLEASE BAM 12 BETWEEN MUTANT *pm11* AND ITS SIBLING WILD-TYPE LINE C_1.

Plasmids containing the Bam 12 fragments from mutant *pm11* and line C_1 were compared using digestions of BamHI combined with one of the following enzymes: EcoRI, HindIII, HinfI, TaqI and HaeIII (Figure 3.1). The double digestion with BamHI was necessary in order to release the insert from the plasmid and eliminate any differences in the restriction endonuclease patterns which may have been due to the orientation of the insert during ligation. Each one of the bands representing the cpDNA insert appear to be identical between the two clones (Figure 3.1).

Figure 3.1. Comparison of cloned Bam 12 fragments between mutant *pm11* and its sibling wild-type. Cloned Bam 12 insert fragments of wild-type line C_1 (pOjo4), mutant *pm11* (pPm11) and vector (V) plasmid pBR322 control were digested with BamHI and one of the following enzymes: HinfI, TaqI, or HaeIII. DNA was electrophoresed on 8% PAGE.

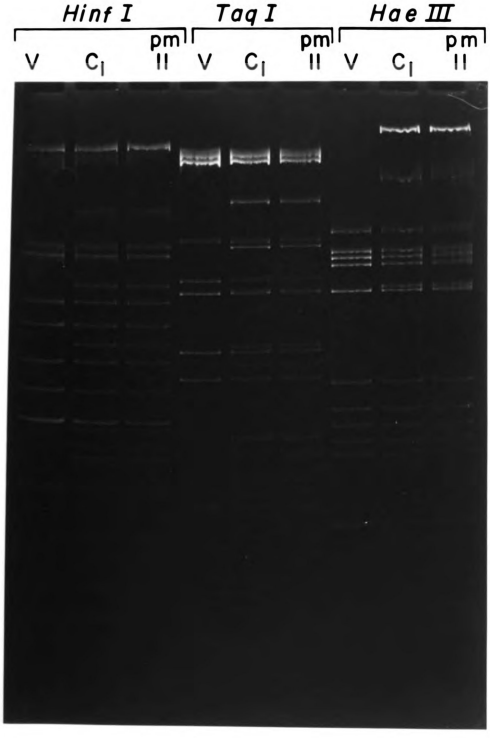


Figure 3.1

3.2.3. *Pm* MUTANTS FALL INTO TWO DISCRETE CLASSES.

In a collaborative project with Ellen Johnson, Linda Schnabelrauch and Ruth Wolfson, cpDNAs from a number of *pm* mutant lines were digested with restriction endonucleases BamHI and HaeIII, electrophoretically separated on a 2% agarose gel, Southern blotted and probed with ^{32}P nick translated total cpDNA from wild-type line C_1. One of these autoradiograms is shown in Figure 3.2. Examination of the bands indicated by arrows in the figure reveals that the mutant cpDNAs fit into two discrete classes: Mutants *pm7* and *pm8* do not resemble either wild-type line D or line C_1 particularly in the mobilities of bands 3b and 12. As suggested by the restriction digestion of fragment Bam 12 described in section 3.2.2, mutant *pm11* seems to be identical to line C_1.

These results indicated that the cpDNA differences observed previously were not correlated with the occurrence of particular mutations. Rather, the existence of two classes of cpDNAs suggests that cpDNA polymorphisms were pre-existing among the descendants of Epp's original *pm* line. To compare cpDNA from mutant *pm8* with a wild-type cpDNA representing this polymorphism group, it was first necessary to identify and recover plants carrying the appropriate wild-type plastome. To this end, seeds were germinated to reisolate line C_2 which had been used for the

Figure 3.2. *Plastome Mutator* induced Plastome I mutants and their sibling wild-types fall into two distinct classes. BamHI digested cpDNA from wild-type lines D, C_1 and from plastome mutants *pm7*, *pm8*, and *pm11* was run on a 0.6% agarose gel. Plastome III cpDNA also digested with BamHI was used as a comparison. DNA was Southern blotted onto nitrocellulose and probed with ^{32}P nick translated total cpDNA from wild-type line D.

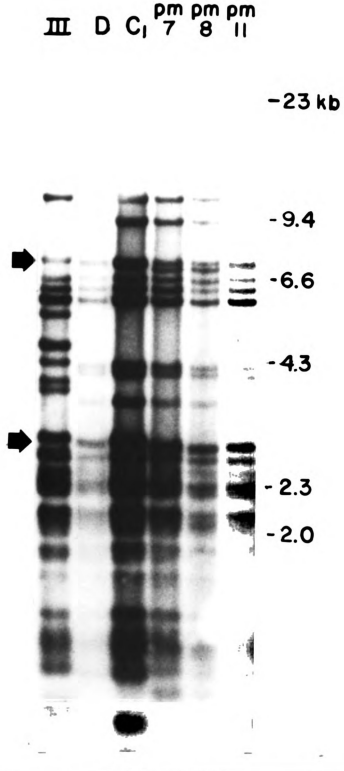
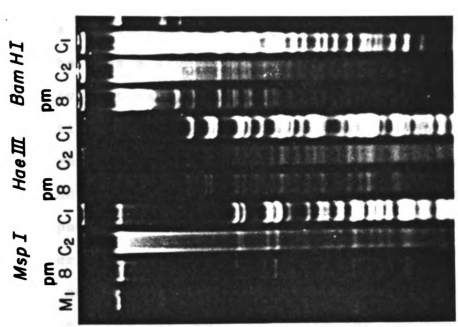


Figure 3.2

initial studies conducted by Sears in Duesseldorf (refer to Figure 2.1). It was hoped that these plants would be the correct wild-type control since they resulted from self-crossing a plant known to be a sibling of the plant on which the *pm8* mutation arose (Figure 2.1). cpDNA from mutant *pm8* and wild-type line C_2 were isolated using the modified whole cell DNA method. cpDNAs were digested with frequently cutting enzymes (Figure 3.3A) The DNAs were blotted to nitrocellulose filters and were hybridized using the variable Bam 3b fragment as a probe (Figure 3.3B). No major alteration in cpDNA between mutant *pm8* and wild-type C_2 could be detected. Some degradation of cpDNA of wild-type line C_1 is evident (BamHI digestion in Figure 3.3).

Figure 3.3. Comparison of cpDNA between mutant *pm8* and its sibling wild-type line C_2 using restriction endonuclease digestion and Southern hybridization. Panel A: Digestion of *pm8*, wild-type line C_2, and wild-type line C_1 with MspI, HaeIII, and BamHI. DNA was electrophoresed on a 2% agarose gel with marker 1 (M_1). Panel B: Autoradiogram of the gel from panel A using ^{32}P nick-translated Bam 3b insert from plasmid pOj118 as the hybridization probe.


Msp I
HaeIII
Bam HI
pm
M1 8 C2 C1
pm
8 C2 C1
pm
8 C2 C1
A.


Msp I
HaeIII
Bam HI
pm
8 C2 C1
pm
8 C2 C1
pm
8 C2 C1
21 Kb
5.0
3.5
2.0
1.5
0.9
B.

Figure 3.3

3.3. DISCUSSION

Mutants *pm8* and *pm11*, as well as the other mutants resulting from the activation of the *plastome mutator* gene have different phenotypes. In all of the mutants studied to date, except for one, *pm7*, no specific lesions have been identified. The experiments described in this chapter indicate that cpDNA of mutant *pm11* has the same restriction endonuclease patterns as its sibling wild-type line C_1. This mutation *pm11*, therefore, is most likely the result of a point mutation which does not give rise to a RFLP for the restriction enzymes used in this study.

Mutant *pm8* shows essentially the same cpDNA restriction endonuclease patterns as wild-type line C_2. In Figure 3.3, there is a hint that the third largest band hybridizing to the probe has a slower mobility and thus a larger size than the equivalent band of wild-type line C_2. However, careful examination of the gel revealed that all of the bands in lane 2 run slightly faster than the bands in lane 1. These differences are thus due to an artifact of the electrophoresis and do not represent a physical characteristic of the DNAs. Some factors which are known to affect mobility of DNA in gel electrophoresis include: Quality of the DNA, salt concentration and imperfections in the gel (Sealey and Southern 1982, and Maniatis et al. 1982). A comparison of cloned DNAs of this fragment from

mutant *pm8* to its sibling wild-type line C_2 was not possible due to the difficulty of obtaining a clone of Bam 3b from line C_2 (discussed more extensively in chapter 4).

The discovery that our *pm* line contained two different plastid types caused us to trace back a pedigree for the origin of these plant lines (Figure 2.1). The pedigree starts with a *pm pm* plastome I line derived from the original E-15-7 plant described by Epp (1973). Seeds resulting from self-crossing of this line were sent by Epp to Stubbe (University of Duesseldorf) and were placed in the field in two different years: 1976 (# 76/119a) and 1978 (# 78/311). The plants in both of these years were self-crossed. It is not known how many plants were used in this set of self-crosses in either season. Seeds from the 76/119a line were planted in 1980 and given the designation 80/959. Seeds from line 78/311 were planted in 1980 and 1981 and were designated 80/960 and 81/124 respectively. The plants in this F_2 generation, grown in two different field seasons, gave rise to sectors containing mutant plastids from which the Sears lab *pm* mutants were derived. It is this F_2 generation to which we can trace back the two different wild-type plastomes which we have observed.

However, since all of the plant lines can be traced back to a single plant (Epp's original *pm pm* plastome I plant) it is possible that the two plastid types could have been present from the start of the pedigree. It has been demonstrated that different branches of the same plant may

contain different plastid types when plastome mutations occur and sort out during subsequent cell divisions (Stubbe and Herrmann 1982, Epp 1972, 1973). Possibly, the cpDNA differences resulted from neutral mutations caused by the *pm* gene or by the original EMS mutagenesis. The possibility also exists that the two distinct plastid types appeared first in the F_2 generation resulting from cpDNA alterations caused by the action of the plastome mutator. (Figure 2.1). Because it is not known how many plants in lines 76/119a and 78/311 were used in self crosses to produce the F_2 generation and because seeds for those plant lines are no longer available, it is impossible to say at which point the two different plastid types arose. Line C_1 was recovered from a limited number of seed packets from the crosses of 81/124. Each seed packet represents a different plant from the field. With such a limited sample size, it would be premature to conclude that the 81/124 line was homoplastidic.

Wild-type line D is from a different stock maintained in Duesseldorf for many years by Stubbe, but both the Cornell and Duesseldorf lines have apparently descended from Cleland's original *O. johansen* strain (Stubbe, personal communication). The differences between cpDNAs of lines D and C may be due to spontaneous variation, but it should also be noted that line D had never been through a mutagenesis treatment. As mentioned previously, mutagenesis of the Cornell line could have caused the cpDNA alterations.

The remainder of the thesis shows the characterization of the regions of variability in the cloned Bam 3a and Bam 12 from the wild-type cpDNAs.

CHAPTER 4

COMPARISON OF CLONED cpDNA RESTRICTION ENDONUCLEASE FRAGMENTS BamHI 3b AND 12 FROM WILD-TYPE PLASTOME I LINES OF *OENOTHERA HOOKERI* STRAIN JOHANSEN

4.1. INTRODUCTION

The original intention of my thesis was to investigate cpDNA alterations in plastome mutants of *Oenothera* induced by the *plastome mutator* gene characterized by Epp. Although the characterizations described in chapter 3 indicated that no major DNA changes could be correlated to the mutation events, the restriction fragment analyses did suggest that two DNA fragments in the BamHI pattern were variable. Differences between cpDNAs of lines D and C may be due to naturally occurring variation. The variation in fragment sizes may be similar to that which is seen when *Oenothera* plastome types I - V are compared (Gordon et al. 1982). The purpose of this part of the study was to locate the BamHI variable fragments on the physical map of the *Oenothera* cpDNA, to characterize the areas of variability between the wild-type lines C_1, C_2 and D, and to investigate any possible homology between the two regions.

4.2. RESULTS

4.2.1. CLONING.

Characterization of the areas of interest in the cpDNA require workable amounts of DNA. The best way to obtain sufficient quantities is by cloning. The cloning of Bam 12 fragment from wild-type line C_1 has been described in chapter 3. cpDNA from wild-type line C_2 was prepared using the protoplast method, and band Bam 12 was cloned from this line also. The BamHI 3b band was also cloned as described in chapter 2, using a modification of the Hanahan transformation procedure. Bam 3b is larger than Bam 12 and thus required a higher transformation efficiency. Clones and subclones are listed in Table 4.1.

Plasmid DNAs were isolated using the physical shearing or the chemical lysis method as described in chapter 2.

Table 4.1. Recombinant plasmids with cpDNA inserts from *Oenothera johansen*. The number given for the BamHI fragment refers to the relative size of the band in the cpDNA restriction pattern generated by the enzyme.

Plasmid Clone Name	BamHI Fragment	Plant Line
pOjl19	12	D
pOjo4	12	C_1
pOejl	12	C_2
pOjl18	3b	D
pOjo6	3b	C_1

During the pursuit of the Bam 3b clones, colony hybridizations were used to screen for the wild-type line C_1 clone, using the Bam 3b insert DNA from plasmid pOj118. Following this initial screen, the identity of the correct clone was confirmed by a comparison of BamHI + EcoRI digestions of plasmid pOj118 and Southern hybridization. The Bam 3b clone from line C_1 was obtained at a very low frequency of one out of 3,000 recombinant colonies screened. In contrast, Bam 3b did not appear to be difficult to clone from line D: Out of six clones carrying BamHI fragments of this size (7.5 kb), one contained the 3b fragment, while five carried the 3a fragment. It is not clear why the Bam 3b fragments of wild-type lines C_1 and C_2 have been difficult to clone. Screening for the fragment Bam 3a was carried out at the same time 3b was being sought. These two fragments are the same size and were co-purified from agarose gels. Colony screening for Bam 3a has in three consecutive experiments consistently detected over 75 positive signals per 300 colonies, whereas Bam 3b gave no positive hybridization except to the positive controls. It could be that the insert DNA may be in some way toxic to the host bacterial cell. The DNA may have contained sequences that are more recombinogenic in lines C_1 and C_2 than they are in line D. In order to try to eliminate the possibility of such a problem, *rec A*$^-$ host *E.coli* HB101 was used, but without success. The problem also may be due to the quality

of the DNA (Maniatis et al. 1982). This reason is unlikely because of the relative ease with which other clones have been obtained using the same procedures.

4.2.2. LOCATION OF THE BAMHI 3b AND 12 VARIABLE REGIONS ON THE PLASTOME I cpDNA.

A physical map of cpDNA from *O. hookeri* was constructed by Gordon et al. (1981, 1982) using the infrequently cutting enzymes SalI, KpnI, and PstI. These enzymes create 10 to 13 fragments in *Oenothera* cpDNA, while BamHI creates approximately 65 fragments. Because BamHI creates so many fragments, no complete BamHI map of cpDNA is yet available for *Oenothera*. Thus, in order to locate the Bam 3b and 12 fragments on the cpDNA physical map, it was necessary to prepare a Southern blot having cpDNA digested with SalI, KpnI, or PstI.

The Bam 3a and 3b fragments had already been localized on the cpDNA by Ellen Johnson. The Bam 3b probe prepared from pOjl18 hybridized to PstI band 1, SalI bands 5 and 7, and KpnI band 5 as indicated in Figure 4.1. This result demonstrated that Bam 3b is located within the large single copy region.

In separate experiments, I hybridized the Bam 12 1.6 kb subcloned fragment and a smaller piece of the subcloned 1.6 kb fragment (a 280 bp restriction fragment from pOjl19a

Figure 4.1. Physical map of *Oenothera hookeri* strain *johansen* plastome I. Adapted from Gordon et al (1981, 1982), using chloroplast gene names proposed by Hallick and Bottomley (1983) listed below.

Gene	Protein
rbcL	large subunit of RUBISCO
atpA	alpha subunit of coupling factor
atpB	beta subunit of coupling factor
atpE	epsilon subunit of coupling factor
atpF	I subunit of coupling factor
atpH	proton-translocating subunit of coupling factor
petA	cytochrome f
petB,D	cytochrome b6, subunit 4 of cyt. b6f complex
psbA	32 kilo dalton herbicide binding protein
psbB	51 kilo dalton chlorophyll a binding protein
psaA	PSI $_{p}$700 chlorophyll a appoprotein

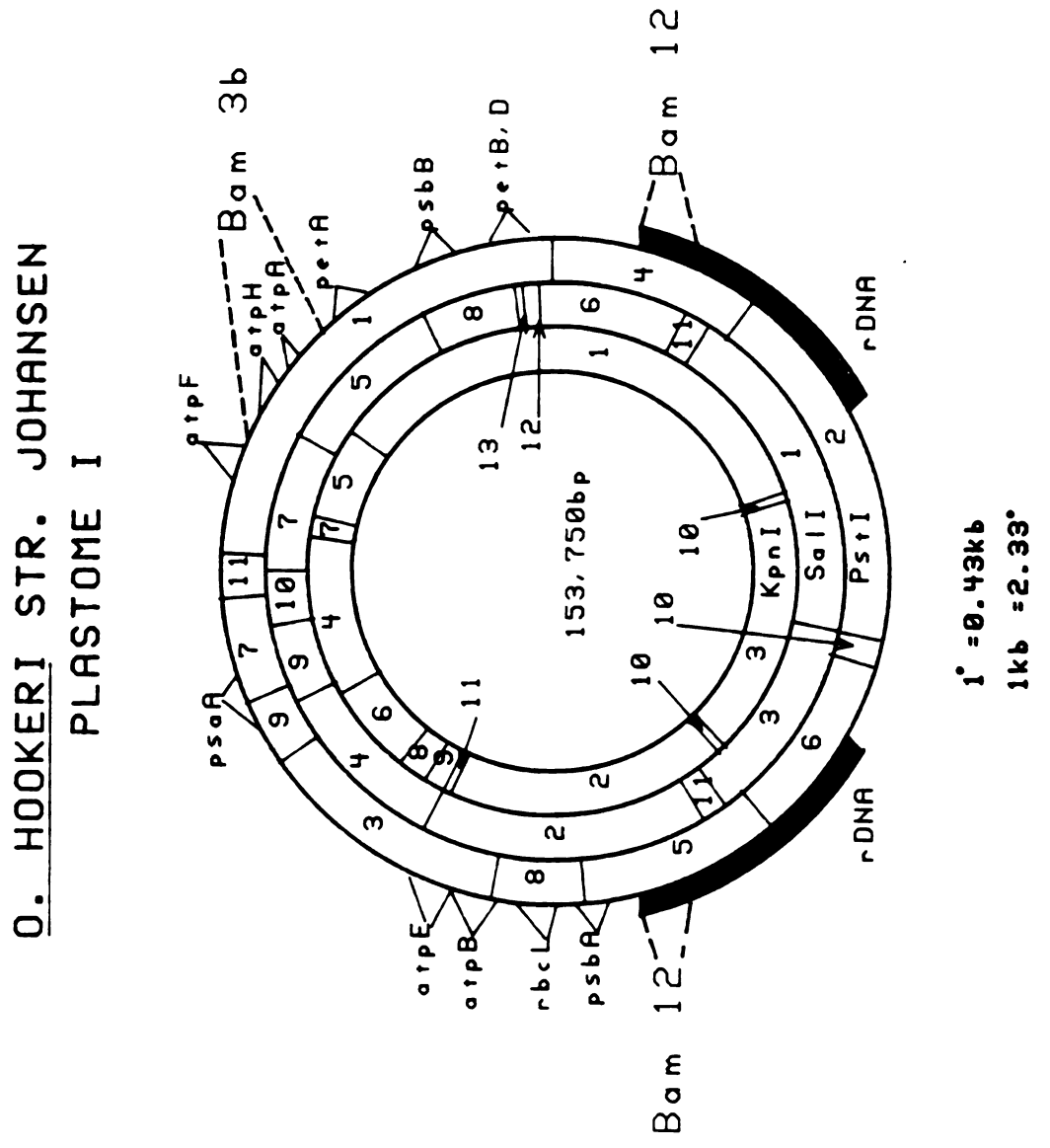

O. HOOKERI STR. JOHANSEN
PLASTOME I
153,750bp
Bam 3b
Bam 12
Bam 12
rDNA
rDNA
psaA
atpF
atpH
atpA
petA
psbB
petB,D
atpE
atpB
rbcL
psbA
KpnI
SalI
PstI
1° =0.43kb
1kb =2.33°

Figure 4.1

which defines the variable region) to a Southern blot of total cpDNA from line C_1 digested with BamHI, SalI, KpnI, PstI, and MspI (Figure 4.2B). These two probes both hybridized to PstI bands 4 and 5, SalI bands 2 and 6, and KpnI bands 1 and 2. Their positions are indicated on the physical map (Figure 4.1). The SalI, KpnI and PstI fragments which hybridized to the probes span the border between the inverted repeat and the large single copy region of the cpDNA molecule. Figure 4.2 also shows the hybridization pattern of the probes to cpDNA fragments produced by more frequently-cutting enzymes BamHI and MspI. The band which hybridizes to the pOjl19 probe is double molar. These results suggest that the entire Bam 12 fragment including the variable region is part of the inverted repeat (Figure 4.1.).

Figure 4.2. Southern Hybridization of Bam 12 insert DNA to restriction endonuclease digested *Oenothera* plastome I cpDNA. Panel A: Restriction endonuclease digestion of cpDNA from line C_1 digested with BamHI, SalI, KpnI, PstI, and MspI. Samples were electrophoresed on a 0.6% agarose gel and transferred to nitrocellulose. Panel B: Autoradiogram of the gel from panel A probed with ^{32}P nick translated 280bp HinfI fragment from line D (pOj119a).

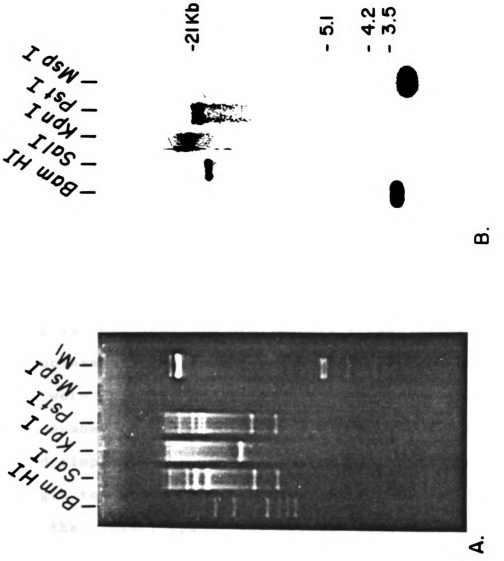

Msp I
Pst I
Kpn I
Sal I
Bam HI
-21Kb
- 5.1
- 4.2
- 3.5
B.
M1
Msp I
Pst I
Kpn I
Sal I
Bam HI
A.

Figure 4.2

4.2.3. CONSTRUCTION OF A PHYSICAL MAP FOR THE BAM 12 FRAGMENT OF THE THREE WILD-TYPE PLASTOME I LINES USING RESTRICTION ENDONUCLEASES.

In order to determine if the variation in fragment size was due to creation of new restriction endonuclease sites, discrete insertion/deletion events, or a dispersed alteration of sequences, restriction endonuclease digestion experiments were performed utilizing the cloned DNA described in Table 4.1.

4.2.3.1. Preliminary Mapping of the Entire 3.0 kb BamHI 12 Fragment.

Since the insert in plasmid pOjl19 is the largest of the three, it was used in preliminary screening of restriction endonucleases. The first set of restriction endonucleases which were tested recognize 6 bp sequences and generate a small number of bands (0 to 3) from the vector pBR322. This helped simplify the initial mapping process and allowed me to begin to define a more discrete region of variability. Since the insert cpDNA was cloned into the BamHI site of pBR322, plasmid pOjl19 was first digested with BamHI in order to release the insert from the vector, and was then digested with one of each of the restriction endonucleases shown in Table 4.2. Those enzymes which were shown to digest the pOjl19 insert DNA were then used in digestion experiments of the line C_1 Bam 12 insert plasmid DNA.

Table 4.2. Restriction endonucleases tested for digestion of the Bam 12 insert DNA. Enzymes which showed no digestion of the insert were tested for and demonstrated activity on phage lambda DNA.

Restriction endonuclease	# of insert fragments
EcoRI	3
ClaI	3
BglII	2
PvuII	no digestion
NruI	no digestion
XbaI	no digestion
XhoI	no digestion

(The Bam 12 fragment of Line C_2 was in the process of being cloned and was not yet available for comparison at this point.) Only one sub-fragment differed in mobility in every comparison (Table 4.3).

These results indicated that a discrete region of the Bam 12 fragment varied between the two lines. To facilitate fine-mapping with enzymes which cut more frequently, I decided to subclone the region of variability.

The variable region is contained entirely within a 1.6 kb fragment liberated during the BamHI/EcoRI digestion. The entire Bam 12 fragment contains two EcoRI sites. In order to be able to determine the cut sites which define the 1.6 kb subfragment, it was necessary to find the orientation of that fragment within the insert. To determine if it is internal (defined by two EcoRI sites) or if it is located at an end of the insert (defined by one EcoRI site and one BamHI site).

In order to be able to define these fragments, a series of end-mapping experiments was carried out. End-mapping experiments take advantage of the known locations of restriction sites on the vector DNA since the entire vector has been sequenced (Maniatis et al. 1982, BRL reference catalogue). The restriction site for EcoRI on plasmid pBR322 is located at base 0 (4362). The single BamHI site is located at base 376. Insertion of the BamHI cpDNA fragment into the vector results in a duplication of that

Table 4.3. Single and double digestions of Bam 12 inserts from pOjl19. A * indicates the band which contained the variable region when compared to the same digestions of pOjo4.

Restriction endonuclease	# of fragments	approximate size (kb)
EcoRI	3	1.60* 0.95 0.75
ClaI	3	1.50* 1.40 0.10
BglII	2	1.80* 1.40
EcoRI/ClaI	5	1.40* 0.95 0.56 0.19 0.10
BglII/ClaI	4	1.40* 1.40 0.20 0.10
EcoRI/BglII	4	1.60* 0.95 0.60 0.15

single BamHI site. Using this information, and combining it with restriction endonuclease digestions, the orientation of the insertion and the definition of the end fragments could proceed.

Plasmids pOjl19 and pOjo4 were digested with EcoRI alone. Fragments located at the end of the insert would have pBR322 DNA attached. Determination of the orientation of the ends was possible because of the uneven amount of pBR322 DNA on either side. The left end would have an additional 376 bp of pBR322, the right end would have about 4000 bp. The plasmids pOjl19 and pOjo4 were also digested with both EcoRI and BamHI for comparison. The results are shown in Table 4.4. The resulting map is shown later in the chapter in Figure 4.8, panel A.

The variable region is contained within a 1.6 kb EcoRI/BamHI fragment which was convenient for subcloning using pBR322. Subcloning was necessary to simplify the fine mapping of the variable region. The BamHI 12 1.6 kb subfragments were subcloned into plasmid pBR322 as described in chapter 2. The subclones use the same clone name with the addition of an "a" (pOjl19a, pOjo4a, pOejla).

Table 4.4. End mapping experiments. Plasmids pOjl19, pOjo4 were digested with EcoRI or both EcoRI and BamHI for determination of the orientation of the variable region and the end fragments. A * indicates the region which varies in size when pOjl19 and pOjo4 are compared. In the double digestion, vector pBR322 bands are indicated by parentheses. Fragments which change in size and hence are located at the ends are underlined. L = left end, R = right end. (Figure 4.8,A).

restriction endonuclease	# of fragments	approximate size (kb)
EcoRI	3	5.70 R *
		1.27 L
		0.70
BamHI/EcoRI	5	4.00 (pBR322)
		1.60 R *
		0.90 L
		0.70
		0.376 (pBR322)

4.2.3.2. Fine mapping of the variable region of Bam 12.

Insert DNA was isolated from the plasmids pOjl19a, pOjo4a, and pOejla and was digested with a number of restriction endonucleases (summarized in Table 4.5).

Size differences were estimated for the three variable regions by a digestion of the subclone plasmid DNAs with HincII, shown in Figure 4.3. In order to accurately measure the differences between the DNAs, digestion products were compared to size standards run on the same gel. These differences were estimated to be 95 bp between line D and line C_1, 76 bp between line C_1 and C_2, and 170 bp between line D and C_2. The HincII digestion also allowed localization of the single HincII site of the 1.6 kb insert on the physical map (Table 4.6) using the same reasoning as in the plasmid DNA end-mapping experiments.

The smallest restriction endonuclease fragment containing the variable region in pOjl19a (line D) is a 280 bp HinfI fragment shown in Figure 4.4. The corresponding bands were calculated to be approximately 185 bp in pOjo4a (line C_1 and 109 bp in pOejla (line C_2).

Table 4.5. Single digests of the 1.6 kb insert of subclone pOj119a. An * indicates the variable fragment; no digestion indicates lack of restriction site for that enzyme within the insert DNA. The activity of enzymes which did not cut the insert DNA was confirmed by digesting plasmid pBR322 DNA.

restriction endonuclease	# of fragments	approximate size (bp)
HinfI	11	443 280* 180 160 138 104 72 4<50
TaqI	10	610* 295 240 178 135 110 4<50
MaeIII	4	620* 460 320 180
RsaI	2	1500* 115
ClaI	2	1515* 100
HincII	2	1050* 550
DraI	no digestion	
HaeIII	no digestion	
MspI	no digestion	
ScrfI	no digestion	

Figure 4.3. Restriction endonuclease digestion and agarose gel electrophoresis of plasmid DNAs from pOj119a, pOjo4a, pOejla enabling the calculation of the sizes of the BamHI 12 subclone variable bands. Plasmid DNAs containing the 1.6kb Bam 12 subfragments from the three wild-type lines D (pOj119a), C_1 (pOjo4a), C_2 (pOejla) and pBR322 (P) were digested with DraI or HincII. The DNA was electrophoresed on a 0.8% agarose gel with markers 1 (M_1) and 3 (M_3).

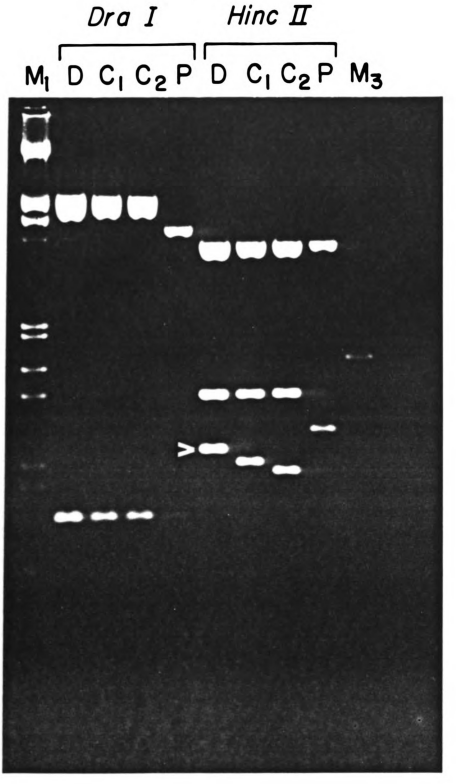


Figure 4.3

Figure 4.4. Restriction endonuclease double digestions of the Bam 12 1.6kb subclones. 1.6kb insert DNA from wild-type lines D (pOj119a) C_1 (pOjo4a) and C_2 (pOejla) were digested with HinfI (H), HinfI + AluI (Al) and HinfI + MboI (Mb) and electrophoresed on 10% PAGE with markers 3 (M_3) and 4 (M_4).

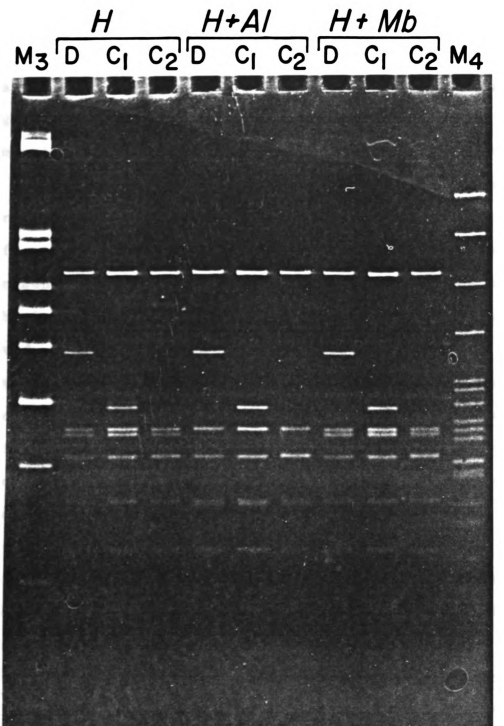


Figure 4.4

As summarized in Table 4.5, digestion of the 1.6 kb inserts with HinfI revealed at least 11 bands (Figure 4.4). Digestion with TaqI revealed at least 10 bands (Figure 4.5). Digestion with MaeIII revealed 4 bands (Figure 4.6) and digestion with RsaI revealed 2 bands (Table 4.5). To begin assigning map locations to the subfragments, a series of end-mapping experiments was conducted. These experiments were based upon the same reasoning as the end-mapping experiments of the entire 3.0 kb insert with the following modifications. Because the enzymes used in the fine mapping experiments generate many fragments in the vector as well as in the insert, a large portion of the vector was physically removed by selecting a fragment after digestion of the plasmids with SalI and PstI followed by gel electrophoresis. These two enzymes, which each cut the vector once without cutting the insert, liberate the insert DNA with vector DNA on both sides (refer to Figure 4.8, panel B). The EcoRI end has an additional 748 bp attached and the BamHI end has an additional 276 bp of pBR322 DNA attached. When this DNA is subsequently digested with another restriction endonuclease and compared to insert DNA lacking the vector DNA ends, but digested with the same enzyme, two fragments should differ in size. This difference should correspond to the original subfragment of the insert plus the piece of attached vector

DNA. The amount of attached DNA is calculated using the published information containing the location of restriction sites in pBR322 (Maniatis et al. 1982, BRL reference catalogue).

This set of end-mapping experiments was performed using the enzymes HinfI, TaqI, MaeIII, and RsaI. Results of these digestions are shown in Table 4.6. Figure 4.5 shows a gel of the HinfI and TaqI digestions. The bands with altered mobility were identified and then assigned an end position on the map (Figure 4.8, panel B).

End cut sites were assigned for MaeIII and RsaI in the same manner. These bands were also placed on the map, as shown in Figure 4.8, panel B.

The end-mapping digestions with HinfI and TaqI demonstrate the bands at the ends do not contain the variable region, since the variable band does not change its size whether insert alone or insert plus vector ends is cut.

Table 4.6. End mapping for comparison of the 1.6 kb inserts from all three lines with and without attached vector DNA. The inserts alone and the inserts plus pBR322 SalI and PstI vector ends were digested with the enzyme indicated to yield a number of fragments. Sizes are shown for fragments from pOj119a. Bands from vector pBR322 are indicated by parentheses. A * indicates the variable band and underlined bands indicate fragments located at an end. L = left side and R = right side of the physical map (Figure 4.8, panel B).

Restriction endonuclease	aprox. size 1.6 kb	aprox. size PstI/SalI
HinfI	443 R	840 L
	280 *	640 R
	180 L	280 *
	138	138
	104	104
	72	72
	4<50	4<50
TaqI	610*	610*
	295	540 L
	240	400 (pBR322)
	178 L	370 R
	135	295
	110 R	240
		135
	4<50	4<50
MaeIII	620 L *	958 L *
	460	596 R
	320 R	460
	180	205(pBR322)
		180
		170(pBR322)
RsaI	1500 L *	1800 L *
	115 R	600 R
		240(pBR322)
HincII	1050 R	
	550 L *	

Figure 4.5. Restriction endonuclease digestions of Bam 12 1.6kb subclone inserts to define the end fragments. 1.6kb insert DNA (i) and insert DNA with the asymmetric vector ends (v) from the three wild-type lines D (pOjl19a), C_1 (pOjo4a), and C_2 (pOejla) were digested with HinfI (H), TaqI (T) or both and run on 10% PAGE with marker 2 (M_2). Fragments located at the ends of the insert in one HinfI digestion experiment and one TaqI digestion experiment are indicated by arrows. Bands which are the result of partial digestion are indicated with "p"'s.

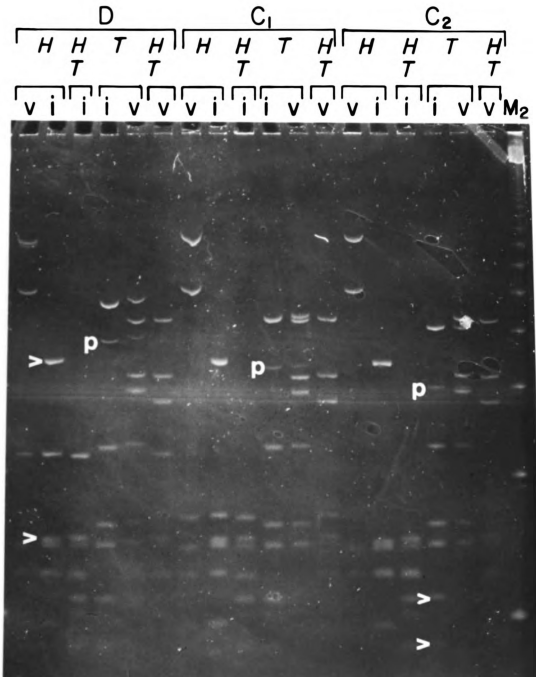


Figure 4.5

The TaqI digestions were plagued with partial digestion, which occurred more frequently than with the other enzymes. Partial digestion is indicated by the appearance of faint bands in the gels (Figure 4.5 and Figure 4.6). These partial bands made the TaqI digestion difficult to interpret. TaqI is sensitive to methylation when the guanosine in the recognition site is methylated, TCG^{me}ATC (Manitis et al. 1982 and BRL techline, personal communication). Although cpDNA is not methylated, restriction endonuclease digestion experiments were performed on cloned cpDNA which had been isolated from Me^{+} bacterial cells.

One particular TaqI partial band appeared in every digest to different degrees, and is indicated with "p"'s in Figure 4.5. This band is approximately 110 bp smaller than the largest band. By comparing this band with the other two subclones, I could determine that this partial band contains part of the variable region (compare Figure 4.6 TaqI digestions below band 1). This partial band indicates the presence of a TaqI site 110 bp from one side of the largest TaqI fragment, probably on the right side of the variable region (Figure 4.8 panel B), because double digestion with TaqI and MaeIII did not cut this 500 bp fragment (Figure 4.6).

Using the information generated from the end-mapping experiments, restriction sites were assigned to the physical

Figure 4.6. Single and double digestions of the Bam 12 1.6kb insert DNAs. Cloned 1.6kb insert DNAs from wild-type lines D (pOj119a), C_1 (pOjo4a), and C_2 (pOejla) were digested with TaqI (T), MaeIII (M)), HincII (Hc), or combinations of the enzymes. DNA was electrophoresed on 8% PAGE with markers 2 (M_2) and 4 (M_4).

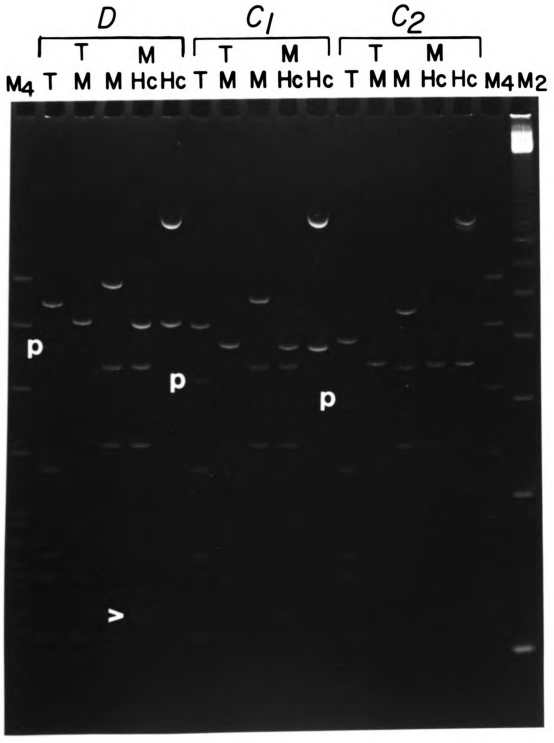


Figure 4.6

map (Figure 4.8, panel B). Analysis with these enzymes showed that the variable region is contained within a 620 bp MaeIII fragment which was mapped to the EcoRI end of the 1.6 kb insert. HinfI produced too many restriction fragments to allow for direct placement of the 280 bp variable fragment on the physical map. Assignment of this fragment was then undertaken in a more indirect manner.

The next set of mapping experiments was done with double digestions using combinations of HinfI, TaqI, HincII and MaeIII. This allowed determination of the order of some of the HinfI, TaqI and MaeIII fragments. Figure 4.6 shows digestions using combinations of MaeIII, TaqI and HincII. Examination of these digestions shows that HincII, which cuts the insert only once has its restriction site within the largest MaeIII band. In the MaeIII/HincII double digestions, the 620 bp MaeIII fragment is cleaved into fragments of 475 bp and 145 bp. A comparison of the same digestions of all three subclones (compare between lines D, C_1, and C_2 of Figure 4.6) reveals that the region of variability is located on the larger of these subfragments.

Double digestion experiments with HinfI and HincII are shown in Figure 4.7. It was hoped that since HincII digests the 1.6 kb fragment only once, a double digest with HincII + HinfI would be able to show which of the HinfI fragments contains the HincII site. That result could have helped in the assignment of the 280 bp HinfI fragment on the map.

Figure 4.7. Single and double digestions of the Bam 12 1.6kb insert. 1.6kb insert DNAs from wild-type lines D (pOj119a), C_1 (pOj4a), and C_2 (pOejla) were digested with HinfI (H), HincII (Hc), or both, and electrophoresed on 10% PAGE with Markers 2 (M_2) and 4 (M_4).

Figure 4.7

However, the bands in the HinfI and HinfI + HincII lanes of Figure 4.7 looked identical. It could not be determined which HinfI fragment is cut with HincII. Examination of recognition sequences of the two enzymes revealed overlapping cut sites. It is possible, then, that the right HinfI border of the variable region also contains a HincII site. (This is considered more thoroughly in the discussion.)

On the left side of the variable region, HinfI and TaqI sites map very close to each other. Examination of HinfI and TaqI recognition sequences also have revealed overlapping cut sites. The variable region, therefore is located between a 280 bp HinfI/TaqI, HinfI/HincII fragment.

In an attempt to cut into the variable region, double digestions using HinfI with either AluI or MboI were performed. Figure 4.4 shows that these two additional enzymes did not cut into the variable region. Since MboI and AluI did not cut into the variable region, no further mapping using these enzymes was pursued.

Figure 4.8. Restriction endonuclease map of the cloned BamHI 12 fragment. Restriction endonuclease map generated from restriction endonuclease digestions is shown in two parts. Panel A: Map of the BamHI 12 3.0kb cloned region. Panel B: map of the 1.6kb variable region with bordering pBR322 DNA from the PstI site on the left and from BamHI to its SalI site on the right. The variable regions are shown as blocked areas. The TaqI site determined by partial digestion is indicated in parentheses. The main map is of DNA from pOjl19 and pOjl19a. Corresponding areas of variation are shown as blocks below the variable region of pOjl19 and pOjl19a. All other areas are of the same size in all three plasmids. An asterisk indicates a restriction site with two possible locations.

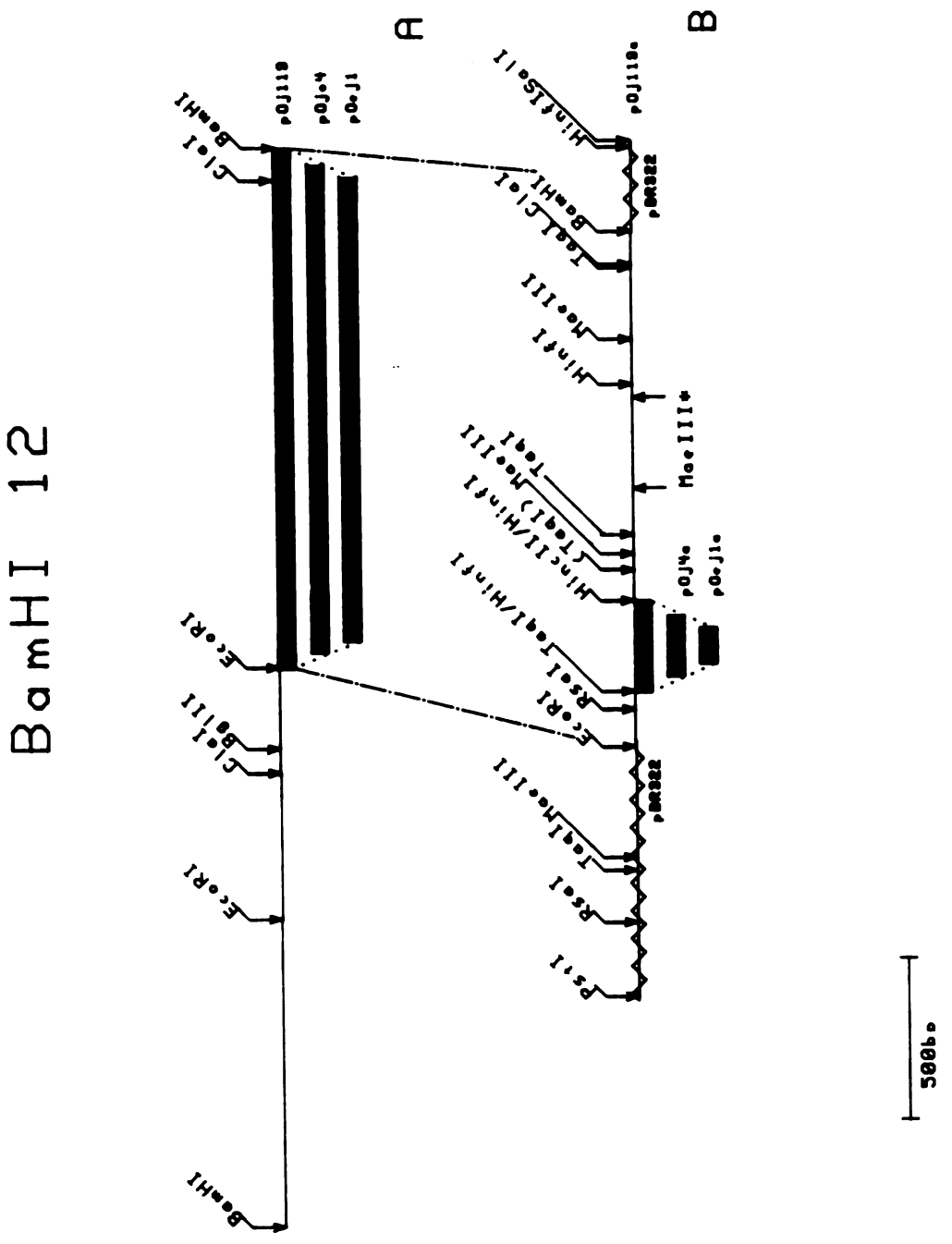


Figure 4.8

4.2.4. CONSTRUCTION OF A PHYSICAL MAP OF BAM 3b DNAs FROM PLASMIDS pOj118 AND pOjo6 USING RESTRICTION ENDONUCLEASES.

Since the insert DNA of plasmid pOj118 appeared to be larger than the insert DNA from pOjo6, insert DNA from pOj118 was used in preliminary screening of restriction endonucleases. The first set which was tested consisted of 6 bp recognizing enzymes which generate a small number of fragments from vector pBR322 DNA. The insert DNA was released from the vector by initial digestion of the plasmid with BamHI. The DNA was then digested with one of each of the restriction endonucleases shown in Table 4.7.

Those enzymes which were shown to digest the pOj118 insert DNA were subsequently used in digestion experiments of insert DNA isolated from plasmids pOj118 and pOjo6. Table 4.8 lists the endonucleases used, the number of fragments they generated and the sizes of pOj118 and pOjo6 fragments. In the comparison of the two clones, which represent two of three of the plant lines compared previously, the initial digestions revealed two nearly compensating variable regions within the Bam 3b fragment. These regions have been designated "A" and "B". "A" is contained within a 2.0 kb BamHI/EcoRI fragment and is larger in pOj118, (line D). Region "B" is contained within a 1.4kb BglII(HindIII)/BglII fragment and is larger in pOjo6 (line C_1).

Table 4.7. Restriction endonucleases tested for digestion of the Bam 3b insert DNA. Enzymes which showed no digestion of the insert were tested and confirmed for their activity on phage lambda DNA.

Restriction endonuclease	# insert fragments
AccI	4
BglII	3
EcoRI	7
HincII	3
HindIII	4
NruI	2
SalI	2
SphI	2
XbaI	2
BclI	no digestion
ClaI	no digestion
NdeI	no digestion
SacI	no digestion
SmaI	no digestion
SstI	no digestion
XhoI	no digestion

Table 4.8. Digestions of Bam 3b inserts from plasmids pOj118 and pOjo6. "A" indicates one of the variable regions, "B" indicates the other variable region.

Restriction endonuclease	# of fragments	approx. size pOj118 (kb)	aprox. size pOjo6 (kb)
AccI	4	4.00 A	3.90 A
		1.90 B	1.95 B
		1.80 B	1.85 B
		0.45	0.45
BglII	3	5.00 A	4.90 A
		1.40 B	1.45 B
		1.00	1.00
EcoRI	7	2.10 A	2.00 A
		1.85	1.85
		1.10 B	1.15 B
		0.94	0.94
		0.72 B	0.74 B
		0.61 B	0.62 B
		0.55	0.55
HincII	3	2.90 B	2.95 B
		2.70 A	2.60 A
		1.90 B	1.95 B
HindIII	4	2.75	2.75
		2.50 A	2.40 A
		2.40 B	2.45 B
		0.41	0.41
NruI	2	5.20 B	5.25 B
		2.00 A	1.90 A
SalI	2	6.00 A	5.90 A
		1.90 B	1.95 B
SphI	2	4.40 B	4.45 B
		3.00 A	2.90 A
XbaI	2	6.10 A	6.00 A
		1.40 B	1.45 B

Digestion with other enzymes used in this study generated more than one fragment containing variable region B.

In order to determine the orientation of the variable regions within the Bam 3b fragment, a series of end-mapping experiments was performed using insert DNAs compared with whole plasmid DNAs digested with AccI, EcoRI and HindIII. Figure 4.9, lanes 2 - 5 shows end-mapping digestions using EcoRI. Table 4.9 lists the sizes of pOj118 DNA sub-fragments and the assignment of end fragments to the map in Figure 4.11.

The end-mapping experiments demonstrated that the two variable regions are located at opposite sides of the Bam 3b fragment approximately 3.6 kb apart from each other. The two regions have opposing types of alterations. This made it possible to assign restriction sites for the other endonucleases without the need for further end-mapping experiments.

The digestions with enzymes SalI, AccI and HincII, (Table 4.8) all produced a 1.90 kb band which is larger in line C_1. Comparison of SalI, AccI and HincII recognition sequences revealed that the sequences may overlap. The AccI 1.9 kb restriction site was mapped to the right end of the Bam 3b insert (Table 4.9) SalI only cuts the 3b fragment once and defines the two variable regions. The 1.9 kb SalI fragment is larger in line C_1 and contains region B and thus corresponds to the 1.9 kb AccI fragment.

Table 4.9. End-mapping for comparison of the Bam 3b inserts from lines D (pOj118) and C_1 (pOjo6). Plasmids containing the Bam 3b inserts and the Bam 3b inserts alone were digested with the enzyme indicated to yield a number of fragments. Sizes are shown for pOj118 DNA. Bands from vector pBR322 alone are indicated by parentheses. End fragments are underlined. L = left side of the insert, R = right side of the insert. A indicates one variable region, B indicates the other. See map, Figure 4.11.

Restriction endonuclease	aprox. size Bam 3b insert	approx. size whole plasmid
AccI	4.00 A	4.00 A
	1.90 R B	3.20 R B
	1.90 B	2.40 L
	0.45 L	1.90 B
		1.60(pBR322)
EcoRI	2.10 L A	4.40 R
	1.85	2.45 L A
	1.10 B	1.85
	0.94	1.10 B
	0.72 B	0.94
	0.61 B	0.72 B
	0.55 R	0.61 B
HindIII	2.75	5.60 R B
	2.50 L A	2.75
	2.40 R B	2.74 L A
	0.41	0.41

Figure 4.9. Restriction endonuclease digestions of the Bam 3b 7.5 kb inserts and plasmid DNAs. Plasmid DNA containing the cloned Bam 3b insert was isolated from wild-type lines D (pOj118) and C_1 (pOjo6). The four lanes indicated as digested by EcoRI show isolated insert DNA alone (i) or the insert still contained within the plasmid vector (v). The two lanes indicated as digested with BglII contain subfragments isolated from these two cloned DNAs as follows: Insert DNAs were digested with HindIII, and the 2.4 kb double bands which contain insert DNA of both ends of the 7.5 kb fragment was purified and digested with BglII. Bands located within variable region A are indicated by "a", bands located within variable region B are indicated by "b". DNA was run on 1% agarose with markers 1 (M_1) and 3 (M_3).

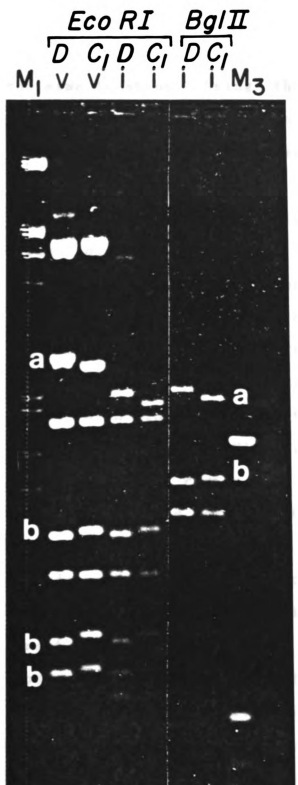


Figure 4.9

As discussed in section 4.3, later in this chapter, HincII will always recognize SalI cut sites. Thus, the cut sites of SalI and HincII which both delineate region B can be placed at the same location. Using this information, in conjunction with that of the location of the variable regions, the remaining HincII sites were placed on the physical map (Figure 4.11).

HindIII cuts the Bam 3b inserts into four fragments (Table 4.8). The location of the end fragments has been described above. Location of the two internal HindIII sites was accomplished by single and double digestions of the insert DNAs with HincII and HindIII. Figure 4.10 shows the results of the digestions. The internal 2.75 kb band of HindIII is not cut with HincII, whereas the other internal HindIII band of 0.41 kb is cut by HincII. The 2.7 kb HincII band located within region A is cut with HindIII. This result enabled the mapping of the 0.41 HindIII band next to the 2.4 kb HindIII band in region A. The sites for the restriction endonucleases are indicated on the physical map (Figure 4.11). Other restriction sites were placed on the map in Figure 4.11 in the same manner.

Another method was used to better define the variable regions of the 3b fragment. Digestion with HindIII revealed two bands of approximately 2.4 kb located at the ends of the 3b fragment (Table 4.9 and Figure 4.10). These two HindIII fragments contained the variable regions.

Figure 4.10. Single and double digestions of the 7.5kb BamHI 3b insert. Cloned Bam 3b 7.5kb insert DNAs from wild-type lines D (pOj118) and C_1 (pOjo6) were digested with HindIII (HIII), HindIII + AccII (Ac), HindIII + HincII (Hc), and HindIII + SphI (Sph). The DNA was run on a 0.8% agarose gel with markers 1 (M_1) and 3 (M_3). Bands which are the result of partial digestion are indicated with "p"'s.

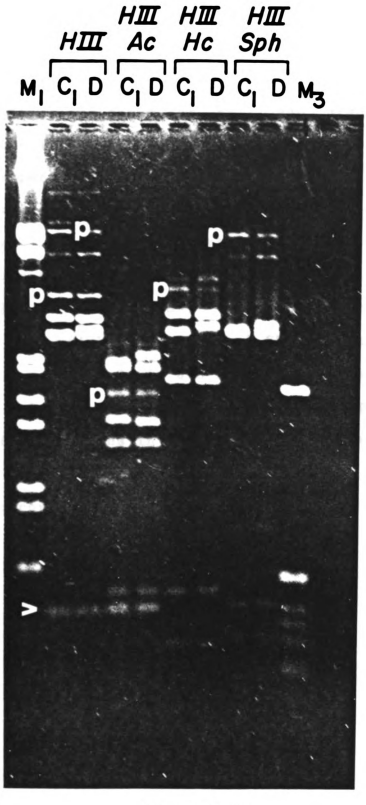


Figure 4.10

The two bands were isolated from a gel and digested with a number of enzymes including BglII. Figure 4.9 shows the result of a digestion of both 2.4 kb HindIII subfragments. Region B is shown to be contained within a 1.4 kb BglII fragment. Since the sites for BglII and HindIII map close together, it is not known at this point which enzyme best defines the left side of region B (refer to Figure 4.11).

To summarize, the Bam 3b fragment contains two regions of variability. The size variations of these two regions nearly compensate for each other. Region A is contained within a 2.0 kb BamHI/ EcoRI fragment and can be further defined by AccI which generates a 1.65 kb fragment with an EcoRI end. Region B is 3.6 kb away from region A and is defined by a 1.4 kb BglII(HindIII)/ BglII fragment (band 2 of the BglII digestions in Figure 4.10). Region A is larger in pOjl18 by approximately 100 bp, region B is larger in pOjo6 by approximately 50 bp.

Figure 4.11. Restriction endonuclease map of the cloned BamHI 3b 7.5kb inserts. The restriction endonuclease map was generated from restriction digestions. The main map is of pOj118 insert DNA. Corresponding areas of variation are shown as blocked areas below the variable regions. All other regions map the same in both plasmid inserts. Asterisks indicate restriction sites which have two possible locations.

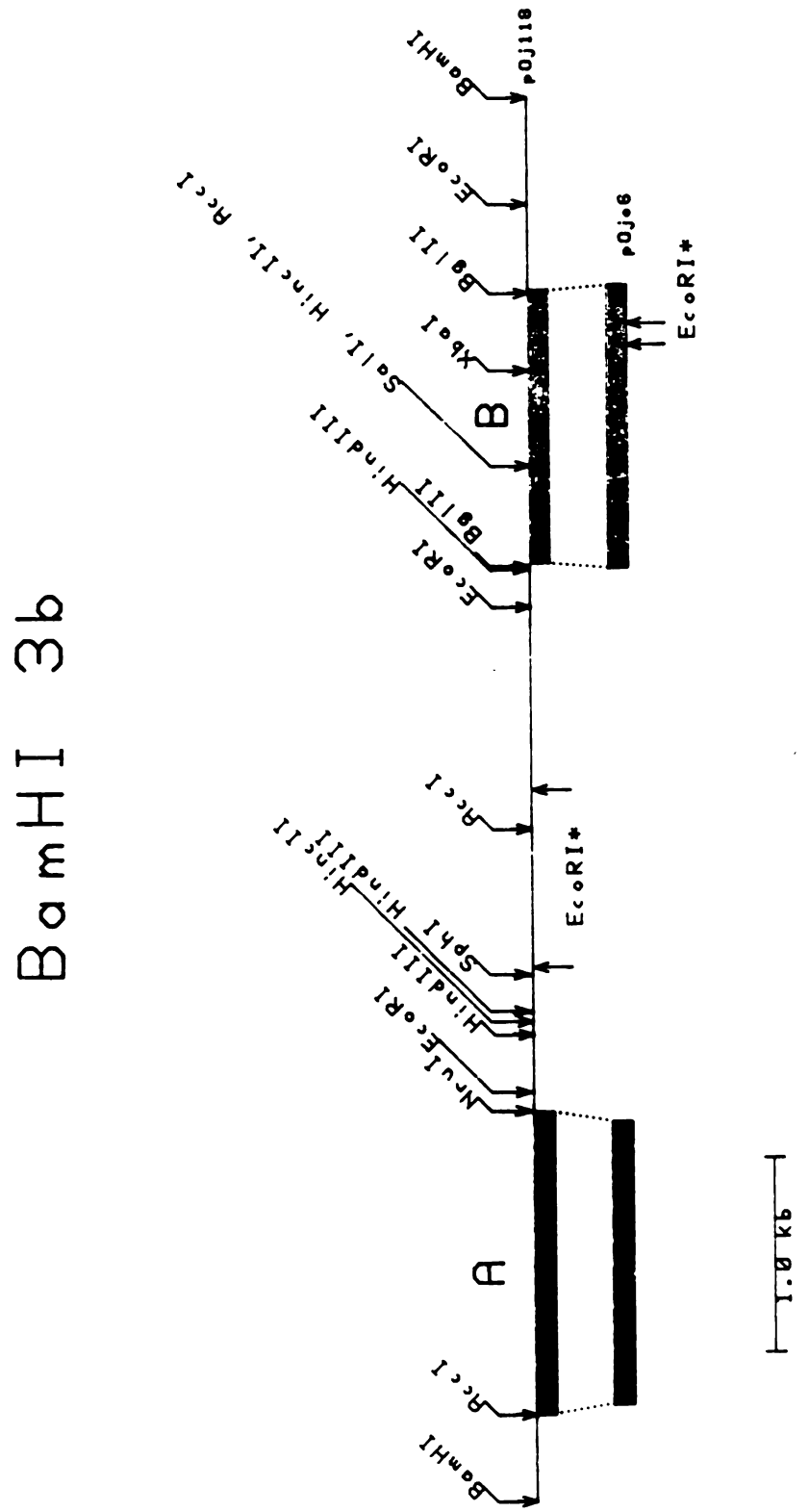

BamHI 3b
pOj118
pOj8
BamHI
EcoRI
Bg/II
XbaI
SalI, HincII, AccI
HindIII
Bg/II
EcoRI
AccI
EcoRI*
HincII
HindIII
SphI
HindIII
NruI
EcoRI
AccI
BamHI
B
A
1.0 kb

Figure 4.11

4.2.5. Comparison of DNA in the variable regions of Bam 3b and Bam 12.

Since both the Bam 3b and Bam 12 cpDNA fragments differed among the wild-type lines by small changes which seem to be due to discrete insertions or deletions, it was necessary to determine if the variable regions shared any DNA sequences. Preliminary evidence from colony hybridizations indicated that the two regions are not homologous.

The polyacrylamide gel in Figure 4.12 shows insert DNAs which were digested with HinfI and TaqI. Replica samples were digested and separated on a 4% Nusieve agarose gel, followed by transfer to a nitrocellulose filter and Southern hybridization of the DNA using the 280p variable HinfI fragment from pOj119 as the probe. No hybridization of the probe to the BamHI 3b insert DNA was detected, whereas hybridization to DNA from pOj119a and pOjo4a did occur (data not shown).

These data indicate that no direct homology exists between the two BamHI variable regions in the cpDNAs of the wild-type lines examined.

Figure 4.12. Polyacrylamide gel electrophoresis of restriction endonuclease digested Bam 3b and Bam 12 inserts. Insert DNAs from wild-type lines D (pOj118, Bam 3b and pOj119a, Bam 12) and C_1 (pOjo6, Bam 3b and pOjo4a, Bam 12) were digested with HinfI. 7.5kb insert from pOj118 and pOjo6 were also digested with TaqI. DNA was run on 8% PAGE with marker 3 (M_3).

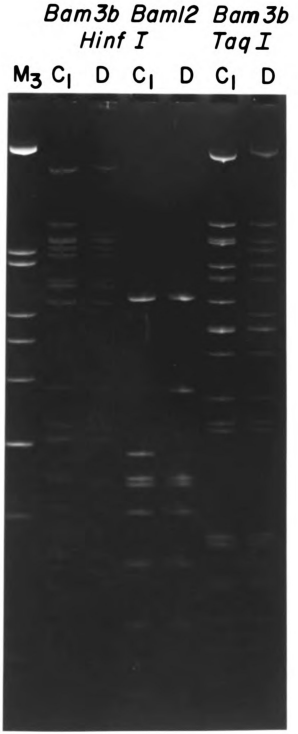


Figure 4.12

4.3. DISCUSSION

Restriction endonuclease mapping analysis of the two variable BamHI fragments from cpDNA revealed small differences between plastome I cpDNAs of *Oenothera hookeri* strain johansen wild-type lines D, C_1, and C_2. Southern hybridization experiments allowed for the localization of these bands on the physical map of the chloroplast genome. Bam 3b is located in the large single copy region in the vicinity of the atpA, atpH and atpF genes (Figure 4.1). Bam 12 is located within the inverted repeat at or near the junction of the large single copy region (Figure 4.1).

The physical mapping of the variable fragments described above has allowed the identification of more specific sites of variability within those larger fragments. The results of fine mapping the Bam 12 fragment are illustrated in Figure 4.8, while a preliminary map for the Bam 3b fragment has been compiled in Figure 4.11.

The enzymes TaqI and HinfI each cut the Bam 12 fragment into about ten subfragments. Although the fragments at the ends and in the variable region could be mapped, the exact cut sites for the others have not been determined. At the left border of the variable region, each enzyme has one cut site very near the other. In fact, HinfI and TaqI have recognition sequences which could overlap in the following ways:

HinfI G↓ANTC overlaps with *TaqI* T↓CGA

HinfI G↓ANTC overlaps with *TaqI* T↓CGA

If the DNA of this region had one of the above overlapping sequences, initial digestion with HinfI would eliminate the TaqI site, thereby making the site unavailable to the enzyme. The second enzyme might not be able to bind and make a cut so close to the end, but even if the second digestion was successful, initial digestion with TaqI would leave a HinfI cut site only one base pair from the end of the fragment. This difference in fragment size would not be detectable on 10% PAGE. As mentioned in the results section (4.2.3.2.), HinfI and HincII also have recognition sequences which may occur in very close proximity. The recognition sites are:

G↓ANTC *HinfI*

GT(T or C)↓(A or G)AC *HincII*

At least two possible sequences could create overlapping restriction sites of these two enzymes:

HinfI G↓AGTC A(G)C

G AGTC↓A/GC *HincII*

HincII GTC↓G ACTC

GTC G↓ACTC *HinfI*

The larger BamHI fragment also provided a challenge to restriction mapping. Three enzymes, AccI, HincII and SalI, gave a fragment of 1.9 kb as indicated in Table 4.8 and on the restriction map (Figure 4.11). Examination of the recognition sequences for these enzymes indicated that they have overlapping recognition sequences.

GT↓(A or C)(G or T)AC	*AccI*
GT(T or C)↓(A or G)AC	*HincII*
G↓TCGAC	*SalI*

The regions of variability within the Bam 3b and Bam 12 fragments do not show any homology when examined using Southern hybridizations. The regions which contain these fragments (near the border of the inverted repeat for Bam 12 and near the ATPase genes of the large single copy region for Bam 3b) have been shown to be sites of rearrangements within the chloroplast genome of higher plants (Palmer 1985b, Mubumbila et al. 1984). These DNA rearrangements are seen when cpDNAs from spinach, lettuce, legumes and the *Onagraceae* are compared.

Possibly, as more sequence data become known, including DNA sequences from the areas of variation of the *Oenothera*

cpDNA which have been mapped in this study, it will be possible to find characteristics such as secondary structures of the DNAs which may lend themselves to rearrangements.

The types of alterations seen in cloned cpDNA from the wild-type lines in this study suggest that insertion/deletion events have occurred in the cpDNA of *Oenothera*. Similar cpDNA alterations have been recognized in broader comparisons among different species within a genus: *Oenothera*, subsection *Euoenothera* (Gordon et al. 1982), *Oenothera* subsection *Munzia* (von Stein and Hatchtel 1986) *Epilobium*, (Schmitz et al. 1986), *Triticum* and *Aegilops* (Bowman et al. 1983), *Brassica* (Palmer et al. 1983), legumes (Michalowski et al. 1987), and *Zea* (Doebley et al. 1987). Some of these studies have led to the observation that the insertions/deletions in cpDNA occur more frequently near the junction of the large single copy region and the inverted repeat region, and that these junctions appear to be more subject to change than is the rest of the genome (Mubumbila et al. 1984, Palmer 1985b, Whitfeld and Bottomley 1983 and Gordon et al. 1982).

The insertions/deletions which were detected in the *Oenothera* plants in this study are small (approximately 100 bp or less). This observation is consistent with the size, nature and location of the insertion/deletions which have been described between *Oenothera* plastome types I -V

(Gordon et al. 1982) and for some of the other insertions/deletions which have been found in other plants also by restriction endonuclease analysis. Restriction endonucleases rarely detect point mutations because they recognize only a small subset of bases, making the probability of detecting a point mutation very low. Thus, the occurrence of point mutations cannot be ruled out by the studies described hare. However, point mutations cannot be the cause of the differences which have been characterized here since each restriction endonuclease tested gives an identical number of fragments in all of the cloned cpDNAs tested, and each one shows discrete and consistent alterations in fragment sizes. Before restriction endonuclease mapping and DNA sequencing techniques were available to test the possibility, Kutzelnigg and Stubbe (1974) hypothesized that the genetically-defined plastome types might be further subdivided.

The differences which have been seen between cpDNAs occur between wild-type lines of *Oenothera hookeri* strain johansen plastome I. Two of these wild-type lines were isolated following Epp's EMS mutagenesis experiments (Figure 2.1). Thus, we do not known if the differences in the cpDNA are the result of natural events or if they were caused by the EMS mutagenesis. EMS is a chemical mutagen which is known to produce mainly point mutations in chromosomes of *Drosophila* (Grigliatti, 1986). These point mutations are

usually in the form of transitions from GC to AT in prokaryotic DNA (Kreig, 1963), or GC to AT and AT to GC in eukaryotes (Auerbach, 1976). Studies of base-specific mutagens of nuclear genes on tomato have shown that EMS primarily affects GC base pairs (Jain and Raut, 1966). Areas of chromosomes rich in AT sustain less damage than do regions containing more GC. A possible explanation of the changes in cpDNA of lines C_1 and C_2 is that EMS is able to cause small deletions or insertions in cpDNA. Alternatively, initial treatment of the plants with the mutagen may have introduced some GC to AT changes in the cpDNA. The altered base composition of the region may have been changed enough to create rearrangement "hot spots" thereby making further insertions/deletions possible. One way to start investigating the possibility is to see if other *Oenothera* plastome I species such as *Oenothera hookeri* and *O. elata* contain small insertion/deletions in their cpDNAs. Another test may be to perform mutagenesis experiments and look for small changes compared to untreated lines over time. An EMS treated line may undergo alterations at a more accelerated rate.

Based on the information from the many studies on cpDNA organization, it appears that many small changes may occur in cpDNA without resulting in any obvious phenotypic changes. This would suggest that those particular regions do not have a coding, regulatory or structural function. It

may be possible that many small regions of variation are missed by restriction endonuclease mapping. Indeed, if region B of the Bam 3b fragment had been a bit larger, it may have compensated for the variable region A, thus making detection of any differences possible only at the DNA sequence level, or through examination of cpDNA with a battery of enzymes.

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LITERATURE CITED

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APPENDIX

RESTRICTION ENDONUCLEASES

AND THEIR RECOGNITION SEQUENCES
(adapted from the BRL reference catalogue)

AluI	AG↓CT	MspI	C↓CGG
AccI	GT↓CT^AG AC	NdeI	CA↓TATG
BamHI	G↓GATCC	NruI	TCG↓CGA
BclI	T↓GATCA	PvuII	CAG↓CTG
BglII	A↓GATCT	RsaI	GT↓AC
ClaI	AT↓CGAT	SacI	GAGCT↓C
DraI	TTT↓AAA	SalI	G↓TCGAC
EcoRI	G↓AATCC	ScrfI	CC↓NGG
HaeIII	GG↓CC	SmaI	CCC↓GGG
HincII	GTPy↓PuAC	SphI	GCATG↓C
HindIII	A↓AGCTT	SstI	GAGCT↓C
HinFI	G↓ANTC	TaqI	T↓CGA
MaeIII	↓GTNAC	XbaI	T↓CTAGA
MboI	↓GATC	XhoI	C↓TCGAC

DNA MARKER BAND SIZES

Marker 1 (M_1): Phage lambda (λ) DNA digested with EcoRI + HindIII. Adapted from the BRL Reference catalogue.

Band Number	Size (bp)
1	21,226
2	5,148
3	4,973
4	3,530
5	2,027
6	1,904
7	1,584
8	1,330
9	983
10	831
11	564
12	125

Marker 2 (M_2): BRL 123 bp ladder

Increments of 123 bp from 4,182 to 123 bp.

Marker 3 (M_3): Plasmid pBR322 DNA digested with HinfI

Band Number	Size (bp)
1	1,632
2	517
3	506
4	396
5	344
6	298
7	221
8	220
9	154
10	75

Marker 4 (M_4): Plasmid pBR322 DNA digested with MspI

Band Number	Size (bp)
1	622
2	527
3	404
4	309
5	242
6	238
7	217
8	201
9	190
10	180
11	160 X 2
12	147 X 2
13	123
14	110
15	90
16	76