MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 00548 0334
LIBRARY
Michigan State
University

This is to certify that the

dissertation entitled

Physiological and Molecular Characterizations of Plastids from a Plastome Mutator-Induced Mutant of Oenothera hookeri str Johansen

presented by

Ellen Margaret Johnson

has been accepted towards fulfillment of the requirements for

Doctor of Philosophy degree in Botany and Plant Pathology and the Program in Genetics

Barbara B. Sears
Major professor

Date May 26, 1988

MSU is an Affirmative Action/Equal Opportunity Institution

O-12771

MSU
LIBRARIES

RETURNING MATERIALS:
Place in book drop to remove this checkout from your record. FINES will be charged if book is returned after the date stamped below.

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF PLASTIDS FROM A PLASTOME MUTATOR-INDUCED MUTANT OF OENOTHERA HOOKERI STR JOHANSEN

By

Ellen Margaret Johnson

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology
and
the Program in Genetics

1988

ABSTRACT

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF PLASTIDS FROM A PLASTOME MUTATOR-INDUCED MUTANT OF OENOTHERA HOOKERI STR JOHANSEN

By

Ellen Margaret Johnson

Pm7 is one of a number of chloroplast mutants derived from the plastome mutator (pm) line of Oenothera hookeri, strain Johansen. This mutant appears chlorotic, and displays little or no internal membrane development in the plastid. Biochemical analysis of pm7 has shown that the mutant has reduced but detectable amounts of chlorophyll, and levels of carotenoids, fatty acids and lipids which are within the range of those found in the wild-type. Immunoblotting analysis has shown that the mutant membrane fraction contains a number of gene products which are larger in size than the corresponding mature proteins. Three proteins appear to be improperly processed in pm7: cytochrome f and the 23-kD and 16-kD subunits of the oxygen-evolving sub-complex of photosystem II. Additionally, the D1 subunit of photosystem II and a 28-kD chlorophyll-binding protein from photosystem II were found to be completely absent in the mutant plastids. These proteins may be separated into catagories representing both chloroplast and nuclear

DNA gene products. The pm7 mutation is inhertited in a non-Mendelian fashion, thus restriction patterns of mutant and wild-type cpDNAs, as well as the DNA sequence of the wild-type and pm7 petA regions which encode cytochrome f, were examined and no differences were found. Since the mutation can undergo reversion in the homozygous pm nuclear background, these results suggest that a plastid DNA-encoded function is required for appropriate processing and subsequent chloroplast development.

To all the Johnsons and the Caspars,
in recognition of their encouragement
and support.

ACKNOWLEDGMENTS

This project would not have been possible without the generous contribution of antibodies from a large number of researchers: Terry Bricker, Alice Barkan, Christer Jansson, Yossi Hirshberg, Charles Yocum, Cathy Chia, Richard Malkin, Sylvia Darr, Dave Husic, Micha Volakita, Nancy Artus, Jack Preiss, John Boynton, Ken Keegstra, Erin Bell, Carol Schumann, Rick Vierstra and Tom Fox. Barbara Sears, Hans Kende, Shauna Somerville, Tom Friedman and Charles Arntzen have each served as valuable members of my guidance committee. I would especially like to thank Barbara and Charlie and the other past and present members of the Sears and Arntzen laboratories who provided enjoyable and constuctive environments in which to work. I would also like to acknowledge Karen Klomperans, Nancy Artus, Ljerka Kunst, Mike Stine and Dan Keathley for helpful advise and instruction in various experimental techniques and analysis. Finally, Tim Caspar deserves my great respect and gratitude for his undying support and encouragement for this endeavor.

TABLE OF CONTENTS

List of Tables. ix
List of Figures. x

Chapter

1 Introduction. 1-5

Use of Chloroplast Mutants. 1

Plastome Mutator System of Oenothera. 2

Introduction to Thesis. 5

2 Examination of Heteroplasmy among Wild-type and Mutant cpDNAs from Oenothera Plastome Mutator Lines. 6-37

Introduction. 6

Materials and Methods. 9
Plant Material. 9
Isolation of Chloroplasts and cpDNA. 13
Restriction and Hybridization Analysis. . . . 14

Results. 19
Preliminary Analysis of Restriction Patterns of pm7 cpDNA. 19
Analysis of Variable Regions in Plastome Mutator Lines. 24

Discussion. 32

3 Immunochemical and Molecular Analysis of the Phenotypes of pm7 and a Putative Revertant of pm7. 38-105

Introduction. 38

Materials and Methods. 46
Plant Material. 46
M13 Cloning and Dideoxy Sequencing Strategy. 46
Chloroplast Preparation and Fractionation of Membrane/Soluble Proteins. 50
SDS-Polyacyrylamide Gel Electrophoresis 51
Preparation and Sources of Antibodies Specific for Chloroplast Proteins. 51
Immunoblotting Analysis. 54

Results. 55
Cloning of Fragments from the Bam 3a Region of pm7 and Wild-type cpDNAs. . . . 55
Analysis of Mutant and Wild-type DNA Sequences in the petA Region. 55
Analysis of Putative cyt f Precursor Specific Antibodies. 58
Analysis of Heme Staining Activity 62
Immunoanalysis of Wild-type and Mutant Chloroplast Proteins. 64
Immunoanalysis of a Putative Revertant of pm7. 73

Discussion. 77

4 Physiological Analysis: Structure, Compostion and Activity of Mutant and Wild-type Plastids. 106-135

Introduction. 106

Materials and Methods. 108
Plant Material. 108
Photosynthetic Activity Measurements. . 108
Methods for Ultrastructural Analysis. . . . 109
Determination of Chlorophyll Content. . 110
Determination of Carotenoid Content. . 110
Determination of Fatty Acid Content. . . . 111
Measurement of Lipid Synthesis. 111

Results. 113
Photosynthetic Electron Transport Activity. 113
Ultrastuctural Analysis. 113
Analysis of Chlorophyll and Carotenoid Accumulation. 116
Analysis of Fatty Acid Accumulation and Lipid Synthesis. 122

Discussion. 129

5 Summary and Conclusions. 136-141

Bibliography. 142-159

LIST OF TABLES

Table

1 Sources of antibodies used in immunoanalysis of pm7 and wild-type chloroplast proteins. 53

2 Summary of immunological analysis of pm7 plastid proteins. 74

3 Photosynthetic electron transport activity in Oenothera wild-type and pm7 mutant chloroplasts. 114

4 Results of statistical analysis using split plot analysis of variance tests. 119-120

LIST of FIGURES

Figure

1 Pedigree of plant lines. 10

2 Restriction map of the variable Bam 12 region of the Johansen strain of Oenothera hookeri from lines D, C_1 and C_2. 16

3 Restriction map of the variable Bam 3b region of the Johansen strain of Oenothera hookeri from lines D, C_1 and C_2. 17

4 Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with total wild-type cpDNA. 20

5 Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a spinach fragment containing the petA gene. . .21

6 Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a Bam 3a. 22

7 Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a Bam 3b. 23

8 Physical map of Oenothera hookeri plastome I. 24

9 Restriction digest of cpDNA from (green) wild-type variants C_1 and C_2. 26

10 Pedigree of plant lines, indicating C_1 and C_2 classes of plastome mutator line. 28

11 Restriction digest and Southern hybridization with Bam 3b. 29

12 Southern hybridization of HinfI-digested pm11, pm7, and C_2 wild-type cpDNA with Bam 3b and Bam12 probes. 31

13 Coomassie blue staining and immunoanalysis with cyt f antibodies of mutant and wild-type chloroplast proteins. . . 39

14 Map of the Bam 3a region from the Johansen strain of Oenothera hookeri cpDNA. 48

15 Strategy employed to sequence the 5' end and region upstream of petA from pm7 and line C_2 wild-type cpDNA. . 49

16 Partial sequence of petA and upstream region of cpDNA from Nicotiana tabacum and the Johansen and Standard strains of Oenothera hookeri. 56-57

17 Immunoblot of wild-type and pm7 total chloroplast proteins probed with antibodies against synthetic cyt f precursor-conjugate and cyt f. 60

18 Heme-stained SDS-polyacrylamide gel (10-15% acrylamide gradient, 4 M urea) of mutant and wild-type chloroplast proteins. 63

19 Coomassie-stained gel of membrane and soluble fractions of mutant and wild-type chloroplast proteins. 65

20 Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of the cyt b_6/f complex. 66

21 Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of the OEC subcomplex of PS II. 68

22 Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of PS II and PS I. 70

23 Immunoblot of pm7 and wild-type membrane (A) or soluble (B) fractions probed with antibodies against a ribosomal protein and two stromal proteins. 72

24 Immunoblot of pm7 and wild-type chloroplast proteins in stable and unstable plastome mutator nuclear backgrounds probed with antibodies against PS II-22, cyt f and OEC-23. .75

25 Amino acid sequences of the N-terminal extensions of the precursors of cyt c_1, cyt f, and the components of the OEC subcomplex of PS II. 95-96

26 Hydrophilicity plots for the presequences of cyt f and the components of the OEC subcomplex of PS II. 97-98

27 Model for processing of cyt f, OEC-23, and OEC-16 in the chloroplast. 101

28 Hydrophilicity plots for the presequences of cyt f and cyt c_1. 103

29 Electron micrographs of plastids from leaf-tip cultures of mutant (A, B) and wild-type (wt, C). 115

30 Chlorophyll content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. 118

31 Total carotenoid content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. 121

32 Major carotenoids in tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. 123-124

33 Fatty acid content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. 125-126

34 Lipid synthesis in tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. 128

CHAPTER 1

Intoduction

Use of Chloroplast Mutants

The genome of higher plant chloroplasts encodes approximately 50 known genes for various soluble and membrane-bound chloroplast proteins involved in photosynthesis, various components of the plastid translational system, a complete set of tRNA genes and a number of yet uncharacterized open reading frames (ORFs) (Parthier, 1982; Herrmann et al., 1985; Rochaix, 1985; Shinozaki et al., 1986; Ohyama et al., 1986). As a consequence of this, there are ample "targets" for mutagenesis of the plastid genome (or "plastome"). Mutation analysis can be a valuable complement to standard biochemical and gene cloning techniques in the analysis of structure and function of gene products and chloroplast development. Thus, the ability to increase mutation levels beyond the naturally low frequency of spontaneous mutation could be advantageous because it would allow the isolation of valuable plastome mutants.

A number of plastome mutants have been very useful in elucidating the identities and functions of chloroplast gene products (Börner and Sears, 1986). These mutants were recovered by isolating rare spontaneous mutants or by screening chemically mutagenized populations. Another alternative to these approaches may be found in

several higher plant isolates which have elevated rates of plastome mutation, including Arabidopsis thaliana (Redei and Plurad, 1973), maize (Shumway and Weier, 1967; Walbot and Coe, 1979; Thompson et al., 1983), Petunia (Potrykus, 1973) and the evening primrose, Oenothera (Epp, 1973).

Plastome Mutator System of Oenothera

Confirming the initial findings of Epp (1973), the plastome mutations analyzed in our laboratory are inherited in a non-Mendelian fashion and represent a variety of phenotypes. Different mutants show varying degrees of chlorosis (Epp, 1973), similar to the chloroplast mutator-induced mutant of Arabidopsis thaliana and in contrast to the mutants induced by the iojap type mutators. They also differ in the extent of plastid development (Epp and Parthasarthy, 1987) and in the accumulation of chloroplast proteins (Sears, personal communication).

The maize plastome mutants induced by the iojap mutator system show only a single phenotype. These mutants and similar mutants induced by the barley albostrians system (Börner et al., 1976) all display a complete loss of chloroplast ribosomes. It has been proposed that the nuclear gene product encoded by iojap is essential for assembly or function of chloroplast ribosomes, based upon the lack of chloroplast polyribosomes, 16S and 23S chloroplast rRNAs and plastid protein synthetic capacity (Walbot and Coe, 1979). In the absence of chloroplast ribosomes, none of the cpDNA-encoded products can be synthesized, including a number of chloroplast ribosomal proteins.

Since these proteins cannot be made, even if the plastids are removed from the iojap nuclear background, they will never be able to regenerate a functional ribosome nor a functional chloroplast. Hence, these mutator systems result in an irreversible and heritable alteration of the chloroplast, presumably without affecting cpDNA.

In the plastome mutator system of Oenothera hookeri str Johansen, plants homozygous for a recessive allele at the pm locus show mutation frequencies for plastome genes which are 1000-fold higher than spontaneous levels (Epp, 1973). A large collection of plastome mutator-induced chloroplast mutants has been generated by several laboratories (Epp, 1973; Epp et al., 1987; Sears, 1983). All of the mutants isolated thus far have been recognized initially by the chlorotic sectors which appeared in plants homozygous for the pm allele.

Plastome mutator activity appears to be independent of a variety of growth conditions (Johnson et al., 1988). For example, a comparison of field-grown and greenhouse-grown plants showed no differences in the mutation frequencies of plants grown in these two environments. Furthermore, when plant lines which are inactive for the plastome mutator activity (pm^+/pm^+ or pm^+/pm) are grown in shoot-tip culture, they are extremely stable. In seven years of growing shoot-tip cultures of Oenothera, we observed only one chlorotic sector in a heterozygous plant line. In contrast, after about four months of cultivation as shoot-tip cultures, homozygous pm/pm lines produce mutations at a high frequency (Johnson et al., 1988). Although it is

not possible to accurately compare the frequency of appearance of sectors in such clones to the frequency of appearance of sectors in field-grown plants, the activity of plastome mutator is clearly not dependent upon conditions exclusive to any one of these growth regimes.

There are a number of possible mechanisms by which the plastome mutator system could induce such mutations. Just as certain components of the chloroplast translational apparatus are encoded by nuclear genes, so are a number of components essential to cpDNA replication and repair (Possingham and Lawrence, 1983). Preliminary experiments designed to address this possibility and identify a candidate for a pm gene product are currently underway in the Sears laboratory. A purified product of the pm gene could ultimately be used to help clone the pm gene itself. If the pm gene encoded a defective form of one of these elements, mutations could be introduced into cpDNA, creating a plastome mutant. A point mutation in the cpDNA of such a mutant could be the result of increased levels of errors in cpDNA replication or defective cpDNA repair processes. Evidence of these mutations may or may not be obvious at the level of cpDNA restriction patterns, depending upon the nature and location of the mutation event. An alternative mode of action of the plastome mutator sytem involves the possibilty that the pm gene product regulates the activity of a chloroplast transposable element. Such a mechanism would most likely be detectable as major alterations in cpDNA restriction patterns.

Introduction to Thesis

The primary objective of the work presented in this thesis is to utilize a number of different approaches to characterize one particular plastome mutator-induced mutant of Oenothera hookeri. Initial analyses explored the possibility that major alterations in the mutant cpDNA pointed to the site of the primary lesion and were indicative of the mechanism by which plastome mutator acts. Various aspects of chloroplast protein accumulation and processing were then addressed as well as the extent to which these proteins assemble into functional complexes. The levels of other major components of mature chloroplasts, such as chlorophyll, carotenoids, fatty acids and lipids have also been determined as part of this characterization.

CHAPTER 2

Examination of Heteroplasmy among Wild-type and Mutant cpDNAs from Oenothera Plastome Mutator Lines

Introduction

The plastome mutator-induced mutants in our collection were initially recognized as chlorotic sectors in plants which were homozygous for pm, and hence displayed active plastome mutator function. Secondary screening of these mutants was performed by Dr. Sears by analyzing two characteristics of plastids isolated from pure mutant cultures. A number of mutants showed variability in the restriction patterns of their cpDNA in comparison to cpDNA isolated from descendants of the wild-type, green plastome mutator line. These variable regions containing the restriction fragment length polymorphisms (RFLPs) were subsequently localized to the Bam 12 and Bam 3b fragments of Oenothera cpDNA for two plastome mutator-induced mutants, pm8 and pm11 (Kaplan, 1987). The 3-kilobasepair (kb) Bam 12 fragment was shown to be located completely within the inverted repeat region of the cpDNA molecule of Oenothera hookeri (Kaplan, 1987; Johnson et al., 1988).

A second screening procedure involved an immunological analysis of cpDNA-encoded proteins present in the mutant plastids. This examination revealed that one particular plastome mutator-induced

mutant accumulated a high molecular weight protein which was antigenically related to cytochrome f (cyt f). Cytochrome f is a thylakoid membrane protein encoded on cpDNA (Doherty and Gray, 1979; Alt et al., 1983; Willey et al., 1983; Phillips and Gray, 1984). It is also known to be translated as a precursor with an amino-terminal extension in a number of plants, including Oenothera (Alt and Herrmann, 1984; Willey et al., 1984a, 1984b; Tyagi and Herrmann, 1986). Aberrant processing of the mutant cyt f may occur as a result of a lesion in the petA gene which encodes this protein . Further analysis of chloroplast proteins in this mutant, denoted pm7, is the subject of the following chapter. However, this mutant was not included in the initial RFLP screen and so it was necessary to purify cpDNA from pm7 tissue in order to compare the mutant and wild-type cpDNA restriction patterns and look for evidence of major rearrangements in pm7 cpDNA.

Lindenhahn et al. (1985) have suggested that chlorotic sectors arising in an Oenothera hookeri line containing an active plastome mutator background were the result of contaminating cross pollination and subsequent hybrid plastome-genome incompatibility, where a given Oenothera plastid type (I, II, III or IV) fails to develop normally in a particular nuclear background (Stubbe, 1959). In response to this assertion, Epp et al. (1987) have recently shown that the cpDNAs isolated from several different plastome mutator-induced sectors all show the plastome I type restriction pattern (Gorden et al., 1982) and are identical in restriction pattern to that the wild-type. Major differences in cpDNA restriction patterns reported by Lindenhahn et

al. were probably, as they proposed, the result of contamination with pollen with plastome III type cpDNA. In contrast, the results described here show cpDNAs from another group of plastome mutator-induced mutants and their wild-type analogs digested with more frequently cutting enzymes than those used by Epp et al. This analysis revealed the occurrence of RFLPs among individual plants all of which were derived directly from Epp's original pm/pm line. However, the subsequent analyses and examination of the pedigree presented in this chapter suggest that the presence of more than one type of cpDNA, or heteroplasmy, occurred independently of plastome mutator-induced mutagenesis.

Genes encoded on cpDNA appear to be unusually conserved (Curtis and Clegg, 1984; Zurawski and Clegg, 1987). However the existence of heteroplasmy in Oenothera suggests there may be a limitation to the conserved nature of cpDNA. Other comparisons of the entire cpDNA molecule between closely related plants have indicated that frequent changes have occurred through insertion/deletion events (Gorden et al., 1982; Salts et al., 1984; Schmitz and Kowallik, 1986; Doebley et al., 1987) and by rearrangements (Palmer, 1985). Similarly, evolutionary comparisons of mitochondrial DNA (mtDNA) have revealed many rearrangements (Sederoff, 1984); many mtDNA polymorphisms within animal species can be explained by sequence duplications.

Materials and Methods

Plant Material

The original pm/pm isolate (E-15-7) was obtained after EMS (ethyl methane sulfonate) mutagenesis of an inbred line of Oenothera hookeri strain Johansen grown at Cornell University (Epp 1973). This species has an A/A nuclear background and a type I plastome, according to the classification scheme of Stubbe (1959). The original pm/pm line was inbred several generations to produce the plastome mutator seed stocks maintained by Prof. W. Stubbe (University of Düsseldorf). Figure 1 indicates the pedigree for the plastome mutator-induced mutants (pm7, pm8, pm9, pm11, pm12, pm32 and pm35) isolated from progeny of further self-crosses. In 1982, green siblings of plants containing pm32 and pm35 were crossed as the female parent with pollen from Oenothera hookeri strain Johansen carrying plastome IV in order to produce a pm^+/pm line containing wild-type green plastids of plastome type I for use as a control. The progeny are heterozygous at the pm locus and carry the A/A nuclear composition which is fully compatible with plastome I (Stubbe 1959). As described further in the results, this wild-type line is classified as the C_1 type, after the Cornell origin of Epp's pm/pm line of O. hookeri, and the cpDNA is referred to as type I-C_1.

Another wild-type line (C_2), carrying a second chloroplast type (I-C_2) was recovered from the 1980 field season, when plants containing the

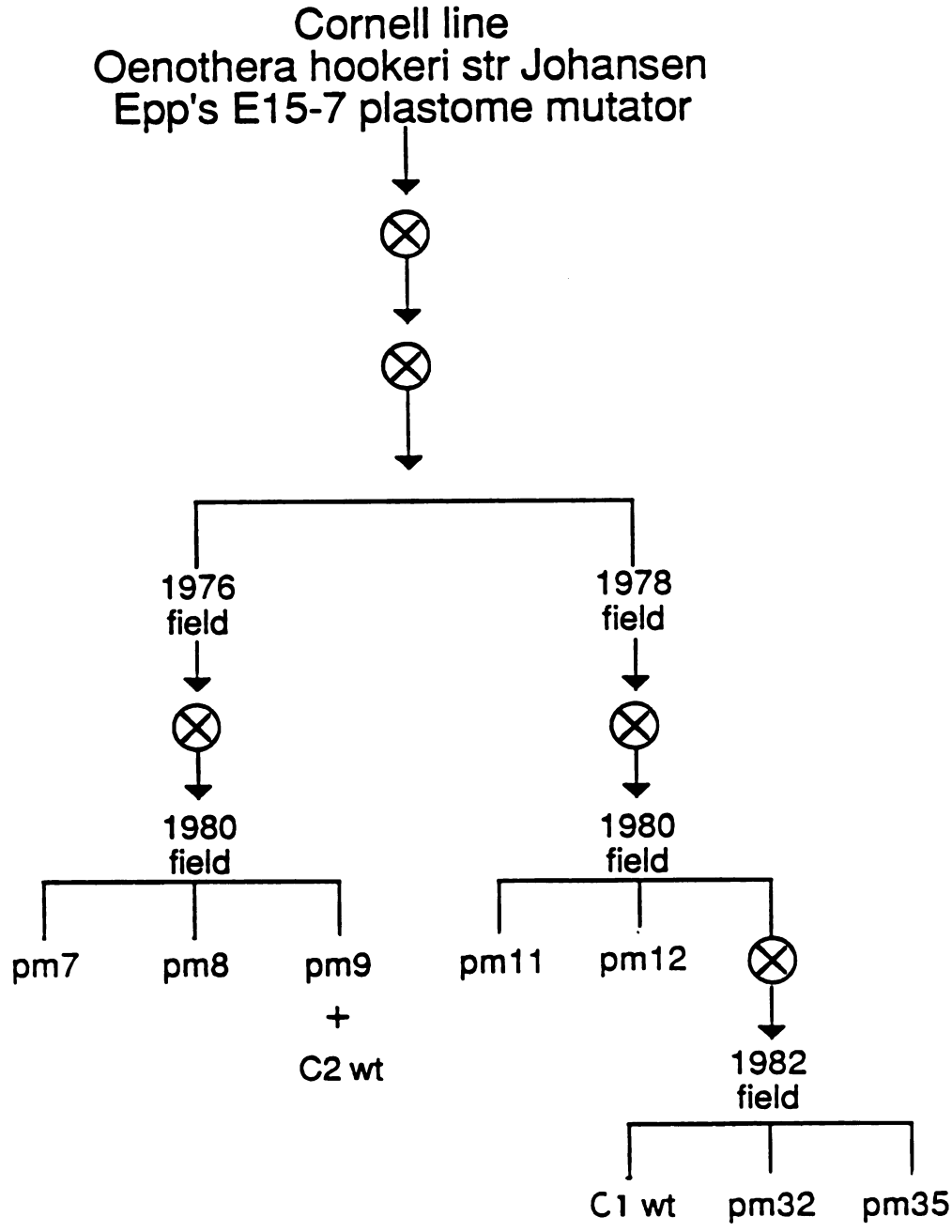


Figure 1. Pedigree of plant lines. Pedigree of plastome mutant and wild-type lines descended from Epp's Cornell Oenothera hookeri pm/pm isolate E-15-7. The circled x's denote self-crosses. Line C_2 wild-type (wt) was recovered by the selection and purification from variegated pm9 seedlings of light green tissue containing wild-type plastids, as described in the Materials and Methods.

newly arisen plastome mutant pm9 were crossed as the female parent to albicans/percurvans (A/C) plants with plastome IV. As described by Cleland (1972), the albicans genome is an alpha-complex (egg-transmitted) while the percurvans genome is a beta-complex (pollen-transmitted); therefore, the seeds resulting from this cross have the genotype johansen/percurvans (A/C). In this particular cross, both the newly arisen mutant plastids and green plastids were transmitted through the egg, and plastome IV plastids were transmitted by the pollen, producing progeny carrying different combinations of three plastid types, as described previously by Stubbe (1957). As shown by Stubbe (1959), plastome I fails to develop normally in this nuclear background, resulting in a pale green leaf color. In the seedlings produced by this cross, three types of tissue were observed: white, light green and dark green. Respectively, these tissues contained the pm9 mutant plastome, wild-type plastome I transmitted from the female parent, and wild-type plastome IV from the male parent. The light green line was selected and maintained in shoot-tip culture, as a wild-type control for the appropriate plastome mutants from line C_2.

Wild-type C_1 plants were grown in the greenhouse in sandy soil. However, as described above, the C_2 cpDNA was available only in an A/C nuclear background. Since plants of plastome type I are not as vigorous in combination with this nuclear background as they are in their natural A/A nuclear background (Kutzelnigg and Stubbe 1974), they were grown as shoot-tip cultures on a modified NT medium (Nakata and Takabe 1971, Stubbe and Herrmann 1982) which contained a ratio of 2:1 (w/w) 6-benzyl-amino purine (13 μM) to

naphthalene acetic acid (8 μM). Cultures of pure mutant tissue were maintained by transferring them to fresh media every four weeks.

Isolation of Chloroplasts and cpDNA

Oenothera leaf tissue (50-500 g) was homogenized in ice-cold buffer containing 330 mM sorbitol, 6 mM EDTA, 1 mM ascorbic acid, 3 mM cysteine, 0.15% (w/v) PVP (polyvinyl pyrrolidone, Mr=40,000), 0.1% (w/v) BSA (bovine serum albumin) and 50 mM Tris, pH 7.5, (Sigma Chemical Co.) as given by Herrmann (1982), with modifications adapted from Somerville et al. (1981), Loomis (1974) and Galliard (1974). All manipulations were performed at 4°C. Following filtration through 100 μm nylon mesh and two layers of Miracloth (Calbiochem) and differential centrifugation at 6000 x g for 5 minutes, the crude chloroplast preparation was washed in the homogenization buffer lacking PVP. This preparation was further purified by layering on a 10 - 80% sucrose step gradient, buffered with 50 mM Tris-HCl, 6 mM EDTA and 330 mM sorbitol, pH 7.5, which was then centrifuged at 22,500 x g for 10 minutes in a Sorvall SS90 vertical rotor. Chloroplast bands were removed from the gradient, diluted with 20 mM EDTA, 50 mM Tris, pH 7.5 and pelleted by centrifugation at 16,500 x g for 10 minutes. The chloroplast pellets were resuspended in equal volumes of NET (5 mM NaCl, 100 mM EDTA, 50 mM Tris, pH8.5) for subsequent lysis in 1% (w/v) sarkosyl and 1 unit/ml pronase (Calbiochem) with gentle agitation at 4°C for 4 hours. When only limited quantities of plant material were available (line C_2 wild-type and some mutant lines), the sucrose gradient was omitted. In both cases, CsCl was added to give a refractive index of approximately 1.400 and the mixture was centrifuged for 45 minutes at 10,000 x g in a Sorvall HB4 rotor. The chlorophyll/protein pellicle which bands at

the top of the gradient was discarded while the clear supernatant was processed further. Bisbenzimide (Hoeschst 33258) was added to a final concentration of 0.01 mg/ml (after Hudspeth et al. 1980) and the refractive index was adjusted to 1.3960. After buoyant density equilibrium centrifugation, achieved in 10 -12 hours with a Sorvall TV865 vertical rotor at 42,000 rpm at 19° C., the upper (cpDNA) band in the gradient was collected. If the lower band of nuclear DNA was so broad that it contaminated the upper cpDNA band during its removal from the gradient, the cpDNA band was further purified on a second gradient under the same conditions. Bisbenzimide was removed by three extractions with NaCl-saturated isopropanol, the remaining cpDNA-CsCl mixture was diluted 10-fold with water, precipitated by the addition of sodium acetate to 300 mM and two volumes of ethanol or one volume of isopropanol. The resulting cpDNA pellet was washed with 70% ethanol and dissolved in 10 mM Tris, 0.1 mM EDTA, pH 8.0.

Restriction and Hybridization Analysis

Restriction endonuclease digests were performed according to the manufacturers' specifications with enzymes purchased from Bethesda Research Laboratories, Boehringer Mannheim or New England Biolabs. Fragments were separated by agarose gel electrophoresis and visualized with ethidium bromide staining according to Maniatis et al. (1982), using HindIII-digested lambda phage DNA fragments as size markers. The Bam 3a fragment was located on the Oenothera cpDNA map using a heterologous probe from spinach containing the petA

gene (a gift from Dr. Warwick Bottomley, CSIRO, Canberra, Australia). Fragments 3a and 3b were isolated from BamHI digests of mutant or wild-type Oenothera cpDNA by cutting the fragment out of a 0.6% agarose gel, freezing in a dry ice-ethanol bath for 10 minutes, then manually crushing using a sterile spatula. An equal volume of redistilled phenol, pH 8.0 (Maniatis et al., 1982) was added to the resulting slurry which was frozen for 10 minutes in dry ice-ethanol. The aqueous phase was recovered from the mixture by centrifugation (13,000 xg, 5 min.) and the phenol phase was re-extracted with an equal volume of 10 mM Tris, 0.1 mM EDTA, pH 8.0 buffer. The two aqueous phases were pooled and extracted three times with phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1) and three times with chloroform:isoamyl alcohol (24:1). This was followed by ethanol precipitation (Maniatis et al., 1982).

These fragments were cloned by conventional procedures (Maniatis et al. 1982) into the plasmid vectors pBR322 or pUC 18/19 (Norrander et al., 1983) and nick-translated with α-^{32}P-dATP or α-^{32}P-dCTP (800 Ci/mMol, New England Nuclear) (Rigby et al. 1977) and used as probes in Southern hybridization experiments. Figures 2 and 3 show restriction maps which illustrate the additional Oenothera Bam 12 (Kaplan, 1987; Blasko et al., 1988) and Bam 3b (Pyung-Ok Lim, personal communication) sub-fragments also used as probes against HinfI-digested cpDNA. Transfer of cpDNA from agarose gels to nitrocellulose (Millipore) was performed as described by Maniatis et al. (1982). The nitrocellulose membranes were hybridized, washed and autoradiographed following procedures described by Southern

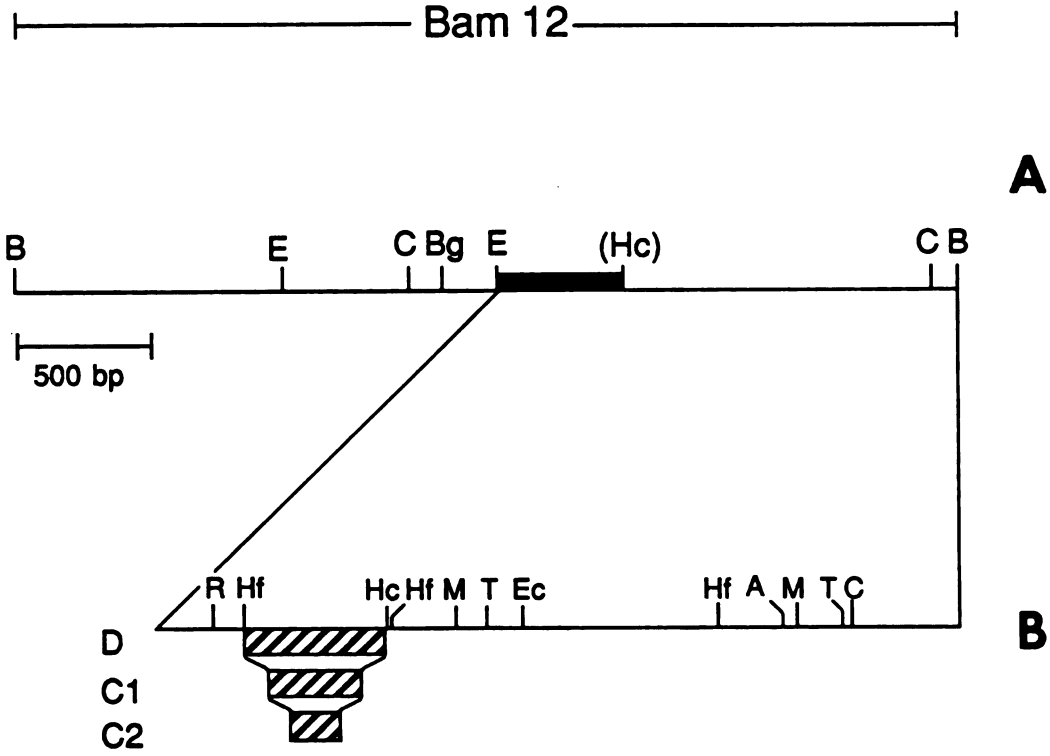


Figure 2. Restriction map of the variable Bam 12 region of the Johansen strain of Oenothera hookeri from lines D, C_1 and C_2. The restriction map of the entire 3-kb Bam 12 fragment is shown (A) as well as a 50% enlargement of the a 1.6-kb portion of this fragment (B). Both maps were generated by the work of Kaplan (1987) and Blasko et al. (1988) . The fragment outlined by a filled box contains the region of variability (striped boxes in B) in the C_1, C_2 and D plastome mutator lines. C_1 and C_2 are named for the Cornell University origin of the plastome mutator line; D refers to a plastome mutator line maintained at the University of Düsseldorf, which is also variable in the Bam 12 region (Kaplan, 1987). The variable regions from these three lines have been further characterized by DNA sequencing (Blasko et al., 1988). This has shown that the size variability in the Bam12 fragment is caused by the amplification (or elimination) of a 24-bp repeat segment within a region homologous to ORF 1708 from N. tabacum (Shinozaki et al., 1986). B=BamHI, E=EcoRI, C=ClaI, Bg=BglII, Hc=HincII, R=RsaI, Hf=HinfI, M=MaeIII, T=TaqI, Ec=EcoRV, A=AvaII.

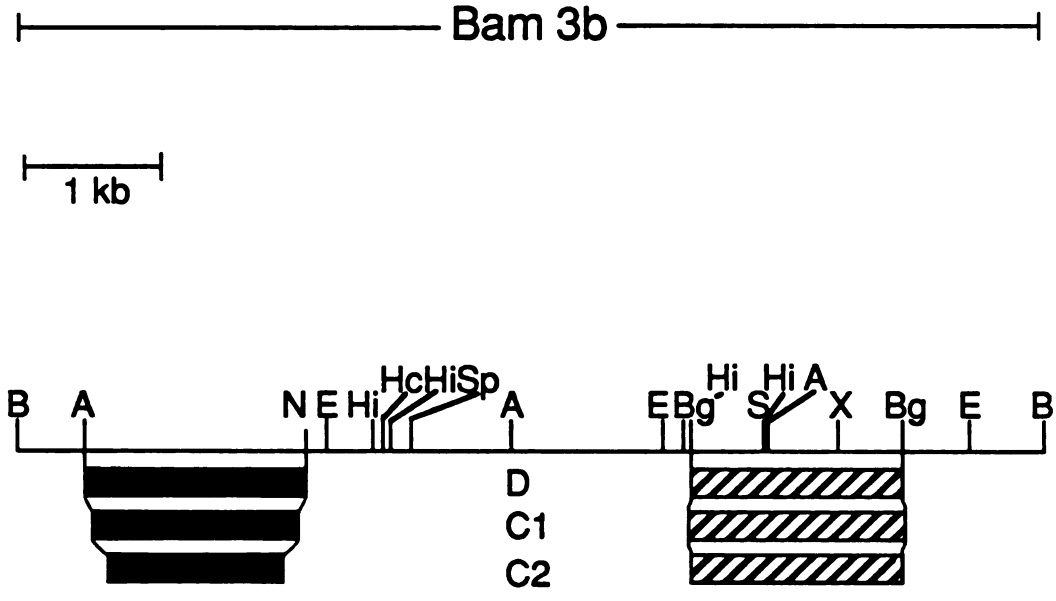


Figure 3. Restriction map of the variable Bam 3b region of the Johansen strain of Oenothera hookeri from lines D, C_1 and C_2. The restriction map of the 8-kb Bam3b fragment is shown (Kaplan (1987) and P. O. Lim (personal communication). Black and striped segments denote the two regions of variabiltity in this fragment (Kaplan, 1987). Clones containing these two fragments were used as probes in Southern hybridization experiments. C_1 and C_2 are named for the Cornell University origin of the plastome mutator line; D refers to a plastome mutator line maintained at the University of Düsseldorf, which is also variable in the Bam 12 region (Kaplan, 1987). B=BamHI, A=AccI, N=NruI, E=EcoRI, Hi=HindIII, Hc=HincII, Sp=SphI, Bg=BglII, S=SalI, X=XbaI.

(1975) and Maniatis et al. (1982) with prehybridization and hybridizations carried out at 65°C in the absence of formamide.

Results

Preliminary Analysis of Restriction Patterns of pm7 cpDNA

Initial comparisons of restriction digests of cpDNA isolated from pm7 and our wild-type line of the Johansen strain of Oenothera hookeri indicated that several restriction fragments varied in size (Figure 4). These differences were seen in comparisons of digests using a number of different restriction enzymes, including BamHI, EcoRI, HaeIII, MspI and TaqI. These differences are more easily seen when probes for specific regions of cpDNA are used. Variable fragments which hybridized to the 3-kb Bam12 fragment have been characterized by Kaplan (1987) and Blasko et al. (1988). Because of the phenotypic difference between wild-type and pm7 plastids with respect to cyt f (discussed further in Chapter 3), the first region of cpDNA used as a probe in this series of Southern hybridization experiments was a spinach fragment containing the cyt f (petA) gene (Figure 5), followed by the homologous 8-kb Oenothera fragment (Bam 3a) which contains petA (Figure 6). Examination of Figure 6 reveals that the Bam 3a probe hybridized to variable fragments in BamHI- and EcoRI-digested cpDNAs, but not to variable fragments in other lanes showing cpDNAs digested with HaeIII, MspI or TaqI. Additionally, when the Bam 3a fragments from mutant and wild-type cpDNA were subcloned into plasmid vectors and cut with endonucleases, no differences were seen in the restriction patterns of the cloned DNAs (data not shown). Figure 7 shows the same restricted cpDNAs from wild-type and

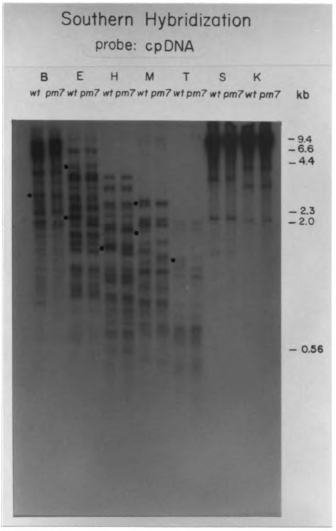


Figure 4. Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with total wild-type cpDNA. Pm7 and C_1 wild-type (wt) cpDNAs were digested with the enzymes indicated (B=BamHI, E=EcoRI, H=HaeIII, M=MspI, T=TaqI, S=SalI, K=KpnI), separated on a 1.2% agarose gel, transferred to nitrocellulose and probed with nick-translated total cpDNA from the C_1 wild-type. The wild-type cpDNA was subsequently identified as the I-C_1 type. Fragments which vary between the two cpDNAs are marked to the left of each pair of lanes with a filled circle.

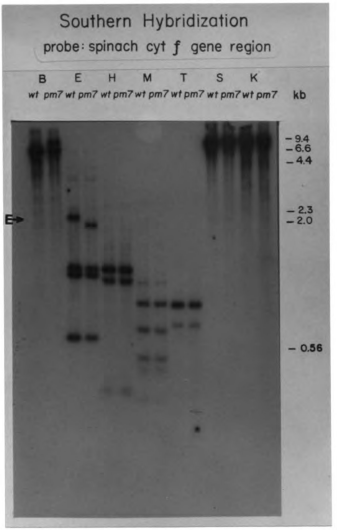


Figure 5. Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a spinach fragment containing the petA gene. Fragments of variable size can be seen in the EcoRI lanes only. The same filter shown in Figure 4 was washed and used for hybridization with nick-translated cloned spinach fragment Bam7.

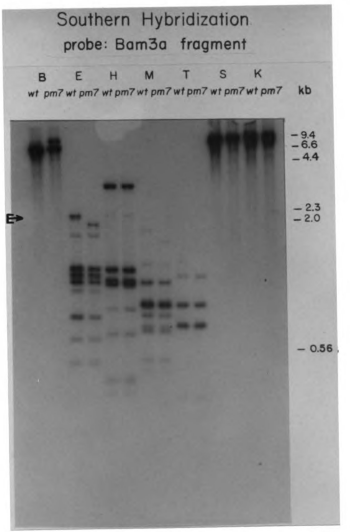


Figure 6. Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a Bam 3a. Fragments of variable size can be seen in the EcoRI lanes only. The same filter shown in Figure 4 was washed and used for hybridization with nick-translated cloned Oenothera wild-type fragment Bam 3a, which contains the petA gene.

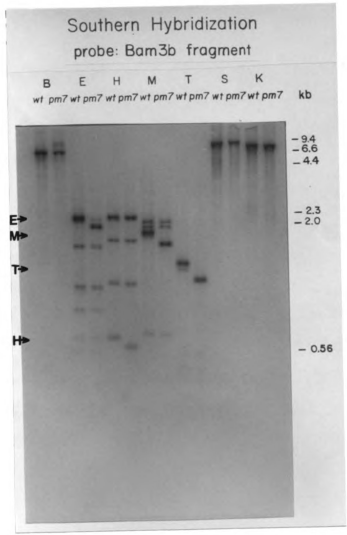


Figure 7. Southern hybridization of pm7 and C_1 wild-type cpDNAs probed with a Bam 3b. Fragments of variable size can be seen in the EcoRI, HindIII, MspI, and TaqI lanes. The same filter shown in Figure 4 was washed and used for hybridization with nick-translated cloned Oenothera wild-type fragment Bam 3b.

mutant probed with an Oenothera Bam 3b fragment. In this case, the same variable bands in the BamHI, MspI and EcoRI digests which were hybridized by the Bam 3a probe are recognized by Bam 3b. In addition, Bam 3b also recognizes bands in the HaeIII and TaqI digests which differ in size between the mutant and wild-type. In each case, the mutant fragment is approximately 200-basepairs (bp) smaller than the corresponding fragment in the wild-type. The implication of the results of the hybridization analysis was that Bam 3a and 3b were adjacent fragments; thus, the variable EcoRI fragment recognized by both the 3a and the 3b probes spans the junction of the two Bam fragments. By further use of Southern hybridization analysis, the Bam 3b fragment was localized to the large single copy region of the Oenothera hookeri cpDNA molecule, neighboring the Bam 3a fragment (Figure 8). It appeared therefore that the region which defined the deletion in pm7 cpDNA mapped to an area just upstream of the petA gene.

Analysis of Variable Regions in Plastome Mutator Lines

Further comparisons of cpDNA from various plastome mutator-induced mutants of plastome I indicated that the mutants all fall into two general classes with respect to their cpDNA restriction patterns (Figure 9). The two classes have been named C_1 and C_2 after the Cornell University origin of Oenothera hookeri, strain Johansen, Epp's original mutator line (Epp, 1973); the plastome I cpDNAs from each of these lines are called I-C_1 and I-C_2 respectively. Of the seven mutants which have been carefully examined, three belong to the I-C_1

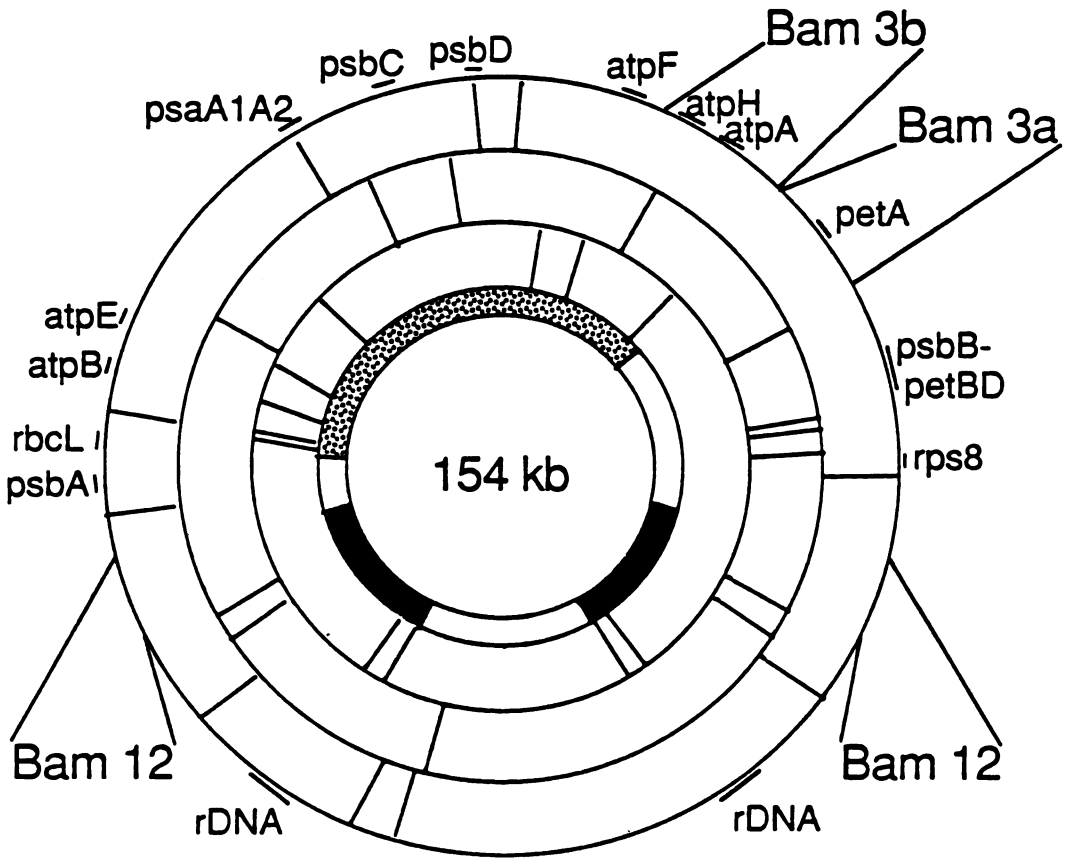


Figure 8. Physical map of Oenothera hookeri plastome I. Adapted from Gordon et al. (1982), the map shows the location of the two variable regions (Bam fragments 3b and 12) and the fragment containig the petA gene (Bam fragment 3a) in classes C_1 and C_2 of our plastome mutator line of Oenothera hookeri strain Johansen. Inverted repeat regions are outlined by black arcs. Region of the Oenothera cpDNA molecule which is inverted with respect to spinach cpDNA is denoted by a stipled arc.

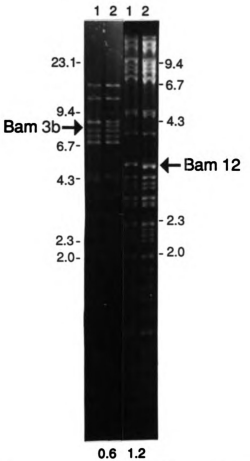


Figure 9. Restriction digest of cpDNA from (green) wild-type variants C_1 and C_2. CpDNA from C_1 (1) and C_2 (2) were digested with BamHI and fragments were separated in the presence of ethidium bromide on 0.6% or1.2% agarose gels. Two variable fragments from the C_1 and C_2 classes are identified as Bam 3b and Bam 12.

class, while the other four contain cpDNA which is of the I-C_2 type (indicated in Figure 10). The RFLPs which distinguish these classes do not correlate with any particular phenotype of individual mutants, and in fact, we have been able to recover green, photosynthetic representatives of these two classes (C_2-wt and C_1-wt in Figure 10). All molecular analyses of plastome mutator-induced mutants have been done in comparison with the appropriate wild-type variant.

All of the plants descended from the Cornell strain and examined thus far may be grouped into one of two major classes in terms of a 3-kb BamHI fragment (Bam12) which is located completely within the inverted repeat region or a second variable region defined by the 8-kb BamHI fragment (Bam 3b)(Kaplan, 1987; Johnson et al., 1988). A comparison is shown in Figure 11 for cpDNAs from two plastome mutants pm7 and pm11 and their corresponding wild-types, lines C_2 and C_1, respectively. For pm7, when BamHI and EcoRI digests of cpDNA are compared to those of the cpDNA of the proper wild-type (C_2), no differences are seen in ethidium bromide-stained gels nor in Southern hybridization experiments using Bam 3b as a probe (Figure 11). However, the cpDNA from pm11 does differ slightly in comparison to I-C_1. A variable fragment in the pm11 lane is indicated with the upper arrowhead in the ethidium bromide-stained gel of digested cpDNA. However, this variability is even more clearly demonstrated in the Southern hybridization analysis of EcoRI digests probed with Bam 3b, where the pm11 fragment appears to be intermediate in size between the corresponding fragment in the I-C_1 wild-type and either I-C_2 wild-type or pm7.

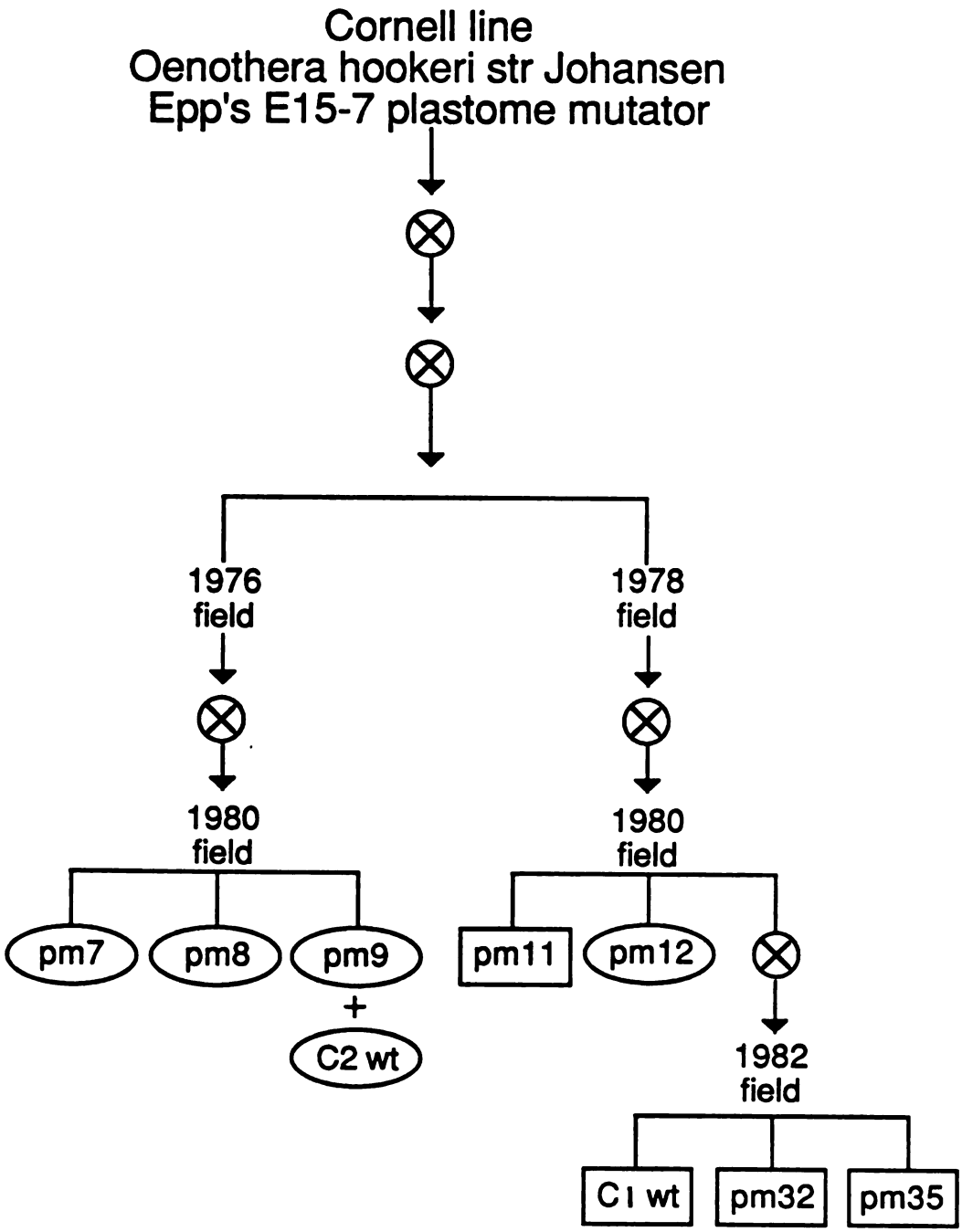


Figure 10. Pedigree of plant lines, indicating C_1 and C_2 classes of plastome mutator line. Pedigree of chloroplast mutant and (green) wild-type lines descending from Epp's Cornell Oenothera hookeri pm/pm isolate, E-15-7. (The circled x's represent self-crosses. According to their cpDNA restriction patterns, mutants in the C_1 class are boxed; mutants in the C_2 class are circled.)

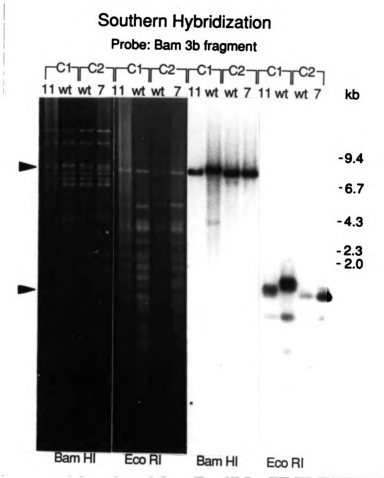


Figure 11. Restriction digest and Southern hybridization with Bam 3b. Chloroplast DNA was digested with EcoRI and fragements separated on a 0.6% agarose gel and stained with ethidium bromide. Fragments were transferred to nitrocellulose and probed with nick-translated plasmids containing cloned Bam 3b fragment from the C_1 class. Variable fragments between the C_1 and C_2 classes are marked with arrowheads. Results of the C_1 and C_2 variability and the pm11 analysis have been previously reported by Kaplan (1987).

Fine mapping of the Bam 12 fragment has shown that HinfI defines the regions of variability (Kaplan, 1987; Blasko et al., 1988). Since HinfI also cuts the Bam3b fragment a number of times, it was used to more carefully assess the variability of both of the regions in a number of mutants isolated from this pm/pm line. The left panel of Figure 12 shows HinfI-digested cpDNAs from two mutants and C_2 wild-type, probed with a mixture of two subcloned fragments from the Bam 3b region (Figure 3). The approximately 800-bp fragment which appears to be variable between the cpDNAs from C_1 and C_2 classes is marked with an arrowhead. There is also a slight difference in the migration of the largest band in the pm7 lane in comparison to the corresponding band in the C_2-wt lane. The right panel of Figure 12 shows the autoradiogram resulting when the same filter was probed with a 600-bp fragment from the Bam 12 fragment containing the region of variability (Figure 2). The most obvious difference between the I-C_1 and I-C_2 cpDNAs is the approximately 50-bp size variability in the smallest fragment in this panel. The larger of these variable fragments is indicated with an arrowhead. However, upon close examination, there is some indication of more subtle differences existing within the I-C_2 class, seen in the migration of the 120-bp and 260-bp fragments in the last two lanes of Figure 12.

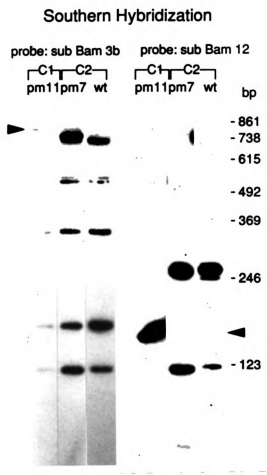


Figure 12. Southern hybridization of HinfI-digested pm11, pm7, and C_2 wild-type cpDNA with Bam 3b and Bam12 probes. Pm11, pm7, and C_2 wild-type cpDNA was digested with HinfI, separated on a 5% polyacrylamide gel, transferred to nitrocellulose and probed with fragments isolated from the variable region of Bam 3b (determined by S. Kaplan and P. O. Lim, shown in Figure 3) and from Bam 12 (determined by S. Kaplan and K. Blasko, shown in Figure 2). Variable regions between C_1 and C_2 types are noted with arrowheads.

Discussion

In the course of the molecular analysis of our plastome mutator lines, we determined that one of our pm/pm lines contained variants of plastome I (preliminary results reported by Sears, 1983; Sears and Kaplan, 1984; Kaplan and Sears, 1986; Kaplan, 1987). We have distinguished plants carrying two major cpDNA types by grouping them as lines C_1 and C_2, thus named after the Cornell University origin of Epp's line of Oenothera hookeri, strain Johansen. The variable regions in these lines appear to be the result of insertion/deletion events, similar to those which have been reported between more distantly related plants (Gordon et al., 1982; Salts et al., 1984; Schmitz and Kowallik, 1986, Doebley et al., 1987). Since we have been able to recover wild-type representatives of these two classes, we now know that the cpDNA RFLPs which are observed in a comparison of lines C_1 and C_2 are not correlated with the various plastome mutator-induced mutations. Hence, any preliminary differences observed between "mutant" and "wild-type," were due to the existence of cpDNA restriction fragment polymorphisms between plants descended directly from Epp's original pm/pm stock.

Various examples of "heteroplasmy" (the presence of two distinguishable types of organelle DNA in a single individual) have occasionally been observed in a number of organisms including Drosophila (Solignac et al., 1983, 1984), crickets (Harrison et al., 1985; Rand and Harrison, 1986), sea scallops (Snyder et al., 1987)

and American shad (Bentzen et al., 1988). In another case, heteroplasmy of a Holstein cow was deduced after an analysis of her descendents showed they carried two different mtDNA types (Hauswirth and Laipis, 1982). In some of these cases, direct descendents of the heteroplasmic individual were heteroplasmic also (e.g. Solignac et al.,1983; Harrison et al.,1985) and in other cases, the descendents were homoplasmic, containing only one or the other type of organelle DNA (Hauswirth and Laipis, 1982; Solignac et al., 1983). Additionally, Moon et al. (1987) have found individual rice plants which contain two co-existing populations of cpDNAs. In contrast, we have found that our individual plant lines (both mutant and wild-type) contain only one type of cpDNA, although we have concluded that a close ancestor must have been heteroplasmic for the cpDNA polymorphisms.

By constructing the pedigree (Figure 10) for the plastome mutants and the green pm/pm plants from which they were derived, we can narrow the occurrence of the event(s) leading to the chloroplast DNA variations to within two generations. The plastome mutator plants in Professor Stubbe's collection were all derived directly from self-crosses of Epp's pm/pm plant line (established from the original pm/pm isolate, E-15-7) containing plastome type I. Some seeds from Epp were planted in 1976 and others in the summer of 1978. Seeds resulting from self-crosses from both field seasons were planted in 1980. The former yielded three mutants which we have analyzed carefully: all contain cpDNA type C_2. Two mutants were analyzed from the latter; one represents type I-C_1 while the other represents

type I-C_2 cpDNA (Figure 9). From the second field accession, green seedlings were self-crossed for maintenance of the pm/pm strain and all of the progeny analyzed to date (two new mutant lines and progeny derived from at least five different green plants of the next generation) contain cpDNA type I-C_1. This homogeneity of our current pm/pm stock could have resulted from a founder effect due to the use of a limited number of plants in the self-crosses to perpetuate the line, and possibly from sampling only a small subset of the seeds produced by the previous generation for the subsequent planting.

Since the original seed lots from Epp and from the 1976, 1978 and 1980 field seasons are no longer available, one can only speculate as to the nature of the event(s) which resulted in the cpDNA heteroplasmy. Various explanations may be considered. Possibly, the variations in chloroplast DNA which define classes C_1 and C_2 are a consequence of the plastome mutator activity itself. However, since we have recovered both mutant and green plants from both the C_1 and C_2 plant lines, these major cpDNA variations are clearly not associated with the mutation events in the mutants analyzed. In the cpDNA comparisons within each line, it appears that two or possibly three regions contain hypervariable sites, while no other fragments seem to change. However, the variable regions found within the plastome mutator line do not correlate with the observed phenotype of any plastome mutator-induced mutants. Thus, one is led to the conclusion that mutations caused by the plastome mutator are most likely point mutations. Alternatively, although the other cpDNA changes seem to involve insertion/deletion events, a relationship of the plastome

mutator activity with variation in these specific cpDNA regions can be imagined. If, for example, the point mutations induced by plastome mutator are the result of a defective cpDNA repair system, this same defect may also increase the occurrence of other types of mutations resulting in the hot spots of cpDNA variability. Furthermore, it is also possible that the mutagenic activity of plastome mutator could be accompanied by a recombinogenic activity which produces this variability in cpDNA in the Bam 12 and Bam 3b regions.

Another possibility is that the plastome differences could have been due to contamination of the self-crosses with pollen from another plant carrying a different plastome type, as had been suggested by Lindenhahn et al. (1985). This is not likely because the results presented here demonstrate that these cpDNAs differ only at two specific sites, and are thus much more similar to each other than they are to any other plastome type (Gordon et al., 1982; Epp et al., 1987).

A more likely possibility is that the heteroplasmy may stem from the original EMS (ethyl-methane-sulfonate) mutagenesis. Although EMS is believed to induce predominantly point mutations in nuclear DNA (Legator and Flamm, 1973), conceivably the EMS treatment was responsible for the deletion/insertion events which appear to constitute the differences between the two cpDNA variants. Alternatively, the EMS may have caused point mutations, which in turn could have created a rearrangement 'hot spot' and the subsequent deletion/insertion. More detailed comparisons are currently under way in the Sears laboratory in order to further characterize the nature

of the cpDNA changes which have occurred in this plastome mutator line of Oenothera.

The existence of cpDNA heteroplasmy within our plastome mutator line underscores the necessity for using the correct wild-type in comparisons of mutant and wild-type cpDNAs. Using Bam 12 and Bam 3b as probes in Southern hybridization experiments to compare C_1 and C_2 wild-type cpDNAs to plastome mutator-induced mutants from each of these lines initially indicated only two distinct classes of cpDNA patterns within our plastome mutator line (Kaplan, 1987, Johnson et al., 1988). However, results of the investigations described here indicate the strong possibility that further variability may indeed exist within line C_1, between cpDNA from pm11 and C_1 in the Bam 3b region (Figure 12) and perhaps between pm7 and C_2 in the Bam 12 region (Figure 12). Less striking but still notable are the apparent subtle differences between HinfI-digested cpDNA from pm7 and its corresponding wild-type, C_2 (Figure 12). It is not clear from these preliminary investigations whether any further differences within line C_1 or C_2 are related to the activity of the plastome mutator. Further investigation of these preliminary observations is necessary in order to rule out the possibilities of partial restriction digestion or anomalous migration of DNA fragments in the electrophoresis conditions.

It is clear from these results that efforts to identify the site of the primary lesion in any one particular plastome mutator-induced mutant must employ other approaches besides cpDNA analysis. For example,

these mutants may be examined using immunological techniques to detect the presence or absence of various important chloroplast proteins. Various physiological assays may be carried out to determine whether the chloroplast proteins present are indeed functional in the mutant plastids. The degree of mutant plastid development may be assessed by ultrastructural analysis as well as direct measurement of the accumulation of certain chloroplast components such as chlorophyll, carotenoids, fatty acids and lipids. The fact that the plastome mutator system is also active in shoot-tip culture conditions provides the opportunity for isolating revertants of plastome mutator-induced mutants. Efforts by Dr. Linda Schnabelrauch to recover such revertants of one plastome mutator-induced mutant, pm7, have been successful, thus providing an additional valuable tool for use in characterization of this particular mutant.

CHAPTER 3

Immunochemical and Molecular Analysis of the Phenotypes of pm7 and a Putative Revertant of pm7

Introduction

Using the plastome mutator system of Oenothera, a large number of plastome mutants have now been isolated, following the appearance of chlorotic sectors in plants containing an active plastome mutator background (pm/pm). Screening of this collection, utilizing a battery of antibodies raised against various individual chloropast proteins, allowed the identification of a particular mutant with a unique phenotype in cytochrome f (cyt f). Cytochrome f is a thylakoid membrane-bound protein which functions in the cyt b_6/f complex as an intermediate electron carrier in photosynthetic electron transport (cytochrome structure and function reviewed by Hauska et al., 1983; Bendall, 1982). The mutant, pm7, accumulates a protein which is cross-antigenic with cyt f but is approximately 5-kD larger than the wild-type cyt f (Figure 13). This observation is intriguing since the gene for cyt f (petA) is a cpDNA-encoded gene, translated on chloroplast ribosomes as a precursor of a molecular weight which is also approximately 5-kD larger than the mature protein. This gene has been cloned and sequenced from a number of higher plants including pea (Willey et al., 1984a), wheat (Willey et al., 1984b),

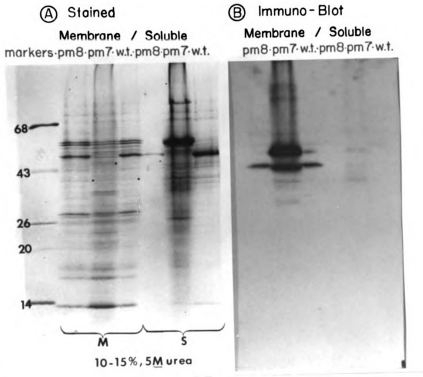


Figure 13. Coomassie blue staining and immunoanalysis with cyt f antibodies of mutant and wild-type chloroplast proteins. Results of an immunological screen performed by Dr. Sears on several the plastome mutator-induced mutants. Panel A shows a stained, denaturing polyacrylamide gel containing membrane and soluble fractions of wild-type (wt) and mutant (pm7 and pm8)chloroplast proteins. To standardize the loading of the membrane fractions, approximately equal amounts of the α and β subunits of the chloroplast ATPase were loaded in both mutant and wild-type lanes. These proteins were separated on denaturing polyacrylamide gels, electrophoretically transferred to nitrocelluose, probed with an anti-cyt f antibody, followed by ^{125}I-protein A (panel B). The arrow in the figure indicates a high molecular weight protein which is cross-antigenic with cyt f which is seen exclusively in the membrane fraction of pm7. (The signal at the migration noted by the arrow in the wild-type membrane lane in B is not reproducible and is probably due to "spillover" from the neighboring pm7 lane in this gel.)

spinach (Alt and Herrmann, 1984), rice (Wu et al., 1986) and Oenothera (Tyagi and Herrmann, 1986). The sequence, including the presence of a 4-5-kD amino-terminal extension which is not found in the mature protein, has proven to be highly conserved in many species.

Various hypotheses can be put forth to explain this observation in pm7. A number of these hypotheses involve the possibility that a lesion may exist in the petA gene itself. Conceivably, the unusually large cyt f protein in pm7 is an unprocessed precursor of cyt f. For example, a site of protein processing has been proposed for various higher plant species (Willey et al., 1984a, 1984b; Alt and Herrmann, 1984; Wu et al., 1986; Tyagi and Herrmann, 1986) by comparing the deduced amino acid sequence of the precursor to cyt f (pre-cyt f) to the actual N-terminal amino acid sequence of cyt f from spinach (Ho and Krogman, 1980). If there were a lesion at this site in the pm7 petA gene, it could result in the defective processing of cyt f. Another highly conserved region of the petA gene encoding two cysteine residues has been suggested as a site of heme binding in cyt f (Nelson and Neumann, 1972; Nelson and Racker, 1972; Siedow, 1980). The covalently bound heme group in cyt f is essential for the proper functioning of mature cyt f in photosynthetic electron transport (Nelson and Neumann, 1972; Nelson and Racker, 1972). However, it is not known at which point in maturation of the protein the heme is attached. Therefore, it is possible that a lesion in petA which alters the heme binding site could have a secondary inhibitory effect on the processing of the pre-cyt f protein.

An alternative explanation for the observed cyt f phenotype in pm7 is that perhaps a mutation is present upstream of the petA gene which results in an overexpression of the gene. Such an "up" mutation could cause the expression of the gene to outpace the activity of the processing enzyme. Such an explanation would be consistent with the apparently "leaky" nature of the phenotype (Figure 13), that is, the presence of a cross-reacting protein that has a migration similar to cyt f. Each of these possibilities involving a lesion directly in the petA gene were tested by DNA sequence analysis of the mutant and wild-type genes.

Conceivably, the 40-kD protein which cross-reacts with cyt f antibodies is not a precursor to this protein. Several other explanations can be considered which could account for a larger protein which is antigenically related to cyt f. Multiple sizes of RNAs containing the cyt f message have been found in pea (Willey, personal communication), spinach (Alt and Herrmann, 1984) and a closely related Oenothera line (Tyagi and Herrmann, 1986), indicating that the transcript of the wild-type petA gene may be processed in a complicated manner. The petA message may be on a polycistronic transcript because no strong consensus transcriptional start sequences for petA exist in a number of species (Willey et al., 1983, 1984b; Alt and Herrmann, 1984, Wu et al., 1986, Tyagi and Herrmann, 1986). Additionally, an ORF has been found directly upstream from petA in pea (Willey et al., 1984a), spinach (Alt and Herrmann, 1986) and tobacco (Shinozaki et al., 1986). It is

conceivable that improper RNA processing of this message results in the use of an upstream translational start site and thus leads to the accumulation of an unusually large-sized cyt f protein in pm7. However, all ATG codons within 200-bp upstream of petA are soon followed by a stop codon, making this alternative unlikely.

Altered splicing could also result in partial or complete inhibition of protein processing because the protein processing site is no longer present or not recognized in pm7 due to an alteration in the N-terminal sequence.

A translation product of increased size could also result from the in-frame insertion of an approximately 100-bp element in the petA gene. An in-frame insertion of this size should be detectable in a restriction analysis of the region containing the petA gene using a number of restriction endonucleases. A frame-shift mutation caused by a small insertion or deletion is not likely to result in a large cyt f product due to the many stop codons in the other two reading frames (Tyagi and Herrmann, 1986).

Read-through of the authentic termination codon of the petA message, due to an altered tRNA or stop codon could yield an aberrant translation product which could be processed at the amino terminus as usual, but would still result in the accumulation of a protein which is cross-antigenic with cyt f but larger than the expected size. Again, this is an unlikely explanation since there are numerous other stop codons near the end of the petA gene.

In addition to proper processing of the precursor form of the protein, proper association with heme and the other protein components of the cytochrome complex as well as integration into the thylakoid membrane are presumably required in order to achieve the active, mature form of cyt f. A number of other higher plant mutants with altered phenotypes in the cyt b_6/f complex have shown the accumulation of one member of a complex may depend upon the presence of other subunits of the complex (Maroc and Garmier, 1981; Mets et al., 1983; Bendall et al., 1986; Barkan et al., 1986; Lam and Malkin, 1985; Lemaire et al., 1986; Olive et al., 1986). In this context, another hypothesis arises which suggests that in the absence of heme or another component of the cyt b_6/f complex, both the processing and assembly of other subunits could be aborted. These possibilities were addressed by using a gel staining technique to detect heme bound to protein and immunoblotting techniques to look for the accumulation of the other components of the cyt b_6/f complex: cyt b_6 and subunit IV, each encoded by chloroplast genes, and the Reiske Fe/S protein, which is translated on cytoplasmic ribosomes.

It is also conceivable that the larger pm7 cyt f protein is a ubiquitinated form of the mature cyt f. The difference between the sizes of the mature and the aberrant proteins in pm7 is consistent with this possibility. Immunoanalysis with an antibody which recognizes plant ubiquitin was used to test this hypothesis. However, no evidence to support this possibilty was found, since the antibody reacted only with a very high molecular weight smear (data not shown)

and did not detect any proteins of sizes similar to the aberrant cyt f protein in pm7.

Finally, the aberrantly sized cyt f protein in pm7 may be the result of defective processing machinery in the mutant plastids. According to this hypothesis some element essential to chloroplast protein processing is encoded by a plastid gene, since pm7 is a non-Mendelian mutant. It is possible that such a processing deficiency could affect other proteins in the chloroplast besides cyt f. To test this, several other cpDNA-encoded proteins could be examined since evidence indicates they are initially synthezed as larger precursors. The post-translational processing of the D1 protein of photosystem II (PS II) has been well studied (Greenbrier et al., 1978; Riesfeld et al., 1982; Marder et al., 1984; Minami and Watanabe, 1985) and is known to involve an acylation step as well as specific proteolytic cleavage in order to achieve the mature protein product of approximately 32-kD in size (Mattoo and Edelman, 1987). Early reports (Langridge, 1981) have also proposed the existence of a precursor form of the large subunit of ribulose bis-phosphate carboxylase/oxygenase (RuBisCO LSU). Thus, determining the status of these other chloroplast proteins in pm7 could indicate whether a general processing defect is involved.

One could also ask whether the processing of nuclear-encoded chloroplast proteins is affected in the mutant. Most proteins which are imported from the cytoplasm contain an amino-terminal transit peptide which is proteolytically cleaved during or after import into the

organelle. Furthermore, some proteins are processed a second time within the organelle, presumably during passage across the thyakoid membrane (Smeekens et al.,1986; Hageman et al., 1986). In order to address the issue of post-translational processing in pm7, a series of immunoblotting experiments was performed using a battery of antibodies raised against various individual chloroplast proteins, including a collection of imported proteins as well as a number of proteins which are translated on chloroplast ribosomes. This approach is made possible by the fact that chloroplast genes are highly conserved between many higher plant species and hence antibodies raised against their gene products often cross-react.

Materials and Methods

Plant Material

Plant material was maintained as shoot-tip cultures as described in Chapter 2, illuminated with 20 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ photosynthetically active radiation (PAR). All wild-type tissues and tissue containing pm7 in the A/C nuclear background were heterozygous for the pm allele, and thus inactive for the plastome mutator function. However, tissue containing pm7 plastids in the A/A nuclear background was homozygous for the mutant pm allele. In this nuclear background the potential exists for reversion, as discussed in Chapter 2. Thus, in the maintenance of the A/A pm7 leaf tip cultures, all tissue was discarded which showed any evidence of reversion (appearance of green sectors).

Spinach leaves for purification of cyt b_6/f particles were purchased from a local farmer's market.

M13 Cloning and Dideoxy Sequencing Strategy

As described in Chapter 2, Bam 3a fragments containing the petA gene encoding cyt f were isolated from wild-type and mutant cpDNA using a heterologous probe from spinach (a gift from Dr. W. Bottomley, CSIRO, Canberra) and cloned into plasmid vectors pBR322 and pUC18/19 (Norrander et al., 1983). More detailed data on DNA restriction patterns were obtained using general methods for restriction digest

and agarose gel electrophoresis described by Maniatis et al. (1982) according to suppliers' specifications (Bethesda Research Laboratories (BRL), Boehringer Mannheim, New England Biolabs).

Segments of the Bam3a fragment (Figure 14) were further cloned into M13 vectors, according to specifications given in the BRL Instruction Manual on M13 Cloning/Dideoxy Sequencing. M13 vectors mp18 and mp19 (Norrander et al., 1983; Yanisch-Perron et al., 1985) and the bacterial host JM 107 (Yanisch-Perron et al., 1985) were employed in the cloning and sequencing stategy originated by Sanger et al. (1977). Figure 15 summarizes the composition of the clones which were constructed and the sequencing strategy followed. Preparative quantities of the double-stranded replicative form (RF) of these phage DNAs for both the vectors and the vectors with inserts were obtained using standard culture prodcedures for M13 phage (Messing,1983) and plasmid preparation protocols given by (Kahn et al., 1979). The DNA sequence of the inserts into these vectors was determined using both ^{32}P- and ^{35}S-labelled Klenow sequencing reactions on single-stranded templates, as described in the BRL M13 Cloning Dideoxy/Sequencing Manual. A total of 700-bp was sequenced on both strands using the universal 17-bp primer (BRL) and two synthetic primers prepared by Dr. C. R. Somerville (Michigan State Univ.) using an Applied Biosystems 380A DNA Synthesizer.

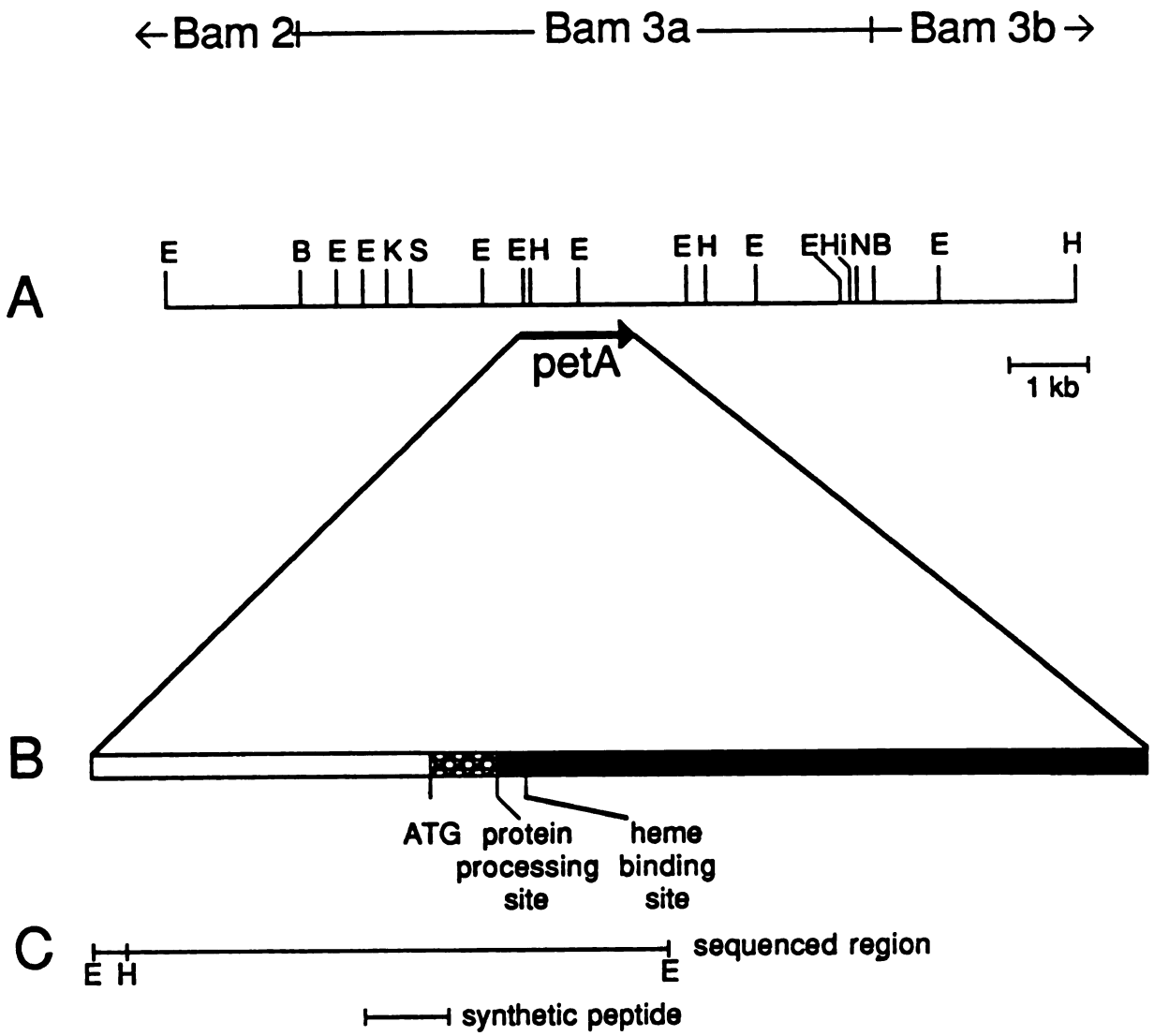


Figure 14. Map of the Bam 3a region from the Johansen strain of Oenothera hookeri cpDNA. Part A shows a restriction map of the Bam 3a region from pm7 and its corresponding wild-type. E=EcoRI, B=BamHI, K=KpnI, S=Sal, H=HindIII, Hi=HincII, N=NdeI. Part B is a 10-fold enlargement of the region containing the petA gene and the upstream sequences. The translation initiation codon is indicated along with the location in the DNA sequence of the protein processing site and the heme binding site. Part C represents the region of the DNA sequenced in pm7 and the wild-type.

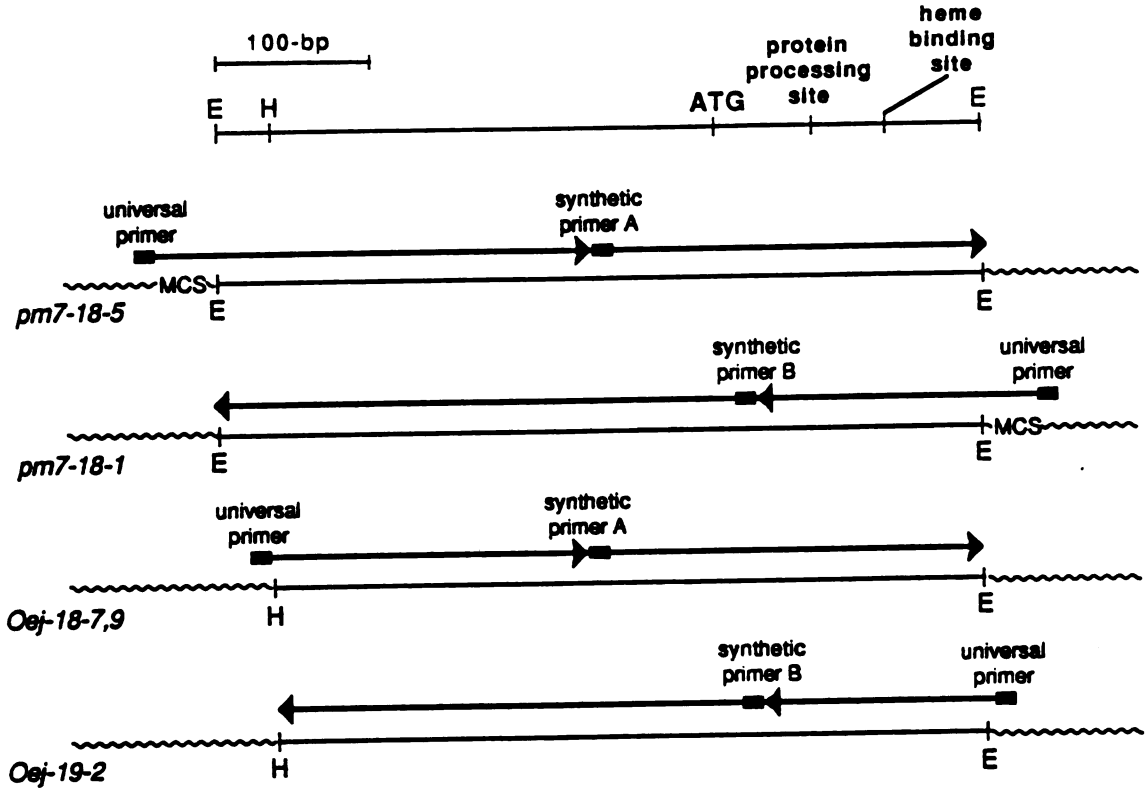


Figure 15. Strategy employed to sequence the 5' end and region upstream of petA from pm7 and line C_2 wild-type cpDNA. Clones were constructed in M13 vectors mp18 (pm7-18-5, pm7-18-1 and Oej-18-7,9) or mp19 (Oej-19-2). This pair of vectors contains the multiple cloning site (MCS) in opposite orientations, thus allowing the production of single-stranded templates containing either sense (pm7-18-5 and Oej-18-7,9) or anti-sense (pm7-18-1 and Oej-19-2) strands of the cloned DNA. Arrows denote the direction of sequencing proceeding from one of 3 different primers, shown as black boxes on the arrows. Th "universal" 17-bp primer (5'-GTAAAACGACGGCCAGT-3') was purchased from Bethesda Research Laboratories; synthetic primers A (5'-TCCAGTAAACCGATTTCAGT-3') and B (5'-TATACATCAGTGATAGGGAAAT-3') were prepared by Dr. C.R. Somerville (MSU) as described in the text. Wavy lines represent vector sequence; thin straight lines denote Oenothera cpDNA sequence.

Chloroplast Preparation and Fractionation of Membrane/Soluble Proteins

Tissue (10-40 g leaf material) was harvested 3-4 weeks after transfer to fresh media. Washed chloroplasts were prepared by homogenization and differential centrifugation as in Chapter 2. Membrane and soluble protein fractions of these chloroplast preparations were isolated in the following manner. Plastid pellets of approximately 100 µl volume were resuspended with vigorous agitation in 3 volumes 50 mM Tris, pH 8.0 and centrifuged 5 minutes in a microfuge. Supernatants were saved in fresh tubes and the pellets were resuspended as before in 3 volumes 100 mM NaCl, 50 mM Tris, pH 8.0. This mixture was centrifuged as before and the resulting supernatant pooled with the previous supernatant sample. This step was repeated once more and the three pooled supernatants were precipitated with 3 volumes of 50% (w/v) trichloro-acetic acid (TCA) on ice for 30 minutes. TCA precipitates were collected by 5 minute microfuge centrifugation, washed twice with 80% acetone and resuspended to a final protein concentration of 0.75 $\mu g \cdot \mu l^{-1}$ in SDS-polyacrylamide gel sample buffer (50 mM Tris, pH 6.8, 0.28 M β-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol) and made to a final concentration of 2 mM phenol methyl sulfonyl fluoride (PMSF). The washed chloroplast membrane pellet was resuspended in sample buffer to a final protein concentration of 2.5 $\mu g \cdot \mu l^{-1}$. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad publication #81-0525), performed on aliquots of the membrane or soluble protein preparations resuspended in water.

SDS-Polyacrylamide Gel Electrophoresis

Membrane and soluble chloroplast protein fractions in 50 µl (wild-type) and 75 µl (mutant) volumes were analysed using the discontinuous buffer system of Laemmli (1970) and 10-15 % (w/v) gradient SDS-polyacrylamide gels containing 5 M urea. Improved resolution of Oenothera chloroplast polypeptides has been achieved by using one-half the originally recommended concentration of bis-acrylamide in the gel recipes (Dr. P. Westhoff, personal communication). Gels were stained with Commassie Blue (Sigma Chemical Co.) to visualize the total protein pattern or tetramethylbenzidine/H_2O_2 (Sigma Chemical Co.) for the detection of covalently-bound heme (Thomas et al., 1976). Except where specifically indicated, all protein sizes discussed are apparent molecular weights as determined by comparison to protein markers of known sizes (Sigma Chemical Co.): bovine serum albumin (68-kD), ovalbumin (45-kD), carbonic anhydrase (29-kD) and cytochrome c_1 (12.4-kD).

Preparation and Sources of Antibodies Specific for Chloroplast Proteins

Cyt b_6/f complex was isolated from 500 g fresh spinach leaves using octylglucoside and cholate extraction according to a procedure published by Hurt and Hauska (1981; Gray and Phillips, 1982; Clark and Hind, 1983). Cytochrome f was purified from this protein

complex by electroelution using a CBS Scientific electroeluter apparatus as directed by the manufacturer. New Zealand white rabbits were initially immunized as directed by Chua et al. (1982) with 500 μg purified cyt f antigen in 1 ml 0.15 M NaCl, 10 mM Na phosphate, pH 7.0 (PBS), mixed with 1 ml complete Freund's adjuvant (Sigma Chemical Co.); injections were repeated biweekly using incomplete Freund's adjuvant. Sera from successive bleedings were screened by Western blotting analysis and serum aliquots were stored at -20°C.

For use in preparation of an antibody specific for the cyt f precursor, a synthetic peptide of the first 17 residues of unprocessed cyt f was prepared by Dr. H. Wolfe at E. I. DuPont Co. using the amino acid sequence deduced from the DNA sequence of Oenothera hookeri (Tyagi and Herrmann, 1986). This peptide was conjugated by C-terminal cys cross-linking to a carrier protein, keyhole limpet hemocyanin (KLH). A mixture of 0.5 - 1.0 mg peptide-KLH conjugate and 1 ml of trehalose dimycolate (TDM) emulsion (RIBI Immuno Chem Research, Inc), reconstituted according to the supplier's specifications, was injected biweekly into each of two white New Zealand rabbits. Serum was collected and screened as described above.

A number of other antisera against particular chloroplast proteins were generously donated by various researchers. The source for each of the antibodies is given in Table 1.

Table 1. Sources of antibodies used in immunoanalysis of pm7 and wild-type chloroplast proteins. Abbreviations for protein names are given in the text.

Antigen	Origin	Source of Antibody
cyt f	spinach	E. Johnson, MSU, E Lansing
cyt f	rape	T. Bricker, Univ MO, Columbia
SU IV(cyt b_6/f)	maize	A. Barkan, Univ CA, Berkeley
FeS(cyt b_6/f)	"	"
OEC-33	spinach	C. Jansson, MSU, E. Lansing
OEC-23	"	"
OEC-16	"	"
D1(PS II)	Amaranthus	J. Hirschberg, MSU, E. Lansing
PS II-22-kD	spinach	C. Yocum, Univ MI, Ann Arbor
PS II43-kD, 47-kD	tobacco	C. Chia, MSU, E. Lansing
PSI	"	R. Malkin, Univ CA, Berkeley
LHC II-29-kD	barley	S. Darr, Univ IN, Bloomington
Carbonic anhydrase	spinach	D. Husic, M. Volakita, N. Artus, MSU, E. Lansing
ADP-glucose pyrophosphorylase	spinach	J. Preiss, MSU, E. Lansing
Ribosomal proteins L21, L29	Chlamydomonas	J. Boynton, Duke Univ, Durham
Plastocyanin	spinach	K. Keegstra, Univ WI, Madison
RubisCO small subunit	maize	E. Bell, MSU, E. Lansing
"	alfalfa	C. Schumann, MSU, E. Lansing
Ubiquitin	oat	R. Vierstra, Univ WI, Madison
Mitochondrial cytochrome oxidase subunit II	yeast	T. Fox, Cornell Univ, Ithaca

Immunoblotting Analysis

Proteins separated on SDS-PAGE were transferred to nitrocelluose membrane filters with a Transphor Electrophoresis Cell (Hoefer Scientific Instruments, San Fransisco, CA) at 4°C for 3-5 hours at 150 V using 25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3 (Towbin et al., 1979). Filters were blocked by shaking overnight at room temperature in 5 % (w/v) non-fat dry milk (Spartan Brand), 0.2 % Na azide (blocking buffer) (Kaufmann et al., 1987). Filters were incubated in antibodies dissolved (at dilutions ranging from 1/100 to 1/1000, depending upon the antibody titer) in blocking buffer for 2 hours to overnight at room temperature. Filters were washed four times (15 minutes each) with 0.05 % Non-idet-P40 (NP40) in PBS (phosphate-buffered saline). The nitrocelluose sheets were then incubated 1 hour at room temperature with rocking in the PBS-NP40 solution containing 5-10 μCi of ^{125}I-labelled protein A (New England Nuclear). Filters were subsequently washed four times (15 minutes each) with the PBS-NP40 solution, dried and exposed to Kodak X-Omat XAR-5 film for autoradiography. Preliminary immunoblots utilized an alkaline phosphotase-protein A detection system based upon the procedure of Leary et al. (1983). For a number of antisera, however, radiolabelling gave a stronger signal and thus ^{125}I-detection was the method of choice for these experiments.

Results

Cloning of Fragments from the Bam 3a Region of pm7 and Wild-type cpDNAs

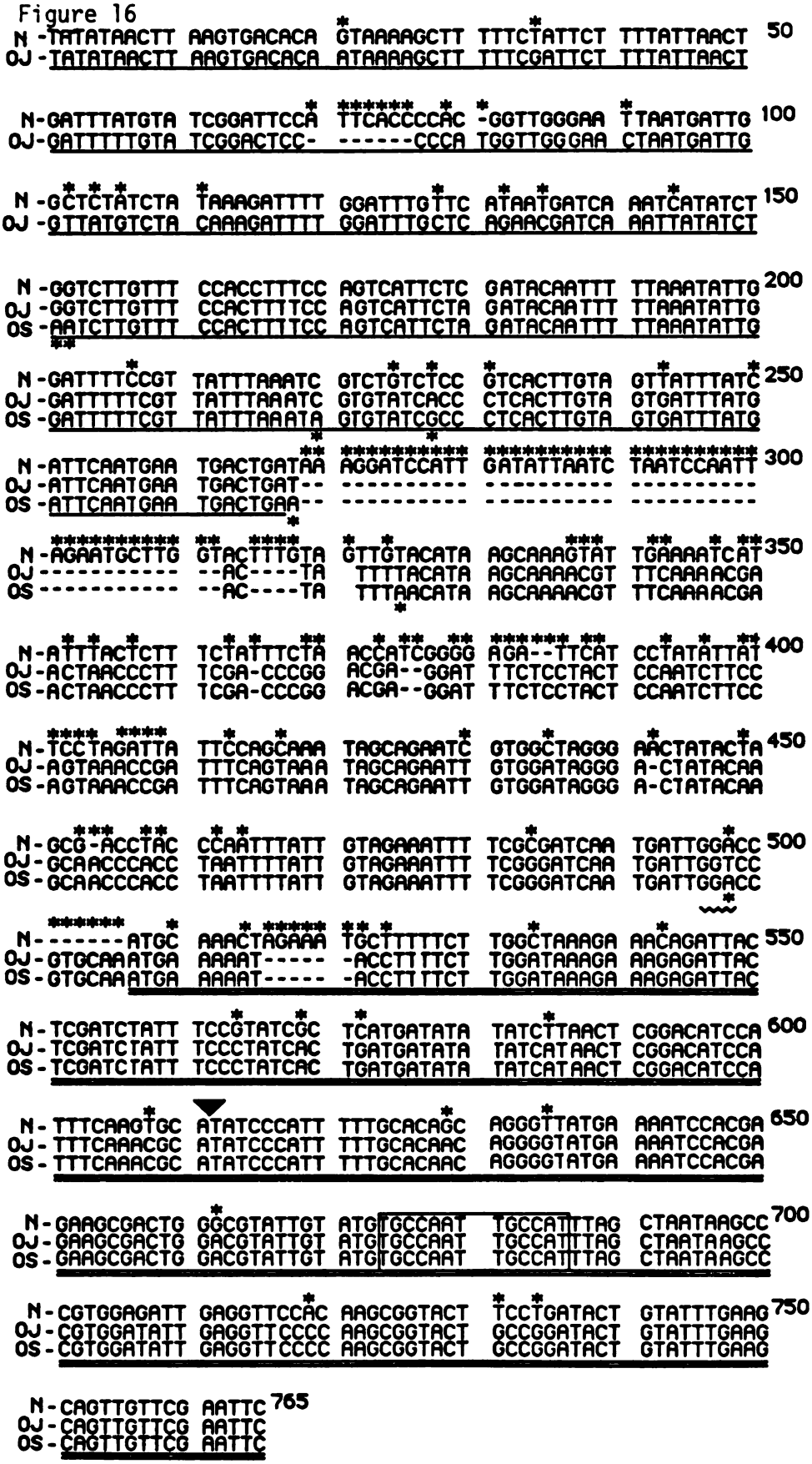
Figure 14 shows the restriction map developed for the Bam 3a region from both the pm7 mutant and the C_2 wild-type. Approximately 700-bp was sequenced from pm7, using the fragment shown in Figure 14C and defined by an EcoRI site within petA and an upstream EcoRI site. The segment sequenced in the wild-type is about 50-bp smaller since the HindIII site shown in Figure 14A was used for subcloning. Both fragments were subcloned into M13 vectors mp 18 or mp19; these clones are shown in Figure 15, including the orientation of the insert with respect to the sense strand of the petA gene. Figure 15 also shows the sites where the synthetic primers anneal.

Analysis of Mutant and Wild-type DNA Sequences in the petA Region

The DNA sequence which encodes a portion of the petA gene and extends approximately 450-bp upstream from this gene in the pm7 mutant and Oenothera wild-type is presented in Figure 16. Regions encoding the proposed processing and heme binding sites for the protein product are marked. No sequence changes are found in these areas between pm7 and its correct wild-type progenitor, line C_2, but there are differences when the sequence from these two isolates is compared to the hookeri Standard strain sequenced by Tyagi and

Figure 16. Partial sequence of petA and upstream region of cpDNA from the Nicotiana tabacum and Johansen and Standard strains of Oenothera hookeri. The top line shows the N. tabacum (N) sequence (Shinozaki et al., 1986); the second line shows the sequence for both pm7 and wild-type I-C_2 from the Johansen strain of O. hookeri (OJ), with nucleotide #1 representing the 5' end of the sequence determined for the mutant and wild-type isolates from the Johansen strain; the third line, which begins at nucleotide #151, contains the available sequence from the Standard strain of O. hookeri (OS) as determined by Tyagi and Herrmann (1986). Astericks above or below the lines of sequence denote differences between the Johansen strain and the tobacco and the Standard strain sequences, respectively. Sequence for 268-bp from the 3' end of ORF 229 is single underlined; sequence for approximately 300-bp from the proposed 5' end of petA is double underlined. A putative ribosome binding site for petA (at nucleotide #496) is underlined with a wavy line. The site at which cyt f protein processing occurs is marked with an arrowhead. The boxed region encodes the proposed heme binding site for cyt f.

Figure 16


N -TATATAACTT AAGTGACACA GTAAAAGCTT TTTCTATTCT TTTATTAACT 50
OJ -TATATAACTT AAGTGACACA ATAAAAGCTT TTTCGATTCT TTTATTAACT
N-GATTTATGTA TCGGATTCCA TTCACCCCAC -GGTTGGGAA TTAATGATTG 100
OJ-GATTTTTGTA TCGGACTCC- ------CCCA TGGTTGGGAA CTAATGATTG
N -GCTCTATCTA TAAAGATTTT GGATTTGTTC ATAATGATCA AATCATATCT 150
OJ -GTTATGTCTA CAAAGATTTT GGATTTGCTC AGAACGATCA AATTATATCT
N -GGTCTTGTTT CCACCTTTCC AGTCATTCTC GATACAATTT TTAAATATTG 200
OJ -GGTCTTGTTT CCACTTTTCC AGTCATTCTA GATACAATTT TTAAATATTG
OS -AATCTTGTTT CCACTTTTCC AGTCATTCTA GATACAATTT TTAAATATTG
N -GATTTTCCGT TATTTAAATC GTCTGTCTCC GTCACTTGTA GTTATTTATC 250
OJ -GATTTTTCGT TATTTAAATC GTGTATCACC CTCACTTGTA GTGATTTATG
OS -GATTTTTCGT TATTTAAATA GTGTATCGCC CTCACTTGTA GTGATTTATG
N -ATTCAATGAA TGACTGATAA AGGATCCATT GATATTAATC TAATCCAATT 300
OJ -ATTCAATGAA TGACTGAT-- ---------- ---------- ----------
OS -ATTCAATGAA TGACTGAA-- ---------- ---------- ----------
N -AGAATGCTTG GTACTTTGTA GTTGTACATA AGCAAAGTAT TGAAAATCAT 350
OJ ---------- --AC----TA TTTTACATA AGCAAAACGT TTCAAAACGA
OS ---------- --AC----TA TTTAACATA AGCAAAACGT TTCAAAACGA
N -ATTTACTCTT TCTATTTCTA ACCATCGGGG AGA--TTCAT CCTATATTAT 400
OJ -ACTAACCCTT TCGA-CCCGG ACGA--GGAT TTCTCCTACT CCAATCTTCC
OS -ACTAACCCTT TCGA-CCCGG ACGA--GGAT TTCTCCTACT CCAATCTTCC
N -TCCTAGATTA TTCCAGCAAA TAGCAGAATC GTGGCTAGGG AACTATACTA 450
OJ -AGTAAACCGA TTTCAGTAAA TAGCAGAATT GTGGATAGGG A-CTATACAA
OS -AGTAAACCGA TTTCAGTAAA TAGCAGAATT GTGGATAGGG A-CTATACAA
N -GCG-ACCTAC CCAATTTATT GTAGAAATTT TCGCGATCAA TGATTGGACC 500
OJ -GCAACCCACC TAATTTTATT GTAGAAATTT TCGGGATCAA TGATTGGTCC
OS -GCAACCCACC TAATTTTATT GTAGAAATTT TCGGGATCAA TGATTGGACC
N -------ATGC AAACTAGAAA TGCTTTTTCT TGGCTAAAGA AACAGATTAC 550
OJ -GTGCAAATGA AAAAT----- -ACCTTTTCT TGGATAAAGA AAGAGATTAC
OS -GTGCAAATGA AAAAT----- -ACCTTTTCT TGGATAAAGA AAGAGATTAC
N -TCGATCTATT TCCGTATCGC TCATGATATA TATCTTAACT CGGACATCCA 600
OJ -TCGATCTATT TCCCTATCAC TGATGATATA TATCATAACT CGGACATCCA
OS -TCGATCTATT TCCCTATCAC TGATGATATA TATCATAACT CGGACATCCA
N -TTTCAAGTGC ATATCCCATT TTTGCACAGC AGGGTTATGA AAATCCACGA 650
OJ -TTTCAAACGC ATATCCCATT TTTGCACAAC AGGGGTATGA AAATCCACGA
OS -TTTCAAACGC ATATCCCATT TTTGCACAAC AGGGGTATGA AAATCCACGA
N -GAAGCGACTG GGCGTATTGT ATGTGCCAAT TGCCATTTAG CTAATAAGCC 700
OJ -GAAGCGACTG GACGTATTGT ATGTGCCAAT TGCCATTTAG CTAATAAGCC
OS -GAAGCGACTG GACGTATTGT ATGTGCCAAT TGCCATTTAG CTAATAAGCC
N -CGTGGAGATT GAGGTTCCAC AAGCGGTACT TCCTGATACT GTATTTGAAG 750
OJ -CGTGGATATT GAGGTTCCCC AAGCGGTACT GCCGGATACT GTATTTGAAG
OS -CGTGGATATT GAGGTTCCCC AAGCGGTACT GCCGGATACT GTATTTGAAG
N -CAGTTGTTCG AATTC 765
OJ -CAGTTGTTCG AATTC
OS -CAGTTGTTCG AATTC

Herrmann (1986). Eight single base pair changes are noted which differentiate these two strains and all of them precede the initial methionine codon. A putative ribosome binding site is indicated by wavy underlining. However, as was previously reported by Tyagi and Herrmann (1986), there are no clear consensus sequences for transcriptional regulatory sites such as a "Pribnow box," expected approximately 10-bp upstream or a second RNA polymerase recognition region expected approximately 35 upstream of the petA gene.

Sequencing comparison extended farther upstream of petA for pm7 and the wild-type line C_2 than has been reported for the petA gene from O. hookeri analysed by Tyagi and Herrmann (1986). Thus, a 260-bp sequence was encountered which was 92% homologous to the 3' end of ORF 229 which has been previously reported in pea (Willey et al., 1984b), spinach (Alt and Herrmann, 1984) and tobacco (Shinozaki et al., 1986). Again, no differences were seen between the mutant and the wild-type in the 3' end of ORF 229 of the intergenic region. This region is also shown in Figure 16. The divergent bases in Nicotiana are noted by asterisks above the line of sequence. The spacing between the petA gene and ORF 229 was 186-bp in line C_2 of Oenothera and 231-bp in Nicotiana.

Analysis of Putative cyt f Precursor-Specific Antibodies

Parallel to the molecular analysis of the petA gene, an attempt was made to test the hypothesis that the mutant protein represented an

unprocessed form of pre-cyt f. A DNA sequence of the Oenothera hookeri petA gene was available (Tyagi and Herrmann, 1986). The deduced amino acid sequence of the amino-terminal extension of cyt f was analysed to choose a sequence for a synthetic peptide. Dr. H. Wolfe (E.I. DuPont Co.) synthesized a peptide based upon the first 17 amino acids of the presumed cyt f presequence, representing 50% of the total N-terminal extension. The final product of this synthesis however contained an unexpected arginine residue (Wolfe, personal communication) Nonetheless, this peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits in order to produce a "precursor-specific" poloyclonal antibody (referred to as the "anti-peptide" antibody). Membrane proteins from wild-type and pm7 mutant plastids were isolated from tissue maintained in shoot-tip culture and subjected to immunoblotting procedures using the anti-peptide antibody. The results of this Western blotting experiment are shown in Figure 17A. This antibody, which should recognize only the cyt f precursor, gives a single faint signal in both the mutant and the wild-type lanes, at a size approximately 15 kD larger than the mature cyt f. It is likely, based upon the especially large size of this protein and also because only a very weak signal was achieved even when the antibody was used at a titer of 1:100, that the anti-peptide antibody does not show specificity for the precursor but gives only a "background" signal. Furthermore, this same nitrocellulose filter was reprobed with the cyt f antibody so that the abberant cyt f band in the mutant could be compared to the protein band recognized by the anti-peptide antibody. Figure 17B shows that these bands do not

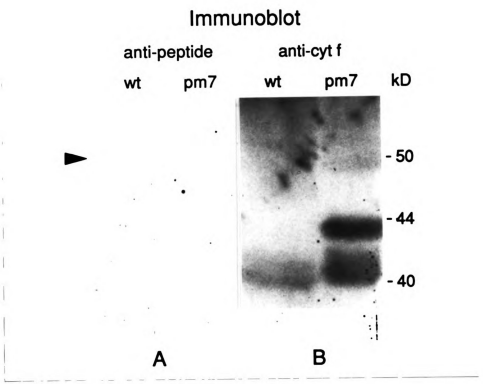


Figure 17. Immunoblot of wild-type and pm7 total chloroplast proteins probed with antibodies against synthetic cyt f precursor-conjugate and cyt f. The arrowhead indicates a cross-reacting polypeptide present in pm7 and wild-type (wt). Reprobing of the filter from panel A with cyt f antibodies shows that the protein recognized by anti-peptide immunoglobilin does not comigrate with the aberrant cyt f in pm7.

comigrate, that is, the two antibodies do not recognize the same proteins.

An alternative explanation for the lack of recognition of a protein in pm7 which is the size expected for pre-cyt f is that perhaps the suggested presequence (Tyagi and Herrmann, 1986) is not the authentic N-terminal sequence of the cyt f precursor. The presequnce proposed by Tyagi and Herrmann (1986) is based on the agreement between the size of the in vitro translation product of the petA message and the predicted size of cyt f with this additional N-terminal extension. Additionally, a putative ribosome binding site is present 8-bp upstream of the AUG codon proposed by Tyagi and Herrmann (1986). Nonetheless, it is possible that wild-type cyt f is the translation product of a message in which the codon encoding the known N-terminal residue of the mature protein (Tyr) is spliced to mRNA from a region even farther downstream than the proposed (contiguous) presequence. In pm7, altered mRNA splicing could disrupt the region recognized by the protease and protein processing activity may be affected. This could result in a mutant cyt f which is larger than the mature product, but not detected by the antibody prepared against the synthetic peptide from the presumed presequence of Tyagi and Herramnn (1986) such as was seen in this experiment.

Ubiquitin (UBQ) is a small protein which becomes bound to a variety of proteins as a signal for their degradation (Finley and Varshausky, 1985). The size of UBQ (2-3-kD) is similar to the increased size of the

aberrant cyt f in pm7. Although UBQ has not been found to be present in chloroplasts, the possibilty that the aberrant cyt f in pm7 is a UBQ-conjugate was investigated using an antibody raised against oat UBQ (Shanklin et al., 1987). The only cross-reacting material detected was a smaear of high molecular weight (greater than 80-kD) bands from total protein preparations from both mutant and wild-type (data not shown).

Analysis of Heme Staining Activity

Denatured chloroplast proteins from the mutant and wild-type were separated electrophoretically on polyacrylamide gels along with the purified proteins of the spinach cyt b_6/f complex and analysed by heme staining. The mutant does not show heme staining activity in the region of the gel where either the mature or the putative pre-cyt f is expected. There is also no stain in the mutant lanes at a size which corresponds to cyt b_6. (This is not unexpected however since the TMBZ procedure does not reliably stain non-covalently bound heme proteins, such as cyt b_6.) Occasionally heme-staining bands were observed in lanes having proteins from the mutant (Figure 18) and sometimes also in lanes containing proteins from the incompatible wild-type (data not shown). However, none of these bands comigrated with mature cyt f (38-40-kD in our gel system) nor do they migrate at a size expected for the cyt f precursor (43-45-kD).

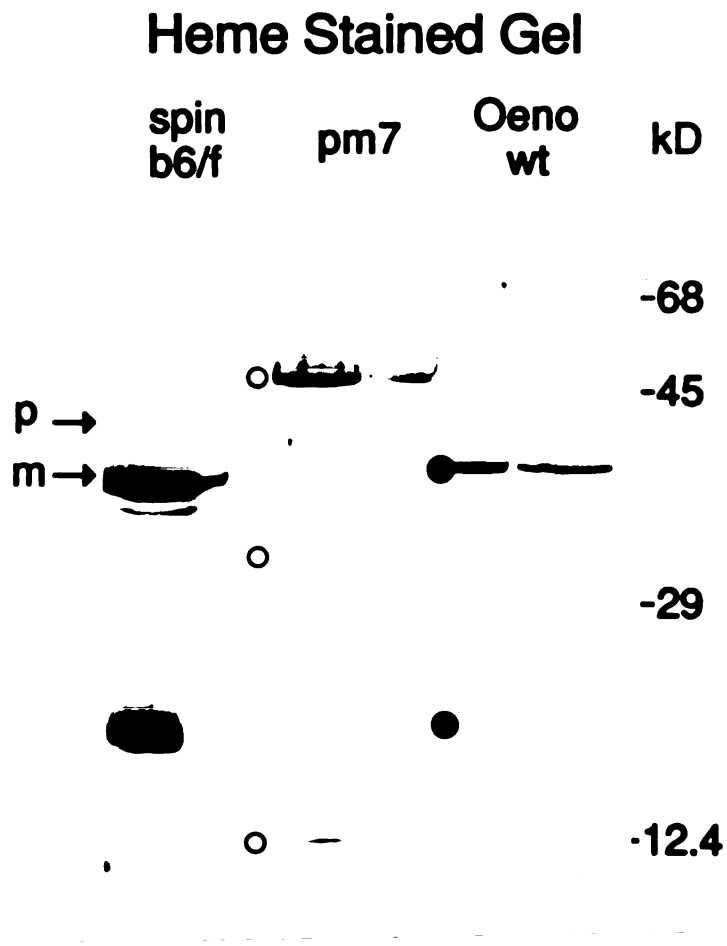


Figure 18. Heme-stained SDS-polyacrylamide gel (10-15% acrylamide gradient, 4 M urea) of mutant and wild-type chloroplast proteins. Pairs of wild-type (wt) and pm7 lanes represent independent preparations of chloroplast proteins. Filled circles indicate the location of heme-staining proteins in the wt; open circles indicate heme-staining proteins in the mutant. A purified cyt b_6/f preparation from spinach is included for reference. The positions of the mature cyt f and its putative precursor in Oenothera are labelled "m" and "p", respectively.

Immunoanalysis of Wild-type and Mutant Chloroplast Proteins

Immunological screening with a variety of different antibodies revealed additional alterations for several other chloroplast proteins. Each antibody was used to probe either both membrane and soluble fractions of chloroplast proteins isolated from mutant and wild-type tissues or, in a few cases, the membrane fractions only. Mutant lanes were loaded with approximately 1.5-fold more protein than wild-type lanes in the gels used for this immunoblotting analysis (Figure 19). All western blots were performed on mutant and wild-type proteins in both A/A and A/C nuclear backgrounds. The same results were observed in both cases and therefore only the A/C data are presented here.

Figure 20 is a composite figure showing the results of several immunoblotting experiments probing plastid membrane proteins with antibodies which are specific for three proteins of the cyt b_6/f complex. Cyt f appears to be the only protein in this group which is qualitatively altered in pm7. Cyt f antibodies reproducibly recognize a protein in pm7 which migrates at the size expected for the mature cyt f protein. Earlier analyses also showed that the fourth member of this complex, cyt b_6, is also present, in wild-type quantities, in the mutant chloroplast membrane fraction (Sears, personal communication) Figure 20B,C shows that the two other proteins of the cyt b_6/f complex (the subunit IV and the Reiske FeS protein) are present. Like the cyt f protein, subunit IV is translated on chloroplast ribosomes, but with no terminal extension. The FeS protein, however,

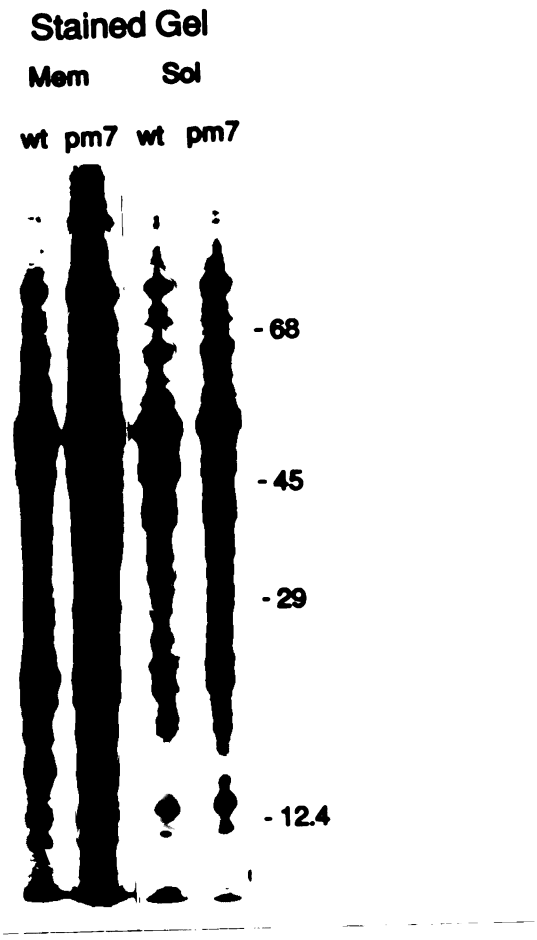


Figure 19. Coomassie-stained gel of membrane and soluble fractions of mutant and wild-type chloroplast proteins. A 10-15% SDS-polyacrylamide gel containing 5 M urea was used to separate mutant and wild-type (wt) membrane and soluble proteins.

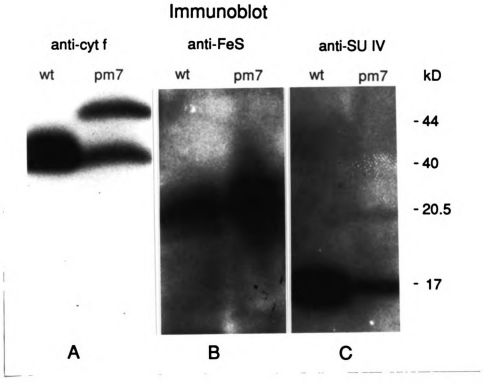


Figure 20. Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of the cyt b6/f complex. Panel A shows that pm7 plastids accumulate a protein which cross-reacts with spinach cyt f antibodies at a size which is consistent with that of pre-cyt f (44-kD). Both and mutant and wild-type (wt) plastids accumulate proteins which cross-react with spinach cyt f (A), maize Fe/S (B) and maize SU IV (C) at the predicted mature sizes. (SU IV is not synthesized a high molecular weight precursor [Heinemeyer et al., 1984].)

is translated on cytoplasmic ribosomes as a precursor with a proposed transit peptide of 68 amino acid residues (Titggen et al., 1986; Steppuhn et al., 1987). Although both proteins appear to be reduced in quantity in pm7, there are no indications of any significant accumulation of unprocessed precursor to the FeS protein in either pm7 or the wild-type plastids.

Western blotting also shows that the 33-kD subunit of the oxygen evolving complex (OEC-33) is found at the expected mature size and does not appear to be lower in quantity in the mutant in comparison to the wild-type (Figure 21). In contrast, the remaining two imported proteins associated with this complex show qualitative differences in the mutant compared to the wild-type. Antibodies raised against the 23-kD and the 16-kD components of the spinach OEC (OEC-23 and OEC-16) cross-react with proteins of approximate sizes 28-kD and 20-kD, respectively, in the mutant, while recognizing proteins of about 23-kD and 16-kD in the wild-type (Figure 21B,C). In other systems, a protein of about 32-kD recognized by the OEC-16 antibody has been suggested to be a dimer (Dr. C. Jansson, personal communication). However, the absence of a 16-kD monomer in pm7 suggests that the cross-reacting 32-kD protein is not a dimer of 16-kD units.

Antibody analysis of a third group of chloroplast proteins, including a number of those associated with either of the two photosystems in the thylakoid membrane, is shown in Figure 22. The D1 protein of the PS II complex is the one other chloroplast protein known to be translated on chloroplast ribosomes as a precursor (Greenbrier et al., 1978;

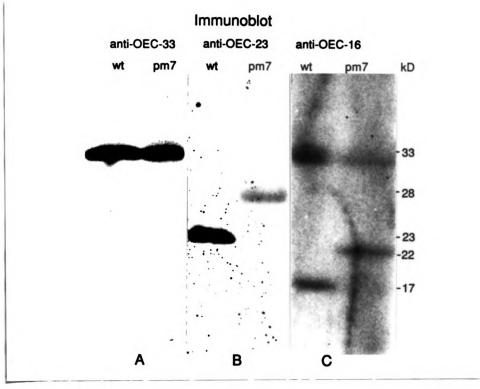


Figure 21. Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of the OEC subcomplex of PS II.

Riesfeld et al., 1982; Marder et al., 1984; Minami and Watanabe, 1985). A D1-specific antibody detected no mature-sized product (32-kD) in the mutant, but instead a possible degradation product around 23-kD (panel A). Panel B shows an immunoanalysis using a monoclonal antibody which was made against the imported 29-kD (minor) apoprotein of the barley light harvesting chlorophyll complex (LHC) for PS II (Darr et al., 1986). The analogous protein in Oenothera appears to be accumulated in lesser amounts in the mutant than in the wild-type, but at the expected size for the mature protein product.

The chloroplast-translated 43-and 47-kD chlorophyll-binding PS II proteins, as well as the 22-kD non-chlorophyll-binding core protein of PS II (Ljungberg et al., 1984, 1986; Ghanotakis et al., 1987a) and a 28-kD component of PS II, proposed to be a chlorophyll-binding core protein (Ghanotakis et al., 1987b), are all recognized by a single antibody preparation made against the purified 22-kD protein from spinach (panel C). Inspection of panel C in this figure indicates that the only one of these proteins appears to be either altered or deficient in the mutant - the 28-kD component of PS II. This protein, as well as the 22-kD PS II protein, are each thought to be products of nuclear genes and imported into the chloroplast (Dr. C. Yocum, personal communication). The upper two proteins in panel C probably represent the Oenothera analogs of the spinach 47-kD and 43-kD chlorophyll proteins of PS II, which are recognized by this antibody (Dr. C. Yocum, personal communication). A protein of approximately 45-kD surprisingly appears to be greatly increased in quantity in pm7

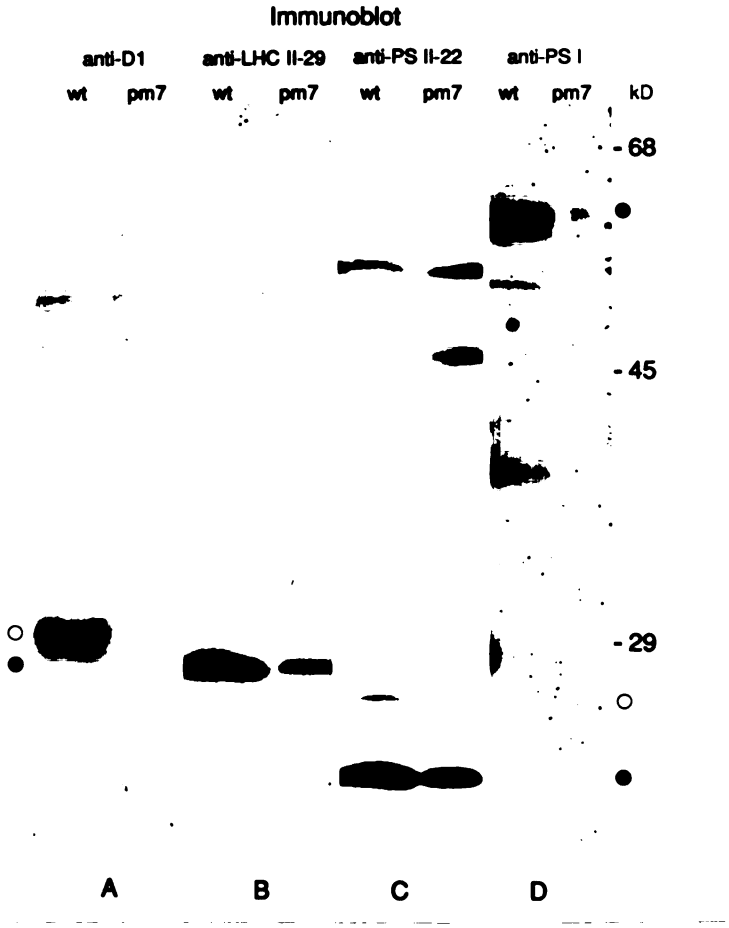


Figure 22. Immunoblot of pm7 and wild-type membrane fractions probed with antibodies against components of PS II and PS I. Open circles indicate the absence in pm7 of a protein at that position; filled circles indicate the presence of a protein in pm7 at that position. Circles on the left margin are indicators for panels A and B; circles on the right margin are indicators for panels C and D.

in this gel but this quantitative relationship was not consistently observed in other immunoblots with this antibody.

Also depicted in this figure is the result of immunoblotting analysis with polyclonal antibodies for the spinach P700 chlorophyll-binding reaction center protein of the photosystem I (PS I), the gene for which is encoded on cpDNA (panel D). Like the 29-kD LHC-II, this protein appears to be reduced in quantity in the mutant but, migrates at the mature, wild-type size. Further deficiencies in other unidentified cross-reacting proteins in pm7 may represent the lack of additional chlorophyll-binding subunits of PS I.

All of the proteins discussed thus far are ultimately located in the thylakoid membrane itself or in the lumen of the thylakoid membrane. Figure 23 shows the results of an analysis of a number of cytoplasmically-translated chloroplast proteins which, following import into the chloroplast, are eventually destined for the stroma. Two of these proteins, the L21 subunit of the chloroplast ribosomal complex (Schmidt et al., 1984, 1985) and the soluble stromal enzyme, carbonic anhydrase (Jacobsen et al., 1975; Reed, 1979; Tsuzuki et al., 1985), both appear to be present but at reduced levels in the mutant. Both subunits of another enzyme normally found in the stroma, ADP-glucose pyrophosphorylase (PYRO) (Krishnan et al., 1986) were also found at the expected mature sizes and did not appear to be greatly lowered in abundance in the mutant.

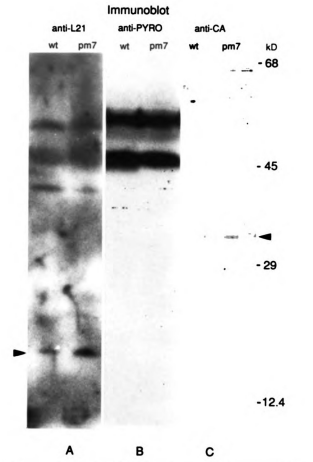


Figure 23. Immunoblot of pm7 and wild-type membrane (A) or soluble (B,C) fractions probed with antibodies against a ribosomal protein and two stromal proteins. Both mutant (pm7) and wild-type (wt) plastids accumulate proteins which cross-react with antibodies against Chlamydomonas chloroplast ribosomal protein L21 (A), spinach ADP-glucose pyrophosphorylase (PYRO, B), and spinach carbonic anhydrase (CA, C) at the predicted mature sizes.

A summary of the combined results of these immunological screens is provided in Table 2. This table indicates the location of the genes for these proteins in nuclear or chloroplast DNA and also shows the final suborganellar destination of the proteins themselves. As well, the range of sizes for the mature proteins in a variety of plants and similar "generalized" sizes for the precursor proteins, if appropriate, are given.

Immunoanalysis of a Putative Revertant of pm7

A possible revertant of the pm7 plant line was isolated by Dr. Linda Schnabelrauch by propagating tissue containing the mutated pm7 plastome in a homozygous pm/pm nuclear background and in leaf-tip culture until green revertant sectors were observed. These sectors were separated and subcloned until sufficent material was accumulated to allow protein purification from the putative revertant. (The revertant is referred to as "putative" because, although the tissue is green, it does not appear to be fully photosynthetically competent. Although roots can be induced, attempts to transfer the plant material to medium lacking sucrose or to a soil medium for growth in the greenhouse have not been successful (Sears, personal communication). Figure 24 presents the results of an immunoanalysis of these proteins with three of the antibodies which showed alterations in the analysis of the pm7 mutant: anti-cyt f, anti-OEC-23 and anti-PS II-22 (Figures 19, 20 and 21, respectively). Cytochrome f is a representative of a class of cpDNA-encoded proteins made as precursors for which the processing is altered in pm7; OEC-23 is a member of a class of

Table 2. Summary of immunological analysis of pm7 plastid proteins. Gene locations in cpDNA or nuclear DNA (nucDNA) are given. The final location of the protein product in the plastid is also specified. Protein sizes refer to range of apparent molecular weights known for these proteins from various plant species. Proteins found in the mutant as cross-reacting polypeptides which are larger in size than the mature wild-type proteins are indicated by ">". The presence of mature-sized cross-reactive material in pm7 is signified by "+". The total absence of any signal at the mature size or greater is shown by "-". Three antibodies were tested but did not cross-react with Oenothera wild-type or pm7; the results of these analyses are denoted by a " ?." In some cases, mutant proteins were localized to the membrane (M) or the soluble (S) fraction of the chloroplast. The analyses of cyt b6, RubisCO LSU (large subunit) and chloroplast ATP synthase (cpATPase) subunits were included in a preliminary analysis performed by Dr. Sears. Protein abbreviations are given in the text. Where possible, comprehensive reviews are given as references.

Protein	Gene location	Protein location	Precursor size	Mature size	Reference [a]	pm7 phenotype	pm7 location
cyt f	cpDNA	thylakoid	38-43	33-38	1	>	M
cyt b6	cpDNA	thylakoid	--	20-23	1	+	-
b6/f-SU IV	cpDNA	thylakoid	--	15	1	+	M
b6/f-FeS	nucDNA	thylakoid	25-26	19	2	+	M
OEC-33	nucDNA	lumen	38-39	33-34	2	+	S/M
OEC-23	nucDNA	lumen	33(28)	23	2	>	M/S
OEC-16	nucDNA	lumen	26(?)	16-17	2	>	M/S
PS II-D1	cpDNA	thylakoid	34	32	1	-	M(wt)
PS II-51	cpDNA	thylakoid	--	49-51	1	+	M
PS II-44	cpDNA	thylakoid	--	41-46	1	+	M
PS II-22	nucDNA?	thylakoid	32?	22	3	+	M/S
PS II-28	nucDNA?	thylakoid	?	28	3	-	M/S(wt)
LHC II-29	nucDNA	thylakoid	32-33	28-29	2	+	M
PS I	cpDNA	thylakoid	--	69-70	1	+	M
cpATPase-α	cpDNA	thylakoid	--	58-59	1	+	-
cpATPase-β	cpDNA	thylakoid	--	56-57	1	+	-
PC	nucDNA	lumen	17	11	2	?	?
RubiscoLSU	cpDNA	stroma	53-58?	52-56	1	+	S
RubiscoSSU	nucDNA	stroma	20-21(18)	14-16	4	?	?
CA	nucDNA	stroma	?	32-33?	5	+	M/S
PYRO	nucDNA	stroma	73/76	51/54	6	+	S
rp-L21	nucDNA	cp-ribosome	17.4(16.3)	14.6	7	+	M
rp-L29	nucDNA	cp-ribosome	14.4	12.2	7	?	?

[a] 1. Crouse et al., 1985; 2. Titggen et al., 1987; 3. Dr. C. Yocum, pers. comm.; 4. Schmidt and Mishkind, 1986; 5. Dr. M. Volakita, pers comm.; 6. Krishnan et al., 1986; 7. Schmidt et al., 1984,1985.

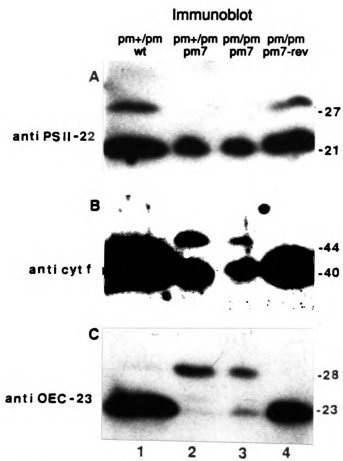


Figure 24. Immunoblot of pm7 and wild-type chloroplast proteins in stable and unstable plastome mutator nuclear backgrounds probed with antibodies against PS II-22, cyt f and OEC-23. Plastid proteins were isolated from wild-type (wt, lane1) or mutant (lanes 2,3) in the stable (pm+/pm) nuclear background (lanes 1,2) or the unstable (pm/pm) nuclear background (lane 3). Lane 4 contains plastid proteins isolated from a putative (green) revertant of pm7 in the unstable (pm/pm) nuclear background. Immunoblots were probed with antibodies against PS-22 (A), cyt f (B), or OEC-23 (C). In all cases the revertant material shows a protein pattern similar to that of the wt.

nuclear-encoded proteins made as precursors and then imported into the chloroplast, the processing of which is also affected in pm7; PS II-28 is a representative of a class of nuclear-encoded proteins which is absent in pm7. All three antisera tested gave similar results in that the protein pattern seen in the mutant analysis has returned to a wild-type pattern in the putative revertant. In contrast to previous results (Figure 21) with the OEC-23 antiserum, the mutant lanes appear to contain a small amount of mature-sized protein. This would not be surprising in the homozygous background, as it is possible that some reversion may have already occurred. However, this protein was not reproducibly observed in either background and probably represents spillover from the neighboring wild-type or revertant lanes in this particular gel.

Discussion

Initial analysis of pm7 showed an accumulation of a polypeptide which was about 5-kD larger than but also cross-antigenic with cyt f (Figure 13). A number of mutational lesions could give such a result, but in all cases, at least part of the cyt f reading frame must be intact since the protein is recognized by cyt f antibodies. This protein may represent an unprocessed precursor to cyt f, due to a lesion in the gene itself or due to a defect in the chloroplast protein processing machinery. For example, a lesion in the upstream regulatory region of the petA gene could potentially create an 'up' mutation, resulting in the overexpression of the gene. Alternatively, the lesion could be a large in-frame insertion in the gene or an insertion or deletion which fuses cyt f to another reading frame.

A number of lines of evidence including the lack of any strong transcriptional start sites and the presence of an ORF approximately 200-bp upstream suggest that the petA gene may be transcribed as a polycistronic message. Altered RNA processing of this message and the aberrant use of an upstream initiation codon could conceivably lead to a larger than normal petA gene product. The lesion in pm7 could also be modified so as to allow read-through of the stop codon which normally terminates translation of the petA message, for example, through the use of an altered tRNA. This could result in a protein of a size greater than wild-type. The molecular analyses described in this chapter were designed to explore the possibility that the primary

lesion in pm7 was located in or near petA, the gene encoding cyt f. Subsequent analysis of the mutant phenotype at the level of various proteins addressed the specificity of the pm7 lesion for cyt f.

The possibility that a sizable insertion or deletion could have created a major alteration in the reading frame of petA was addresssed through the use of restriction endonucleases. Analysis of cpDNA restriction patterns from the mutant and wild-type Bam 3a region showed no differences between the two regions (Figures 14) and thus, no evidence of major insertions or deletions in the petA gene. Furthermore, the existence of many termination codons in the two alternate reading frames (Tyagi and Herrmann, 1986) renders the possibilities a frame-shift mutation or a change in the stop codon unlikely.

Translation from an upstream initiation codon resulting in a petA gene product of increased size is improbable since the potential upstream start codons are each closely followed by a stop codon, and thus, would not yield a protein product of the size seen in pm7. Furthermore, as mentioned above, premature initiation of translation would have to maintain the normal reading frame of cyt f in order to give cross-antigenic products. It is also possible that altered splicing of the upstream portion of the the petA message could subsequently affect the protein processing site and lead to the accumulation of a large mutant cyt f protein. The merits of this possibility are addressed below in the context of the analysis of the pm7 revertant.

The results discussed thus far have addressed the question of whether various factors affecting petA gene expession, acting in either a 'cis' or a 'trans' fashion, could be responsible for the accumulation of an unusually large petA gene product in pm7. Each of these factors, including insertion or deletion events in the petA gene and alterations to components of the chloroplast translational apparatus, such as an altered tRNA which permits read-through of the normal termination codon may be ruled out based on the results of the restriction analysis of the region containing the petA gene and the examination of the sequence for the Oenothera petA gene itself. The remaining viable hypotheses all involve the possibility that the aberrantly-sized cyt f in the mutant is an unprocessed precursor of cyt f. Perhaps the precursor is not processed as a result of a mutation which directly alters the DNA encoding the processing site or the heme-binding site of cyt f. Alternatively, factors other than the gene encoding cyt f could be altered and consequentially interfere with proper processing of cyt f. These may be other components of the cyt b_6/f complex, such as the heme group or other protein subunits, or perhaps the processing apparatus itself. If some factor other that the petA gene is responsible for the altered cyt f in pm7, then the processing of other proteins besides cyt f may also be altered. This, it is worthwhile to investigate whether the processing of other chloroplast protein precursors is also affected in pm7.

Initial investigations focused on the confirmation of the identity of the large mutant cyt f protein as a cyt f precursor. Unfortunately, the attempts to prepare an antibody which specifically recognizes the

precursor region of cyt f by immunizing rabbits with a conjugate of KLH and a synthetic peptide derived from the cyt f presequence were unsuccessful. It is possible that the synthetic peptide created from the deduced amino acid sequence from wild-type Oenothera cyt f did not maintain the necessary epitope(s) which are found in this region in the "intact" precursor. Furthermore, amino acid analysis had shown that the synthetic peptide either contained an error or was a mixture of two peptides, since an unexpected arginine residue was found to be present. Addition of this highly basic residue may have significantly affected structure and maintenance of the epitope(s) and thus the peptide antibody may not recognize the cyt f precursor. In spite of the neagtive results with this antibody, the close correlation in the sizes of the abberant cyt f in the mutant and the proposed size of the Oenothera precursor, based on the deduced amino acid sequence of the wild-type Oenothera cyt f sequence would argue that they are identical. Furthermore, the existence of a putatve ribosome binding site at the correct position, (Tyagi and Herrmann,1986) favors the identification of the large cyt f protein in the mutant as an unprocessed precursor to cyt f. (The possibility remains that altered mRNA splicing may be involved. This will be discussed further below.) The primary gene product of petA was determined to be about 35-kD by the immunoprecipitation of in vitro-translated poly A^- mRNA from pea (Willey et al.,1984a), wheat (Willey et al.,1984b) and spinach (Alt and Herrmann, 1984). This compares to a mature cyt f of aproximately 31-kD in each of these plants (Willey et al.,1984a,b; Alt and Herrmann, 1984), indicating that about 4-kD of pre-cyt f is removed during processing. This size closely agrees with the 4-kD

difference in size between the cyt f species in pm7 and wild-type Oenothera. Since the Oenothera sequences are colinear (and approximately 90% homologous) with the petA sequences from the aforementioned plants, the size of the large cyt f product is consistent with the proposal that it is a precursor to cyt f.

A sequence change in the area encoding the protein processing site, the heme binding site or an upstream regulatory region could conceivably be responsible for the accumulation of a precursor in pm7. To test this hypothesis, critical regions of petA were sequenced from both the mutant and wild-type cpDNA. The results shown in Figure 16 indicate that petA is not likely to be the site of the primary lesion, since no differences were found between the mutant and the wild-type genes in the region encoding the processing site and the heme-binding site, ruling out the possibility that protein processing is abnormal because of an alteration in the processing site or the site at which the heme group is covalently bound. The lack of any DNA differences as far as 450-bp upstream of the petA gene argues against the possibility that a change in the 5' regulatory region results in overexpression in pm7. Since no good transcriptional start sites were found in this region in the mutant or the wild-type, it is possible that such an 'up' promoter mutation could be located 5' to the upstream ORF or could even be in a trans-acting regulator. However, results of the immunoanalysis discussed below make the possibility of transcriptional overexpression of the petA gene in pm7 improbable as the primary defect and thus, the point mutation in pm7 is most likely elsewhere in the chloroplast genome. The results of this sequence

analysis are sufficient to rule out several of the hypotheses proposed for the aberrant cyt f phenotype in pm7. Since no differences were found between the mutant and wild-type petA sequences in the regions encoding the processing site or the heme binding site, if altered processing of pre-cyt f occurs in the mutant it is not due to changes in these sites. As well, the absence of mutations within the region approximately 500-bp upstream of the gene gives no evidence for altered regulation of petA expression.

Besides proper protein processing, the maturation of cyt f requires the covalent binding of a heme group. DNA sequencing showed that no differences existed in the DNA encoding the heme binding site of cyt f. Results of heme staining of plastid proteins indicated that wild-type plastids in the compatible A/A nuclear background (Stubbe, 1959) contain heme proteins at the expected size for cyt f and (much more faintly) at the predicted size for cyt b_6, which contains a non-covalently-bound heme group (Haehnel, 1984). In the mutant, heme was present but not bound to proteins of sizes expected for either mature cyt f nor precursor cyt f (Figure 18). Wild-type plastids in a less compatible A/C nuclear background (Stubbe, 1959) sometimes contained the aberrantly-sized heme proteins as well (data not shown). Trivial explanations could explain the aberrant sizes of these heme-staining bands in pm7. One possibility is that the chloroplast preparations were contaminated with other organelles which contain heme-staining proteins. For example, mitochondrial cytochromes or peroxisomal catalases would, if present, produce a positive TMBZ stain (Thomas et al., 1976). However, it is unlikely that the relatively low

speed centrifugation (6000 x g, 5 minutes) used to pellet the chloroplasts would pellet the significantly smaller mitochondria and peroxisomes. In fact, 25,000 x g (30 minutes) is required to pellet mitochondria from Oenothera (Dr. B Sears, personal communication). Additionally, Western blotting experiments with an antibody raised against yeast cytochrome oxidase subunit II and succinate dehydrogenase activity assays showed no evidence of mitochondrial contamination in pm7 plastid preparations (data not shown), whereas enriched spinach mitochondrial preparations gave positive results. Furthermore a native gel stain specific for the presence of catalase, failed to indicate peroxisomal contamination of pm7 plastids when compared to the positive staining of purified catalase (Sigma Chemical Co.) (data not shown). These particular heme-staining bands were probably the result of occasional incomplete solubilization of the mutant or incompatible wild-type protein samples or perhaps the association of free heme with more abundant chloroplast proteins. Having ruled out the possibility that the aberrant heme-staining bands were due to contamination of the plastid fraction, I have concluded that heme is indeed present in the mutant plastids, although it is not associated with proteins of sizes expected for mature cyt f nor its precursor.

There are other reports of examples of abberant sized cytochrome proteins in chloroplasts. In one case (Bhaya and Castelfranco, 1986), pea chloroplasts were fed radioactive precursors of heme biosynthesis which were incorporated into a 43-kD protein. These results were interpreted to indicate the existence of a novel chloroplast

cytochrome, as opposed to a cyt f precursor, since no cross-reactivity occurred with a cyt f antibody (Dr. P. Castelfranco, personal communication). Allred and Staehelin (1986a) also reported the presence of abnormally sized heme-staining bands in pea which they attributed to pools of cyt f which remained unassociated with the cyt b_6/f complex. In this case, the size observed was too small to be the unprocessed precursor of cyt f (Willey et al., 1984a). Possibly pea chloroplasts process cyt f to an intermediate prior to the processing which produces a mature product. However, the existence of a totally unprocessed cyt f appears to be an observation unique to the pm7 mutant of Oenothera hookeri.

In contrast to the complex protein patterns resulting from non-discriminating protein stains such as Coomassie Blue, immunoanalysis provides a means by which one may specifically determine which proteins are affected in the mutant chloroplasts. In this case not only the presence or absence of a protein in pm7 was informative, but also whether any precursor forms of the proteins accumulated in the mutant. The results of the Western blotting experiments, summarized in Table 2, allow the classification of these chloroplast proteins into three categories, those which were present at expected mature sizes in both the mutant and the wild-type, those which were absent in the mutant chloroplasts and those which were found in the mutant at sizes larger than expected for the mature wild-type polypeptides. Table 2 also shows that there were proteins in each category which are known to be the products of cpDNA genes as well as proteins which are known to be imported from the cytoplasm. Since the immunoblotting

analyses were performed on material which had been separated into membrane and soluble protein fractions, further conclusions may be drawn as to the intraorganellar location of the mature or precursor-sized proteins in pm7. All proteins analysed which were present in pm7 were found in the fraction in which they would be expected to be found in the wild-type plastids. In some cases, as with carbonic anhydrase and all of the imported subunits of PS II, proteins were found in both membrane and soluble fractions. This indicated that either cross-contamination of the two fractions had occurred, proteins were located in both fractions in the mutant or perhaps they were only loosely associated with the membranes and were easily 'lost' to the soluble fraction. The latter possibility is more likely since other membrane proteins were found only within the 'membrane fraction.'

In contrast to cyt f, the proteins in the mutant which cross-react with the OEC-23 and OEC-16 antibodies are intermediate in size between the mature size and the presumed primary precursor size. One explanation for these aberrantly sized proteins in pm7 is that they represent intermediate processing products. Similarly, in lut-1, a non-Mendelian mutant of tobacco, proposed intermediate processing products of the OEC-34 and the OEC-23 proteins have been shown to accumulate (Chia and Arntzen, 1986). However, lut-1 appears to process OEC-16 and cyt f normally. cDNA clones for genes encoding each of the three proteins in this sub-complex of PS II have since been isolated from spinach (Tittgen et al., 1986) and the DNA and deduced amino acid sequences for all three have been determined (Tyagi et al., 1987; Jansen et al., 1987), along with the actual N-terminal amino

acid sequences for OEC-23 and OEC-16 (Vater et al., 1986). Amino-terminal transit peptides of approximately 5-kD for OEC-34 and 10-kD for OEC-23 and OEC-16 are indicated by the sequence analyses, as well as by the sizes of the proteins immunoprecipitated from in vitro translation products for these proteins (Westhoff et al., 1985).

The most unexpected results of the immunological analysis of pm7 are those which show that three different chloroplast protein precursors accumulate in the mutant. In contrast, the results showing reduced amounts of other chloroplast proteins are less surprising in such a chlorotic mutant. Similarly reduced levels of a number of chloroplast proteins have quite often been seen in other non-green plastome mutator-induced mutants (Sears, personal communication). In addition, the D1 protein, which is one of the two proteins missing in pm7, is known to turn over rapidly in chloroplasts (Mattoo et al., 1984, Wettern and Galling, 1985; Greenberg et al., 1987). One could have possibly anticipated the rapid degradation of any unprocessed precursor of this protein (conceivably, the faint cross-reactive material seen in the mutant in Figure 22A). A similar speculation could be made for the other chloroplast protein which appears to be completely depleted in pm7, the 28-kD chlorophyll-binding core protein of PS II, however much less is known about the stability of this protein. It is consistent with the chlorophyll deficiency of this mutant that a chlorophyll-binding protein is absent from the plastids. On the other hand, other chlorophyll-binding proteins (LHC-II-29, PS I, etc) are still present but in lesser amounts in the mutant than in the wild-type.

A number of the proposed hypotheses for the cyt f phenotype can be ruled out by the results of these immunological analyses. The observation that altered protein patterns of both a chloroplast-encoded gene product (cyt f) and a chloroplast protein imported from the cytoplasm (OEC-23) have returned to wild-type patterns in a putative revertant of pm7 (Figure 24) links these traits to a single mutation which affects the processing of particular subset of chloroplast proteins. Furthermore, the presence of the PS II 22-kD protein in plastids of the revertant suggests that the pm7 mutation also affects the accumulation of other chloroplast proteins. Therefore, the lesion in pm7 must affect some process which is not unique to cyt f. This reasoning, together with the petA DNA sequence analysis, suggests that the aberrant accumulation of a number of putative chloroplast protein precursors in pm7 is not due to a lesion in the petA gene itself. As well, the possibility that the altered cyt f phenotype is due to improper processing or translational termination of the petA message is unlikely, since the mRNAs for the OEC polypeptides are cytoplasmically processed and translated, whereas cyt f is completely synthesized within the chloroplast.

The hypothesis that maturation of cyt f could have been affected because of the absence of other subunits in the cyt b_6/f complex is also not supported by these immunoblotting results. All other members of the cyt b_6/f complex were present in pm7, although the quantity of these proteins may have been significantly reduced in the mutant. However, even if the quantitative reduction in subunit IV and the FeS

protein were important to the processing of cyt f, it is not clear how or why this should affect the processing of OEC-23 and OEC-16. Furthermore, the third member of the PS II oxygen-evolving sub-complex, OEC-33, was present in apparently normal amounts. These results do not completely rule out the possibilty that one of the proteins which does appear to be totally deficient in pm7 is critical to the processing of other chloroplast proteins. However, such an explanation requires that the complete development of the chloroplast thylakoid membrane is a highly intergrated process, and the disruption of one critical component of this membrane may have a deleterious effect on other components of the maturation process.

Thus, the remaining hypothesis seems the most likely: some essential element of the processing function for chloroplast proteins must be defective in pm7 and furthermore this processing function is shared by certain chloroplast and nuclear gene products. This element could be a component of the processing machinery itself or some other factor which regulates the maturation of this subset of chloroplast proteins. The question remains as to what features are shared by these three proteins (cyt f, OEC-23 and OEC-16) so that they display such a related response to the mutational lesion in pm7 despite the differences in gene locations, protein functions and ultimate destinations in the chloroplast.

These three proteins (cyt f, OEC-23 and OEC-16), are similar to a number of other chloroplast proteins in that they must enter or traverse the thylakoid membrane in order to reach the sites where

they ultimately function in photosynthetic electron transport. It is possible that a thylakoid-bound protease similar to the one recently proposed to process a PC intermediate (Hageman et al., 1986) may serve to process this subset of thylakoid and lumen proteins. The simplest explanation for the apparent abberant processing in pm7 is a deficiency in this protease activity in the mutant. Alternatively, perhaps this protease activity is sensitive to the status of the membrane in which it probably resides and functions.

A thylakoid-located protease activity has also been proposed for the processing of the D1 protein (Marder et al, 1984). In contrast to the other proteins discussed here, this processing is thought to occur from the carboxy-end. However, little information is available for the biochemical nature of this protease.

Like D1 (Sayre et al., 1986), cyt f must move from the chloroplast ribosomes to become at least partially embedded in the thylakoid membrane where it associates with other components of its protein complex (Schmid et al., 1976; Cox and Andersson, 1981; Shaw and Henwood, 1985; Manfield and Andersson, 1985; Allred and Staehelin, 1985). According to a structural model based on the analysis of the deduced amino acid sequence for cyt f (Willey et al., 1984a,b; Alt and Herrmann, 1984; Wu et al., 1986; Tyagi and Herrmann, 1986), the majority of the protein resides in the thylakoid lumen with only one membrane-spanning region and a short C-terminal stretch located in the stroma. Other models based on primary sequence analysis for OEC-23 and OEC-16 suggest that these

extrinsic proteins are ultimately located in the lumen of the thyakoid membrane (Jansen et al., 1987). Therefore, it is conceivable that these three proteins are all subject to processing within the thylakoid membrane.

In this context, it is worth noting that along with the precursor-sized cyt f which accumulated in the mutant membranes, a significant amount of mature-sized cyt f was reproducibly found in in the membrane fraction of the pm7 plastids. This suggests that the pm7 phenotype may be 'leaky' with respect to the processing of cyt f, perhaps because the cyt f precursor in pm7 is still recognized by the protease, although the processing activity is less efficeint than in wild-type plastids. The observation that both the presumed precursor and the presumed mature protein are located in the membrane fraction of pm7 is strong support for the suggestion that pre-cyt f is processed by a membrane-localized protease. It is also clear that processing is not an essential pre-requisite for the insertion of cyt f into a membrane.

Extensive analysis of protein uptake and processing by organelles has shown that transit peptides, and presumably sequences and/or structures within them, play a critical role in correct targeting, uptake and subsequent processing of various imported proteins (Neupert and Schatz, 1981; Sabatini et al., 1982; Hay et al., 1984; Schmidt and Mishkind, 1986). Although little homology is seen in primary amino acid sequences for complete transit peptides for many imported chloroplast proteins, a common amino acid framework for a "generalized" transit peptide has been proposed (Karlin-Neumann and

Tobin, 1986). These authors suggest that short "blocks" of shared homology at the beginning, middle and end of the presequence represent regions which are important to proper protein uptake, processing and/or localization. Other researchers have reported little evidence of homology among various chloroplast and mitochondrial transit peptides (Schatz and Butow, 1983; Coleman and Robinson, 1986; Hurt et al., 1986).

An alternative model for transit peptide function has been developed based upon the apparent conservation of amino acid structural "domains." In chloroplasts and mitochondria, the N-terminal region of the transit peptide often contains a short stretch of predominantly basic residues. In a number of imported chloroplast proteins, amino acid sequences are capable of forming β-pleated sheet structures at or near the site proposed for the initial cleavage event (von Heijne, 1983; 1984 a,b,c; Roise et al., 1988). This event results in a processing product which is intermediate in size between the precursor and the mature protein, and would be the first stage in a two-step processing system. In addition, each transit peptide sequence contains an internal stretch of 15-20 hydrophobic residues in the C-terminal half of the presequence, immediately proximal to the terminal processing site (van Loon and Schatz, 1987; Singer et al., 1987). This sequence has been proposed to function as a "stop-transfer" signal for transport across the inner mitochondrial membrane and the thlyakoid membrane in chloroplasts. According to this theory, this hydrophobic stretch of amino acids could serve to anchor the protein in the membrane, as the precursor is being threaded through the membrane.

Alternative theories assume that the precursor moves across the membrane in a globular form, through membrane channels, independent of interaction between the membrane and the hydrophobic regions of the transit peptide.

A two-domain model for two-step processing of proteins which are imported into the chloroplast has been proposed, based upon recent work with the chloroplast protein plastocyanin (PC) (Smeekens et al., 1986; Hageman et al., 1986). This model shares a number of features with models for cytoplasmically translated mitochondrial proteins. To reach its final destination, the initial precursor to PC (pre-PC) has to cross a total of three chloroplast membranes: the outer and inner membrane of the chloroplast envelope and the thylakoid membrane.

The model proposes that the initial processing step occurs during or following the uptake of the precursor (mediated by the 'chloroplast import domain') via a specific protease located in the stroma. This step results in an intermediately-processed protein which still retains a fraction of the presequence, the "thylakoid transfer domain" (Hageman et al., 1986). Subsequently, the complete processing of the intermediate to the mature-sized protein is accomplished by a second protease located in the thylakoid or perhaps in the lumen itself (Hageman et al., 1986). This model does not address the issue of where the addition of particular prosthetic groups such as the copper in PC occurs.

A certain potential for regulatory control is provided for in the model as the thylakoid-located protease activity appears to be specific for the intermediate and does not recogonize the primary precursor of PC in in vitro experiments (Hageman et al., 1986). Preliminary analysis of this protease also indicates that the protein is extractable from the thylakoid with gentle detergent treatment and once freed from the membrane, the water soluble protein retains its specific proteolytic activity (Hageman et al., 1986). The example of PC processing is especially applicable to the results presented here since like the OEC polypeptides, it is also ultimately directed to the lumen of the thylakoid (Haehnel, 1984).

A prediction of the amino acid residue(s) implicated in the intermediate processing events of OEC-23 and OEC-16 has been made based upon the potential ability of these "cleavage domains" to form β-pleated sheet structures (Jansen et al., 1987). The theoretical sizes proposed for the intermediate processing products are in close agreement with the sizes of the proteins in pm7 which cross-react with OEC-23 and OEC-16 antibodies. Figure 25 shows the deduced amino acid sequence for the transit peptides of spinach OEC-23 and OEC-16 (Jansen et al., 1987) and the presequence of cyt f . The OEC-33 presequence (Tyagi et al., 1987) is included for comparison since it is evidently processed properly in pm7. The comparisons are presented with the N-terminal residue of the mature sequence of cyt f (+1) aligned with the N-terminal residues of the mature sequences of the imported proteins. Thus, the sequences of the transit peptides

from the imported proteins extend farther than does the presequence of cyt f. Visual inspection of these sequences (Figure 25) suggests that with the possible exception of the arginine residue directly preceding the conserved hydrophobic region and the alanine residue at -1, no extensive homology exists between these sequences. Computer-assisted searches for homology between cyt f and these imported chloroplast proteins were performed (Microgenie Software Systems, Beckman). These allowed consideration of differences in length of the presequences, and also of residues which are not identical but rather conserved with respect to charge or polarity. However, the results of these analyses indicated that even in the more restricted cases, cyt f, OEC-23 and OEC-16 are no more similar to each other than they are to OEC-33.

A possible pattern is evident however, if one compares the hydrophilicity plots of the C-terminal portions of the OEC-23 and OEC-16 transit peptides with that of the presequence of cyt f (Figure 26). Comparison of these plots reveals that all of these peptides share a hydrophobic stretch near the C-terminal of the peptides. This hydrophobic sequence has been proposed to serve as a possible signal for transport across the thylakoid membrane (Singer et al., 1987). Although the general structural similarity is intriguing, again, it does not distinguish these three proteins from other proteins which also contain N-terminal transit peptide extensions.

Antibodies raised against spinach PC did not cross-react with any Oenothera proteins which migrated at sizes expected for the prePC or

Figure 25. Amino acid sequences of the N-terminal extensions of the precursors of cyt c_1, cyt f, and the components of the OEC subcomplex of PS II. Sequences were deduced from the published DNA sequences for yeast cyt c_1 (Sadler et al., 1984), spinach OEC-23, OEC-16 (Jansen et al., 1987), spinach OEC-33 (Tyagi et al., 1987) and Oenothera cyt f (this work). The presequences are aligned at the presumed site of the terminal processing event (residue -1). Hydrophobic stretches are double-underlined; charged resudues preceding the hydrophobic stretches are marked with an asterisk; conserved alanine residues at -1 are boxed; regions capable of forming β-pleated sheet structures are single underlined.

Figure 25.

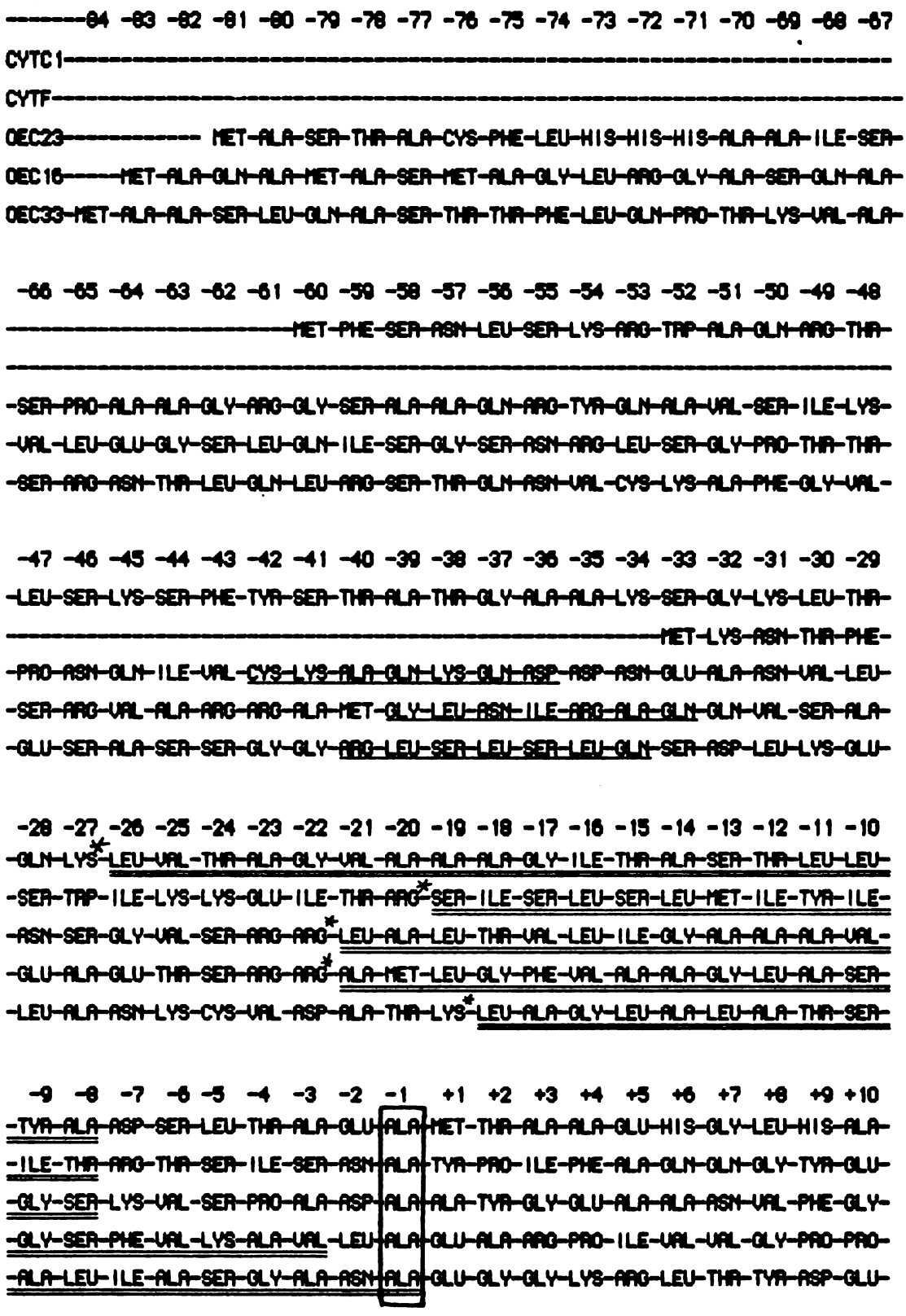
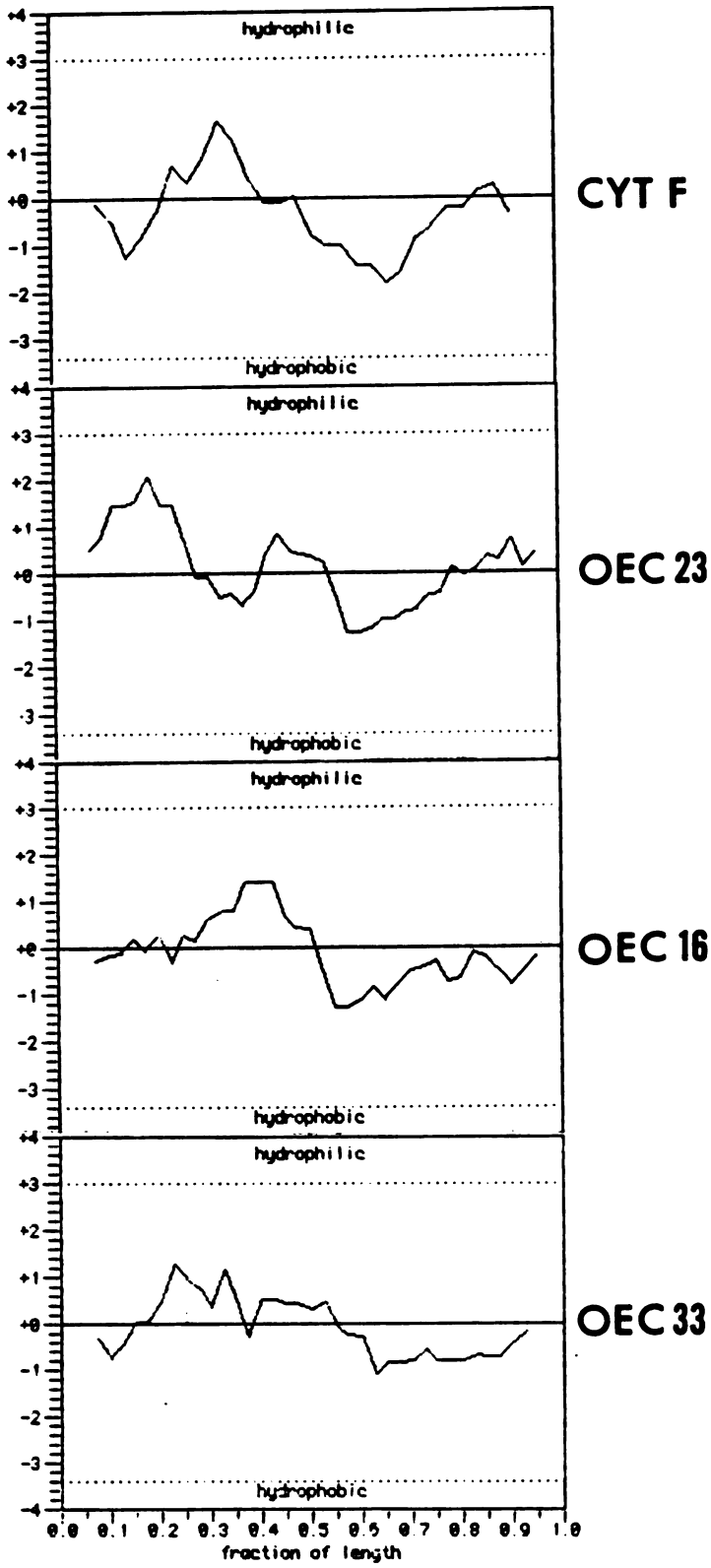

-------84 -83 -82 -81 -80 -79 -78 -77 -76 -75 -74 -73 -72 -71 -70 -69 -68 -67
CYTC1----------
CYTF----------
OEC23---------- MET-ALA-SER-THR-ALA-CYS-PHE-LEU-HIS-HIS-HIS-ALA-ALA-ILE-SER-
OEC16-----MET-ALA-GLN-ALA-MET-ALA-SER-MET-ALA-GLY-LEU-ARG-GLY-ALA-SER-GLN-ALA-
OEC33-MET-ALA-ALA-SER-LEU-GLN-ALA-SER-THR-THR-PHE-LEU-GLN-PRO-THR-LYS-VAL-ALA-
-66 -65 -64 -63 -62 -61 -60 -59 -58 -57 -56 -55 -54 -53 -52 -51 -50 -49 -48
----------MET-PHE-SER-ASN-LEU-SER-LYS-ARG-TRP-ALA-GLN-ARG-THR-
----
-SER-PRO-ALA-ALA-GLY-ARG-GLY-SER-ALA-ALA-GLN-ARG-TYR-GLN-ALA-VAL-SER-ILE-LYS-
-VAL-LEU-GLU-GLY-SER-LEU-GLN-ILE-SER-GLY-SER-ASN-ARG-LEU-SER-GLY-PRO-THR-THR-
-SER-ARG-ASN-THR-LEU-GLN-LEU-ARG-SER-THR-GLN-ASN-VAL-CYS-LYS-ALA-PHE-GLY-VAL-
-47 -46 -45 -44 -43 -42 -41 -40 -39 -38 -37 -36 -35 -34 -33 -32 -31 -30 -29
-LEU-SER-LYS-SER-PHE-TYR-SER-THR-ALA-THR-GLY-ALA-ALA-LYS-SER-GLY-LYS-LEU-THR-
----------MET-LYS-ASN-THR-PHE-
-PRO-ASN-GLN-ILE-VAL-CYS-LYS-ALA-GLN-LYS-GLN-ASP-ASP-ASN-GLU-ALA-ASN-VAL-LEU-
-SER-ARG-VAL-ALA-ARG-ARG-ALA-MET-GLY-LEU-ASN-ILE-ARG-ALA-GLN-GLN-VAL-SER-ALA-
-GLU-SER-ALA-SER-SER-GLY-GLY-ARG-LEU-SER-LEU-SER-LEU-GLN-SER-ASP-LEU-LYS-GLU-
-28 -27 -26 -25 -24 -23 -22 -21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11 -10
-GLN-LYS-LEU-VAL-THR-ALA-GLY-VAL-ALA-ALA-ALA-GLY-ILE-THR-ALA-SER-THR-LEU-LEU-
-SER-TRP-ILE-LYS-LYS-GLU-ILE-THR-ARG-SER-ILE-SER-LEU-SER-LEU-MET-ILE-TYR-ILE-
-ASN-SER-GLY-VAL-SER-ARG-ARG-LEU-ALA-LEU-THR-VAL-LEU-ILE-GLY-ALA-ALA-ALA-VAL-
-GLU-ALA-GLU-THR-SER-ARG-ARG-ALA-MET-LEU-GLY-PHE-VAL-ALA-ALA-GLY-LEU-ALA-SER-
-LEU-ALA-ASN-LYS-CYS-VAL-ASP-ALA-THR-LYS-LEU-ALA-GLY-LEU-ALA-LEU-ALA-THR-SER-
-9 -8 -7 -6 -5 -4 -3 -2 -1 +1 +2 +3 +4 +5 +6 +7 +8 +9 +10
-TYR-ALA-ASP-SER-LEU-THR-ALA-GLU-ALA-MET-THR-ALA-ALA-GLU-HIS-GLY-LEU-HIS-ALA-
-ILE-THR-ARG-THR-SER-ILE-SER-ASN-ALA-TYR-PRO-ILE-PHE-ALA-GLN-GLN-GLY-TYR-GLU-
-GLY-SER-LYS-VAL-SER-PRO-ALA-ASP-ALA-ALA-TYR-GLY-GLU-ALA-ALA-ASN-VAL-PHE-GLY-
-GLY-SER-PHE-VAL-LYS-ALA-VAL-LEU-ALA-GLU-ALA-ARG-PRO-ILE-VAL-VAL-GLY-PRO-PRO-
-ALA-LEU-ILE-ALA-SER-GLY-ALA-ASN-ALA-GLU-GLY-GLY-LYS-ARG-LEU-THR-TYR-ASP-GLU-

Figure 26. Hydrophilicity plots for the presequences of cyt f and the C-terminal portions of the presequences for components of the OEC subcomplex of PS II. Hydrophilicity values (Hopp and Woods, 1981) have been plotted against the fraction of the length of the amino acid sequence of this portions of the deduced sequences for cyt f (this work)(residues -33 through-1), OEC-23 (residues -42 through -1)(Jansen et al., 1987), OEC-16 (residues -39 through -1)(Jansen et al., 1987) and OEC-33 (residues -40 through -1)(Tyagi et al., 1987).

Figure 26.


hydrophilic
hydrophobic
CYT F
OEC 23
OEC 16
OEC 33
+4
+3
+2
+1
+0
-1
-2
-3
-4
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
fraction of length

mature PC in either mutant or wild-type plastids. Antibodies specific for D1, as shown in Figure 22A, recognize (very faintly) only presumed degradation products in pm7. It is not known if this degradation of D1 was preceded by precursor processing. So no conclusions may be drawn as to whether the previously reported thylakoid proteases which process pre-D1 (Mattoo and Edelman, 1987) and the PC intermediate (Hageman et al., 1986) are the same as those which appear to be deficient in activity in pm7. However, previous reports of such protease activities in the thylakoid are consistent with the suggestion that the apparent lack of processing of certain precursors in pm7 could occur because this processing is dependent upon proteases normally found in the thylakoid, which in turn may not be properly developed in this mutant. However, pm7 does process some other imported proteins to their correct mature sizes. As noted above, these proteins include the OEC-33, which is eventually both functionally and structurally associated with OEC-23 and OEC-16 in the thylakoid lumen (Andersson, 1986; Ghanotakis et al., 1987) and the L21 chloroplast ribosomal protein, which undergoes a two-step processing in Chlamydomonas (Schmidt et al., 1984; 1985). In order to reconcile these results, allowance must be made for different classes of precursors to be processed by different mechanisms, quite possibly by different proteases with different activity requirements. Cyt f is the only known thylakoid protein encoded by a chloroplast gene which is post-translationally processed from the N-terminal end. Perhaps the maturation of cyt f depends on a protease which also processes certain imported proteins. Regulation conferring specificity may be achieved by simply utilizing different proteases which

recognize particular precursor substrates. Alternatively, processing of a number of precursors might rely upon the activity of a single protease which has different affinities for different precursors in different suborganellar locations, in the presence of different regulating elements or at different active sites on the enzyme. The simplest of these possiblities which is consistent with the results of the pm7 analysis is illustrated in Figure 27. This model proposes that pre-cyt f and the processing intermediates of OEC-23 and OEC-16, although originally synthesized in different subcellular compartments, may utilize a common processing function in the thylakoid membrane for final maturation. This model is based on the one proposed by Hageman et al., (1986), with the additional suggestion that cpDNA-encoded precursors may share processing functions with imported chloroplast proteins. Specifically, the shared function could be the second step in this processing scheme, which presumably facilitates the passage into or through the thylakoid membrane of intermediately processed imported proteins or cpDNA-encoded precursors. Neither this nor the original model address the issue of the stability of the peptide fragments processed away from the mature proteins. Furthermore, the severity of a lesion affecting this second processing step may differ depending on where the gene product originated or where it is destined to reside. Regulation such as this could explain why the cpDNA-encoded, thylakoid protein cyt f is also present at the size expected for the mature product while there are no mature-sized proteins present for the nuclear DNA-encoded, lumen proteins, OEC-23 and OEC-16.

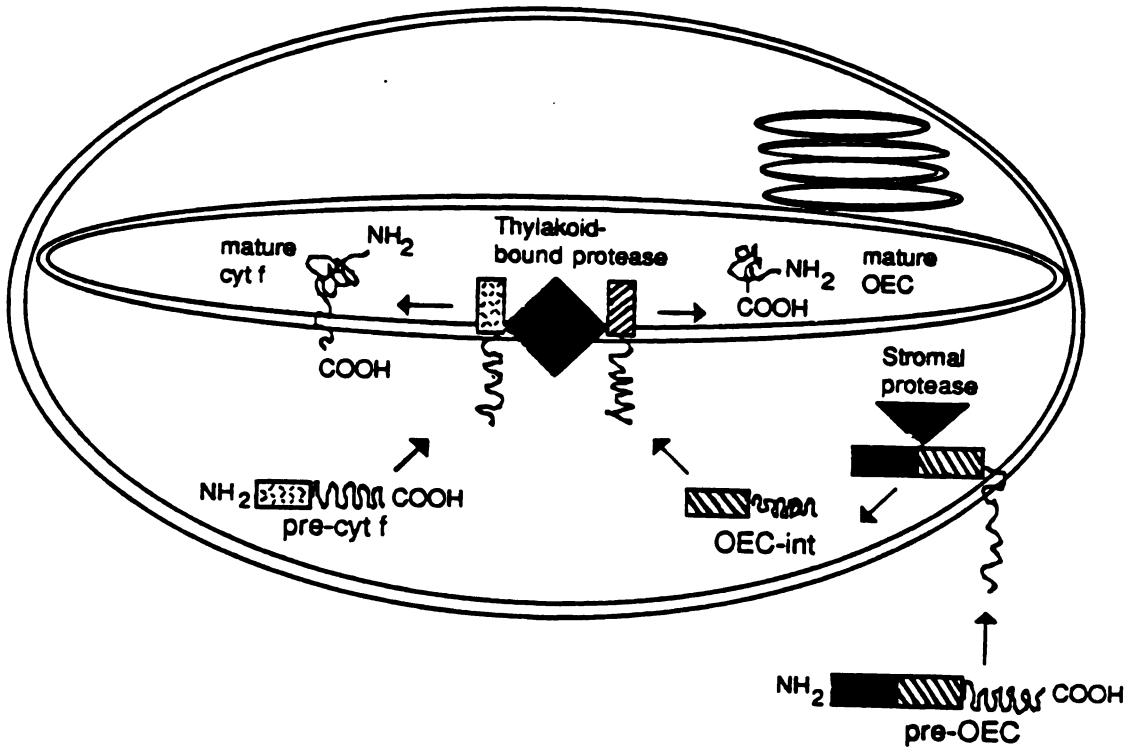


Figure 27. Model for processing of cyt f, OEC-23, and OEC-16 in the chloroplast. The proposed processing pathway of the OEC proteins is similar to that described for plastocyanin (Hageman et al., 1986; Smeekens et al., 1986), as discussed in the text. The additional feature of this model is the dependence of the processing of pre-cyt f and the intermediate (int) forms of OEC-23 and OEC-16 on a common thylakoid-bound protease.

The possibility remains that although DNA sequence analysis shows that the heme-binding site of cyt f if normal in pm7, and heme-staining of plastid proteins indicates that the mutant is capable of making heme, this heme may be altered so that it is unable to be properly bound by chloroplast cytochromes, including cyt f. If the heme moiety were altered in such a way, perhaps cyt b559, a component of PS II with a proposed function in water oxidation (Pakrasi et al., 1988) could also be missing its non-covalently bound heme. This could subsequently affect the assembly of PS II and perhaps the processing of components of the OEC sub-complex of PS II. Antibodies specific for cyt b559 were not tested in these experiments, and the presence of a non-covalently bound heme is generally poorly detected by the TMBZ/H_2O_2 heme stain, so this proposal remains only speculative.

The suggestion that heme could act as a regulator of processing is reminiscent of the observation that cyt c_1 is one of a number of imported mitochondrial proteins which are post-translationally processed in at least two steps (van Loon et al., 1986; Hartl et al. 1987). This is of particular interest since cyt c_1 is the mitochondrial "analog" of cyt f in the chloroplast. Although cyt c_1 is a nuclear gene product (Wakabayashi et al., 1980; Sadler et al., 1984) while the gene for cyt f is encoded in cpDNA, Figure 28 shows that the mature proteins share some structural similarity (Willey et al., 1983; Alt and Herrmann, 1983). The most striking similarities are seen in the two hydrophobic stretches: one proximal to the terminal cleavage sites (I) and one near the carboxy-terminus of the mature protein (II) (Figure

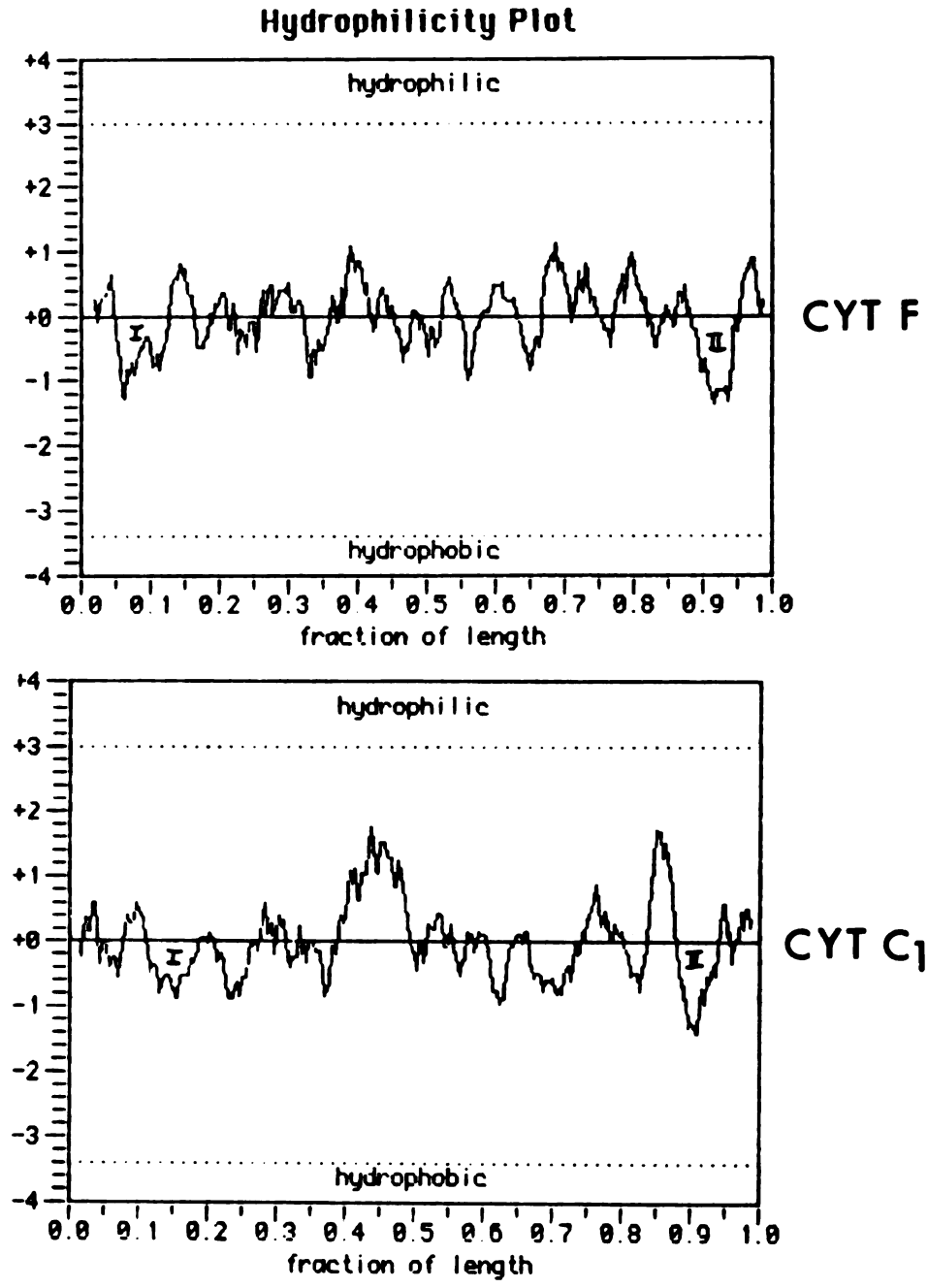


Figure 28. Hydrophilicity plots for the presequences of cyt f and cyt c_1. Hydrophilicity values (Hopp and Woods, 1981) have been plotted against the fraction of the length of the amino acid sequence for Oenothera cyt f (Tyagi and Herrmann, 1986) and yeast cyt c_1 (Sadler et al., 1984). "I" and "II" indicate two hydrophobic stretches of likely significance.

28). Furthermore extensive functional similarity also exists since both proteins act as intermediate electron carriers through their associated heme (Bendall, 1982; Hauska et al., 1983). Hartl et al. (1987) proposed that mechanisms similar to those used to process cyt c_1 may act on precyt f in chloroplasts. Figure 25 also shows the complete amino acid sequence of cyt c_1 (Sadler et al., 1984) Thus the cyt c_1, cyt f, and the OEC precursor polypeptides can be compared. Again, only a stretch of hydrophobic residues at the carboxy-terminus of the presequence, preceded by a charged residue is common between cyt c_1 and the other sequences.

Another pertinent aspect of the cyt c_1 analogy involves a yeast mutant which is deficient in heme biosynthesis. This mutant accumulates the intermediate processing product of cyt c_1, indicating that heme attachment is necessary for the second step of the processing of this protein (Lin et al., 1982; Gasser et al., 1982; Ohashi et al., 1982). However, this yeast mutant is not exactly analogous to pm7 since pm7 plastids are capable of synthesizing heme. Furthermore, respiratory electron transport, which also depends on heme appears to be normal in pm7 since heterotrophic growth of the plants in culture suggests that mitiochondrial electron transport is fully functional. Finally, there is recent evidence that, in maize, mitochondrial and chloroplast hemes may share a biosynthetic pathway (Scheegurt and Beale, 1986). This would also favor the conclusion that pm7 is capable of synthesizing heme in the plastid although it does not eliminate the possibility that the binding of this heme to chloroplast cytochromes may be inhibited in some way.

The following chapter presents a further chacterization the physiological activity and structure of pm7 plastids. Further examination of membrane structure is especially pertinent since significant amounts of mature-sized cyt f were seen in the membrane fraction of the mutant plastids. If some proper processing of cyt f is occurring in pm7 and the resulting mature-sized product is found in a membrane fraction, it is worthwhile to investigate the nature of these chloroplast membrane fractions in pm7. The results of analyses designed to determine whether levels of any of several known chloroplast components were critically altered in pm7 will be discussed in terms of whether any of these components may serve to regulate protein processing and chloroplast development in pm7.

CHAPTER 4

Physiological Analysis: Structure, Composition and Activity of Mutant and Wild-type Plastids

Introduction

Immunological analyses of proteins from pm7 and wild-type plastids have revealed evidence which suggests a major alteration in chloroplast protein processing in this plastome mutator-induced mutant of Oenothera. Proteins affected include one encoded by a chloroplast gene (cyt f) as well as the nuclear gene products, OEC-23 and OEC-16. The suggestion that the processing of these three proteins in pm7 may be inhibited by a lesion in some shared feature of the proteolytic machinery is supported by the observation that all three proteins appear to be normally processed in a putative revertant of pm7.

Experiments described in this chapter are concerned with further characterization of pm7 mutant plastids, including the status of other non-protein components of the chloroplast. A number of such aspects of thylakoid development may be addresssed experimentally, including an analysis of the extent of photosynthetic electron transport present in the mutant, the ultrastructural development of the mutant plastid and the biochemical analysis of various components of mature chloroplasts such as chlorophyll, carotenoids, fatty acids and lipids.

The results of these experiments are then discussed in the context of various possible connections between chloroplast protein processing, accumulation of chloroplast components and the degree of thylakoid development in pm7.

Materials and Methods

Plant Material

Plant materials containing C_1 wild-type plastids or C_2 pm7 plastids in the A/A nuclear background and either C_2 wild-type or pm7 mutant chloroplasts in the A/C nuclear background were maintained as shoot-tip cultures as described in Chapter 2. Tissue containing C_2 wild-type plastids in the A/A nuclear background has only recently become available and was used in the wild-type ultrastructural analysis. Shoot-tip cultures were illuminated with either 2, 20 or 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR), as specified for each particular parameter measured. For all analyses discussed in this chapter, tissue which had been maintained in culture for 3-4 weeks since the previous transfer was used.

Photosynthetic Activity Measurements

Pm7 and wild-type plastids were analysed for photosynthetic electron transport activity. Whole chain and PS I-dependent photosynthetic rates were determined by measuring the methyl viologen-mediated Mehler reaction as oxygen uptake using a water-jacketed oxygen electrode (Hansetech) (Chia et al., 1986). Chloroplasts (25 or 50 µg chlorophyll, measured in 80% acetone, as given by MacKinney, 1941) were added to the assay mixture containing 50 mM Tricine, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sorbitol, 1 mM NH_4Cl, 0.1 µM M

gramicidin, 0.5 mM Na azide, and 1 mg (3000 units) ml^{-1} superoxide dismutase and illuminated with 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. Whole chain activity was measured using water as an electron donor to PS II and 0.5 mM methyl viologen (MeV) as an artificial electron acceptor from PS I. Values for PS I-dependent activity were analysed as oxygen consumption derived from oxygen electrode assays using N,N,N',N' tetramethyl-p-phenylenediamine ($TMPDH_2$) reduced with 2.5 mM ascorbate as an artificial electron donor to PS I and 0.5 mM MeV as an artificial acceptor in the presence of 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). DCMU-sensitive PS II-mediated electron transport activities were determined spectrophotometrically with an Hitachi 100-60 as earlier described (Steinback et al., 1979) using water as a source of electrons and 40 μM DPIP (2,6-dichlorophenolindolphenol) as an artificial electron acceptor from PS II.

Methods for Ultrastructural Analysis

Pm7 leaf tissue was prepared for thin-sectioning and ultrastructural analysis by fixation in 3% (v/v) glutaraldehyde in Na cacodylate buffer (pH 7.2) for 1 hour, post-fixation in 2% (w/v) OsO_4 in the same buffer, dehydration through a series of graded ethanol solutions and embedding in Spurr's resin (Spurr, 1969). Blocks containing embedded pm7 samples were sent to Dr. M. Epp (ARCO) for further processing as described in Epp and Parthasarathy (1987). Wild-type tissue was embedded and sectioned for electron microscopic analysis by Dr. K. Klomperans according to standard procedures.

Determination of Chlorophyll Content

Extraction of chlorophyll for quantitative analysis was performed on 0.2 - 0.5 g samples of wild-type and pm7 leaves (three replicates each) in dimethyl formamide (DMF) under green safe-light conditions, as directed by Moran and Porath (1980). Absorbance values obtained from spectral analysis with a Perkin-Elmer spectrophotometer were quantified using equations presented by Moran (1982).

Determination of Carotenoid Content

Total plant carotenoids were isolated by extraction of 0.3 - 0.7 g of mutant or wild-type leaf tissue (two replicates each) in 10 ml ice-cold acetone in the presence of Na bicarbonate (1g/10g sample) and 1% (w/v) butylated hydroxytoluene (BHT) for approximately 5 minutes in reduced light conditions (Britton, 1985). Extracts were filtered through Whatman type 1 filter paper and concentrated to known volumes under a stream of N_2 gas. Total carotenoid levels were determined by spectral analysis with an Hitachi Model 110 using a general 1% extinction coefficient for carotenoids of 2500 at 450 nm (Davies, 1976). Any remaining sediment was removed by forcing samples through Sep Pac18 cartridges (Millipore) before injecting 50 µl aliquots into a C-18 long column (4.6 x 20 cm, Varian, MCH-4-n-cap) for separation of individual carotenoids by reverse phase high pressure liquid chromatography (HPLC) (Varian 5000 LC) using a linear gradient of 90% aqueous methanol to 100% ethylacetate over

20 minutes at 1 ml/min (Casadero et al., 1983). Peaks were detected and quantified by using a Varian electron detector.

Determination of Fatty Acid Content

Methyl ester derivatives of mutant and wild-type fatty acids from leaves were prepared according to Browse et al. (1986a), employing a 1 hour treatment of 0.2 - 0.5 g leaf samples in methanolic-HCl at 80°C, followed by phase separation via the addition of 1 volume 0.9 % (w/v) NaCl and 1 volume hexane spiked with 17:0 fatty acid methyl ester as a standard. Three replicate extractions were made for each mutant and wild-type genotype. Fatty acid methyl esters in the organic phase were quantitated by gas chromatography and flame ionization detection (Browse et al., 1986a).

Measurement of Lipid Synthesis

Rates of lipid synthesis were determined for wild-type and pm7 tissues by following the incorporation of Na ^{14}C-acetate into plant lipids according to methods given by Browse et al. (1986b). For the '16:3' plant, Arabidopsis thaliana, a 2 hour labelling period is sufficient (Browse et al., 1986b). However, because Oenothera is an '18:3' plant which uses the eukaryotic pathway of lipid synthesis (see Heinz and Roughan, 1983 for review), labelling of two replicate mutant or wild-type shoot-tip cultures was allowed to proceed for 48 hours under moderate light conditions of 50 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ PAR. Following this period of uptake, tissues were frozen in liquid N2 and leaf lipids

extracted with chloroform:methanol (1:1) as described in Browse et al. (1986b). Individual lipids were separated on one-dimensional thin layer chromatography (TLC) plates (Baker Si 250 PA, 200 μm) impregnated with $(NH_4)_2SO_4$ (Khan and Williams, 1977). TLC plates were autoradiographed to locate lipid spots, which were then quantified by liquid scintillation as described in Browse et al. (1986b).

Results

Photosynthetic Electron Transport Activity

To date, it has been difficult to measure significant levels of electron transport in subcellular fractions of many Oenothera tissues (Hallier and Heber 1977; Hallier et al., 1978). Despite extensive attempts at modification of the organelle isolation procedures (for example, the inclusion of polyvinyl pyrrolidone [Schneider and Hallier, 1970] in homogenization buffers) and varying assay protocols, the best wild-type levels reached were approximately 50 percent of rates optimally achieved with other more conventional plant tissues (Haehnel, 1984) (Table 3) . In these experiments, the pm7 plastid membrane preparations consistently showed no detectable electron transport activity.

Ultrastructural Analysis

Oenothera wild-type and mutant tissue proved to be quite amenable to ultrastructural and biochemical analysis however. Pm7 tissue from leaf tip cultures was embedded and sent to Dr. M. Epp (ARCO Plant Cell Research Institute) for electron microscopic analysis. This analysis revealed a striking lack of developed internal membrane structure (Figure 29A,B). For comparison, Figure 29C shows an Oenothera type I-C_2 wild-type chloroplast in the A/A nuclear background also grown in shoot-tip culture (tissue prepared and

Table 3. Photosynthetic electron transport activity in Oenothera wild-type and pm7 mutant chloroplasts. "Whole chain" assays were performed using an oxygen electrode with water or diphenyl carbazol (DPC) as an electron donor to PS II and methyl viologen (MeV) as an artificial electron acceptor from PS I. Values for PS I-dependent (dichloromethyl urea [DCMU] insensitive) activity were derived from oxygen electrode assays using reduced tetramethyl phenamine diamine ($TMPDH_2$) as an artificial electron donor to PS I and MeV as an artificial acceptor. DCMU-sensitive PS II-dependent activities were determined spectrophotometrically with water or DPC as electron donors and DPIP (dichlorophenylindoyl phenol) as an artificial electron acceptor for PS II. Rates presented represent the range of results for several independent experiments.

Genotype	Whole Chain (μmol O_2/ mg chl/hr)	PS II-dependent (μmol DCPIP/ mg chl/hr)	PS I-dependent (μmol O_2/ mg chl/hr)
wt	37-94	116-285	37
pm7	0	0	0

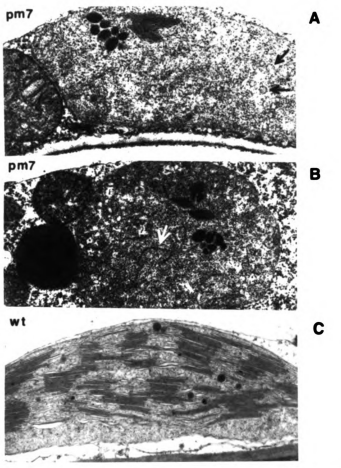


Figure 29. Electron micrographs of plastids from leaf-tip cultures of mutant (A, B) and wild-type (wt, C). Arrows in panel A point to small vesicles; the arrow in panel B points to an elongated membrane-like structure. Magnification of all panels is appoximately 15,000x. Ultrastructural data for the pm7 plastids have been previously published (Epp and Parthasarthy, 1987).

sectioned by Dr. K. Klomparens, Michigan State Univiversity). Some but not all mutant sections contained prominant layered structures which have been interpreted as isolated "granal stacks" (Epp and Parthasarthy, 1987) and numerous small vesicles (black arrows in Figure 29A). A few sections also contained structures resembling single elongated membranes (indicated by a white arrow in Figure 29B). However, in light of the lack of electron transport activity and the deficiency or complete lack of a number of thylakoid membrane proteins, it seems unlikely that these structures are fully developed or fully functional granal or stromal lamellae. Alternatively, the "stacked" structures may represent protein aggregates. The micrographs do however show the presence of a double outer membrane surrounding the plastid, probable chloroplast ribosomal units, plastoglubuli and a large number of vesicular structures. A similar lack of thylakoid development is observed in the ultrastructure of pm7 plastids when the mutant plastids are maintained as chlorotic sectors in variegated greenhouse-grown plants (Epp and Parthasarthy, 1987). In this case, there were fewer plastoglobuli present and larger single vesicles but still little evidence of thylakoid development.

Analysis of Chlorophyll and Carotenoid Accumulation

In order to study a number of more specific aspects of chloroplast development in pm7, chlorophyll and carotenoid levels were determined for both wild-type and mutant tissues which had been maintained in shoot-tip culture under a range of light levels. Type I-C_2 mutant and wild-type plastids were each examined in two different

nuclear backgrounds, resulting in either the A/A-I compatible plastome-genome combination or the A/C-I incompatible combination.

Figure 30 confirms what was easily seen by simple visual inspection of the plants, that is, that when grown under a variety of light conditions, the mutant accumulates significantly less chlorophyll on a fresh weight basis than does the wild-type ($p<0.01$). Chlorophyll concentrations, as well as values for all of the other parameters measured were subjected to statistical analysis using an analysis of variance (Table 4). This analysis allows one to make comparisons between mutant and wild-type chloroplasts, comparisons between the two nuclear backgrounds, and comparisons among the light regimes. For chlorophyll levels, Table 4 shows that the nuclear background has a significant effect and furthermore, chlorophyll levels in both the mutant and the wild-type appear to be sensitive to the higher light conditions. This photosensitivity seems to be especially acute in the mutant where chlorophyll levels approach the limits of detection.

One main function proposed for carotenoid pigments in plants is to provide protection from photo-damage under high light conditions (Casadoro et al., 1983; Oelmüller and Mohr, 1985; Mayfield et al., 1986a, 1986b). Figure 31 presents values for the concentration of total carotenoids found in mutant and wild-type material. As tested by the ANOVA shown in Table 4, any differences between mutant and wild-type or between the two nuclear backgrounds are not statistically significant ($p>0.1$). Furthermore, when four major plant carotenoids - ß-carotene, neoxanthin, violoxanthin and lutein - were separated and

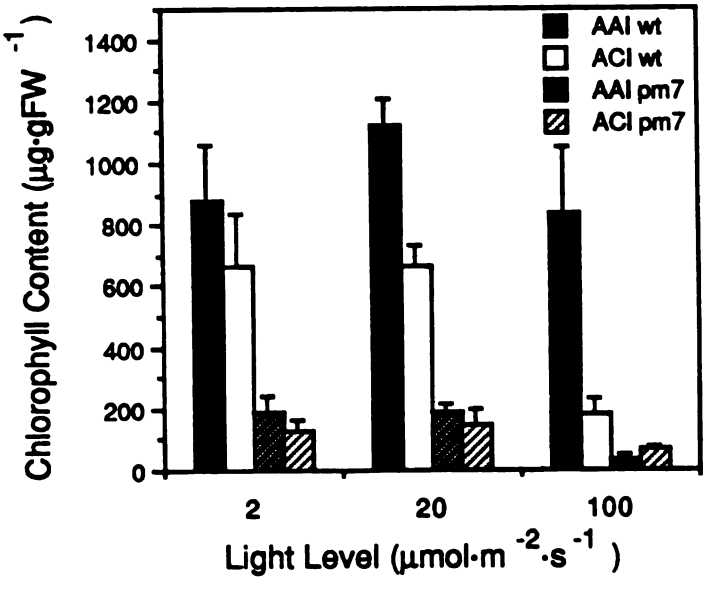


Figure 30. Chlorophyll content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. Plants were grown in 3 different light regimes for 3 weeks and chlorophyll content was then determined. Bars represent the mean ± SD (n=3).

Table 4. Results of statistical analysis using split plot analysis of variance tests. ANOVA treatment (Snedecor and Cochran, 1967) of pigment, fatty acid and lipid data from Oenothera wild-type and pm7 mutant plastids in A/A and A/C nuclear backgrounds. C = chloroplast genome, N = nuclear genome, L = light, * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$

Parameter	Single variables	F ratio	Interactive variables	F ratio
Chlorophyll	C	111.47***	CxN	1.24
	N	34.80**	CxL	0.16
	L	15.12	NxL	0.09
			CxNxL	0.19
Carotenoid	C	6.12	CxN	0.04
	N	0.18	CxL	0.01
	L	1.31	NxL	0.12
			CxNxL	0.11
16:0 fatty acid	C	186.50***	C x N	0.00
	N	2.15	C x L	0.03
	L	5.20*	N x L	0.10
			C x N x L	0.00
18:0 fatty acid	C	25.11**	C x N	0.01
	N	0.10	C x L	0.10
	L	21.90***	N x L	0.06
			C x N x L	0.01
18:1 fatty acid	C	0.25	C x N	0.04
	N	0.18	C x L	0.01
	L	1.31	N x L	0.12
			C x N x L	0.11
18:2 fatty acid	C	23.29**	C x N	0.02
	N	0.71	C x L	0.00
	L	2.27	N x L	0.12
			C x N x L	0.11
18:3 fatty acid	C	11.66*	C x N	0.04
	N	0.11	C x L	0.03
	L	28.03***	N x L	0.25
			C x N x L	0.00
MGD	C	0.06	CxN	0.19
	N	0.00		
PG	C	0.16	CxN	0.74
	N	1.81		

Table 4. (con'td)

Parameter	Single variables	F ratio	Interactive variables	F ratio
DGD	C	0.54	CxN	0.00
	N	0.34		
SL	C	1.65	CxN	2.37
	N	2.86		
PE	C	29.26	CxN	2.79
	N	0.18		
PC	C	1.78	CxN	12.01
	N	15.19		
PI	C	7.69	CxN	0.00
	N	0.62		

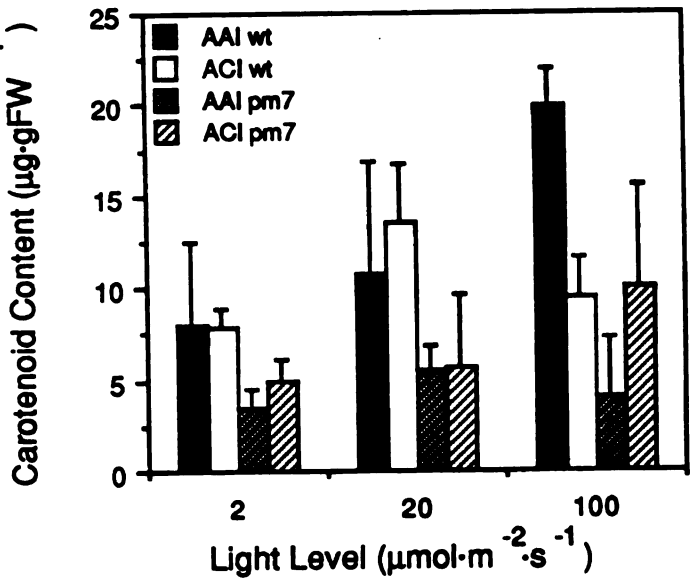


Figure 31. Total carotenoid content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. Plants were grown in 3 different light regimes for 3 weeks and total carotenoid content was then determined. Bars represent the mean ± SD (n=2).

analysed, no major differences were seen for any of these specific carotenoids in plant tissue grown at high light levels (Figure 32). In fact, the only obvious differences in carotenoids at any light level were the decrease in the percent β-carotene and the increase in the percent neoxanthin in mutant tissue grown in low light. Since these values are each expressed as a percent of total carotenoid, they are not independent measurements and cannot be statistically analysed. However, in neither case is the one type of carotenoid totally deficient and thus no low light-sensitive lesion in carotenoid synthesis is indicated. Hence, there was no evidence indicating a lesion in pm7 in the biosynthesis or accumulation of these specific carotenoids.

Analysis of Fatty Acid Accumulation and Lipid Synthesis

Another aspect of chloroplast development which was measured in both wild-type and pm7 mutant tissue is the degree of accumulation of fatty acids and the ability of the mutant to synthesize lipids from these fatty acids. Mutant and wild-type tissues were maintained in shoot-tip culture under light levels ranging from 2 - 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fatty acids in crude extracts were derivatized to methyl esters, extracted with hexane, separated and quantified by gas-liquid chromatography. The comparisons of fatty acid composition for mutant and wild-type are shown in Figure 33. For three of the five fatty acids examined, 16:0, 18:0, and 18:2, significant differences in accumulation between mutant and wild-type were observed($p<0.001$, $p<0.05$ and $p<0.05$, respectively).

Figure 32. Major carotenoids in tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. Plants were grown in 3 different light regimes for 3 weeks and the presence of major carotenoids was then determined. Bars represent the mean ± SD (n=2). High, medium and low light refers to 100, 20 and 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, respectively.

Figure 32.

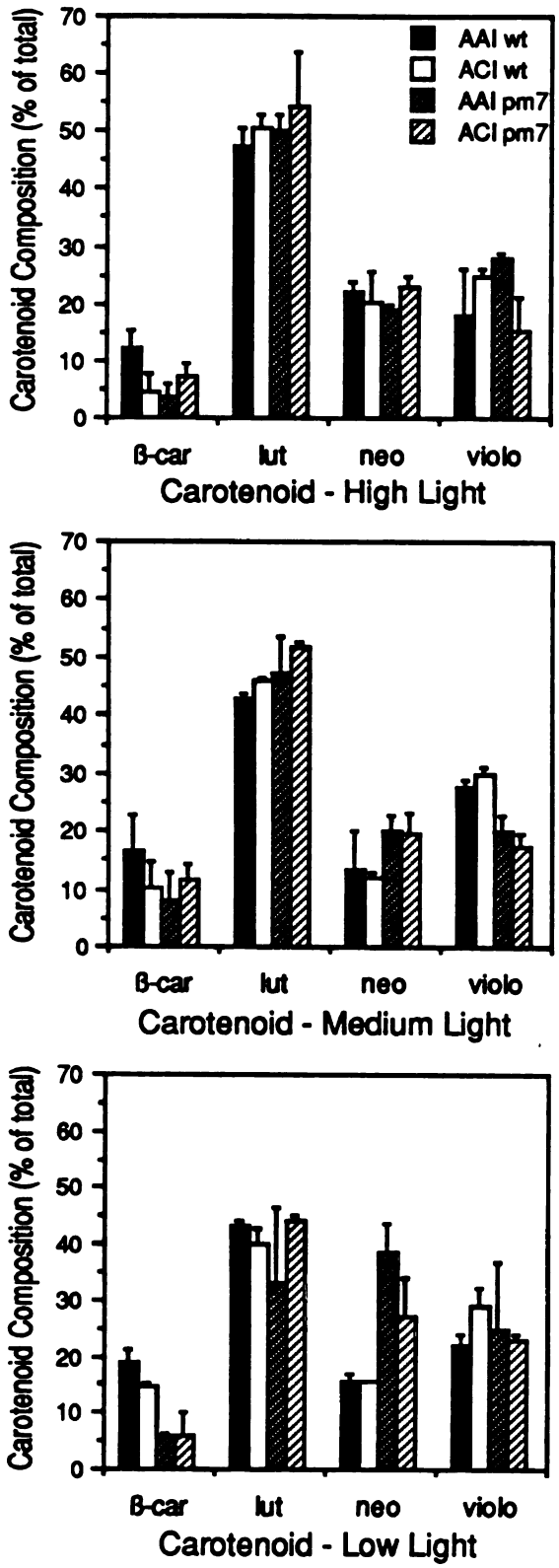
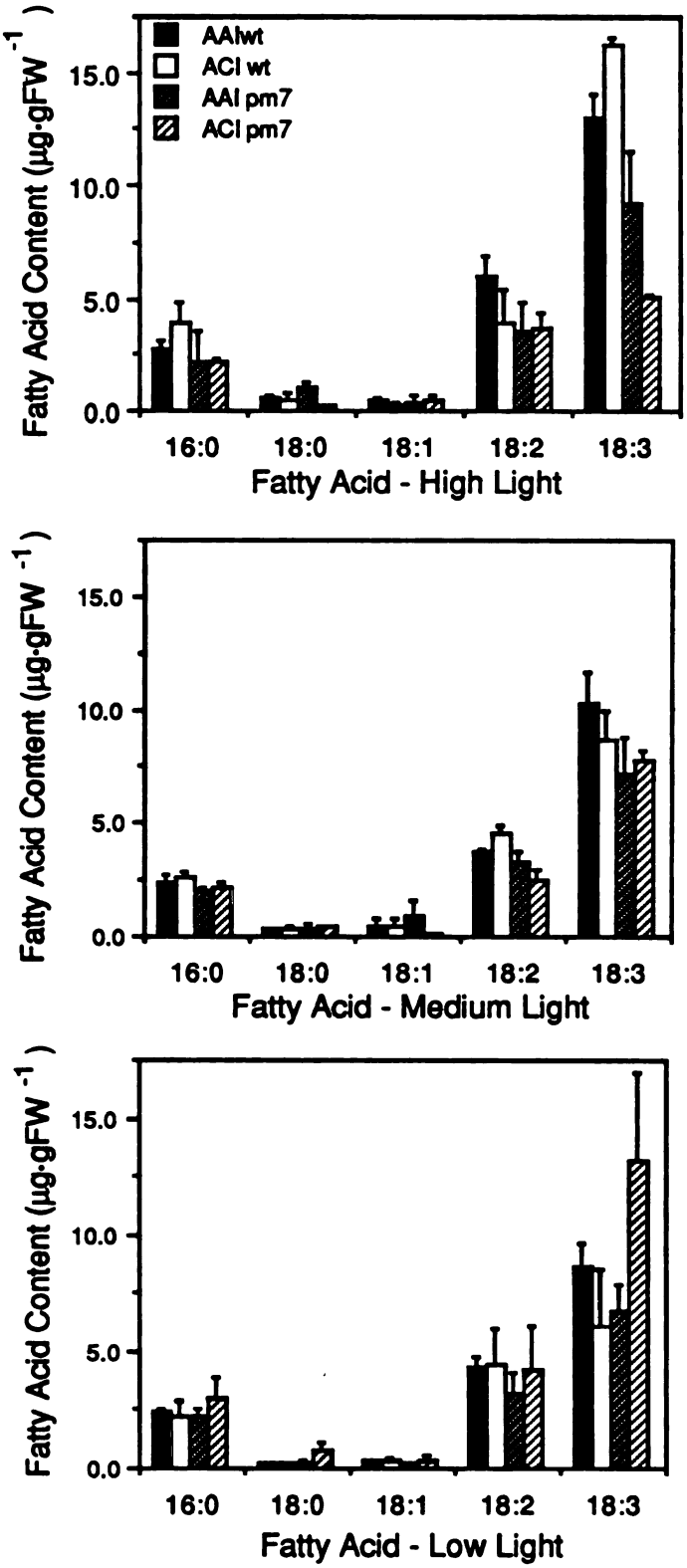

AAl wt
ACl wt
AAl pm7
ACl pm7
Carotenoid Composition (% of total)
0
10
20
30
40
50
60
70
β-car
lut
neo
violo
Carotenoid - High Light
Carotenoid Composition (% of total)
Carotenoid - Medium Light
Carotenoid Composition (% of total)
Carotenoid - Low Light

Figure 33. Fatty acid content of tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. Plants were grown in 3 different light regimes for 3 weeks and chlorophyll content was then determined. Bars represent the mean ± SD (n=3). High, medium and low light refers to 100, 20 and 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, respectively.

Figure 33.


AAIwt
ACI wt
AAI pm7
ACI pm7
Fatty Acid Content (µg·gFW -1)
15.0
10.0
5.0
0.0
16:0
18:0
18:1
18:2
18:3
Fatty Acid - High Light
Fatty Acid Content (µg·gFW -1)
15.0
10.0
5.0
0.0
16:0
18:0
18:1
18:2
18:3
Fatty Acid - Medium Light
Fatty Acid Content (µg·gFW -1)
15.0
10.0
5.0
0.0
16:0
18:0
18:1
18:2
18:3
Fatty Acid - Low Light

The extent to which these fatty acids were potentially converted into lipids in the mutant was addressed by applying ^{14}C-labelled acetate to shoot-tip cultures of pm7 and wild-type tissues, then allowing 48 hours for the synthesis into various lipids. Figure 34 illustrates the amount of radioactivity incorporated into monogalactosyldiacylglycerol (MGD), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DG), sulphoquinovosyldiacylglycerol (SL), phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Analysis of variance revealed no significant differences between levels of lipid synthesis in the mutant and the wild-type ($p>0.1$) or in comparisons between the A/A and the A/C nuclear backgrounds ($p>0.1$). Again, these results indicate that there is no biosynthetic lesion in the mutant with respect to lipid synthesis.

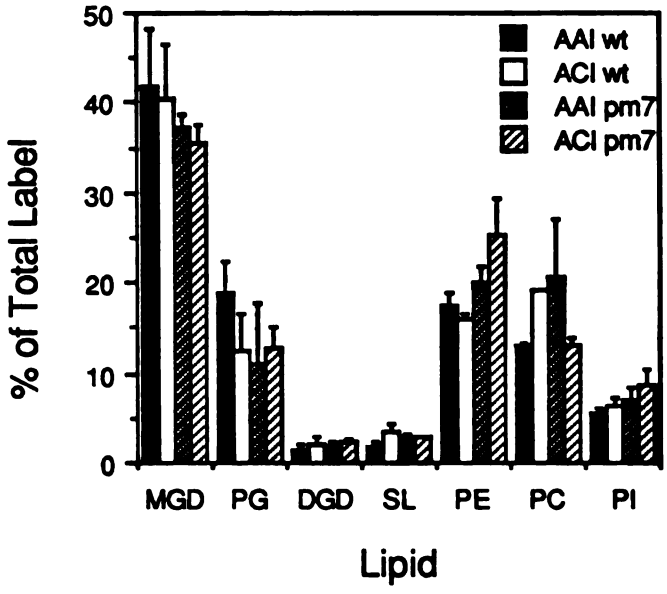


Figure 34. Lipid synthesis in tissue containing wild-type and pm7 plastome type I plastids in A/A and A/C nuclear backgrounds. Plants were fed ^{14}C-acetate for 48 hours in a light regime of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR before the samples were harvested. Bars represent the mean $\pm$ SD (n=2).

Discussion

Examples of proteins which are not properly processed or those which are not accumulated in the plastids of the plastome mutator-induced mutant pm7 were discussed in the preceding chapter. Results of investigations discussed in Chapter 4 reveal that in addition to differences in chloroplast proteins, little internal membrane structure was found in the mutant plastids and not surprisingly, no detectable electron transport activity was present in the pm7 plastids. Futhermore, a lack of chlorophyll was found in the mutant tissue in comparison to wild-type, as well as significant differences in levels of three fatty acids - 16:0, 18:0 and 18:2 fatty acids. However, these analyses did not reveal any evidence of significant differences in total carotenoid levels or in the synthesis of seven chloroplast lipids.

The pigment, fatty acid and lipid measurments were also performed on two different nuclear genotypes in combination with plastome type I chloroplasts. The A/A nuclear background is more compatible with plastome I (Stubbe, 1959) and chloroplasts are present. The A/C nuclear background, however, is less compatible with plastome type I, and tissue containing this plastome-genome combination is pale green, containing plastids which are not fully developed (Drillisch, 1975). Comparisons of chlorophyll, carotenoid, fatty acid and lipid levels in A/A I and A/C I tissues revealed significant differences in chlorophyll concentrations only ($p<0.05$)

The observation of a lack of activity in whole chain electron transport assays is not unexpected for a mutant that is not photosynthetically competent. However, despite the accumulation of a number of components of the various protein complexes, specifically the cyt b_6/f complex and PS I, there was no evidence of partial electron transport through either photosystem. This result is reasonable since the heme staining described in Chapter 3 showed that the cytochrome complex components lacked bound heme to act as an electron carrier.

Consistent with this lack of physiological activity was the clear absence of developed thylakoid membrane structure in the mutant plastids as shown by the electron microscopic analysis of pm7 leaf tissue. It seems more probable that this deficiency is the result of aborted development in pm7 plastids as opposed to the senescence of developed chloroplasts in these cells. This argument is based upon the observation that the mutant tissue does not go through stages of greening before becoming chlorotic but rather appears pale yellow from very early stages of "development" in shoot-tip culture. Epp and Parthasarthy (1987) have shown that pm7 plastids lack developed thylakoids when they are maintained as chlorotic sectors in wild-type greenhouse grown plants. Hence, the lack of thylakoid membrane development is not a consequence of maintaining the mutant in shoot-tip culture, as has been suggested for aberrant plastid development under various other tissue culture conditions (Parthier, 1979; Colijn et al., 1982).

The ultrastructural analysis suggested the usefulness of a more detailed examination of specific chloroplast components. These analyses were performed using mutant and wild-type plastids in two different nuclear backgrounds. The incompatible genotype combinations were included because pm7 plastids were only available in an A/A nuclear background which was also homozygous for the pm gene, and thus unstable in terms of plastome mutator activity. Including both nuclear backgrounds in these experiments allows the analysis of one type of plastome-genome incompatibility as well as the comparison of mutant and wild-type phenotypes. The design of the statistical analysis also makes use of the two nuclear backgrounds as another set of replicates for the comparison of the mutant and wild-type chloroplasts.

This more detailed analysis of the levels of various chloroplast components in pm7 included the measurement of chlorophyll and carotenoid pigments, as well as an examination of the fatty acids and lipids found in the mutant. Figure 34 shows that the mutant is not simply deficient in the ability to synthesize chlorophyll. Instead, pm7 displays symptoms of photobleaching under the relatively higher light condition of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Photobleaching, or the loss of chlorophyll pigments under conditions of high light, is a characteristic of many carotenoid-deficient plants (Casadoro et al., 1983; Oelmüller and Mohr, 1985; Mayfield et al., 1986a, 1986b). The lack of any signifigant difference in total carotenoid accumulation (Figure 31) and the presence of four specific carotenoid pigments (Figure 32) demonstrate that the photobleaching seen in pm7 is probably not a

consequence of a substantial loss of photo-protective carotenoid pigments. Since a significant degree of photobleaching is observed in the wild-type as well as the mutant tissue, it seems unlikely that this photosensitivity is the major factor responsible for the absence of thylakoid development in the mutant.

Other major plastid components which are critical to the development of mature wild-type chloroplasts are the fatty acids (made in the plastid for the entire plant cell - Roughan and Slack, 1982, 1984) and the lipids which incorporate these fatty acids. Dorne et al. (1982) reported major alterations in lipid composition in a barley mutant (albostrians) which also displays altered internal membrane structure and the absence of chloroplast ribosomes. The albostrians mutant showed a drastic reduction in the ratio of MGD to DGD. This reduction was attributed to the absence of the thylakoid membranes, the major site of MGD accumulation in chloroplasts (Roughan and Slack, 1982, 1984; Douce and Joyard, 1980).

In order to determine whether a major deficiency in fatty acid or lipid biosynthesis accompanied the aberrant internal membrane structure in pm7, levels of steady state fatty acid accumulation and newly synthesized lipids were measured. Although three types of fatty acids showed significant differences between mutant and wild-type, in the cases of the 18:0 and 18:2 fatty acids, relative concentrations are not consistent between the two nuclear backgrounds. In the case of the 16:0 fatty acids in tissue grown under high light conditions, although analysis of variance suggests that the difference between mutant and

wild-type levels are highly significant ($p<0.001$), the mutant plastids are still able to accumulate 60-80% of the 16:0 fatty acid found in the wild-type chloroplasts.

These chloroplast-synthesized fatty acids are necessary substrates for the synthesis of major plant lipids (Roughan and Slack, 1982, 1984; Douce and Joyard, 1980). Synthesis of four lipids found primarily in the chloroplast, MGD, DGD, PC, and SL (Roughan and Slack, 1982, 1984; Douce and Joyard, 1980) was found to be quite similar in pm7 and wild-type tissue. The remaining lipids analysed (PE, PC and PI) are found outside of the chloroplast (Roughan and Slack, 1982, 1984; Douce and Joyard, 1980) and were also found to have equivalent rates of synthesis in mutant and wild-type (Figure 34). The lipid analysis of the Oenothera wild-type and pm7 mutant described here was of synthesis, measured by assessing the rate of ^{14}C-acetate labelling of plant lipids, rather than a quantitative steady-state measurement as was done by Dorne et al. (1982). This may account for the difference in lipid levels in two mutants both of which lack thylakoids. However, it is important to note that the barley albostrians mutant is also deficient in plastid ribosomes (Börner et al., 1976), a condition not present in pm7. Hence, the conclusion which may be drawn from these results is that the mechanisms for synthesizing chloroplast-specific lipids (MGD, PG, DGD and SL) as well as for exporting fatty acids to the cytoplasm, synthesizing extrachloroplastic lipids (PE, PC and PI), are intact in the mutant. Furthermore, the absence of a significant difference in mutant and wild-type lipid synthesis suggests

that the minimal differences observed in fatty acids (necessary for lipid synthesis) are not fundamental to the altered phenotype of pm7.

Earlier analyses have characterized the extent of possible alterations in chloroplast protein processing in pm7. Subsequently, experiments discussed in this chapter were designed to determine the degree to which other non-protein components of the thylakoid membrane accumulated in the pm7 plastids. It does not seem likely that thylakoid membrane development would progress normally in the absence of fully processed precursors to a number of subunits for various protein complexes. In fact, the lack of processing is accompanied by a complete deficiency in at least two other chloroplast proteins which are components of PS II, as well as decreases in other polypeptides of cyt b_6/f and PS I. A lack of processing could possibly block membrane development, coupled with the loss of a major constituent of the membrane such as chlorophyll. Hence, one hypothesis suggests that a gene encoding some component of this processing function is the site of the primary lesion in pm7. Without proper processing activity in the chloroplast, thylakoid membrane development may be aborted.

Alternatively, the polypeptide precursors which accumulate in pm7 (as well as other precursors) may be dependent on thylakoid-bound proteases for the final processing to mature-sized proteins. The state and abundance of thylakoids could thus affect protease abundance and activity. Thus, the lesion in pm7 could have a primary effect on thylakoid development, for example by inhibiting the accumulation of

chlorophyll to some essential threshold level. As a result, the thylakoid-bound processing machinery responsible for the maturation of a particular set of chloroplast proteins may not be able to function properly. This secondary effect would have to be somewhat specific however, as other proteins do seem to be correctly processed in pm7 and other plastome mutator-induced mutants which do not accumulate chlorophyll do appear to process pre-cyt f (Sears, personal communication).

Results of experiments described here do not allow the discrimination between these two possible hypotheses. Thus, it is not possible to specify a "cause and effect" relationship between chloroplast protein processing and thylakoid membrane development. However, it is clear that, despite the altered processing and accumulation of a number of chloroplast proteins and an extensive lack of chlorophyll accumulation and thylakoid development, a number of other chloroplast components remain present in pm7 plastids at relatively normal levels.

Chapter 5. Summary and Conclusions

One major conclusion which may be drawn from the results of the research presented in this thesis is that the processing of a particular subset of chloroplast proteins can be blocked by a single mutaional event. In the case of pm7, a plastome mutator-induced mutant of Oenothera hookeri, some but not all of the chloroplast proteins encoded in either the plastome or the nuclear genome displayed phenotypes which most likely resulted from faulty protein processing in the mutant. The proposed deficiency in processing of cyt f, OEC-23 and OEC-16 in pm7 may be the result of a lesion in a proteolytic enzyme common to the processing of these three proteins or alternatively, it could be a more indirect lesion in some factor which controls or influences this activity. How closely the lack of internal membrane structure found in pm7 is related to the phenotype of altered processing is not clear.

Another conclusion from this work is that despite the clear lack of development of mature thylakoid structures in this mutant, a surprising number of chloroplast components, including various chloroplast proteins, pigments, fatty acids and lipids, are present at relatively normal levels in the pm7 plastids. A few proteins are absent, chlorophyll levels are quite low and the abundance of a few fatty acids is minimally altered. Possibly one of the missing or deficient components in pm7 is the signal to which the processing function responds.

A more extensive analysis of pm7 is necessary to accurately describe the defect resulting in the accumulation of a number of aberrantly large chloroplast proteins. Cytochrome f is not the only gene product for which a large cross-antigenic product is accumulated in pm7 and subsequently found only as a mature-sized protein in the pm7 revertant. Therefore, it seems unlikely that the primary lesion lies in the petA gene, although it is conceivable that a lesion which alters cyt f processing could have a secondary effect on the processing of other chloroplast proteins. The sequencing data discussed in Chapter 3 ruled out a lesion in the processing site itself and the 5' upstream region. However, it should be noted that the identification of the precursor-specific N-terminal extension is only tentatively based on the presence of a presumed ribosome binding site upstream of the proposed translation start site and the shared homology of the presumed deduced presequence with that from other plants which have been examined using in vitro translation analysis (Alt and Herrmann, 1984, Willey et al., 1984a,b). In fact, on Northern blots, a complex pattern of RNAs hybridize to a petA probe (Tyagi and Herrmann, 1986). Conceivably, RNA splicing could add message encoding the N-terminal portion of the precursor to the petA transcript. A mutation in pm7 in this part of the protein could alter the ability of a protease to recognize the processing site. The existence of a non-adjacent segment which encodes the cyt f precursor N-terminus could explain why the anti-peptide antibodies did not react against the larger protien in pm7. Since the peptide antigen was synthesized to match the amino acid sequence deduced

from the reading frame immediately 5' to the mature cyt f protein sequence. According to this hypothesis, the cyt f N-terminal sequence could actually be quite different.

This possibility could be experimentally addressed by employing the technique of S_1 nuclease protection analysis (Berk and Sharp, 1977) hybridizing an end-labelled single-stranded DNA probe, encoding the presumed transcriptional start site, to the message (Weaver and Weissman, 1979). Digestion of this hybrid with S_1 nuclease would allow the identification of the RNA transciption initiation site for petA. This in turn would show whether transcript processing is involved in petA expression. If processing were indicated, the mutant transcript size and sequence could be analysed for evidence of any faulty mRNA processing.

Results of in vitro translation of wild-type Oenothera messages for cyt f, OEC-23 and OEC-16 would allow the determination of the sizes of these primary gene products for these proteins. This could reinforce the identification of the aberrant proteins in pm7 as unprocessed cyt f and intermediately processed OEC-23 and OEC-16. Ultimately, the most rigorous manner in which to verify the identity of these mutant proteins as incompletely processed precursors is to purify these proteins from pm7 tissue, using a combination of immunoaffinity chromatography (Kristiansen, 1978) and gel purification, and subject the purified material to N-terminal microsequencing. Microsequencing procedures employing a gas phase sequenator (Hewick et al., 1981; Aebersold et al., 1986) are well-suited for use

with small amounts of proteins and the number of residues of of interest here.

Screening of mutant and wild-type chloroplast proteins could also reveal additional critical differences between mutant and wild-type which are related to the pm7 phenotype. It is also possible that such a differential screening of mutant and wild-type chloroplast proteins, either by in vitro transcription/translation of cpDNAs or by in vivo radiolabelling of newly-synthesized proteins, may also provide a means to discover the processing enzyme which may be deficient in pm7. It should be remembered, however, that at least two other plastid proteins, the functions of which are not thought to be related to protein processing, were observed to be missing in pm7, and this could make the interpretation of the differential screening results difficult. Furthermore, it is also possible that a nonfunctional protease may still be present even if there is no activity seen.

In conclusion, it is most likely that petA, the plastid gene encoding cyt f, is probably not itself the site of the primary lesion in pm7. Instead, the point mutation in pm7 indicated by the restriction analysis of pm7 and the wild-type cpDNA probably lies somewhere else in the chloroplast genome. Thus, there is an unidentified plastome gene which encodes a trans-acting factor which affects processing of a subset of both imported and chloroplast-synthesized chloroplast proteins.

A search for the primary lesion in pm7 cpDNA and hence the potential identification of the plastome gene involved in chloroplast protein processing could utilize the following strategy. DNA from mutant and wild-type plastids would be digested with a number of separate restriction endonucleases, melted and allowed to reanneal as a mixture of wild-type and mutant cpDNA fragments. The resulting fragments would represent wild-type reannealed to wild-type strands, mutant to mutant strands or hybrids of wild-type and mutant strands. The hybrid fragments which contain the point mutation should be sensitive to S_1 nuclease at the site of the point mutation (Shenk et al, 1975; Vogt, 1981). Therefore when cpDNA treated in this manner is analyzed electrophoretically, two new fragments should be found in comparison to controls using wild-type or mutant cpDNA alone. Digested fragments of reasonable size (thus the justification for employing more than one restriction enzyme) could then be isolated and cloned for further sequencing analysis in order to locate the point mutation. The possibility that the mismatch found is due to the hypervariability in certain cpDNA fragments of Oenothera (Blasko et al., 1988) may be addressed by also performing this analysis on newly germinated and purified pm7 tissue. This mutant material will have undergone fewer rounds of cpDNA replication than the currently used tissue which has been maintained in shoot-tip culture for seven years. Presumably, this "newer" tissue will be less exposed to events in cpDNA replication or repair which may be involved in the occurrance of hypervariability.

The pm7 mutant may prove to be useful for further study of chloroplast protein processing. The accumulated precursors may be more extensively localized using techniques of immunocytochemistry or protease-sensitivity of right-side-out compared to inside-out membrane preparations (Åkerlund et al., 1982). Organelle uptake studies may be used to follow kinetics of protein maturation as well as the degree of degradation (a feature of the relationship between chloroplast development and protein accumulation which was not addressed in this study). Finally, the pm7 mutant may serve as a valuable source from which to purify precursors or intermediates which are not naturally available from other systems. Purified preparations of intermediates may be subjected to amino acid sequence analysis in order to clearly determine cleavage sites. Such intermediates (or fusion proteins synthesized based upon the knowledge of the sequence of these intermediates) may also be utilized in biochemical studies using reconstitution of subcellular fractions to further investigate the mechanism of and requirements for chloroplast protein processing. Such reconstitution experiments could involve both homologous and heterologous combinations of the pm7 precursors with chloroplast membrane and soluble fractions from Oenothera and other plants in the presence of various amounts of cofactors, energy sources and so forth.

Such future experimental possibilities represent valuable outgrowths from the analysis of the role of chloroplast gene products in chloroplast development using a mutational approach.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aebersold R H, D B Teplow, L E Hood, S B H Kent (1986) Electroblotting onto activated glass. J Biol Chem 261:4229-4238

Ahne F, A M Merlos-Lange, B F Lang, K Wolf (1984) The mitochondrialgenome of the fission yeast Schizosaccharomyces pombe. Curr Genet 8:517-524

Åkerlund H-E, C Jansson, B Andersson (1982) Reconstitution of photosynthetic water splitting inside-out thylakoid vesicles and identification of a participating polypeptide. Biochim Biophys Acta 681:1-10

Allred D R, L A Staehelin (1985) Lateral distribution of the cytochrome b_6/f and coupling factor ATP synthetase complexes of chloroplast thylakoid membranes. Plant Physiol 78:199-202

Allred D R, L A Staehelin (1986) Spatial organization of the cytochrome b_6-f complex within chloroplast thylakoid membranes. Biochim Biophys Acta 849:94-103

Alt J, R G Herrmann (1984) Nucleotide sequence of the gene for pre-apocytochrome f in the spinach plastid chromosome. Curr Genet 8:551-557

Alt J, P Westhoff, B B Sears, N Nelson, E Hurt, G Hauska, R G Herrmann (1983) Genes and transcripts for the polypeptides of the cytochrome b6/f complex from spinach thylakoid membranes. EMBO J 2:979-986

Andersson B (1986) Proteins participating in photosynthetic water oxidation In L A Staehelin, C J Arntzen, eds, Photosynthesis. III. Photosynthetic Membranes and Light Harvesting Systems. Encyclopedia of Plant Physiology New Series, Vol 19. Springer-Verlag, New York, pp 447-456

Backer J, F Foury (1985) Repair properties in yeast mitochondrial mutators. Curr Genet 10:7-13

Barkan A, D Miles, W C Taylor (1986) Chloroplast gene expression in nuclear, photosynthetic mutants of maize. EMBO J 5:1421-1427

Bendall D S (1982) Photosynthetic cytochromes of oxygenic organisms. Biochim Biophys Acta 683:119-151

Bendall D S, M Sanguansermsri, J Girard-Bascou, P Bennoun (1986) Mutations of Chlamydomonas reinhardii affecting the cytochrome bf complex. FEBS Lett 203:31-35

Bentzen P, W C Leggett, G G Brown (1988) Length and restriction site heteroplasmy in the mitochondrial DNA of American shad (Alosa sapidissima). Genetics 118:509-518

Berk A J, P A Sharp (1977) Sizing and mapping early adenovirus mRNAs by gel electrophoresis of S_1 endonuclease digested hybrids. Cell 12:721-733

Bhaya D, P Castelfranco (1986) Synthesis of a putative c-type cytochrome by intact, isolated pea chloroplasts. Plant Physiol 81:960-964

Blasko K, S A Kaplan, K G Higgins, R Wolfson, B B Sears (1988) Variation in copy number of a 24-base pair tandem repeat in the chloroplast DNA of Oenothera hookeri strain Johansen. Curr Genet, submitted

Börner T, B Schumann, R Hagemann (1976) Biochemical studies on a plastid ribosome-deficient mutant of Hordeum vulgare. In T Bücher, W Neupert, W Sebald, S Werner, eds, Genetics and Biogenesis of Chloroplasts and Mitochondria, Elsevier/North Holland Biomedical Press, Amsterdam, pp 41-48

Börner T, B B Sears (1986) Plastome mutants. Plant Mol Biol Report 4:69-92

Britton G (1985) General carotenoid methods. In JH Law, HC Rilling, eds, Steroids and Isoprenoids Part B, Methods in Enzymology, Vol 111, Academic Press, New York, pp 113-149

Browse J, P McCourt, C R Somerville (1986a) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem 152:141-145

Browse J, N Warwick, C R Somerville, C R Slack (1986b) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. Biochem J 235:25-31

Casadoro G, G Høyer-Hansen, C G Kannangara, S P Gough (1983) An analysis of temperature and light sensitivity in tigrina mutants of barley. Carlsberg Res Commun 48:95-129

Chia C P, C J Arntzen (1986) Evidence for two-step processing of nuclear-encoded chloroplast proteins during membrane assembly. J Cell Biol103:725-731

Chia C P, J H Duesing, J L Watson, R Guy, C J Arntzen (1986) Characterization of cytoplasmic mutants of Nicotiana tabacum with altered photosynthetic function. Curr Genet 10:469-479

Chiu W-L, W Stubbe, B B Sears (1988) Plastid inheritance in Oenothera : organelle genome modifies the extent of biparental plastid transmission. Curr Genet 13:181-189

Chua N-H, S G Bartlett, M Weiss (1982) Preparation and characterization of antibodies to chloroplast proteins. In M Edelman, R B Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology, Elsevier Biomedical Press, Amsterdam, pp 1063-1080

Chua N-H, G W Schmidt (1978) Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. Proc Natl Acad Sci USA 75:6110-6114

Clark R D, G Hind (1983) Isolation of a five-polypeptide cytochrome b-f complex from spinach chloroplasts. J Biol Chem 258:100348-10354

Cleland R (1972) Oenothera: Cytogenetics and Evolution, Academic Press, New York p 370

Colman A, C Robinson (1986) Protein import into organelles: hierarchical targeting signals. Cell 46:321-322

Colijn C M, P Sijmons, J N M Mol, A J Kool, H J J Nijkamp (1982) Light and benzylaminopurine induce changes in ultrastructure and gene expression in plastids of Petunia hybrida cell cultures. Curr Genet 6:129-135

Cox E C (1976) Bacterial mutator genes and the control of spontaneous mutation. Ann Rev Genet 10:135-156

Cox R P, B Andersson (1981) Lateral and transverse organization of cytochromes in the chloroplast thylakoid membrane. Biochem Biophys Res Commun 103:1336-1342

Crouse E J, J M Schmitt, H-J Bohnert (1985) Chloroplast and cyanobacterial genomes, genes and RNAs: a compilation. Plant Mol Biol Report 3:43-89

Curtis S E, M T Clegg (1984) Molecular evolution of chloroplast DNA sequences. Mol Biol Evol 1:291-301

Darr S C, S C Somerville, C J Arntzen (1986) Monoclonal antibodies to the light-harvesting chlorophyll a/b protein complex of photosystem II. J Cell Biol 103:733-740

Davies B H (1976) Carotenoids. In The chemistry and biochemistry of plant pigments, Ed 2, Academic Press, New York, pp154-155

Del Giudice L, D R Massardo, F Manna, K Wolf (1986) Isolation and characterization of a conditional mutant in Saccharomyces cerevisiae producing rho° petites at the nonpermissive temperature. Curr Genet 121:201-204

Doebley J F, D P Ma, W T Renfroe (1987) Insertion/deletion mutations in the Zea chloroplast genome. Curr Genet 11:617-624

Doherty A, J C Gray (1979) Synthesis of cytochrome f by isolated pea chloroplasts. Eur J Biochem 98:87-92

Dorne A-J, J-P Carde, J Joyard, T Börner, R Douce (1982) Polar lipid composition of a plastid ribosome-deficient barley mutant. Plant Physiol 69:1467-1470

Drillisch, M (1975) Vergleichhende Untersuchungen an den A-Genotypen von Oenothera. PhD Thesis. University of Düsseldorf, Düsseldorf

Epp M D (1973) Nuclear gene-induced plastome mutations in Oenothera hookeri I. Genetic analysis. Genetics 75:465-483

Epp M D, M V Parthasarthy (1987) Nuclear gene-plastome mutations in Oenothera hookeri . II. Phenotype description with electron microscopy. Amer J Bot 74:143-151

Epp M D, J L Erion, B B Sears, W Stubbe (1987) The plastome mutator of Oenothera continues to function as originally described. Mol Gen Genet 206:515-518

Finley D, A Varshavsky (1985) The ubiquitin system: functions and mechanisms. TIBS 10:343-347

Foury F, J Kolodynski (1983) pif mutation blocks recombination between mitochondrial rho$^+$ and rho$^-$ genomes having tandemly arrayed repeat units in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 80:5345-5349

Foury F, E Van Dyck (1985) A PIF-dependent recombinogenic signal in the mitochondrial DNA of yeast. EMBO J 4:3525-3530

Galliard T (1974) Techniques for overcoming problems of lipolytic enzymes and lipoxygenases in the preparation of plant organelles. In S Fleischers, L Packer eds, Biomembranes, Part A, Methods in Enzymology, vol 31. Academic Press, New York, pp 520-528

Gasser S M, A Ohashi, G Daum, P C Böhni, J Gibson, G A Reid, T Yonetani, G Schatz (1982) Imported mitochondrial proteins cytochrome b_2 and cytochrome c_1 are processed in two steps. Proc Natl Acad Sci USA 79:267-271

Genga A, L Bianchi, L Foury (1986) A nuclear mutant of Saccharomyces cerevisiae deficient in mitochondrial DNA replication and polymerase activity. J Biol Chem 261:9328-9332

Ghanotakis D F, C M Waggoner, N R Bowlby, D M Demetriou, G T Babcock, C F Yocum (1987) Comparitive structural and catalytic properties oxygen-evolving photosystem II preparations. Photosyn Res 14:191-199

Glickman B, P Van den Elsen, M Radman (1978) Induced mutagenesis in dam$^-$ mutants of Escherichia coli : A role for 6-methyladenine residues in mutation avoidance. Mol Gen Genet 163:307-312

Gordon K H J, E J Crouse, H J Bohnert, R G Herrmann (1982) Physical mapping of differences in chloroplast DNA of the five wild-type plastomes in Oenothera subsection Euoenothera. Theor Appl Genet 61:281-296

Gray J C, A L Phillips (1982) Isolation of cytochrome f and a cytochrome b-f complex. In M Edelman, R B Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology, Elsevier Biomedical Press, Amsterdam pp 917-932

Grebanier A E, D M Coen, A Rich, L Bogorad (1978) Membrane proteins synthesized but not processed by isolated maize chloroplasts. J Cell Biol 78:734-746

Greenberg B M, V Gaba, A K Mattoo, M Edelman (1987) Identification of a primary in vivo degradation product of the rapidly-turning-over 32 kd protein of photosystem II. EMBO J 6:2865-2869

Hageman J, C Robinson, S Smeekens, P Weisbeek (1986) A thylakoid processing protease is required for complete maturation of the lumen protein plastocyanin. Nature 324:567-569

Hallier U W, U W Heber (1977) Cytochrome f deficient plastome mutants of Oenothera. Plant Cell Physiol :257-273

Hallier U W, J M Schmitt, U Heber, S S Chaianova, A D Volodarsky (1978) Ribulose-1,5-bisphosphate carboxylase-deficient plastome mutants of Oenothera. Biochim Biophys Acta 504:67-83

Hartl F-U, J Ostermann, B Guiard, W Neupert (1987) Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by bipartite signal peptide. Cell 51:1027-1037

Harrison R G, D M Rand, W C Wheeler (1985) Mitochondrial DNA size variation within individual crickets. Science 228:1446-1448

Hauska G, E Hurt, N Gabellini, W Lockau (1983) Comparative aspects of quinol-cytochrome c/plastocyanin oxidoreductases. Biochim Biophys Acta 726:97-133

Hauswirth W W, P J Laipis (1982) Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Proc Nat Acad Sci 79:4686-4690

Hay R, P Böhni, S Gasser (1984) How mitochondria import proteins. Biochim Biophys Acta 779:65-87

Heinemeyer W, J Alt, R G Herrmann (1984) Nucleotide sequence of the clustered genes for apocytochrome b6 and subunit 4 of the cytochrome b/f complex in the spinach plastid chromosome. Curr Genet 8:543-549

Heinz E, P G Roughan (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol 72:273-279

Herrmann R G (1982) The preparation of circular DNA from plastids. In M Edelman, R B Hallick, N-H Chua (eds) Methods in Chloroplast Molecular Biology, Elsevier Biomedical Press, Amsterdam pp 259-280

Herrmann R G, P Westhoff, J Alt, P Winter, J Tittgen, C Bisanz, B B Sears, N Nelson, E Hurt, G Hauska, A Viebrock, W Sebald (1983) Identification and characterization of genes for polypeptides of the thylakoid membrane. In O Cifferi, L Dure III eds, Structure and function of plant genomes. Plenum Press, New York pp 143-153

Herrmann R G, P Westhoff, J Alt, P Winter, J Tittgen, N Nelson (1985) Thylakoid membrane proteins and their genes. In L van Vloten-Doting, G S P Groot, T C Hall eds, Molecular form and function of the plant genome. Plenum Press, New York pp 233-256

Hewick R M, M W Hunkapiller, L E Hood, W J Dreyer (1981) A gas-liquid solid phase peptide and protein sequenator. J Biol Chem 256:7990-7997

Ho K K, D W Krogmann (1980) Cytochrome f from spinach and cyanobacteria. J Biol Chem 255:3855-3861

Hopp T P, K R Woods (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78:3824-3828

Hudspeth M E S, D S Shumerd, K H Tatti, L I Grossman (1980) Rapid purification of yeast mitochondrial DNA in high yield. Biochim Biophys Acta 610:221-228

Hurt E, G Hauska (1981) A cytochrome f/b6 complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chloroplasts. Eur J Biochem 117:591-599

Hurt E C, A P G M van Loon (1986) How proteins find mitochondria and intramitochondrial compartments. TIBS 11:204-207

Jacobsen B S, F Fong, R L Heath (1975) Carbonic anhydrase of spinach. Plant Physiol 55:468-474

Jansen T, C Rother, H Reinke, K Beyreuther, C Jansson, B Andersson, R G Herrmann (1987) Nucleotide sequence of cDNA Clones encoding the complete '23 kDa' and '16 kDa' precursor proteins associated with the photosynthetic oxygen-evolving complex from spinach. FEBS Lett 216:234-240

Jensen K H, D L Herrin, F G Plumley, G W Schmidt (1986) Biogenesis of photosystem II complexes: Transcriptional, translational, and posttranslational regulation. J Cell Biol 103:1315-1325

Johnson E M, S A Kaplan, K Blasko, R Wolfson, B B Sears (1988) Oenothera plastome mutator: mutation frequencies and chloroplast DNA polymorphisms. Mol Gen Genet, submitted

Karlin-Nemann G, E M Tobin (1986) Transit peptides of nuclear-encoded chloroplast proteins share a common amino acid framework. EMBO J 5:9-13

Kaplan S A (1987) Characterization of chloroplast DNA from wild-type and mutant plastids of Oenothera hookeri, str Johansen. M Sc Thesis. Michigan State Univ, East Lansing

Kaplan S A, B B Sears (1986) Plastome mutator gene induces alterations in chloroplast DNA. Genetics 113:s22.

Kaplan S A, K Blasko, E M Johnson, P O Lim, R Wolfson, B B Sears (1987) Oenothera plastome mutator line is heteroplasmic for chloroplast DNA restriction fragment length polymorphisms. Cold Spring Harbor Meeting on Molecular Biology of Mitochondria and Chloroplasts, Abstracts p 65

Kaufmann S H, C M Ewing, J H Shaper (1987) The erasable Western blot. Anal Biochem 161:89-95

Khan M, R Kolter, C Thomas, D Figurski, R Meyer, E Remaut, D R Helinski (1979) Plasmid cloning vehicles derived from plasmids ColEI, F, R6K and RK2. In R Wu, ed, Recombinant DNA, Methods in Enzymology, Vol 68. Academic Press, New York, pp 268-280

Khan M-U, J P Williams (1977) Improved thin-layer chromatographic method for the separation of major phospholipids and glycolipids from plant lipid extracts and phosphatidyl glycerol and bis(monoacylglyceryl) phosphate from animal lipid extracts. J Chromotogr 140:179-185

Kimball R F (1979) Relationship between repair processes and mutation induction in bacteria. In W M Generoso, M D Shelby, F J de Serres eds, DNA Repair and Mutagenesis in Eukaryotes, Plenum, New York, pp 1-23

Krishnan H B, C D Reeves, T W Okita (1986) ADPglucose pyrophosphorylase is encoded by different mRNA transcripts in leaf and endosperm of cereals. Plant Physiol 81:642-645

Kristiansen T (1978) Matrix-bound antigens and antibodies In O Hoffman-Ostenhof, eds, Affinity Chromatography, Pergamon Press, Oxford, pp191-201

Kutzelnigg H, W Stubbe (1974) Investigations on plastome mutants in Oenothera I. General considerations. Sub-Cell Biochem 3:73-89

Laemmli, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophoge T4. Nature 227:680-695

Lam E, R Malkin (1985) Characterization of a photosynthetic mutant of Lemna lacking the cytochrome b6-f complex. Biochim Biophys Acta 810:106-109

Langridge P (1981) Synthesis of the large subunit of spinach ribulose bisphosphate carboxylase may involve a precursor polypeptide. FEBS Lett 123:85-89

Leary J J, D J Brigati, D C Ward (1983) Rapid and sensitive colorimetric method for visualizing biotin labelled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bioblots. Proc Natl Acad Sci USA 80:4045-4049

Legator M S, W G Flamm (1973) Environmental mutagenesis and repair. Ann Rev Biochem 42:683-708

Lemaire C, J Girard-Bascou, F-A Wollman, P Bennoun (1986) Studies on the cytochrome b_6/f complex. I. Characterization of the complex subunits in Chlamydomonas reinnhardtii. Biochim Biophys Acta 851:229-238

Lindenhahn M M, M Mettzlaff, R Hagemann (1985) Remarkable restriction patterns between green and white branches of 'plastome mutator' plants of Oenothera hookeri . Mol Gen Genet 200:505-505

Ljungberg U, H-E Åkerlund, B Andersson (1986) Isolation and characterization of the 10-kDa and 22-kDa polypeptides of higher plant photosystem 2. Eur J Biochem 158:477-482

Ljungberg U, H-E Åkerlund, C Jansson, B Andersson (1984) Identification of polypeptides associated with the 23 and 33 kDa proteins of photosynthetoc oxygen evolution. Biochim Biophys Acta 767:145-152

Loomis W D (1974) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. In S Fleischers, L Packer eds, Biomembranes PartA, Methods in Enzymology, vol 31. Academic Press, New York, pp528-544

MacKinney G (1941) Absorbtion of light by chlorophyll solutions. J Biol Chem 140:315-322

Maniatis T, E F Fritsch, J Sambrook (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York p 545

Mansfield R W, J M Anderson (1985) Transverse organization of components within the chloroplast cytochrome b-563/f complex. Biochim Biophys Acta 809:435-444

Marder J B, P Goloubinoff, M Edelman (1984) Molecular architecture of the rapidly metabolized 32-kilodalton protein of photosystem II. J Biol Chem 259:3900-3908

Maroc J, J Garnier (1981) Gel electrophoresis of chloroplast membranes of mutants of Chlamydomonas reinhardii which have impaired photosystem II function and lack photosynthetic cytochromes. Biochim Biophys Acta 637:473-480

Mattoo A K, M Edelman (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein. Proc Natl Acad Sci USA 84:1497-1501

Mattoo A K, H Hoffman-Falk, J B Marder, M Edelman (1984) Regulation of protein metabolism: coupling of photosynthetic electron transport to in vivo degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. Proc Natl Acad Sci USA 81:1380-1384

Mayfield S P, T Nelson, W C Taylor (1986a) The fate of chloroplast proteins during photooxidation in carotenoid-deficient maize leaves. Plant Physiol 82:760-764

Mayfield S P, T Nelson, W C Taylor, R Malkin (1986b) Carotenoid synthesis and pleiotropic effects in carotenoid-deficient seedlings of maize. Planta 169:23-32

McCourt P, L Kunst, J Browse, C R Somerville (1987) The effects of reduced amounts of lipid unsaturation on chloroplast ultrastructure and photosynthesis in a mutant of Arabidopsis. Plant Physiol 84:353-360

Messing J (1983) New M13 vectors for cloning. In R Wu, A Grossman, K Moldave, eds, Recombinant DNA, Methods in Enzymology, Vol 101. Academic Press, New York, pp 20-78

Metz J G, D Miles, A W Rutherford (1983) Characterization of nuclear mutants of maize which lack the cytochrome f/b-563 complex. Plant Physiol 73:452-459

Michaelis P (1969) Über Plastiden-Restitutionen (Rückmutationen). Cytologia 34 (suppl):1-115

Minami E, A Watanabe (1985) Detection of a precursor polypeptide of the rapidly-synthesized 32,000 dalton thylakoid protein in spinach chloroplasts. Plant Cell Physiol 26:839-846

Moon E, T-H Kao, R Wu (1987) Rice chloroplast DNA molecules are heterogeneous as revealed by DNA sequences of a cluster of genes. Nuc Acids Res 15:611-630

Moran R (1982) Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide. Plant Physiol 69:1376-1381

Moran R, D Porath (1980) Chlorophyll determination in intact tissues using N,N-dimethylformamide. Plant Physiol 65:478-479

Nakata T, I Takabe (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12-20

Nelson N, J Neumann (1972) Isolation of a cytochrome b6-f particle from chloroplasts. J Biol Chem 247:1817-1824

Nelson N, E Racker (1972) Partial resolution of the enzymes catalyzing photophosphorylation. J Biol Chem 247:3848-3853

Neupert W, G Schatz (1981) How are proteins imported into mitochondria. TIBS 6:1-4

Norrander J, T Kempe, J Messing (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26:101-106

Oelmüller R, H Mohr (1985) Carotenoid composition in milo (Sorghum vulgare) as affected by phytochrome and chlorophyll. Planta 164:390-395

Ohashi A, J Gibson, I Gregor, G Schatz (1982) Import of proteins into mitochondria. J Biol Chem 257:13042-13047

Ohyama K, H Fukuzawa, T Kohchi, H Shirai, T Sano, S Sano, K Umesono, Y Shiki, M Takeuchi, Z Chang, S Aota, H Inokuchi, H Ozeki (1986) Chloroplast gene organization deduced from complete sequence of liverwort Marchantia polymorpha chloroplast DNA. Nature 322:572-574

Olive J, O Vallon, F-A Wollman, M Recouvreur, P Bennoun (1986) Studies on the cytochrome b6/f complex. II. Localization of the complex in the thylakoid membranes from spinach and Chlamydomonas reinhardtii by immunocytochemistry and freeze-fracture analysis of b6/f mutants. Biochim Biophys Acta 851:239-248

Ortiz W, R Malkin (1985) Topographical studies of the subunits of the thylakoid cytochrome b6-f complex. Biochim Biophys Acta 808:164-170

Pakrasi H B, J G K Williams, C J Arntzen (1988) Targeted mutagensis of the psbE and psbF genes blocks photosynthetic electron transport: evidence for a functional role of cyt b559 in photosystem II. EMBO J 7:325-332

Palmer J D (1985) Evolution of chloroplast and mitochondrial DNA in plants and algae. In R J MacIntyre ed, Molecular Evolutionary Genetics, Plenum Publ, New York pp 131-239

Parthier B (1979) The role of phytohormones (cytokinins) in chloroplast development. Biochem Physiol Pflanzen 174:173-214

Phillips A L, J C Gray (1984) Synthesis of components of the cytochrome b-f complex by isolated pea chloroplasts. Eur J Biochem 138:591-595

Possingham J V, M E Lawrence (1983) Controls to plastid division. Int Rev Cytol 84:1-56

Potrykus I (1970) Mutation und Rückmutation extrachromosomale vererbter Plastidenmerkmale von Petunia. Z Pflanzenzuch 63:24-40

Radman M, R E Wagner Jr, B W Glickman, M Meselson (1979) DNA methylation, mismatch correction and genetic stability. In M Alacevic ed, Progress in Environmental Mutagenesis, Elsevier-North Holland, Amsterdam pp 121-130

Rand D M, R G Harrison (1986) Mitochondrial DNA transmission genetics in crickets. Genetics 114:955-970

Redei G P, S B Plurad (1973) Hereditary structural alterations of plastids induced by a nuclear gene in Arabidopsis. Protoplasma 77:361-380

Reed M L (1979) Intracellular localization of carbonate dehydratase (carbionic anhydrase) in leaf tissue. Plant Physiol 63:216-217

Reisfeld A, A K Mattoo, M Edelman (1982) Processing of a chloroplast-translated membrane protein in vivo. Eur J Biochem 1245:125-129

Rigby P W J, M Drekman, C Rhodes, P Berg (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237-251

Rochaix J D (1985) Genetic organization of the chloroplast. Intl Rev Cytol 93:57-91

Roise D, F Thelier, S J Horvath, J M Tomich, J H Richards, D S Allison, G Schatz (1988) Amphiphilicity is essential for mitochondrial preseqence function. EMBO J 7:649-655

Roughan P G, C R Slack (1982) Cellular organization of glycerolipid metabolism. Ann Rev Plant Physiol 33:97-132

Roughan G, R Slack (1984) Glycerolipid synthesis in leaves. TIBS 9:383-386

Sabatini D D, G Kreibich, T Morimoto, M Adesnik (1982) Mechanisms for the incorporation of proteins in membranes and organelles. J Cell Biol 92:1-22

Sadler I, K Suda, G Schatz, F Kaudewitz, A Haid (1984) Sequencing of the nuclear gene for yeast cytochrome c1 precursor reveals an unusually complex amino-terminal presequence. EMBO J 9:2137-2143

Salts Y, R G Herrmann, N Peleg, U Lavi, S Izhar, R Frankel, J S Beckmann (1984) Physical mapping of plastid DNA variation among eleven Nicotiana species. Theor Appl Genet 69:1-14

Sanger F, S Nicklen, A R Coulson (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467

Sayre, R T, B Andersson, L Bogorad (1986) The topology of a membrane protein: the orientation of the 32 kd Qb-binding chloroplast thylakoid membrane protein. Cell 47:601-608

Schatz G, R A Butow (1983) How are proteins imported into mitochondria? Cell 32:316-318

Schmid G H, A Radunz, W Menke (1977) Localization and function of cytochrome f in the thylakoid membrane. Z Naturforsch 32c:271-280

Schmidt G W, M L Mishkind (1986) The transport of proteins into chloroplasts. Ann Rev Biochem 55:879-912

Schmidt R J, N W Gillham, J E Boynton (1985) Processing of the precursor to a chloroplast ribosomal protein made in the cytosol occurs in two steps, one of which depends on a proteins made in the chloroplast. Mol Cell Biol 5:1093-1099

Schmidt R J, A M Myers, N W Gillham, J E Boynton (1984) Chloroplast ribosomal proteins of Chlamydomonas synthesized in the cytoplasm are made as precursors. J Cell Biol 98:2011-2018

Schmitz U K, K V Kowallik (1986) Polymorphism and gene arrangement among plastomes of ten Epilobium species. Plant Mol Biol 7:115-127

Schneegurt M A, S I Beale (1986) Biosynthesis of protoheme and heme a from glutamate in maize. Plant Physiol 81:965-971

Schneider V, U W Hallier (1970) Polyvinylpyrrolidon als Schutzstoff bei der Untersuchung gerbstoffgehemmter Enzymreaktionen. Planta 94:134-139

Schötz F (1954) Über Plastiden konkurrenz bei Oenothera . Planta 43:183-240

Schötz F (1974) Untersuchungen uber die Plastidenkonkurrenz bei Oenothera IV. Der Einfluss des Genoms auf der Durchsetzungsfahigkeit der Plastiden. Biol Zbl 93:41-64

Sears B B (1983) Genetics and evolution of the chloroplast. In P Gustafsson, ed, Stadler Symposium XV, Univ Missouri Press, Columbia, pp 119-139

Sears B B, Kaplan S A (1984) Nuclear gene induces changes in chloroplast DNA. Genetics 107:s96

Sederoff R R (1984) Structural variation in mitochondrial DNA. Adv Genet 22:1-108

Shanklin J, M Jabben, R Vierstra (1987) Red light-induced formation of ubiquitin-phytochrome conjugates: Identification of possible intermediates of phytochrome degradation. Proc Natl Acad Sci USA 84:359-363

Shaw P J, J A Henwood (1985) Immuno-gold localization of cytochrome f, light-harvesting complex, ATP synthase and ribulose1,5-bisphophate carboxylase/oxygenase. Planta 165:333-339

Shenk T E, C Rhodes, P W J Rigby, P Berg (1975) Biochemical method for mapping mutational lesions in DNA with S1 nuclease: the location of deletions and temperature-sensitive mutations in Simian Virus 40. Proc Natl Acad Sci USA 72:989-993

Shinozaki K, M Ohme, M Tanaka, T Wakasugi, N Hayashida, T Matsubayashi, N Zaita, J Chunwongse, J Obokata, K Yamaguchi-Shinozaki, C Ohto, K Torazawa, B Y Meng, M Sugita, H Deno, T Kamogashira, K Yamada, J Kusuda, F Takaiwa, A Kato, N Tohdoh, H Shimada, M Sugiura (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J 5:2043-2049

Shumway L K, T E Weier (1967) The chloroplast structure of iojap maize. Amer J Bot 54:773-780

Siedow J N, L E Vickery, G Palmer (1980) The nature of the axial ligands of spinach cytochrome f. Arch Biochem Biophys 203:101-107

Singer S J, P A Maher, M P Yaffe (1987) On the transfer of integral proteins into membranes. Proc Natl Acad Sci USA 84:1960-1964

Smeekens S, C Bauerle, J Hageman, K Keegstra, P Weisbeek (1986) The role of the transit peptide in the routing of precursors toward different chloroplast compartments. Cell 46:365-375

Snedecor G W, W G Cochran (1967) Statistical Methods, Ed 6, Univ Iowa Press, Ames pp 369-375

Snyder M, A R Fraser, J LaRoche, K E Gartner-Kepkay, E Zouros (1987) Atypical mitochondrial DNA from the deep sea scallop Placopecter magellanicus . Proc Nat Acad Sci 84:7595-7599

Solignac M, M Monnerot, J-C Mounolou (1983) Mitochondria DNA heteroplasmy in Drosophila mauritiana. Proc Nat Acad Sci 80:6942-6946

Solignac M, J Genermont, M Monnerot, J-C Mounolou (1984) Genetics of mitochondria in Drosophila: mtDNA inheritance in heteroplasmic strains of D. mauritiana. Mol Gen Genet 197:183-188

Somerville C R, S C Somerville, W L Ogren (1981) Isolation of photosynthetically active protoplasts and chloroplasts from Arabidopsis thaliana. Plant Sci Lett 21:89-96

Southern E M (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517

Steinback K E, J J Burke, C J Arntzen (1979) Evidence for the role of surface-exposed segments of the light-harvesting complex in cation-mediated control of chloroplast structure and function. Arch Biochem Biophys 195:356-361

Steppuhn J, C Rother, J Hermans, T Jansen, J Salnikow, G Hauska, R G Herrmann (1987) The complete amino-acid sequence of the Reiske FeS-precursor protein from spinach chloroplast deduced from cDNA analysis. Mol Gen Genet 210:171-177

Stubbe W (1957) Dreifarbenpanaschierung bei Oenothera. I. Entmischung von drei in der Zygote vereinigten Plastidomen. Ber Deutsch Bot Gesell 70:221-227

Stubbe W (1959) Genetische analyse des Zusammenwirken von Genom und Plastom bei Oenothera. Z Verebungsl 90:288-298

Stubbe W, R G Herrmann (1982) Selection and maintenance of plastome mutants and interspecific genome/plastome hybrids from Oenothera . In M Edelman, R B Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology, Elsevier Biomedical Press, Amsterdam pp 149-165

Thomas P E, D Ryan, W Levin (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacryamide gels. Anal Biochem 75:168-176

Thompson D, V Walbot, E H Coe Jr (1983) Plastid development in iojap and mutator-affected maize plants. Amer J Bot 70:940-950

Tittgen J, J Hermans, J Steppuhn, T Jansen, C Jansson, B Andersson, R Nechhushtai, N Nelson, R G Herrmann (1986) Isolation of cDNA clones for fourteen nuclear-encoded thylakoid membrane proteins. Mol Gen Genet 204:258-265

Towbin H, T Staehelin, J Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. Proc Natl Acad Sci USA 76:4350-4354

Tsuzuki M, S Miyachi, G E Edwards (1985) Localization of carbonic anhydrase in mesophyll cells of terrestrial C_3 plants in relation to CO_2 assimilation. Plant Cell Physiol 26: 881-891

Tyagi, A K, J Hermans, J Steppuhn, C Jansson, F Vater, R G Herrmann (1987) Nucleotide sequence of cDNA clones encoding the complete "33 kDa" precursor protein associated with the photosynthetic oxygen-evolving complex from spinach. Mol Gen Genet 207:288-293

Tyagi A K, R G Herrmann (1986) Location and nucleotide sequence of the pre-apocytochrome f gene on the Oenothera hookeri plastid chromosome (Euoenothera plastome I). Curr Genet 10:481-486

van Loon A P G M, A W Brändli, G Schatz (1986) The presequences of two imported mitochodrial proteins contain information for intracellular and intramolecular sorting. Cell 44:801-812

van Loon A P G M, G Schatz (1987) Transport of proteins to the mitochondrial inner membrane space: the 'sorting' domain of the cytochrome c_1 presequence is a stop-transfer sequence specific for the mitochondrial inner membrane. EMBO J 6:2441-2448

Vater J, J Salnikow, C Jansson (1986) N-terminal sequence and secondary structure analysis of extrinsic membrane proteins in the water-splitting complex of spinach. FEBS Lett 203:230-234

Vogt V M (1980) Purification and properties of S_1 nuclease from Aspergillus. In L Grossman, K Moldave, eds, Nucleic Acids Part 1, Methods in Enzymology, Vol 65. Academic Press, New York, pp 248-255

von Heijne G (1983) Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133:17-21

von Heijne G (1986a) Mitochondrial targeting sequences may form amphiphilic helices. EMBO J 5:1335-1342

von Heijne G (1986b) A new method for predicting signal sequence cleavage sites. Nucleic Acid Res 14:4683-4690

von Heijne G (1986c) Towards a comparitive anatomy of N-terminal topogenic protein sequences. J Mol Biol 189:239-242

Wakabayashi S, H Matsubara, C H Kim, K Kawai, T E King (1980) The complete amino acid sequence of bovine heart cytochrome c_1. Biochem Biophys Res Commun 97: 1548-1554

Walbot V, E H Coe (1979) Nuclear gene iojap conditions a programmed change to ribosome-less plastids in Zea mays. Proc Natl Acad Sci USA 76:2760-2764

Walker G C (1985) Inducible DNA repair systems. Ann Rev Biochem 54:425-457

Weaver R F, C Weissman (1979) Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 15S β globin mRNA precursor and mature 10S β globin mRNA have identical map coordinates. Nuc Acid Res 7:1175-1183

Westhoff P, C Jansson, L Klein-Hitpaß, R Berzborn, C Larsson, S G Bartlett (1985) Intracellular coding sites of polypeptides

associated with photosynthetic oxygen evolution of photosystem II. Plant Mol Biol 4:137-146

Wettern M, G Galling (1985) Degradation of the 32-kilodalton thylakoid-membrane polypeptide of Chlamydomonas reinhardi Y-1. Planta 166:474-482

Willey D L, A D Auffret, J C Gray (1984a) Structure and topology of cytochrome f in pea chloroplast membranes. Cell 36:555-562

Willey D L, C J Howe, A D Auffret, C M Bowman, T A Dyer, J C Gray (1984b) Location and nucleotide sequence of the gene for cytochrome f in wheat chloroplast DNA. Mol Gen Genet 194:416-422

Willey D L, A K Huttly, A L Phillips, J C Gray (1983) Localization of the gene for cytochrome f in pea chloroplast DNA. Mol Gen Genet 189:85-89

Wu N, J C Côté, R Wu (1986) Nucleotide sequence of the rice cytochrome f gene and the presence of sequence variation near this gene. Gene 50:271-78

Yanisch-Perron C, J Vieira, J Messing (1985) New M13 host strains and the complete sequences of M13mp and pUC vectors. Gene 33:103-119

Zurawski G, M T Clegg (1987) Evolution of higher-plant chloroplast DNA-encoded genes: implications for structure-function and phylogenetic studies. Ann Rev Plant Physiol 38:391-418

MICHIGAN STATE UNIV. LIBRARIES
31293005480334