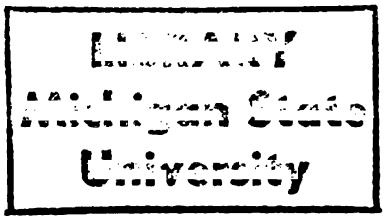

This is to certify that the

dissertation entitled

Instability of Photosystem II Complexes
in a Chloroplast-Encoded Tobacco Mutant

presented by

Catherine Pauline Chia

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biochemistry

Chas. J. Arntzen
Major professor

Date November 25, 1985

INSTABILITY OF PHOTOSYSTEM II COMPLEXES IN A CHLOROPLAST-ENCODED TOBACCO MUTANT

By

Catherine Pauline Chia

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1985

ABSTRACT

INSTABILITY OF PHOTOSYSTEM II COMPLEXES
IN A CHLOROPLAST-ENCODED TOBACCO MUTANT

By

Catherine Pauline Chia

The Photosystem II core complex of chloroplast thylakoid membranes is comprised of six chloroplast-encoded polypeptides. The genetic regulation of the biogenesis and maintenance of this complex may be studied by examining chloroplast-encoded mutants. This thesis describes such an approach using photosynthetic mutants of Nicotiana tabacum.

A collection of fifteen tobacco plastome (chloroplast genome) mutants were generated by ethyl methane sulfonate mutagenesis of Nicotiana tabacum seed. Selected mutants were chlorophyll deficient and displayed the characteristic variegation patterns of a maternally-inherited trait. Reciprocal crosses confirmed the cytoplasmic location of the mutations. The mutants were divided into four classes on the basis of their whole leaf chlorophyll fluorescence properties. All mutants exhibited altered electron transport activities and liquid nitrogen chlorophyll fluorescence spectra compared to wild type tobacco. Polyacrylamide gel electrophoresis of

thylakoid membrane polypeptides indicated a depletion of polypeptides identified as Photosystem II or Photosystem I constituents.

One mutant, lutescens-1, was analyzed in more detail. This Photosystem II mutant had a lesion affecting the stability of Photosystem II complexes in the chloroplast thylakoid membrane, resulting in the loss of Photosystem II complexes during leaf maturation. Functional properties of lut-1 Photosystem II centers were like those of wild type. The depletion of Photosystem II complexes was due neither to altered transcript levels nor to diminished chloroplast protein synthesis, but to a post-translational process which prevented the accumulation of wild type protein levels. An unusual effect of this membrane protein turnover process was the accumulation of intermediate-size species of nuclear-encoded Photosystem II-associated polypeptides. This indicated that two-step processing occurs during assembly of these subunits with the Photosystem II core complex.

To my parents

ACKNOWLEDGEMENTS

There were many people who helped me to acquire and assemble the work described in the following pages. I thank Charles Arntzen for his patience, guidance and confidence, and who allowed me free choice in my endeavors. I especially appreciate Charlie's genuine enthusiasm for the scientific enterprise, an attitude which I hope to emulate and convey to future colleagues. I thank Lee McIntosh, John Wilson, John Wang and Peter Wolk, members of my guidance committee, for their advice and support. I thank Chris Somerville, Shauna Somerville, and members of their laboratories for adopting and welcoming me into their groups this past year. Many others from the past and present Plant Research Laboratory community provided company, suggestions, big and small talk, empathy, coaching and optimism: John Duesing, Dave Kyle, Itzhak Ohad, Herb Nakatani, Joanna Hanks, Rachel Guy, Ellen Johnson, Erin Bell, John Fitchen, Yossi Hirschberg, Christer Jansson. My immeasurable thanks go to Sylvia Darr and Jan Watson, both of whom shared their time and care with me. I also thank Barb Wilson and Ljerka Kunst, my good friends, for their support, especially in these last crazy months.

I acknowledge partial financial support from a Michigan State University Minority Competitive Doctoral Fellowship, a United States Office of Education Professionals Opportunites Program Fellowship and the Department of Energy Plant Research Laboratory.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pairs
BSA	bovine serum albumin
CF_1	coupling factor (chloroplast ATPase)
chl	chlorophyll
CP	chlorophyll-protein
cpDNA	chloroplast DNA
cpRNA	chloroplast RNA
cyt	cytochrome
DCMU (diuron)	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DPIP	2,6-dichlorophenolindophenol
EDTA	ethylenediaminetetraacetic acid
F_m	maximum fluorescence (*in vitro*)
F_o	initial fluorescence
F_p	peak fluorescence (*in vivo*)
F_t	terminal fluorescence
F_v	variable fluorescence
kd	kilodalton

LDS	lithium dodecyl sulfate
LHC	light-harvesting complex
lut-1	lutescens-1
MES	2[N-morpholino]-ethane sulfonic acid
MOPS	3[N-morpholino]-propane sulfonic acid
MV	methyl viologen (N,N'-dimethyl-Y,Y'--dipyridylium chloride
OEC	oxygen evolving complex
PAGE	polyacrylamide gel electrophoresis
PQ	plastoquinone
PS	photosystem
Q_A	the primary quinone electron acceptor of PSII
Q_B	the secondary quinone electron acceptor of PSII
RC	reaction center
SDS	sodium dodecyl sulfate
SSC	150 mM NaCl, 15 mM sodium citrate, pH 7.0 (1X)
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
$TMQH_2$	tetramethyl-p-benzohydroquinone
TP	tobacco plastome
Tricine	N-tris-(hydroxymethyl)-methylglycine
Tris	tris-(hydroxymethyl)-aminomethane
WT	wild type
Z	primary electron donor to PSII

CHAPTER 1

INTRODUCTION

Light absorbed by green plants is converted into chemical energy via a series of electron transfer reactions catalyzed by lipoprotein complexes found in the thylakoid membranes of chloroplasts. The genetic regulation of development of one of these multi-subunit complexes, Photosystem II (PSII), is the topic of this thesis. This chapter serves to introduce the reader to current views on the function and structure of PSII complexes. Readers are also referred to works edited by Barber (12), Govindjee (59) and Staehelin and Arntzen (147), for comprehensive reviews, particularly of PSII reaction center bioenergetics.

A. Functional Analysis of PSII

PSII may be defined functionally as a pigment (primarily chlorophyll) -containing protein complex capable of carrying out the light-induced oxidation of water and subsequent transfer of the electrons to the plastoquinone pool. Freeze fracture electron microscopic analyses and biochemical studies of defined membrane fractions physically locate PSII complexes primarily, but not exclusively, in the appressed membrane regions called grana or grana

stacks while Photosystem I (PSI) is found in the non-appressed membrane regions, called stromal lamellae (6,9). The PSII units in the grana stacks are called α-centers and are considered capable of energy transfer among themselves, whereas those units in the stromal lamellae, called β-centers, do not participate in energy transfer (155). This lateral heterogeneity in the distribution of PSII complexes may be partly responsible for imparting a functional difference to the PSII complexes (112).

A variety of biochemical and biophysical methods are available to monitor the photochemistry carried out by leaves and isolated chloroplast membranes (thylakoids). Basic principles behind some of the techniques used in the studies described in Chapters 2 and 3 are briefly discussed in the next few pages. They include assays of electron transport and room temperature chlorophyll (chl) fluorescence, liquid nitrogen chl emission fluorescence spectroscopy, and the kinetic analysis of O_2 evolution.

1. Electron Transport Activity

Linear electron transport in chloroplasts involves two photosystems. In the first phase, electrons extracted from water by a light-driven oxidation reaction mediated by PSII are passed via plastoquinone to the cytochrome (cyt) b_6/f complex (66). Electrons then move, via plastocyanin, from this complex to PSI, where in a second light-driven reaction, electrons are transferred to $NADP^+$ via ferredoxin. Electron transport activity is routinely assayed with artificial electron donors and/or acceptors, which are compounds exogenously added to thylakoid preparations, that donate or accept

electrons at different regions of the electron transport chain (161,81). Compounds are available which allow either whole chain activity or partial reactions to be measured. For characterization of PSII partial reactions, it is common to assay oxygen evolution, with water as the electron donor (as *in vivo*) and a compound such as benzoquinone acting as the electron acceptor, to quantitatively evaluate PSII activity (81).

Methyl viologen (MV), a dipyridylium salt, because of its low redox potential, accepts electrons exclusively from PSI. This compound is highly auto-oxidizable. Under appropriate conditions, its reduction is easily measured as oxygen consumption (161). Whole chain electron transport may be monitored as oxygen uptake, with water as the donor and MV as the acceptor. When MV is used in conjunction with an electron donor to PSI (such as reduced N,N,N',N'-tetramethyl phenylenediamine (TMPD) which donates to the PSI reaction center) oxygen uptake is a measure of PSI activity. When MV is used with tetramethyl-p-benzohydroquinone ($TMQH_2$) which donates to the plastoquinone pool, oxygen uptake is a measure of the cyt b_6/f to PSI electron transport activity in this region of the chain (82).

If the artificial acceptor or donor is a dye, measurement of aborbance changes as a result of its oxidation or reduction provides a spectrophotometric assay of electron transport. One such compound is 2,6-dichlorophenolindolphenol (DPIP) which is a frequently used PSII acceptor (161).

2. Chl Fluorescence: Room Temperature Induction

Chl fluorescence is a widely used and well-accepted method of monitoring the status of the photosynthetic apparatus (11). At room temperature, chl fluorescence arises mainly from PSII and thus serves as an indicator of PSII function (130). The chl fluorescence yield of leaves or thylakoids is inversely related to the amount of photochemistry (i.e., electron transport) which occurs. Thus chl fluorescence will increase as PSII photochemistry decreases, specifically as PSII traps close (i.e., are unable to pass their electrons to the electron transport chain).

When dark-adapted chloroplasts or leaves are first illuminated, fluorescence rapidly rises to a constant fluorescence yield, F_o, which arises from photochemically inactive chl. Fluorescence then increases as PSII centers become reduced and the intersystem electron transport carriers become "filled" (i.e., reduced). Maximum fluorescence (F_m) (or peak fluorescence (F_p); used when referring to leaves) is achieved when all the PSII traps are closed and the rate constant for photochemistry is reduced to a minimum. Fluorescence will remain low if an electron acceptor, such as ferricyanide, is present, since the acceptor will keep components of the electron chain oxidized. In leaves, fluorescence will fall from F_p because the physiological electron acceptor, $NADP^+$, is present to keep the intersystem electron transport carriers oxidized. The difference between the maximum or peak fluorescence and the constant fluorescence yield is called variable fluorescence, F_v (F_m or F_p minus F_o equals F_v). The variable fluorescence arises exclusively from the PSII reaction centers; assays

of the amplitude of variable fluorescence are therefore diagnostic indicators of the presence or absence of PSII (11,99,130).

If inhibition of electron transport occurs on the reducing side of PSII (i.e. after the primary quinone acceptor, Q_A), the PSII traps rapidly become stably reduced. Under these conditions, the rate of the increase in the fluorescence yield of dark-adapted samples is rapid, and the fluorescence level remains high during continuous illumination. This is the condition produced when PSII inhibitors, such as diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU), are added to leaves or isolated chloroplasts, or when there is a functional block which prevents normal electron transport (1,131).

3. Chl Fluorescence: Liquid Nitrogen (77 K) Emission Spectra

At 77 K, leaves and chloroplasts show three distinct chl fluorescence bands with maxima centering at 680-685, 695 and 735 nm. These bands have been ascribed to the three major chl-protein complexes in chloroplasts: the light-harvesting a/b complex (LHC-II), PSII and PSI (27).

Results of experiments by Butler and colleagues, using analyses of 77 K chl fluorescence emission spectra and light spectroscopy, led to the widely accepted tripartite model of the photosynthetic apparatus (28). Briefly stated, this scheme has the antennae complex LHC-II serving primarily PSII; LHC-II may also sensitize PSI. Divalent cations, which stimulate grana stacking (13), increase the coupling of LHC-II to PSII and reduces the transfer of excitation energy to PSI. This is possibly a means of regulating the distribution of quanta between the two photosystems (additional

postulates of this model can be found in ref. 28). Alterations in chl emission spectra, such as changes in relative fluorescence yield and peak emission wavelengths observed in perturbed (e.g. mutant) systems, can be interpreted as alterations in the energy transfer capabilities of the pigment-protein complexes or the specific loss of individual complexes.

4. Kinetic Analysis of Oxygen Evolution

The oxygen evolving complex (OEC) is an aggregate of proteins, required for the oxidation of water, which is extrinsically bound to the hydrophobic PSII complex. The lability of oxygen evolving activity during isolation of the PSII complex has hampered biochemical investigations of the mechanism of oxygen evolution. However, in the last five years, much has been learned about the polypeptide composition and cofactors of the OEC (7,60).

The current kinetic model for the mechanism of oxygen evolution originated from functional studies of Chlorella (a green alga). The major observation was that when suspensions of Chlorella were illuminated with equally spaced, saturating light flashes, maximum oxygen yield occurred with a periodicity of four (88). After many flashes, the initial oscillatory pattern damped as the oxygen yield per flash became the same (53). These results led to the postulation of a linear four-step mechanism, called the S-state cycle, requiring four consecutive photoacts which promoted the sequential extraction of four electrons from two water molecules to generate one O_2 (88). Two factors were suggested to be responsible for the damping phenomenon. These are double hits, which occur when two electrons are extracted

per flash, and misses, which occur when no electrons are extracted on a given flash. Thus, after many flashes, the population of cycling S-states would be randomized as a result of multiple double hits and misses.

Because oxygen evolution is a mechanistically complex process (60,175), alterations in the oxygen flash yield pattern, and in the parameters for double hits and misses, reflect alterations in PSII reaction center properties.

B. PSII Polypeptides

Identification of the polypeptide constituents of PSII complexes has been aided recently by improved procedures of detergent fractionation of thylakoid membranes, analyses of mutants impaired in PSII activity, and immunological methods. There is general agreement that six polypeptide species comprise the core complex. PSII is defined as being the structural unit capable of transferring electrons from an exogenous donor to the plastoquinone pool. Detergent-derived core complexes (e.g. from spinach) (25,140) contain species, in equimolar ratios, of 51, 44, 34, 32, 9 and 4 kilodaltons (kd)[1], all of which are chloroplast-encoded (48).

---

[1]Molecular weights of polypeptide species, expressed in kilodaltons, are based on their electrophoretic mobility on sodium (or lithium) dodecyl sulfate polyacrylamide gels. These weights vary depending on plant source and electrophoretic system used for analysis.

The 51 and 44 kd polypeptides are the apoproteins of CP47 and CP43 (CP: chlorophyll-protein), the chl-a binding proteins (29,42) comprising the PSII reaction center. In the reaction center, an electron is transferred from P680 (P for pigment; 680 is the wavelength of maximum absorption of a chl molecule made unique by its proteinaceous microenvironment), the primary donor, to pheophytin a, the intermediate electron acceptor. Studies by de Vitry et al. (43) have shown that this primary charge separation between P680 and pheophytin a occurs within CP47 of Chlamydomonas reinhardtii (a green alga). These data are in accord with findings by Nakatani et al. (125) who reported the photobleaching of P680 upon photoreduction of pheophytin in CP47 from spinach. The isolated spinach CP47 also showed a liquid nitrogen (77K) chl fluorescence emission maximum at 695 nm, which is generally attributed to PSII centers (28). In contrast, CP43 did not show the light-induced spectral changes associated with pheophytin reduction and had a 685 nm chl fluorescence emission maximum (at 77K) (ref. 128), which has been attributed to antennae chl-protein complexes (28). CP43 is thus considered to serve a light harvesting role.

In neither of the two above-mentioned studies was the primary quinone acceptor, Q_A, shown unequivocally to be associated with a specific protein species. De Vitry et al. (43), however, showed that photoreduction of the primary quinone required the presence of both the CP47 and CP43 proteins in Chlamydomonas (molecular weights of apoproteins were listed as 50 and 47 kd, respectively) and suggested that the binding of the primary quinone may be shared by the two polypeptides. However, a third set of studies (176,177) showed that a

PSII reaction center complex, CP2-b (isolated from a thermophilic cyanobacterium, Synechococcus sp) lacking the chl-protein complex designated CP2-C (which is believed to be analogous to the higher plant CP43), was capable of undergoing a light-induced charge separation. This suggests that pheophytin and Q_A reside on CP47, and that CP43 is not required for primary photochemistry.

A third PSII core polypeptide migrates at approximately 32 kd and has been identified as the apoprotein for the secondary electron acceptor, Q_B, a quinone. Because of its unusual properties, the function, structure and biogenesis of this 32 kd polypeptide (also called D1 because of its diffuse appearance on autoradiograms when radioactively labeled; ref. 34), have been studied intensively (see ref. 93 for a review). The Q_B-binding protein functions on the acceptor side of the PSII reaction center by binding plastoquinone (a lipophilic electron carrier) which is reduced to plastoquinol by two successive electron transfers. The secondary electron acceptor thus serves as a two electron gate so as to allow pairs of reducing equivalents to enter the quinone pool and intersystem electron transport chain (37).

The phenomenon of the rapid turnover of the 32 kd polypeptide in vivo (93) has raised questions regarding the control of its metabolism. Mattoo et al. (110) suggested that the levels of the 32 kd polypeptide are photoregulated in a process sensitive to rates of photophosphorylation and electron flow. Ohad et al. (127) consider the turnover to be a consequence of a damage, removal and replacement process related to the protein's function as a quinone binding site. The removal and subsequent replacement of the 32 kd polypeptide are

particularly intriguing events because they imply a need to recognize, degrade and synthesize adequate amounts of this protein. Understanding the mechanism and regulation of this turnover process is one of the aims of current photosynthesis research.

Another chloroplast-encoded protein in the molecular weight range of 32 to 34 kd, is called D2. As in the case of D1, D2 had a diffuse appearance on autoradiograms when labeled; ref. 34). The D2 protein has been assigned to the PSII core complex (42,140), although no definite function has been ascribed to it. However, because D2 has regions of strong amino acid homology to the 32 kd Q_B-binding protein, it has been suggested that D2 is involved in quinone binding (93,137), and possibly functions to bind Z, the primary donor to PSII, which has been shown to be a plastoquinone (40,128). Another hypothesis for the function of D2 comes from comparison of the D1 and D2 sequences to the sequences of the L and M PSII reaction center subunits of photosynthetic bacteria (172,173,178). Because significant homology exists between these two pairs of proteins (D1 and L, D2 and M), it has been suggested that D1 and D2 (rather than the 51 kd protein) are the PSII reaction center polypeptides in higher plants (93). At present there are no functional data to support this idea.

The 9 kd polypeptide species found in PSII core complexes has been identified as an apoprotein of cyt $\underline{b}_{559}$, the function of which remains problematic. Cyt $\underline{b}_{559}$ has been suggested to act on the donor side of PSII as well as participating as an electron carrier in the main chain (36). The nucleotide sequence of the spinach apocytochrome gene yielded an 83 amino acid residue protein of 9.4 kd. An open reading frame, beginning eleven bases after the terminal cyt coding

triplet, was found. The nucleotide sequence of this reading frame coded for a 39 residue, 4.4 kd polypeptide. This small polypeptide is usually not seen on SDS polyacrylamide gels. Because cyt $\underline{b}_{559}$ binds a heme prosthetic group co-ordinated via two histidines (10), and the amino acid sequences of the 9.4 and 4.4 kd polypeptides indicate one histidine each, it was suggested that cyt $\underline{b}_{559}$ contains at least two polypeptides. This postulated structure may be a homodimer having identical 9.4 kd protein subunits or a heterodimer consisting of one copy each of the 9.4 and 4.4 kd proteins (72). Further, based on the DNA sequence data, it appears unlikely that cyt $\underline{b}_{559}$ is synthesized as larger protein as previously suggested (179).

A number of other polypeptides are considered part of the PSII complex but are not involved in reaction center activity. These include nuclear-encoded (168) species of 16, 23 and 33 kd which are water soluble and extrinsically bound on the inner thylakoid surface. These polypeptides comprise the OEC (7). Chloride, calcium and manganese ions have been implicated as co-factors of the OEC. Although the functional roles of these ions are not fully defined, manganese is considered to have a redox function as a charge accumulator (a recent review is ref. 60). A fourth polypeptide, possibly involved in water oxidation is a 34 kd species (antibodies prepared against the OEC 33 kd polypeptide do not cross react with this 34 kd species). This polypeptide apparently has an altered mobility (to 36 kd) in a green alga mutant, Scenedesmus obliquus, which is unable to use water as an electron source but has PSII activity comparable to that of the wild type alga when artificial donors are used (115).

An additional three PSII-associated polypeptides were proposed to be involved in the water splitting process by Ljungberg, et al. (106) who used an immunological approach to determine the nearest neighbors of the 23 and 33 kd OEC proteins. Detergent-solubilized PSII preparations (from spinach) were incubated with the monospecific antiserum made against either the 23 or 33 kd polypeptides. The immunoprecipitates contained the target antigens as well as polypeptide species at 24, 22 and 10 kd which were suggested to be additional subunits of the OEC.

In summary, the PSII complex from higher plant chloroplasts has been purified in active form and considerable progress has been made in understanding details of its function and identifying its polypeptide constituents.

C. Synthesis Sites of PSII Polypeptides

The majority of the chloroplast polypeptides are encoded by nuclear genes and are synthesized in the cytoplasm (50,23). The establishment of a protein's site of synthesis is viewed as good evidence for the presence of the gene in the same compartment. This is because there has been no evidence of RNA moving from one compartment to another (50). A variety of methods has established the synthesis (coding) site of chloroplast proteins. A classical genetics approach was taken by Kung et al. (90) who concluded that polypeptides of LHC-II were found to be nuclear-encoded on the basis of their Mendelian inheritance pattern. A biochemical approach was used by Chua and Gillham (34) who labeled C. reinhardtii proteins in vivo plus

or minus protein synthesis inhibitors specific for either 70s (chloroplast) or 80s (cytoplasmic) ribosomes. The technique of translating compartment-specific mRNA populations *in vitro* and immunoprecipitating products with monospecific antibodies, is a method currently used to determine the synthesis site of chloroplast proteins (169).

In the case of the PSII core complex polypeptides (which are the 51 and 44 kd chl-*a* binding reaction center apoproteins, the D1 and D2 proteins of 34-32 kd, and cyt b_{559}; see Section B), *in vivo* and *in vitro* labeling studies revealed their site of synthesis to be in the chloroplast. The 51 and 44 kd polypeptides are considered the higher plant counterparts of *C. reinhardtii* polypeptides number 5 and 6, respectively (33,169). These two species, as well as the D1 and D2 proteins were labeled *in vivo* when *C. reinhardtii* cells were grown in the presence of [^{14}C]acetate and the cytoplasmic protein synthesis inhibitor, anisomycin (34). This antibiotic will not affect the synthesis of polypeptides made on the chloroplast (70s) ribosomes. Confirming these results was the experiment using a *C. reinhardtii* mutant having spectinomycin resistant chloroplast ribosomes. The polypeptides synthesized in the presence of anisomycin were also synthesized in this mutant but not in the wild type cell treated with spectinomycin.

In vitro labeling studies using [^{35}S]Met and isolated intact pea chloroplasts established that D1 was a major chloroplast protein synthesis product (49). Initially referred to as peak D (because it was the fourth labeled peak on cylindrical polyacrylamide gels used to separate the label polypeptides), this protein did not accumulate in

the thylakoid membranes, but appeared to turn over rapidly. The identification of peak D as D1, the Q_B-binding protein was not made until later (these studies are described in Section D).

The synthesis of the fifth PSII core polypeptide, cyt b_{559}, by chloroplasts was also demonstrated by *in vitro* labeling studies using isolated spinach chloroplasts (179). Cyt b_{559} was isolated from radiolabeled chloroplast proteins using established purification protocols. The isolated cyt migrated slightly slower than the authentic coomassie-blue staining polypeptide which led Zeilinski and Price to suggest that the protein was synthesized in a precursor form.

The genes for all five of the PSII core complex polypeptides have been mapped to the chloroplast genome (plastome), confirming the conclusion of the labeling studies described above (45,136,166,167). Their DNA sequences of these genes have also been published (4,72,77,121).

The genes of the 51 kd, 44 kd and cyt b_{559} polypeptides were mapped to the spinach plastome in the following manner (166,167). Isolated chloroplast mRNA was fractionated by hybridization to matrix-bound chloroplast DNA (cpDNA) fragments of known map position. These selected mRNA species were translated *in vitro* and monospecific antisera made against the individual *C. reinhardtii* polypeptides 5 and 6 (shown to cross-react with single pea and spinach proteins, ref. 33) or against the purified spinach cyt b_{559} (171) were used to immunoprecipitate translation products. Those mRNA species yielding immunoprecipitated protein products were matched to the cpDNA fragment. Cell-free transcription-translation of recombinant cpDNA fragments verified the location of the genes.

The gene for the D2 polypeptide was located on the plastome of C. reinhardti by methods analogous to the ones used for the other PSII core polypeptides. Recombinant cpDNA fragments from C. reinhardtii were used in a linked transcription-translation system. Protein products were immunoprecipitated with antibodies prepared against the C. reinhardtii D2 polypeptide (33) and the correct cpDNA fragment was identified (136). The cloned D2 gene was sequenced (137) and was also used as a heterologous probe to find the D2 gene in pea (133).

The relative abundance of the transcript for the 32 kd Q_B-binding polypeptide (D1) allowed the mapping of its gene in a manner different from the one described above for the other PSII core polypeptides. Bedbrook et al. (14) noted that the levels of certain cpRNA transcripts greatly increased upon illumination of etiolated corn seedlings. Chloroplast RNA from these seedlings yielded a major RNA species which hybridized to a specific cpDNA (maize) restriction fragment. Subsequent in vitro translation of the cpRNA showed a major product of approximately 34.5 kd. Similar results were obtained by Driesel et al. (45) who fractionated and translated in vitro cpRNA from young spinach plants. The fraction containing the transcript for a 32 kd protein was labeled and hybridized to restricted cpDNA, thus locating the gene on the cpDNA map.

The gene for D1 has been sequenced in a number of higher plants and in C. reinhardtii (a compilation of chloroplast genes is in ref. 38). As discussed in the next section, mutations in the polypeptide product of this gene are responsible for chloroplast resistance to PSII-directed herbicides.

It has been recently demonstrated that the spinach OEC polypeptides are synthesized in the cytoplasm. Transcripts for the three proteins were found in the cytosolic poly A^+-RNA fraction. This is strong evidence that they are nuclear-encoded (168). DNA sequences of the OEC polypeptides have not been reported. There is no information on the synthesis sites of the other PSII-associated polypeptides of 34 kd (the fourth polypeptide implicated in oxygen evolution; ref. 115), and of 24, 22 and 10 kd which were co-precipitated with either the 23 or 33 kd immunoprecipitates (106).

4. PSII MUTANTS

The analysis of the structure, function and development of photosynthetic membranes has benefited from mutants in higher plants and algae [Miles (116,117), von Wettstein (164), Henningsen and Stumann (68), and Somerville (145)]. Rather than summarize the numerous studies on PSII mutants, it is more useful to describe well-studied examples of three classes of PSII mutants and the important concepts which emerge or are emerging from their analysis. Photosynthetic mutants may be either nuclear- or chloroplast-encoded. Because there have been no extensive studies performed on PSII plastome mutants (except for the PSII herbicide-resistant mutants) discussion of plastome mutants is restricted to the latter. The three categories discussed are:

1. Mutants altered in chloroplast structure
2. Structurally complete mutants with altered function
3. Regulatory mutants

1. Mutants altered in chloroplast structure

PSII-deficient mutants, particularly of C. reinhardtii, have been effectively used as biochemical tools in defining the polypeptide subunits of PSII. To provide examples of the type of analyses previously conducted, three mutants are described below. As will become apparent in subsequent chapters of this thesis, there are several parallels between these studies and the research presented on tobacco plastome mutants.

C. reinhardtii mutant F34 lacks variable fluorescence which indicates the absence of functional PSII centers. Comparison of wild type and F34 thylakoid membrane proteins by SDS-PAGE indicated the absence of polypeptides at 47 and 50 kd (35). This was one of the first indications that these proteins, now believed to correspond to the 44 and 51 kd reaction center polypeptides of higher plants, were part of the PSII core complex. Subsequent biochemical and biophysical analyses of F34 and another PSII-deficient isolate, BF25, identified an additional three polypeptides at 30, 20 and 17 kd as PSII components. Since these species were notably absent in BF25, which was incapable of oxygen production despite relatively high levels of reaction centers (60% of wild type), it was concluded that they were involved in the water oxidation process on the donor side of PSII (16). It is likely that these C. reinhardtii proteins are the counterparts of the higher plant chloroplast OEC proteins at 33, 23 and 16 kd, described earlier.

The mutant F34 was also used by Delepelaire to determine the genetic origin of PSII polypeptides (41). C. reinhardtii cells were

pulse-labeled in the presence or absence of either cytoplasmic or chloroplast protein synthesis inhibitors, and thylakoid membranes were analyzed by gel electrophoresis. The results showed that PSII core polypeptides corresponding to the 51, 44 and 32 (Q_B-binding protein or D1) kd polypeptides are chloroplast-encoded whereas the corresponding OEC subunits are nuclear-encoded. Because D2 was a clearly labeled, chloroplast-synthesized species in wild type C. reinhardtii but lacking in F34 thylakoids, it was assigned to the PSII complex. The other PSII core protein, cyt b_{559}, was not identified as an integral structural protein of PSII through analysis of F34. Its absence was not clearly obvious in the complex polypeptide pattern at low molecular weight regions.

The ultrastructural analyses of photosynthetic mutant membranes supported the conclusion of biochemical studies that EF_s particles (observed when chloroplast membranes are freeze-fractured; ref. 146) consist of PSII core complexes and associated light-harvesting pigment proteins (9). Membranes from the Chlamydomonas mutant F34 which lacks PSII centers, also lacked EF_s particles (129). A suppressed strain of F34, F34SU, had partially restored (50% of wild type) PSII activity which was correlated with an increased level of PSII core polypeptides and increased numbers of EF_s particles (174). A study by Miller and Cushman (118) of a PSII-deficient tobacco mutant NC95 also linked EF_s particles to PSII function.

Mutations affecting protein complexes frequently affect all the subunits of the complex (117,68), i.e., the mutations are pleiotropic. Thus, the thylakoids of F34, a nuclear mutant (32), are depleted in both nuclear- and chloroplast-encoded PSII polypeptides. This

pleiotropism makes it difficult to focus on the primary lesion of the mutation because it is usually not possible to distinguish secondary effects from the primary dysfunction. This limits the usefulness of structural mutants in this class because their primary lesion cannot be defined at the molecular level.

2. Structurally complete mutants with altered function

A second category of PSII mutants are those having altered PSII properties resulting from a minor perturbation of the complex structure. One example is the Scenedesmus mutant discussed earlier. The only difference between mutant and wild type polypeptides was the apparently altered mobility of a 34 kd polypeptide to 36 kd (115). This coincided with the loss of the mutant's ability to evolve oxygen, and the protein was thus assigned to the donor side of PSII.

Herbicide resistant mutants, of which there are many, are another example of this group. Herbicide resistant weeds have been a relatively recent resource to PSII researchers. Since the site of action of certain chloroplast-specific herbicides, e.g. atrazine, has been established to be the 32 kd Q_B-binding protein (93), herbicide-resistant mutants have provided a new dimension to the study of the reducing side of PSII, i.e. quinone binding (163).

Two properties of the 32 kd Q_B-binding protein aided in identifying it as a chloroplast-encoded gene product. The first is that this protein is a rapidly labeled product of chloroplast protein synthesis. The second feature is that it is the site of herbicide-binding. Steinback et al. (151) labeled pea chloroplast proteins separately with [^{35}S]Met, and azido-[^{14}C]atrazine. Trypsin

treatment of the labeled membranes yielded identical peptide fragments, indicating that the two proteins were equivalent.

The 32 kd Q_B-binding protein gene (psbA) from atrazine susceptible and resistant plants (75,76), algae (51,52), and cyanobacteria (57) have been sequenced. In all studies, the mutant genes had single nucleotide base substitutions causing an amino acid change in the protein which was responsible for the resistant phenotype. The mapping of these mutated sites shows that they are clustered over a segment of amino acids which form two membrane-spanning regions (hydrophobic regions defined by hydropathy plots) of the 32 kd polypeptide.

3. Regulatory Mutants

In addition to helping to establish structure/function relationships and the supramolecular organization of PSII complexes, PSII mutants can potentially play a unique role in revealing the regulatory processes governing membrane protein complex formation. Although this approach has had limited use to date, one example has been the extensive analysis of a nuclear maize mutant, hcf-3. This mutant is PSII-defective because its thylakoids lack the major PSII polypeptides (114). Neither nuclear- nor chloroplast-encoded polypeptides accumulate, making hcf-3 (shown to be a single locus mutation; ref. 104) another example of a pleiotropic mutant.

Other photosynthetic functions unrelated to PSII are largely unaffected in hcf-3. This suggests that the PSII complex is a self-contained unit having its own assembly and maintenance capabilities. Work by Leto et al. (103) has indicated that there is a

stabilization process specific for PSII complexes which is affected in hcf-3. The chloroplast-encoded 48 and and 34.5 kd polypeptides in hcf-3 (corresponding to the 51 kd and 32 kd Q_B-binding protein polypeptides in higher plants) were found to undergo an accelerated turnover in mutant thylakoids as compared to the polypeptides in wild type thylakoids. These results confirm the idea that the nucleus codes for components necessary for the biogenesis and maintenance of the PSII complex.

In summary, PSII mutants have been, and will continue to be valuable tools in determining the composition of the complex (Class 1) and specific structure/function relationships (Class 2). Examination of additonal regulatory mutants (Class 3) can reveal mechanisms for controlling PSII biogenesis.

E. Introduction to Thesis

Because the PSII complex is one of the most intensively studied thylakoid protein complexes with respect to its composition, function and biosynthesis, fruitful areas of research include the following: 1) The identification of factors controlling the assembly of the complex and their regulation. Questions to be asked include: Is there stepwise addition of the subunits into the thylakoid membrane or do they insert in unison into the membrane? How do the proteins find each other and what controls their association and membrane insertion? What is the conformation of the individual subunits in the membrane and how do they interact with one another? 2) The identification of factors controlling the biogenesis of PSII. Questions to be asked

include: Since the chloroplast genome is polyploid (numbers of DNA molecules per chloroplast range from 10 to 300; ref. 48), how is the expression of the multiple chloroplast genes controlled? This is part of the broader and fascinating question of how the chloroplast and nucleus communicate since it is clear that the formation of the photosynthetic apparatus requires coordinated gene expression of the nuclear and chloroplast genomes.

An examination of chloroplast-encoded mutants can provide a means of focusing on the role of the plastome in the ontogeny of chloroplast thylakoid membrane complexes such as PSII. In addition, the relatively small chloroplast genome permits analyses of perturbations at the molecular level which are not yet feasible with the nuclear genome. The results of such a project are described in the following chapters. Chapter 2 introduces a collection of tobacco plastome mutants and describes alterations in their photosynthetic function. Chapters 3, 4 and 5 describe the results of studies of one isolate, TP-13, renamed lutescens-1 (lut-1).

CHAPTER 2

Characterization of Cytoplasmic Mutants in Nicotiana tabacum With Altered Photosynthetic Function

Abstract

A series of cytoplasmic mutants of tobacco (Nicotiana tabacum) have been generated and characterized. Compared to wild type tobacco, they have been found to have diminished levels of photosynthetic pigments and a range of functional impairments including modified chl fluorescence properties, loss of PSI and/or PSII electron transport activity, and aberrant ultrastructure. The loss of defined functional activities has been correlated to the depletion of specific thylakoid membrane proteins. All of these mutants exhibited pleiotropic losses of polypeptides, including those known to be nuclear-encoded; this is consistent with the concept that loss of one component of a multi-subunit membrane protein complex results in unstable complex assembly.

The phenotype of two mutants was developmentally regulated, in one case with slow chloroplast development and in the other by premature senescence of PSII centers as a function of leaf development. These mutants should be especially useful in studying membrane protein assembly.

Introduction

The molecular analysis of organellar biogenesis can benefit from the examination of mutants in comparative biochemical studies. Since chloroplast ontogeny and appearance of photosynthetic competence results from an integrated expression of the nuclear and plastid genes, mutations affecting photosynthesis may reside in either genome. The best characterized photosynthetic mutants in higher plants are nuclear-encoded (68,114). The contribution made by the plastid genome (plastome) to chloroplast development and the regulation of its expression has yet to be carefully defined (for a review of plastome mutants, see ref. 21). As a starting point in studies on the functional role of the plastome, we characterize in this chapter a collection of fifteen cytoplasmically inherited plastome mutants of Nicotiana tabacum (TP = tobacco plastome mutant).

Materials and Methods

Mutagenesis and Plant Material

Mutagenesis and genetic crosses were performed by J. H. Duesing. Seeds of Nicotiana tabacum (var. L.C. from the Connecticut Agricultural Experiment Station) were treated with 50 or 100 mM aqueous ethyl methane sulfonate for 16 hours at room temperature. Among the M1 population of plants, fifteen mutants were identified which exhibited chl deficiencies. The mutant leaf tissue appeared as variegating sectors in a cell lineage pattern consistent with the sorting out of mutant chloroplasts. Reciprocal genetic crosses revealed strict maternal inheritance of the mutations, confirming the cytoplasmic location of the mutations. (see ref. 158 for a discussion of non-Mendelian inheritance criteria.)

Mutant plant tissue was maintained as variegated sectorial and/or periclinal chimeras with wild type (WT) N. tabacum. Plants were grown in soil in a greenhouse at 25±5 ^{o}C under natural illumination and watered daily.

Chloroplast Isolation and Photosynthetic Activity Measurements

Thylakoid membranes were prepared at 0-4^{o}C under dim light by grinding washed, chilled and deveined leaves in a Waring blendor for 5 to 8 s at high speed in isolation buffer containing 400 mM sorbitol, 100 mM Tricine-NaOH, pH 7.8, 10 mM NaCl and 2 mM PMSF. The solution was filtered through two layers and then one layer of Miracloth before centrifugation at 3,000 x g for 5 min. The resultant pellet was suspended in 10 mM Tricine-NaOH, pH 7.8 and centrifuged at 3,000 x g for 5 min to yield stroma-free thylakoids. These membranes were

suspended in 100 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$ to approximately 1 to 2 mg chl/ml and stored on ice until use. Chl concentrations were determined by the method of MacKinney (107).

Whole chain, PSI-dependent, and intersystem electron transport activity using water, TMPD (0.5 mM), and $TMQH_2$ (0.5 mM) as donors, respectively, were measured by monitoring the methyl viologen (MV) mediated Mehler reaction as oxygen uptake using a water-jacketed oxygen electrode (Hansetech, U.K.) at 20°C. Chloroplasts (10 to 30 ug chl) were added to the reaction mixture which contained: 100 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, 10 mM NaCl, 5 mM Mg_2Cl_2, 0.5 mM MV, 0.5 mM NH_4Cl, 5 uM diuron, 0.1 uM gramicidin-D, and 5-10 ug superoxide dismutase (ca. 3000 units/mg). Diuron was omitted for whole chain measurements. $TMQH_2$ was prepared as described in Vermaas et al. (163). Saturating white light illumination was provided by a high intensity microscope lamp.

PSII-mediated DPIP reduction was measured at 580 nm using a Hitachi 100-60 using a Hitachi Model 100-60 spectrophotometer modified for direct sample illumination with an actinic beam (131). Chloroplasts (5 to 10 ug chl) were added to 2 ml of a solution containing 100 mM sorbitol, 50 mM potassium phosphate, pH 6.8, 0.4 mM DPIP, 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM NH_4Cl and 0.1 uM gramicidin-D.

Polyacrylamide Gel Electrophoresis (PAGE)

Analysis of membrane polypeptides using LDS-PAGE was carried out using the discontinuous buffer system of Laemmli (97). Detergent was omitted from both the 10-17.5% acrylamide gradient analyzing gel and

the 5% stacking gel. Samples were boiled for 1 min in sample buffer containing 50 mM Tricine-NaOH, pH 7.8, 50 mM dithiothreitol, 10 % (v/v) glycerol, and 2% (w/v) LDS. Bromphenol blue was used as a tracking dye. The upper buffer reservoir contained 0.1% (w/v) LDS and 1 mM EDTA. Electrophoresis was performed at 2.5 W constant power. Molecular weight standards were: bovine serum albumin, 68 kd; ovalbumin, 45 kd; carbonic anhydrase, 29 kd; and cyt c, 12.4 kd. Gels were stained for 45 min in 0.1% (w/v) coomassie blue, 50% (v/v) methanol, 7% (v/v) acetic acid and destained in 20% (v/v) methanol, 7% (v/v) acetic acid, and 3% (v/v) glycerol.

Chl Fluorescence Measurements

Fluorescence induction transients of intact leaf tissue were acquired with a Model SF-10 Plant Productivity Fluorometer (R. Brancker, Ottawa, Canada) as described (1). Transients were recorded with a Nicolet Explorer III digital oscilloscope and plotted with an x-y recorder.

Low temperature (77K) chl fluorescence emission spectra of isolated thylakoids were measured using a System 4800 scanning spectrofluorometer (SLM Instruments, IL). Samples (10 ug chl/ml), in a buffer solution containing 60% glycerol, 50 mM Tricine-NaOH, pH 7.8, 10 mM NaCl and 5 mM $MgCl_2$, were frozen in 0.5 mm i.d. capillary tubes and excited at 440 nm.

Electron Microscopy

Rectangular pieces (1 mm x 5 mm) of chilled leaves were fixed in 1.25% glutaraldehyde in 250 mM Na-cacodylate buffer, pH 7.4 for 2 h on

ice by a modification of the procedure of Karlson and Schultz (85) as described by Sjostrand (144). Efficiency of fixation was improved by vacuum infiltration of primary fixative. Post-fixation was performed in 1% osmium tetroxide in 250 mM Na-cacodylate, pH 7.8. Samples were dehydrated in a graded series (25, 50, 75, 90, 95 and 100 %) of ethanol and embedded in Epon 812 polymerized at 65^{o}C for 48 h. Thin sections were stained in aqueous uranyl acetate (30 min) followed by saturated lead citrate (5 min) and were observed either on a Phillips 201 or 300 transmission electron microscope.

Results

In Vivo Fluorescence Analysis

Whole leaf chl fluorescence induction transients provide a non-destructive means to analyze functionally the photosynthetic electron transport components in chloroplast membranes (130,99). This method has been used by other investigators to select photosynthesis mutants in algae and higher plants (19,20,105,116). The justification used by these and other researchers for using fluorescence detection is straightforward. Alterations in the kinetic parameters of fluorescence emission by mutant chloroplasts reflect changes in the status of the photosynthetic apparatus, thus allowing preliminary characterization of mutants which is rapid and relatively simple.

Our collection of tobacco chloroplast mutants described herein was initially characterized by fluorescence transient analysis and thereby grouped into four main categories as described below. These categories were primarily used to aid in subsequent analysis and discussion, and were not definitive in explaining the underlying mutation mechanism.

The initial fluorescence level, F_o, upon illumination of a normal dark-adapted leaf (Figure 1a), is a measure of the functional coupling of the antennae pigment bed to PSII reaction centers. A low F_o indicates efficient excitation energy transfer, whereas a high F_o implies less efficient transfer (under conditions where the PSII acceptors are oxidized, as in dark-adapted leaves or chloroplasts).

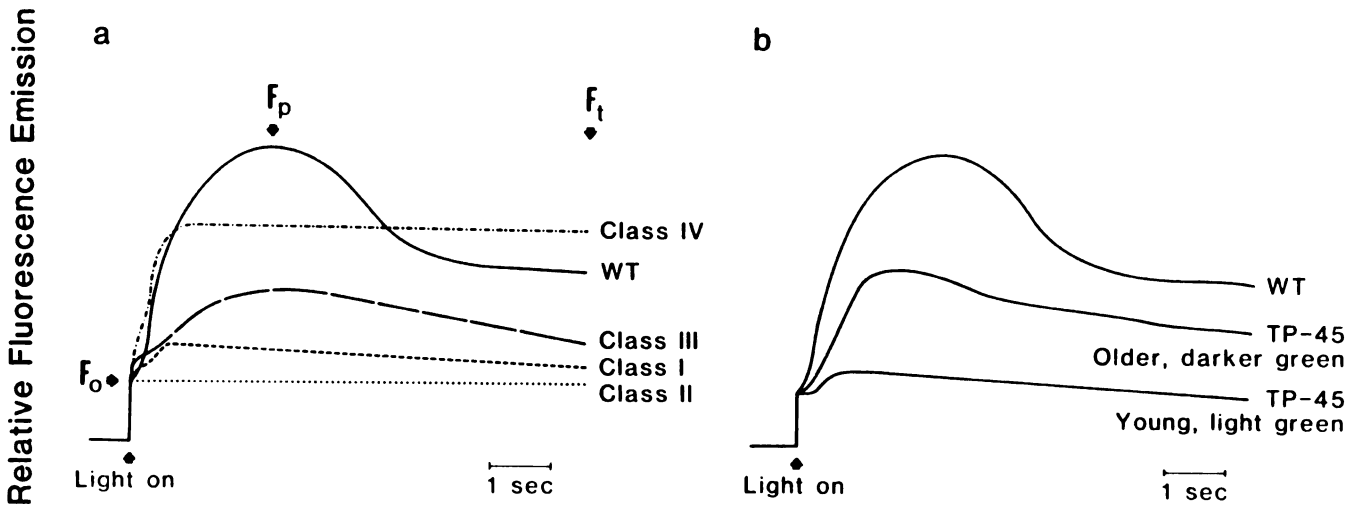

Figure 1 Whole leaf chl fluorescence transients used to classify the collection of tobacco plastome mutants. Traces representing each mutant class are normalized to the same F_o in order to compare more clearly the variable fluorescence component ($F_v = F_p - F_o$) of the classes to WT and to each other. (a) Classes I-IV (b) Developmental mutant TP-45. As its leaves greened, this mutant's variable fluorescence and photosynthetic capabilities increased (See also Table 1).

Following onset of illumination, the chl fluorescence intensity rises from F_o in a biphasic pattern to a peak level (F_p). The variable fluorescence ($F_v = F_p - F_o$) is the change in fluorescence yield which occurs as the primary electron acceptors for PSII, as well as the plastoquinone pool and intersystem carriers, become reduced. The subsequent fluorescence decline to a terminal level (F_t) largely reflects a partial re-oxidation of the primary PSII quinone electron acceptor (Q_A) as well as the formation of a trans-thylakoid pH gradient (78).

All tobacco mutants studied had altered chl fluorescence transients. In addition to having reduced levels of variable fluorescence, mutants typically exhibited F_o values 2 to 2.5 times WT values. Representative transients are shown in Figure 1a where the curves are normalized to the WT F_o in order to show differences in variable fluorescence (F_v). The first and largest group of mutants (defined as Class I) includes TP-10, -12, -16, -26, -32, -40, and -60. These had induction transients characterized by a high F_o, a small variable component (approximately 15% of WT), and a small, time-dependent decrease to F_t. A second mutant class (Class II) included TP-23 and -28; these were identified by high initial fluorescence and lack of a variable component. The mutant TP-21 was the single representative of Class III. Its whole leaf fluorescence curve exhibited a high F_o but continued to show significant variable fluorescence (approximately 40% of WT). The fourth category of mutants (Class IV) includes TP-2, TP-18, and TP-47. This group was characterized by a high F_o, a normal rise to F_p, (F_v = 65% of WT) with little or no decrease to F_t.

Two mutants, TP-13 and TP-45, could not be grouped into a specific class based upon their chl fluorescence properties since their fluorescence transients varied during leaf development. In the case of TP-45, leaf fluorescence showed a high F_0, and a slight variable component in young, light green leaves. The variable fluorescence increased with the expansion and increased chl content of mutant leaves (Figure 1b). The reverse pattern occurred with TP-13, whose leaves, when newly emerged, had a hign F_0 and a F_v which was 80% of WT, but gradually lost variable fluorescence during leaf expansion (data not shown). The physiological and structural data for these mutants, because of their dynamic phenotype, are presented separately in the last section of the results.

Photosynthetic Electron Transport and Light Harvesting Properties

Isolated mutant chloroplast membranes were assayed for their chl a/b ratios and photosynthetic activity in an attempt to identify functional lesions. The results are shown in Table 1 (rates are expressed as % WT activity). No defined pattern emerged for the PSII- and PSI-dependent activity measurements. The predominant phenotype was a severe reduction in PSII activity, as compared to PSI activity, which was responsible for the overall loss of whole chain activity. The relative chl content of mutant tissue (expressed on a % WT basis) are also presented in Table 1. Since all mutants were identified by pigment deficiencies, all chl values were less than WT. Activity measurements were determined on a chl basis which made difficult accurate and fair comparisons of the functional deficiencies of these mutants having such widely varying pigment contents. Instead, the

TABLE 1

Chl a/b Ratios, Chl content[a] and Photosynthetic Activity[a] of Tobacco Plastome Mutants

		Chl a/b[b]	Chl content[c]	Whole Chain	PSII Dependent	PSI-Dependent	
				$H_2O \rightarrow MV$[d]	$H_2O \rightarrow DPIP$[e]	$TMQH_2 \rightarrow MV$[f]	$TMPD \rightarrow MV$[g]
	TP-10	2.2	15	13	14	47	100
	-12	2.0	16	16	12	23	47
	-16	2.6	20	19	12	400	280
Class I	-26	3.0	84	32	24	225	150
	-32	2.4	21	11	17	72	350
	-40	2.7	11	11	12	88	270
	-60	3.9	5	32	19	73	435
Class II	TP-23	2.0	30	19	8	43	60
	-28	2.4	22	22	11	125	28
Class III	TP-21	2.3	63	0	10	55	17
Class IV	TP- 2	2.4	<1	nd[h]	nd	nd	nd
	-18	2.4	<1	nd	nd	nd	nd
	-47	2.4	<1	nd	nd	nd	nd
TP-13	Young leaves	2.9	78	70	74	180	130
	Old leaves	2.1	9	33	28	230	250
TP-45	Young leaves	2.9	2	70	35	60	120
	Old leaves	2.9	15	96	88	52	230

[a] expressed as % WT

[b] WT chl a/b ratio: 2.9 - 3.1

[c] mutant to WT ratios were determined for each experiment

Average WT rates (WT controls were run with each mutant):

[d] 160 umoles O_2 consumed·mg $chl^{-1}·h^{-1}$

[e] 195 umoles DPIP reduced·mg $chl^{-1}·h^{-1}$

[f] 75 umoles O_2 consumed·mg $chl^{-1}·h^{-1}$

[g] 480 umoles O_2 consumed·mg $chl^{-1}·h^{-1}$

[h] not determined

important data are the relative ratios of activities which remain the same using any parameter. None of the mutants had distinct lesions solely on the water oxidizing side of PSII as neither diphenylcarbazide (which donates electrons to PSII after steps involved in oxygen evolution [81]) nor hydroxylamine (whose electron donation would enhance the F_p of *in vitro* chl fluorescence induction kinetics [81]) had any effect on the isolated chloroplasts (data not shown).

Almost all mutants which were designated as Class I based on *in vivo* fluorescence transients had altered chl *a*/*b* ratios, for which there was no consistent pattern. All of the Class I mutants were PSII deficient (Table 1). Mutant TP-12 was also slightly PSI defective as judged by measurement of the TMPD to MV partial reaction. The remaining mutants in this class had high PSI rates as compared to WT, although this was partly a function of expressing electron transport rates on a chl basis since there appeared to be a decrease in PSII-associated chl in mutant tissue.

Liquid nitrogen chl fluorescence emission spectra were used to characterize the chl-protein complexes of isolated mutant thylakoids. In chloroplast membranes of higher plants, the short wavelength emission maximum at 685 nm is ascribed to the light-harvesting chl-protein complex of PSII and the CP43 PSII core protein whereas the 695 nm emmission is attributed to the PSII reaction center (RC) core complex (22,27,125). The PSI antenna had an emission maximum near 735 nm (22,27,122). Figure 2 shows representative low temperature chl fluorescence emission spectra of the mutant classes, as compared to the WT spectrum. All spectra are normalized for equal

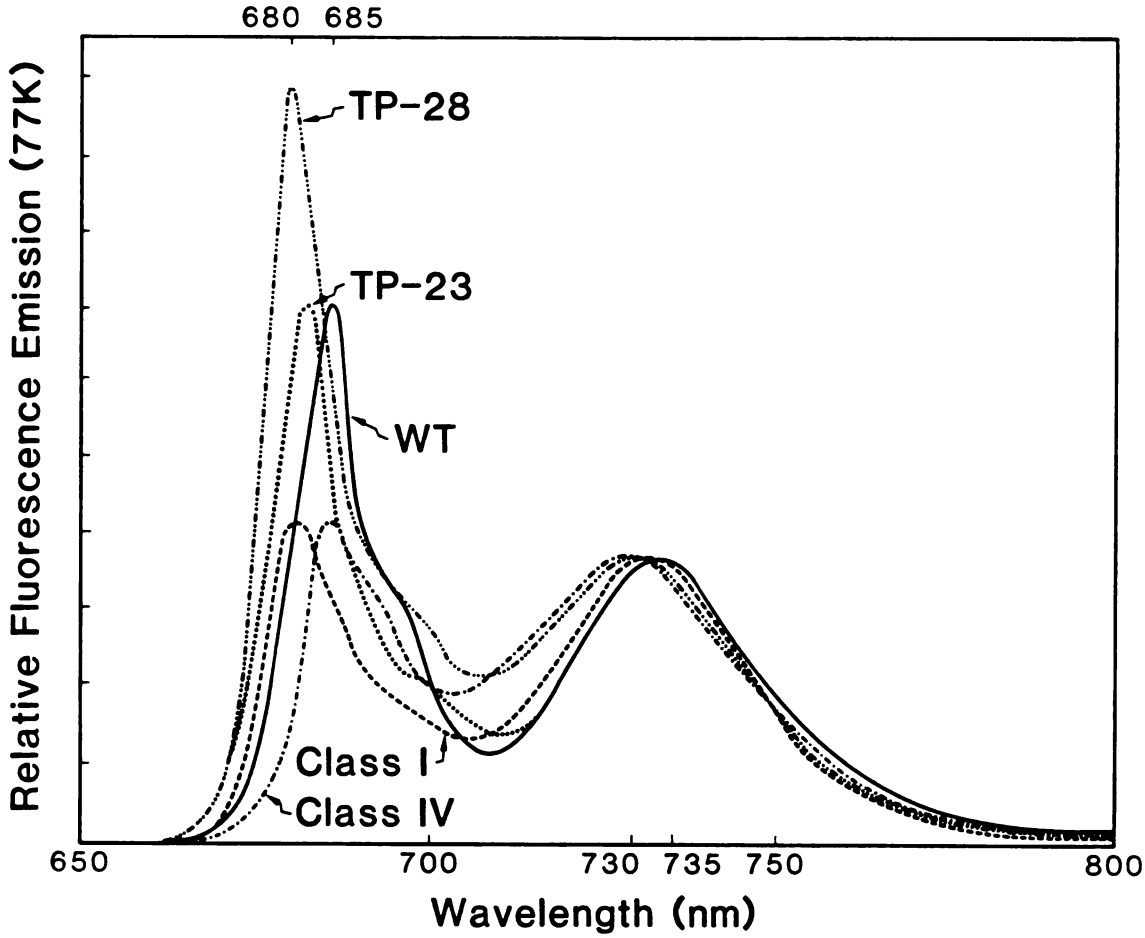

Figure 2 Representative low temperature chl fluorescence emission spectra of mutant classes were normalized to the long wavelength emission peak ascribed to PSI. The relative peak heights indicate whether the short to long wavelength emission peak ratio was less than, greater than or similar to that of WT (See also Table 2). Although both are Class II mutants, TP-23 and TP-28 had different 77K chl fluorescence emission spectra. The Class III mutant, TP-21, had a spectrum similar to that of WT.

emission at the long wavelength (730-735 nm) peak. Table 2 (page 45) lists the low temperature chl fluorescence emission peak wavelengths and the 680-685 nm/730-735 nm peak ratio for each mutant.

The mutants designated as Class I had in common a low temperature emission spectrum that showed a consistent blue shift of both PSI and PSII emission peaks as well as a 680-685 nm/730 nm peak emission ratio less than that of WT (Figure 2). We interpret the blue shift from 685 to near 680 nm as being the result of an increased proportion of the "free" light-harvesting chl a/b pigment protein complex that serves PSII (LHC-II). As has been found in previous PSII-deficient nuclear mutants and with purified LHC-II, the free LHC-II has an enhanced fluorescence emission at 680 nm at 77K (26,102). The reason for the slight blue shift from 733 to 732 nm is not clear, but may suggest a partially decreased content of the LHC-I(the light-harvesting pigment-protein complex serving PSI) (67,122).

Class II mutants, TP-23 and TP-28, were deficient in PSII activity and had chl a/b ratios less than that of WT. TP-23 had diminished PSI activity, but even greater inhibition of intersystem electron flow ($TMQH_2$ to MV). This indicates an impairment in the transfer of electrons between the cyt b_6/f complex (the site of $TMQH_2$ electron donation) and PSI. The short wavelength low temperature chl fluorescence emission peak of both mutants was blue shifted. TP-28 was the most extreme case of all the mutants with a five nanometer shift in its short wavelength emmission maximum to a new peak at 680 nm (Figure 2). Further, it was the only mutant whose short to long wavelength peak emission ratio was greater than that of WT (685 nm/733 nm ratio of 2.2 for WT and 680 nm/731 nm ratio of 2.6 for TP-28).

Mutant TP-23 displayed a chl fluorescence emission peak ratio similar to that of WT.

The Class III mutant, TP-21, had a chl a/b ratio less than that of WT, and was the only mutant in the collection which had no detectable whole chain activity although there were slight but measurable activities for PSII and PSI partial reactions (Table 1). The 77K fluorescence emission spectrum of TP-21 was nearly identical to WT in both emission peak wavelengths and the relative fluorescence emission ratio of 685 nm/730 nm (Figure 2).

Very low chl levels in Class IV mutants made it impossible to isolate sufficient membranes for activity measurements. A typical low temperature emission spectrum of isolated thylakoids of a Class IV mutant, TP-2, is shown in Figure 2. It displayed a relative enhancement of the PSI fluorescence yield relative to the short wavelength fluorescence emission yield.

Structural Characterization

In order to identify thylakoid proteins which were missing or depleted in these tobacco plastome mutants, detergent-solubilized thylakoid membrane complexes were isolated from WT Nicotiana tabacum following published purification procedures (PSII: ref. 17; PSI: ref. 122; CF_1: ref. 18; OEC: ref: 91). The polypeptide profiles of these preparations, resolved by PAGE, were comparable to those from pea and spinach where functional assignments have been made for many of the proteins. (For reviews, see ref. 66,74,84).

Figure 3 presents a sample set of data accumulated during polypeptide analysis of the tobacco plastome mutants. The WT

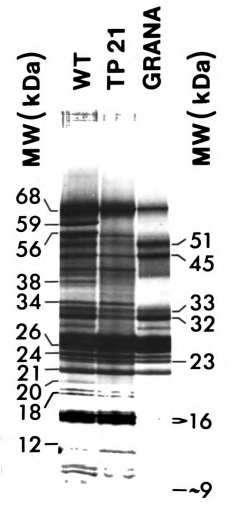

Figure 3 LDS-PAGE analysis of thylakoid membrane polypeptides from WT (lane 1), TP-21 (lane 2) and a grana membrane fraction prepared from WT in the manner of Berthold et al. (17) (lane 3). Equal amounts of protein were loaded in each lane. Identification of protein species is presented in Table 3. The depletion of polypeptides in mutant membranes was determined by comparing mutant polypeptide profiles to the predominant polypeptides in different WT thylakoid protein complex preparations as described in the Results.

thylakoids (Lane 1) resolved into more than 30 protein bands; some of these are labeled by apparent molecular weight. A grana membrane fraction (95) prepared from WT thylakoids is shown in Lane 3; this preparation is depleted of CF_1 polypeptides and greatly reduced in PSI constituents (148). Similar PAGE comparisons were made using PSI, CF_1 and OEC preparations to yield Table 3 (page 46) correlating polypeptide molecular weight (MW) and identity. It should be emphasized that, in many cases, more than one polypeptide migrated in PAGE at the same relative position. For example, a 21 kd polypeptide is isolated in a purified PSI preparation, but also appears as a dominant band in the grana preparation (Figure 3, lane 3) which is depleted in PSI; we conclude that different polypeptides representing each PS co-migrate on PAGE.

The dominant polypeptides used for subsequent comparisons among mutants for PSII included species at: 51 and 45 kd (RC proteins; 140); 32, 23 and 16 kd (proteins implicated in water oxidation; ref. 91). A diffuse staining region at 32-34 kd was tagged by the photoaffinity label azidoatrazine and was concluded to contain the Q_B-binding protein (the apoprotein of the secondary electron acceptor; ref. 93). LHC-II polypeptides ranged from 26-29 kd (84). The predominant polypeptides of the isolated PSI were at: 68 kd (the P700 RC apoprotein); 21 kd (considered to be one of the polypeptides of LHC-I; 98,122); 18 and 13 kd (subunits of the PSI complex involved in electron transfer; 15). The ATPase complex (CF_1; coupling factor) included the subunits (in kd) alpha, 59; beta, 56; gamma, 38; delta, 17.5; and epsilon, 13 (153).

It was possible to visualize two components of the cyt $\underline{b_6}/\underline{f}$ complex by staining for heme proteins (156). Stained bands were observed at 34 kd and 20 kd (cyt $\underline{f}$ and $\underline{b_6}$, respectively; 79) (data not shown). None of the plastome mutants lacked these two heme staining polypeptides.

All mutant chloroplasts were characterized by comparing their electrophoretic profiles to WT membranes. An example is shown in Figure 3, Lane 2. This gel was slightly overloaded in order to bring out minor bands (examples: 21, 18, 16 and 12 kd). As a result, some resolution was lost from dominant bands such as those in the 25-27 kd region, where changes among mutant protein profiles were not observed. TP-21 was found to contain the 68 kd PSI RC polypeptide (as well as the 21, 18 and 12 kd proteins which are isolated in PSI preparations). TP-21 did not contain the alpha, beta, or gamma subunits of CF_1 and had reduced amounts of the 45 and 51 kd PSII RC polypeptides (as evidenced by the reduction in staining intensity compared to WT bands). This pattern of pleiotropic loss of polypeptides was the rule for all mutants analyzed; no mutant was found to have lost only a single thylakoid polypeptide.

The comparison of all plastome mutants for polypeptide content is summarized in Table 2. All Class I mutants were found to be depleted in PSII RC polypeptides, but contained normal levels of the PSI RC. Two Class I mutants (TP-10 and TP-12) showed a concomitant loss of coupling factor subunits.

The polypeptide profiles of TP-23 and TP-28 (Class II mutants) showed the presence of polypeptides co-migrating with PSI, but the depletion of PSII constituents. Mutant TP-28 had the coupling factor

polypeptides; however, mutant TP-23 did not, being similar in this respect to mutants TP-10 and TP-12. The polypeptide profiles for the Class IV mutant indicated the presence of PSII and ATPase complex polypeptides but an extensive depletion of the 68 kd PSI RC polypeptide.

All Class I mutants were examined by thin section electron microscopy of leaf tissue. Thin section micrographs of mutant TP-10 (Figure 4b) showed organized stroma and grana lamellae similar to that of WT (Figure 4a), although there were fewer discs per granum. The ultrastructure of mutants TP-12, TP-16 and TP-26 was similar to that of WT (data not shown). TP-32 had large grana with many discs, and relatively low levels of stromal lamellae. The thylakoids were often oriented parallel to the short, rather than long axis of the organelle (data not shown). Mutants TP-40 (Figure 4d) and TP-60 had predominantly unilamellar membranes.

Class II Mutants TP-23 and TP-28 contrasted greatly in their ultrastructure: TP-23 developed giant grana (Figure 4c). TP-28 chloroplasts lacked an organized thylakoid system, showing only vesicular membrane inclusions. The ultrastructure of Class III mutant, TP-21, was similar to WT. The chloroplast ultrastructure of the Class IV mutants showed limited lamellar development, characteristically without grana and with vesicular body formation (data not shown).

We, and other investigators of photosynthetic mutants (e.g. ref. 164) conclude that it is not possible to establish a clear relationship between alterations in chloroplast ultrastructure/protein composition and photosynthetic dysfunction. Rather, the diversity of

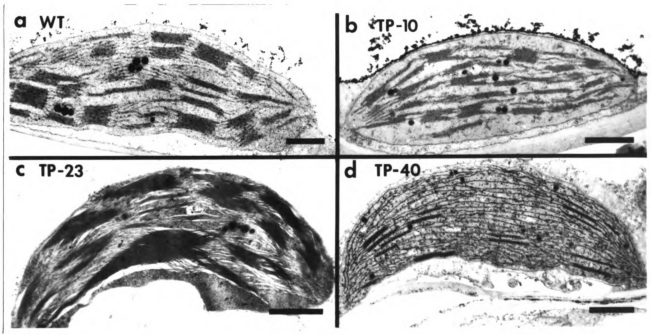

Figure 4 Electron micrographs of chloroplasts from WT tobacco and plastome mutants with irregular ultrastructure: a) WT b)TP-10 (Class I) c) TP-23 (Class II) d) TP-40 (Class I). Bar = 1 um.

functional and structural defects is a testimony to the complex nature of chloroplast biogenesis. Perturbation of the relatively simple chloroplast genome can cause major changes in the photosynthetic apparatus comparable to changes observed in nuclear mutants.

Developmentally Expressed Mutations

The functional properties of the mutants described above were constant in all leaf samples studied. In contrast, the chloroplasts of mutants TP-45 and TP-13 exhibited physiological characteristics which changed as a function of leaf age. They were, therefore, characterized at different stages of leaf expansion to determine if they were suitable candidates for examining regulatory aspects of plastid development which are controlled by the plastome.

TP-45 had a chl a/b ratio equivalent to that of WT throughout its development (chl a/b ratio of 2.9 to 3.0). The young leaves of TP-45 had above normal PSI but low PSII activity (Table 1). With expansion and greening of individual leaves emerging at the shoot apex (a process occuring over a two week period in both mutant and WT plants), PSI- and PSII- dependent activities increased approximately two-fold and four-fold, respectively, based upon rates expressed on a chl basis. Whole chain activity approached WT rates (Table 1) as leaf expansion was completed. This delayed development of photochemical activity is typical of previously described nuclear-encoded "virescens" mutants (86,96).

The low temperature chl fluorescence emission spectrum of TP-45 revealed peak wavelengths which were the same as those of WT at all stages of leaf expansion. The ratio of 685 nm to 730 nm fluorescence

yield changed with leaf age, from being greater than WT (in young leaves) to the same as WT (in mature leaves) (Table 2). During greening of leaves of TP-45, increased photosynthetic activity as a function of leaf maturation was directly correlated to increased levels of thylakoid polypeptides of the same electrophoretic mobility as authentic PSII proteins. Membranes from young leaves were deficient in these PSII proteins; however, the membranes from greened leaves had a polypeptide profile similar to that of WT (data not shown).

Ultrastructure features further demonstrated the developmental progression of these mutant chloroplasts. Young TP-45 plastids had primarily unilamellar membranes; chloroplasts from green mature leaves had a near normal lamellar system, except for slightly smaller grana with fewer thylakoids per stack (data not shown).

The mutant TP-13 displayed a markedly different pattern of phenotypic expression during leaf development. This was observed as a progressive degeneration of photosynthetic capacity during leaf expansion. As its leaves matured, TP-13 leaves lost chl and its chl a/b ratio fell from approximately 2.9 to 2.0. Its variable fluorescence and PSII activity decreased with pigment loss and leaf growth (Table 1). The low temperature fluorescence emission peak maxima of TP-13 were shifted to lower wavelengths with increasing age and the 680-685 nm/730 nm ratio of fluorescence yield increased primarily as a result of the increased fluorescence yield of PSII-associated antennae (Table 2). There was a physical reduction of PSII-associated polypeptides with time (Chapter 3, Results), concomitant with a degeneration in the ultrastructure of chloroplasts

TABLE 2

Summary of Low Temperature Chl Fluorescence and Structural Properties of Tobacco Plastome Mutants

		Chl Fluorescence Emission (77 K)			Polypeptides[a]			Ultrastructure
		Peak Maxima		Peak Ratios				
		Short Wavelength (nm)	Long Wavelength (nm)	680-685 nm/730-733 nm	PSII	PSI	CF_1	
WT		685	733	2.2	+	+	+	
Class I	TP-10	681	732	1.1	-	+	-	Small Grana
	-12	681	732	1.1	-	+	-	Small Grana
	-16	681	732	1.4	-	+	+	Normal
	-26	681	732	1.3	-	+	+	Normal
	-32	681	732	1.2	-	+	+	Normal
	-40	681	732	1.1	-	+	+	Unilamellar
	-60	681	732	1.1	-	+	+	Unilamellar
Class II	TP-23	683	733	2.2	-	+	-	Giant grana
	-28	680	731	2.6	-	+	+	Some grana formation with later vesiculation
Class III	TP-21	685	733	2.2	-	+	-	Normal
Class IV	TP- 2	685	730	1.1	+	-	+	Unilamellar with early vesicular deterioration
	-18	685	730	1.1	+	-	+	
	-47	685	730	1.1	+	-	+	
TP-13	Young leaves	685	733	2.2	+	+	+	Normal
	Old leaves	680	730	2.5	-	+	+	Normal
TP-45	Young leaves	685	733	2.5	-	+	+	Delayed appearance of stacking during development
	Old leaves	685	733	2.2	+	+	+	

[a]Depletion (indicated by a -) or WT levels (indicated by a +) of polypeptides in mutants was judged by comparing the coomassie blue staining intensity of "marker" polypeptides. For PSII, these were the 51 and 45 kd RC polypeptides; for PSI the marker protein was the 68 kd RC polypeptide; for CF_1, the alpha and beta subunits at 59 and 56 kd.

TABLE 3

Identification of WT Chloroplast Membrane Proteins and Their Gene Location

MW (kd)[a]	Identity	Gene Site	Reference[b]
68	PSI RC	Chloroplast	38
59	CF_1 (alpha)	Chloroplast	38
56	CF_1 (beta)	Chloroplast	38
51	PSII RC	Chloroplast	166
45	PSII RC	Chloroplast	166
38	CF_1 (gamma)	Nucleus	38
34	cyt f	Chloroplast	38
33	PSII (Q_B)	Chloroplast	170
32	OEC	Nucleus	168
26	LHC-II	Nucleus	90
24	LHC-1	Nucleus	123
23	OEC	Nucleus	168
21	PSI	Nucleus (?)	123
20	cyt b_6	Chloroplast	38
18	PSI	Unknown	
16	OEC	Nucleus	168
12	PSI	Chloroplast (?)	123
9	cyt b_{559}	Chloroplast	173

[a]Molecular weights were determined by comparison to known standard protein markers (see Materials and Methods).

[b]Where possible, literature references are for recent comprehensive reviews of chloroplast protein genes.

(data not shown). A more detailed analysis of this mutant is presented in the next three chapters.

Discussion

All plastome-encoded mutants characterized in this study were initially identified by pigmentation deficiencies. It was expected that the chl loss at the leaf level would correspond to alterations in the reaction center chl-protein complexes of PSI and PSII and/or changes in light-harvesting pigment protein complexes serving PSI or PSII (LHC-I or LHC-II; ref. 84). The polypeptides of LHC-II and LHC-I are nuclear-encoded (90,123). Two lines of evidence demonstrated that none of the plastome mutants studied herein lacked these complexes. All contained LHC-II polypeptides (26-29 kd) and LHC-I polypeptides (21-24 kd). In the case of Class IV mutants, the LHC-I polypeptides were present even though the mutant was deficient in the PSI reaction center. Presence of the LHC-I was also evidenced in all mutants by the low temperature chl fluorescence emission at 725-730 nm which has been shown to be a property of the isolated LHC-I (67,98).

Analyses of RC activity absence or presence (by combined partial reaction assays, polypeptide analysis, and fluorescence properties) revealed changes in all mutants studied. The accumulated information for these cytoplasmically inherited mutants indicated that greatly reduced PSII activity was the predominant phenotype. There are several possible explanations for the prevalence of PSII defects in our random screening of pigment variants. First, more mutations affecting PSII may have been recovered because many polypeptides of the PSII reaction center complex are chloroplast-encoded. These include two chl *a* binding RC proteins of 51 and 45 kd (166), a 32 kd polypeptide identified as the Q_B-binding protein (151,170) and cyt b_{559} (179). As for PSI, only the gene for the 68 kd RC polypeptide

has been definitively localized to the chloroplast genome (38). There is evidence indicating that another subunit of PSI is also synthesized within the chloroplast, although the majority of the constituents of the PSI complex are apparently nuclear-encoded (123).

All PSII mutants studied expressed pleiotropic effects. Thylakoid membranes were always depleted in multiple PSII polypeptides. This was not always the case with nuclear-encoded proteins (for example, the polypeptides of 16, 23, and 32 kd which have been implicated in water oxidation (92,168). Polypeptides co-migrating with the OEC were observed in some of the TP mutants; The presence of the OEC polypeptide is explored in more detail using immunochemical identification protocols described in Chapters 4 and 5. The phenomenon of pleiotropic effects has been observed in other nuclear (114) and plastome mutants (120). Since the assembly of chloroplast protein complexes is dependent upon the co-ordinated synthesis of their constituent subunits, an alteration of a structural protein gene for a subunit of a complex, regardless of its location, appears to disrupt normal complex assembly.

A second explanation for the preponderance of mutants with a PSII deficient phenotype is the increasing evidence that photoinhibition (loss of PSII activity) is the consequence of any inhibition of electron flow that results in an overall reduction of the intersystem plastoquinone (PQ) pool of electron carriers. Recent work with C. reinhardtii suggests that the primary site of inhibition is on the reducing side of PSII. Specifically, the 33 kd apoprotein of the secondary acceptor of PSII appears to be selectively damaged under conditions where the PQ pool is over-reduced (93). This may occur,

for example, under high light conditions where all PSII centers are turning over at maximum rates but the rate-limiting reaction of linear electron transport (either the oxidation or the diffusion of plastoquinol [66]) maintains the PQ pool in a highly reduced condition. Alternatively, under normal photon flux densities, if the rate of CO_2 fixation is limiting, reducing equivalents are not used and carbon metabolism is curtailed, leading again to a highly reduced PQ pool (132).

What we may be observing, in some of the mutant classes having PSII deficiencies, are lesions that mimic the photoinhibited condition. The malfunction of components on the acceptor side of PSII (not necessarily limited to the electron transport reactions) results in a reduced PQ pool which leads to injury of the 33 kd polypeptide and subsequent degeneration of PSII-associated polypeptides. According to this concept, photoinhibition is effectively accelerated under otherwise normal light conditions because excitation energy is not being utilized in the abberrant chloroplasts due to defects in components functioning in the electron transport chain after PSII, or even in the carbon assimilation pathway.

A third possible explanation for the high occurrence of PSII mutants in our random screening might be a consequence of the fact that, in developing chloroplasts, PSI reaction centers are formed before PSII centers (for a review, ref. 126). Some of the plastome mutants may be blocked in a developmental process leading to a functional PSII sometime after PSI formation occurs. This possibility seems less likely, since specific PSI-deficient mutants have been

detected in other plants such as barley (74) and in C. reinhardtii (35).

Mutants TP-10 (Figure 4b), -12 (Class I) and -23 (Class II) are especially intriguing because two protein complexes, PSII and the ATPase, are depleted from the thylakoid membranes. The limited number of mutants screened for this collection makes the observation of three separate cases of double mutations an unlikely possibility. Other plausible mechanisms include (1) a disruption of di- or poly-cistronic mRNA's for proteins which affected proper translation or (2) a mutation in a gene responsible for an event common to the biogenesis of both complexes, such as posttranslational processing of proteins.

Of these three mutants, TP-23 was unusual in that it had giant grana stacks and low levels of stromal lamellae (Figure 4c). Structural and functional evidence currently suggests that the ATPase complex is localized in stroma-exposed thylakoids (5). A possible explanation for the loss of this complex in TP-23 is that the mutation did not affect the proteins of the CF_1, but rather some aspect of membrane development which blocked the formation of a site for stable integration and assembly of the complex.

This collection of photosynthetic mutants in a common genetic background allows comparative physiological and biochemical studies of the control of membrane protein biosynthesis and assembly exerted by the plastome. Further, the data for mutants TP-45 and TP-13 indicate that these isolates are particularly suitable candidates for studies of events and/or agents governing chloroplast development and maintenance in concert with the nuclear genome. We believe these chloroplast mutants constitute a valuable resource for further studies

on the mechanism of photoinhibition and membrane protein complex formation and interactions. Careful biochemical analyses, including immunological studies and molecular characterizations (such as determining chloroplast mRNA levels) of these mutants will provide a better understanding of plastid gene expression and its control of chloroplast biogenesis.

CHAPTER 3

Developmental Loss of PSII Activity and Structure in Chloroplast-Encoded Tobacco Mutant Lutescens-1

Abstract

Lutescens-1, a tobacco mutant with a maternally inherited dysfunction, displayed an unusual developmental phenotype. In vivo measurement of chl fluorescence revealed deterioration in PSII function as leaves expanded. Analysis of thylakoid membrane proteins by polyacrylamide gel electrophoresis indicated the physical loss of nuclear- and chloroplast-encoded polypeptides comprising the PSII core complex concomitant with loss of activity. Freeze fracture electron micrographs of mutant thylakoids showed a reduced density, compared to wild type, of the EF_s particles which have been previously shown to be the structural entity containing PSII core complexes and associated pigment-proteins. The selective loss of PSII cores from thylakoids resulted in a higher ratio of antenna chl to reaction centers and an altered 77 K chl fluorescence emission spectra; these data are interpreted to indicate functional isolation of light-harvesting chl a/b complexes in the absence of PSII centers. Examination of PSII reaction centers (which were present at lower levels in mutant membranes) by monitoring the light-dependent phosphorylation of PSII polypeptides and flash-induced O_2 evolution patterns demonstrated that the PSII cores which were assembled in

mutant thylakoids were functionally identical to those of wild type. We conclude that the lutescens-1 mutation affected the correct stoichiometry of PSII centers, in relation to other membrane constituents, by disrupting the proper assembly and maintenance of PSII complexes in lutescens-1 thylakoid membranes.

Introduction

The formation of the photosynthetic apparatus in higher plants requires coordinated gene expression of both the nuclear and chloroplast genomes. To evaluate the contribution of the plastome (the plastid genome) to the biogenesis and maintenance of photosynthetic membranes, we have analyzed a series of chloroplast-encoded photosynthetic mutants (Chapter 2). One of these, lutescens-1, was initially characterized by a developmentally expressed phenotype. This mutant is described in more detail herein.

Photosynthetic mutants have been used as tools in numerous studies to understand plastid development and elucidate functional properties of the chloroplast (68,86). In almost all cases, however, the materials studied were nuclear-encoded mutations. For higher plants there have been only a few thorough studies of plastome mutants affecting photosynthetic function. These include pigment-deficient mutants of Oenothera (65), Pelargonium (70), Antirrhinum (69), and Nicotiana (118), and herbicide-resistant mutants of Brassica (39), Amaranthus (75,76) and Solanum (58). In all these cases, uniparental inheritance of the mutation was presumptive evidence of a plastome mutation.

The mutant lut-1 belongs to a collection of tobacco chloroplast mutants, described in Chapter 2, generated through ethyl methane sulfonate mutagenesis of Nicotiana tabacum seed. The mutants were initially identified by the appearance of leaf sectors showing chl deficiencies. Reciprocal genetic crosses showed that this trait was maternally inherited, thereby indicating a mutation in the plastid genome. Preliminary results have shown that most of mutants from this

collection have reduced electron transport activity (primarily in PSII), altered chl a/b ratios, and the loss or depletion of certain thylakoid membrane proteins as compared to WT tobacco grown under the same conditions.

What made lut-1 subject to more intensive examination was the obvious and consistent loss of chl exhibited by mutant leaves as a function of leaf age. This suggested that a developmentally controlled biochemical change, occurring over the lifetime of the mutant chloroplasts, was responsible for the loss of Chl. Similar patterns of photobleaching have been described in other pigment deficient nuclear mutants of maize (134), barley (30) and tobacco (87) but no systematic study has been performed to understand the possible cause(s) of reduced pigment content.

Materials and Methods

In Vivo Analysis

Mature plants of WT and mutant lut-1 (previously designated TP-13, Chapter 2) tobacco (Nicotiana tabacum, var. L.C. from the Connecticut Agricultural Experiment Station) were grown in a greenhouse under natural light conditions (temp. 25±5 ^{o}C) in soil. Plants were watered daily. Mutant plants were maintained as variegated sectorial and/or periclinal chimeras, and pruned to force growth of mutant axillary buds which gave rise to completely mutant shoots suitable for chloroplast analysis.

In vivo chl fluorescence induction transients were monitored with a Model SF-10 Plant Productivity Fluorometer (R. Brancker, Ltd., Ottawa, Canada) as described earlier (Chapter 1). Transients were recorded with a Nicolet Explorer III digital oscilloscope and plotted with an x-y recorder. A minimum of four different sections of a given leaf were assayed; if dissimilar fluorescence transients were found within the same leaf, that leaf was not used for further analysis. Tissue from leaves having similar fluorescence transients were pooled for chloroplast isolation.

Chloroplast Membrane (Thylakoid) Isolation

Thylakoid membranes were isolated as previously described (Chapter 2) in an isolation medium containing 400 mM sorbitol, 100 mM Tricine-NaOH, pH 7.8 and 10 mM NaCl. The chloroplast pellet obtained was washed with 10 mM Tricine-NaOH, pH 7.8, 10 mM NaCl and 5 mM $MgCl_2$. All buffers contained 2 mM phenylmethylsulfonyl fluoride and 1 mM p-aminobenzamidine as protease inhibitors. Chl was assayed by the

method of MacKinney (107). Protein determinations were performed using a modified Lowry assay (109).

Photosynthetic Reactions

PSII-dependent DPIP reduction was measured at 580 nm and whole chain electron transport (H_2O to MV) and PSI-mediated electron transport were measured as described in Chapter 2.

Fluorescence Measurements

Room temperature fluorescence induction transients with stroma-free thylakoids were measured in the absence or presence of 5 uM DCMU as previously described (131). Chloroplast membranes (10 ug chl) were dark-adapted for 10 min in 2 ml of 100 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, 10 mM NaCl and 5 mM $MgCl_2$. Transients were recorded with a Nicolet oscilloscope. A minimum of three replicates of each sample were recorded.

Low temperature (77 K) fluorescence emission spectra were acquired as described in Chapter 2. Sodium fluorescein (1 uM) was used as an internal standard (89).

Flash Induced Oxygen Production

Oxygen evolution patterns induced by a series of single-turnover flashes were measured with a Joliot-type O_2 electrode (163a). Freshly prepared thylakoid membranes (250 ug chl/ml), transferred to the Pt-electrode surface in dim light, were given a train of 40 flashes at 1 Hz and then dark-adapted for 15 min before recording the O_2-evolution patterns with an x-y recorder. Illumination was provided

by a Xenon flash lamp (General Radio Stroboslave Model 1539A). Calculations for double hits, misses and S-state fractions from the O_2-evolution patterns induced by the first 10 to 12 flashes after dark adaptation were made using a computer program, provided by W.F.J. Vermaas, for a Hewlett-Packard 85 computer. Experimental data were fitted to O_2-evolution values calculated from the classical model developed by Kok et al. (88).

Polypeptide Analysis

SDS-PAGE of denatured membrane polypeptides was performed using the discontinuous buffer system of Laemmli (97) as described previously (Chapter 2). Molecular weight markers were: bovine serum albumin, 68 kd; ovalbumin, 45 kd; carbonic anhydrase, 29 kd; and cyt c, 12.4 kd. Extraction of PSII chl-protein complexes was accomplished using octyl-β-D-glucopyranoside following the procedure of Camm and Green (29). For our analyses, isolated membrane fragments were washed twice with 300 mM sucrose, 5 mM MOPS (3-[N-morpholino]-propanesulfonic acid), pH 7.0 before solubilization. Chl-protein complexes were separated on polyacrylamide slab gels run at 4 ^{o}C in the presence of LDS rather than SDS.

Protein Phosphorylation

Phosphorylation of WT and lut-1 thylakoid membrane proteins was carried out using [γ-^{32}P]ATP. The protocol was as previously described (94) except that the final ATP concentration was 100 uM rather than 200 uM and 5 mM NaF was added to prevent dephosphorylation of phosphorylated samples (except as indicated). Samples were

illuminated with white light (500 uE/m^2/s) for 20 min in the presence or absence of of 10 uM DCMU. Samples were analyzed by SDS-PAGE (10 ug chl/lane) and phosphoproteins were detected by autoradiography.

Protein Detection by Immunoblotting

Proteins from denaturing SDS polyacrylamide gels were transferred to nitrocellulose sheets (0.45 um pore size, Schleicher & Schuell, Keene, NH) for 6 to 10 h at 60 v with a Transphor Electrophoresis Cell (Hoefer Scientific Instruments, San Fransciso, CA). The transfer buffer was 25 mM Tris, 192 mM glycine, 20 % v/v methanol, pH 8.3 (160). Nitrocellulose filters were quenched at room temperature for a minimum of 4 h in 20 mM Tris, 0.9 % (w/v) NaCl, pH 7.4 (Tris-saline) and 3 % (w/v) bovine serum albumin. Blots were incubated with primary antibody (see below; typically dilutions of 1/500 or 1/1000) in Tris-saline, 1 % (w/v) bovine serum albumin at 37 ^{o}C for 1 to 3 h. Blots were washed 3 times, 15 min each, with Tris-saline, 0.1 % (w/v) bovine serum albumin, 0.1 % (w/v) Triton X-100 (30 ml per 100 cm^2) before addition of alkaline phosphatase conjugated to protein A (Sigma; 10 units/100 ml) for 3 h at room temperature. Filters were washed 3 times, 15 min each, with 100 mM Tris, pH 7.5, 100 mM NaCl, and 2 mM $MgCl_2$ and then washed twice, 10 min each, in alkaline phosphatase buffer (designated AP 9.5) containing 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ at room temperature. The color detection of the immune complexes (100) was performed using 0.33 mg nitro blue tetrazolium/ml of AP 9.5 and 16.7 mg 5-bromo-4-chloro-3-indoxyl phosphate per 333 ul dimethylformamide per 100 ml of AP 9.5. After 15 min of color development in the dark, the reaction was stopped by

washing filters in 10 mM Tris, pH 7.5, 1 mM EDTA for 15 min. Blots were stored in 10 mM Tris, pH 9.5, 5 mM EDTA before air drying.

Antisera Production

Antisera to the PSII RC polypeptides and a 33 kd polypeptide were prepared as described (33). PSII enriched preparations were derived from detergent fractionation of corn (Zea mays) thylakoid membranes (17). These PSII preparations were subjected to preparative LDS-PAGE (10-17.5% slab gels). Coomassie blue staining bands at 51, 45 and 33 kd were excised and pooled; protein was electroeluted, concentrated and checked for purity by re-running using analytical LDS-PAGE. Antibodies were raised in female New Zealand rabbits. One week prior to immunization, rabbits were bled to obtain pre-immune sera. The initial injection contained 50 ug of the purified polypeptide in 0.5 ml 10 mM sodium phosphate, pH 7.0, and 1% (w/v) SDS mixed with an equal volume of complete Freund's adjuvant. The emulsion was injected into the subcapsular space of each rabbit (3 sites). The injection was repeated using incomplete adjuvant on days 20, 34 and 60. Rabbits were bled 4 d after each booster injection. Antisera to the 32 kd Q_B-binding protein was provided by J. Hirschberg and L. McIntosh, Michigan State University, E. Lansing, Michigan.

Electron Microscopy

Membrane samples were diluted with glycerol to a final concentration of 35% (v/v) glycerol before freezing in liquid N_2 cooled Freon 12. Freeze-fracture replicas were prepared with a

Balzers 360 M device at -115 ^{o}C. Replicas were cleaned with commercial bleach, distilled water and 1:1 chloroform/methanol (v/v). Magnification of micrographs is indicated in the figure legend.

Results

Stage-Specific Changes in Chl Fluorescence in lut-1

The youngest leaves of tobacco plastome mutant lut-1 closely resembled the dark green color of WT tobacco leaves of equivalent age, whereas successively older leaves along a mutant stem had less and less chl. The oldest leaves were nearly colorless. We have investigated whether or not an underlying functional alteration of the mutant chloroplasts preceded this pigment loss (i.e., that pigment loss is a secondary effect of the mutation). To accomplish this, it became necessary to establish adequate criteria to be used in pooling sufficient quantities of leaf material which could be used for biochemical characterization studies.

Chl fluorescence induction transients were used as a non-destructive technique (116) to monitor the photosynthetic status of homoplastidic intact leaves from completely mutant shoots of lut-1. Fluorescence transients of young and mature WT leaves and four leaves of lut-1 of increasing age (designated I-IV) are shown in Figure 5. These traces have been normalized to the same F_o (initial level of chl fluorescence after dark adaptation; indicated by the horizontal arrow). The transients of young and old leaves of WT are considered to be insignificantly different. The transients showed a biphasic increase to F_p (the maximal or peak fluorescence level, achieved at the time when the primary acceptor, a plastoquinone called "Q_A", of PSII is largely reduced). Under our experimental conditions, F_p was reached after 2 to 3 s of illumination of WT leaves, or after 4 to 5 s of mutant leaves. Variable fluorescence (F_v) is defined as $F_p - F_o$ (61).

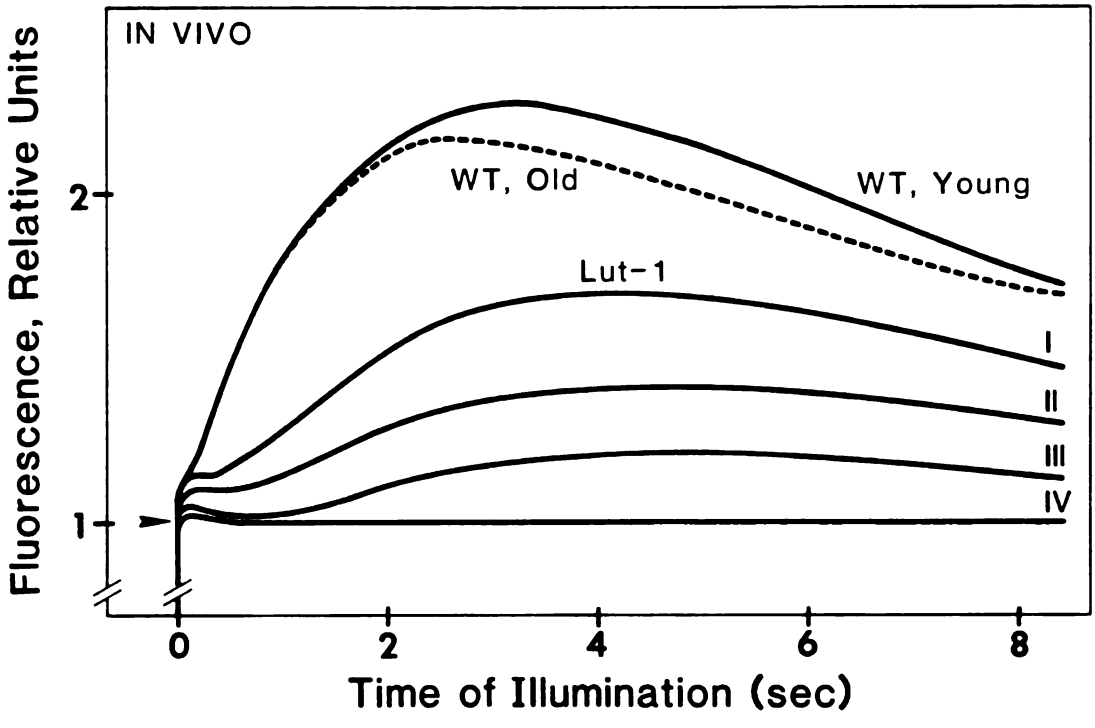

Figure 5 Leaf chl fluorescence induction transients used to establish the classification scheme for homoplastidic mutant lut-1 leaves. Traces have been normalized to the same F_o (arrow) in order to show more clearly the loss of variable fluorescence ($F_v = F_p - F_o$) occurring during leaf maturation. Traces labeled I, II, III and IV are from progressively older leaves from a completely mutant shoot of lut-1. F_p is defined as the peak or maximal intensity of fluorescence observed for each sample. Age did not significantly affect the F_v of WT leaves.

Young leaves of lut-1, with transients showing F_v values of 50% or more of WT values, were designated as Stage I. Mutant leaves with little or no variable fluorescence were grouped into Stage IV. Stages designated as II and III were leaves having a reduced amplitude of variable fluorescence (F_v values of 25-50% and 10-25% of WT, respectively). Changes in F_v were related to leaf age in the mutant (monitored by determining the number of days between emergence of a morphologically distinct leaf from the apex to the time at which samples of that leaf were analyzed); results are presented in Table I. Loss of variable fluorescence was clearly a function of leaf age and was directly correlated with loss of chl on a fresh weight basis. The mutant chloroplasts generally had chl a/b ratios ranging from 2.3 to 2.8 (Table 4). Membranes from younger tissue had a relatively high ratio, whereas those from older leaves had a lower ratio. WT membranes had chl a/b ratios ranging from 3.0 to 3.2. It should be noted that both mutant and WT leaves expanded to maximal surface area by day 20; i.e., the mutation did not affect patterns of leaf cell division or expansion.

The four mutant "stages" of fluorescence transients for whole leaves were easily monitored while harvesting mutant tissue. The technique was therefore used as the basis for pooling leaf tissue to insure sufficient homogeneity of chloroplasts for isolation of comparable samples for biochemical analyses. It is important to emphasize that discrete leaf categories did not exist in vivo; rather these "stages" were arbitrary designations made to provide an initial quantitative classification of mutant leaves. The reduction in variable fluorescence did not appear as a series of single events in

Table 4. Chl Content and Room Temperature Chl Fluorescence (*in vivo* and *in vitro*) of WT and Plastome Mutant *lut-1*

	Leaf age[a] (days)	mg chl/g fw[b]	Chl a/b	Chl Fluorescence[c] in vivo F_v/F_o	in vitro F_o	in vitro F_m	in vitro F_v/F_o
WT	5 to 7	2.2	3.3	1.8 ±.10	920 ± 30	3942 ± 45	3.28
	15 to 20	2.4	3.1	1.7 ±.09	1055 ± 4	4628 ± 16	3.39
lut-1 Stage I[d]	5 to 7	1.8	2.8	0.67 ±.17	3709 ± 23	6581 ±133	0.77
II	8 to 14	1.2	2.6	0.41 ±.08	3871 ±240	6260 ± 75	0.62
III	15 to 20	0.9	2.3	0.25 ±.09	3809 ± 98	5789 ±104	0.52
IV	21 to 28	0.3	2.2	0.06 ±.04	5461 ±134	6973 ±162	0.29

[a] growth during late spring and summer seasons

[b] fw: fresh weight of leaf tissue

[c] measurements are the average of 3 replicates and are given ± SD;
$F_v = F_m - F_o$

[d] "Stages" of mutant leaf tissue, which were established using chl fluorescence data, broadly paralleled leaf age and chl content.

lut-1 but as a progressive alteration in photosynthesis during leaf growth.

Because equivalent amounts of chl were compared, in vitro chl fluorescence measurements (Table 5), allowed a more rigorous assessment of photochemical efficiency than in vivo measurements. These results reinforced the classification scheme which emerged from the leaf fluorescence data. Mutant chloroplasts isolated from successively older mutant leaves exhibited the same progressive reduction in F_v as seen at the intact tissue level. The mutant chloroplasts had high F_o values compared to WT. This indicated that an increased fraction of the excitation energy received by the light-harvesting pigment-proteins (primarily serving PSII) was not used to drive electron transport, but was re-emitted as fluorescence. The high F_m values observed with mutant chloroplasts were related to the high F_o values; i.e., to an increased fluorescence yield from a functionally inactive pigment bed.

To determine if there was an excess of light-harvesting antennae in the mutant, half-rise times of chl fluorescence inductions (measured in the presence of DCMU) of Stage I lut-1 and WT thylakoids, were determined. The values obtained were 2.7 and 4.6 ms, for mutant and WT, respectively. Since the $t_{1/2}$ for inductions is a relative (inverse) measure of the size of the antenna bed serving each PSII trap (113), we can conclude that there were more light-harvesting pigments serving each PSII center in the mutant, with efficient energy transfer to the trap.

At low temperature (77 K), there are three chl fluorescence emission maxima from chloroplast membranes: F685 and F695 which are

Table 5. Protein Content and Photosynthetic Activity of Tobacco Plastome Mutant lut-1 Compared to WT Tobacco

	ug protein/ug Chl[b]	Activity[a] Whole Chain	PSII-dependent	PSI-dependent
lut-1 Stage I	5.1	93	92	130
II	6.2	72	65	230
III	7.4	21	23	280
IV	9.3	6	6	250

[a] Activity measurements are expressed as per cent of WT activity. WT activities: 269 and 415 umoles O_2 mg chl^{-1} h^{-1}, whole chain and PSI-dependent, respectively; 288 umoles DPIP reduced mg chl^{-1} h^{-1}, PSII-dependent.

[b] WT value: 5.5 ug protein/ug Chl

jointly assigned to the light-harvesting and PSII core complexes, and a broad band centered near 730 nm which is assigned to PSI (28). Fluorescence emission spectra at 77 K of WT and mutant thylakoids are shown in Figure 6a. These spectra have offset baselines, but been normalized such that the amplitude of the peak emission at 680-685 was equal in all samples. In parallel experiments using fluorescein as an internal standard (89), the absolute fluorescence yield was found to increase during mutant progression from Stage II to IV (Figure 6b). At the most extreme state of mutant expression, the ratio of emission of lut-1/WT in the 680-685 emission band was found to be 1.8 (in Stage IV thylakoids). In Stage I mutant membranes, the short-wavelength chl emission was centered at 686 nm -- identical to WT samples. This peak shifted progressively to shorter wavelengths in Stages II, III and IV (684, 683 and 682 nm, respectively). This indicated an increased amount of "free LHC-II" in the mutant membranes (the purified form of which shows a peak emission at 680 nm (26); this type of spectral shift has also been reported in a PSII-deficient mutant of maize (102) and was correlated to an increase in "free LHC-II").

Electron Transport Properties of lut-1 Membranes

Assays were performed to determine electron transport partial reactions (on a chl basis) for chloroplasts prepared from Stage I through IV leaves of lut-1 in comparison to those from WT. From Table 5 it can be seen that whole chain electron transport and PSII activity declined from 93-92% of WT activity in Stage I to 6% in Stage IV. In contrast, PSI activity was present at greater than WT levels through all four Stages. Since these activities were measured on a

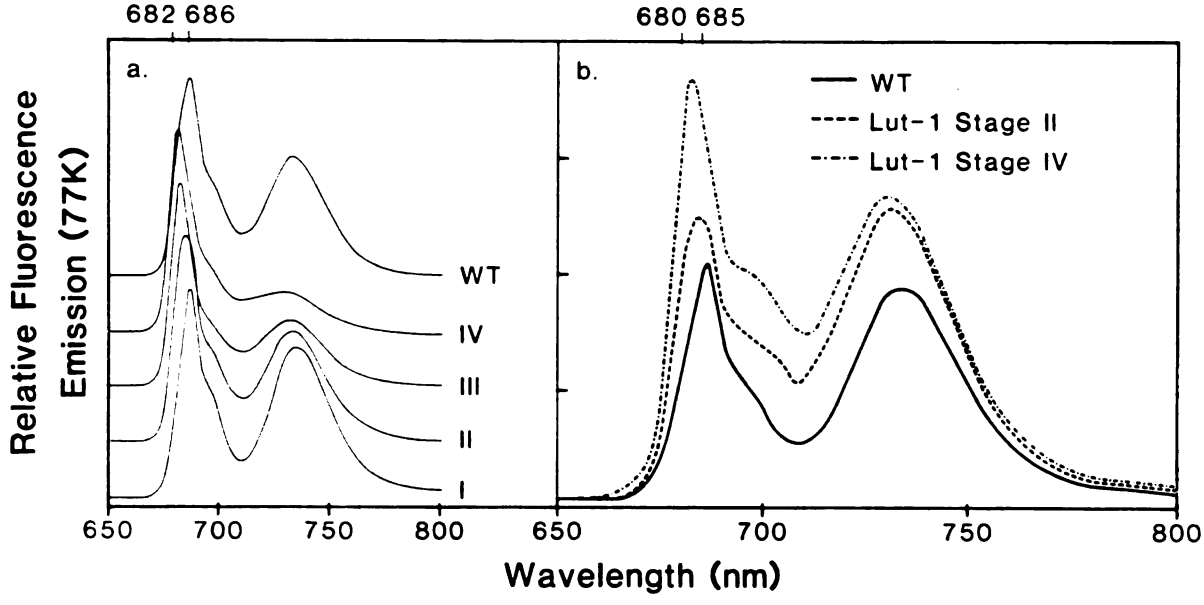

Figure 6 Liquid nitrogen (77 K) chl fluorescence emission spectra of isolated WT and mutant thylakoids: a) normalized at the low wavelength emission peak in order to show more clearly the gradual shift in the peak from 686 nm (WT and Stage I) to 682 nm (Stage IV) with increasing mutant Stage. This blue-shift of the 686 nm chl emission peak, which arises from the light-harvesting complexes serving PSII, is indicative of a functional disconnection between the antennae and RC (26,102). The fluorescence emission yield at the long-wavelength peak appeared to diminish as a result of this normalization process; b) normalized to the emission peak of an internal standard, fluorescein (89) in order to show the increase in fluorescence emission yield at both peak wavelengths. The dramatic increase in yield from the low-wavelength peak is typical of inhibited excitation energy transfer from the antenna chl to the PSII centers. The increase in long wavelength (PSI) emission was probably due to increased PSI sensitization by free LHC-II.

chl basis, however, the increase in PSI only represents a relative proportional increase as PSII activity was lost, and not necessarily a stoichiometric increase in PSI content compared to other thylakoid components.

The ratio of protein to chl (in purified thylakoid preparations) is shown in Table 5. The higher PSI rates in later stages of mutant chloroplasts were correlated to the increased protein to chl ratio. Measurements of chl/PSI RC (chl/P700) made by monitoring chemical oxidation/reduction absorbance changes at 700 nm (data not presented) indicated that WT and mutant chloroplasts had similar ratios of functional P700 to protein in all mutant thylakoid preparations. Since PSI activity on a chl basis (Table 5) increased more than the change in protein/chl, however, we conclude that the electron donor and/or acceptor used in assaying PSI activity had greater access to electron transport components associated with PSI in late stage mutant thylakoids. As the loss of whole chain activity closely paralleled the loss of PSII activity, the decline of the latter was likely responsible for the overall decrease.

Efficiency of PSII-dependent Reactions

Since PSII centers were lost during chloroplast development in lut-1, we were interested to learning whether the centers at early stages of leaf development had some functional impairment not detectable by fluorescence or partial reactions measured at saturating light. Under flash illumination, chloroplasts are known to evolve O_2 in a pattern expressing a periodicity of four (88); this process requires the concerted reactions of several PSII constituents. Except

for the diminished O_2 yield/flash of mutant chloroplasts, the flash yield patterns of mutant (Stage I) and WT chloroplasts were similar (Figure 7). Maximum O_2 yield occurred upon the third flash after dark adaptation, and peaked thereafter every four flashes.

In experiments measuring O_2 flash yield, the periodicity of O_2 evolution damps out after many flashes due to a combination of factors. First, centers can occasionally undergo two turnovers (double hits) during the duration of the flash; this results in an extra advance of the flash yield sequence in a sub-fraction of the total number of centers. Second, some centers may not undergo a charge separation during the flash (misses). Using the classical model of Kok (88) defining the "S-states" of O_2 evolution, the values for misses and double hit probabilities were calculated. Miss probabilities, 0.12 and 0.14, respectively, were similar for lut-1 and WT. The double hit parameter for lut-1 (0.05) was less than one-half of WT (0.11); this finding accounted for the higher probability for a single step transition for lut-1 centers than WT centers (0.83 and 0.75, respectively). In summary, the measurement of O_2 flash yields in lut-1 chloroplasts indicated that the active PSII centers were nearly indistinguishable from WT, and that there were no major changes in the rate constants for the processes involved in electron transport from water to the plastoquinone pool.

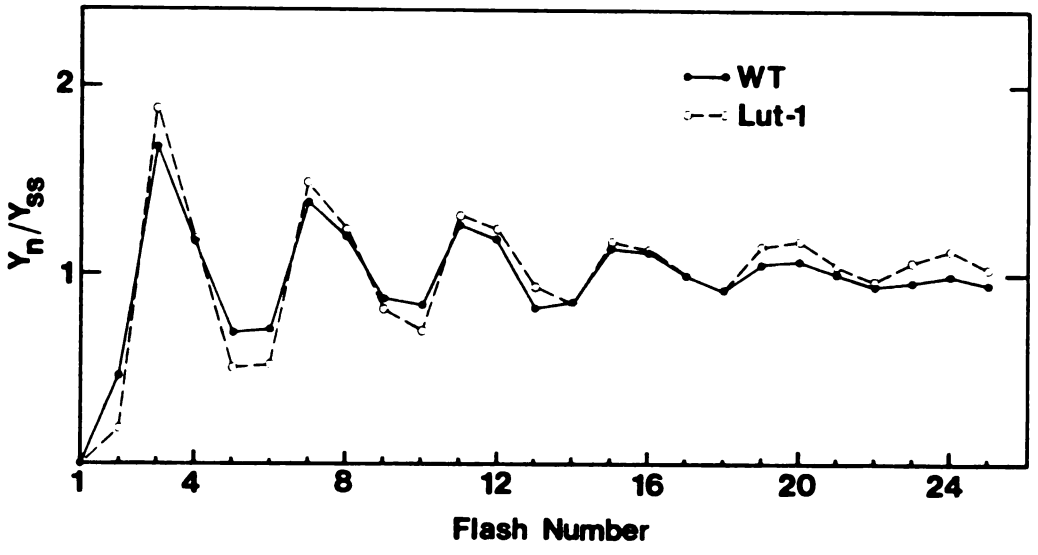

Figure 7 Oxygen yield as a function of saturating 1 Hz light flashes for dark-adapted WT and Stage I lut-1 thylakoids. O_2 yield after each flash (Y_n) was normalized to a steady state flash yield (Y_{ss}), acquired after the oscillations damped out (generally after 40 flashes). The WT and mutant patterns both displayed the typical oscillatory pattern of O_2 evolution, with peaks at the third flash and every fourth flash thereafter.

The ability of isolated WT and mutant chloroplast membranes to phosphorylate several thylakoid polypeptides, due to light activation of a thylakoid-bound kinase (3) was a second PSII-associated property assayed to assess the PSII characteristics of the mutant. Using [γ-^{32}P]ATP, WT and lut-1 (Stage I) thylakoids were phosphorylated in the light, and the membrane proteins separated by SDS-PAGE. The autoradiogram of the gel is shown in Figure 8. Lanes 1 and 5 show that in the absence of DCMU, the same proteins of WT and lut-1 chloroplasts, respectively, were radiolabelled.

The phosphoproteins observed in WT and mutant tobacco thylakoids corresponded to those phosphoproteins observed in other higher plant chloroplasts (149). These included species at 45, 32, 28, 27 and 10 kd, all of which have been identified either as PSII constituents or apoproteins of the LHC-II. The protein kinase is believed to be activated by a reduced PQ pool (3). If the reduction of the PQ pool was prevented, either by inhibition with DCMU or darkness, the kinase is inactive and no phosphorylation was observed in either WT or mutant membranes (Figure 8; plus DCMU: lanes 2,6 [WT and lut-1, respectively]; unilluminated: lane 4 [WT]). A phosphorylated WT sample allowed to de-phosphorylate in the dark was run to show the reversibility of the reaction (lane 3). The interpretation of these data is that the mutant membranes have a normal pattern of protein phosphorylation control; i.e., the mutant, in early stages of development, has an apparently normally functioning PSII complex.

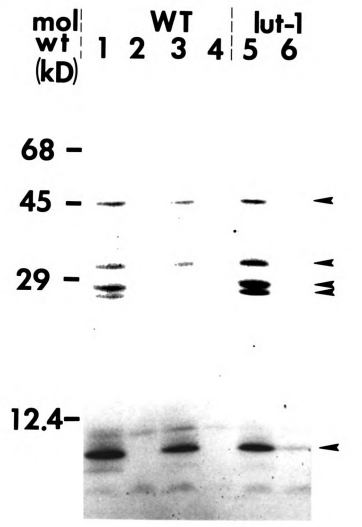

Figure 8 Autoradiogram of a denaturing SDS polyacrylamide gel, containing 4 M urea, showing the radiolabeled phosphoproteins of WT and lut-1 (Stage I) thylakoids. Lanes were loaded on an equal protein basis. Lanes 1 and 5, WT and lut-1, respectively, illuminated samples minus DCMU; lanes 2 and 6, WT and lut-1 respectively, illuminated in the presence of DCMU (phosphorylation was inhibited because DCMU blocks electron transport between the Q_B-binding protein and the plastoquinone pool, keeping the plastoquinone pool largely oxidized). Lane 3 was a sample which was phosphorylated in the light and then allowed to de-phosphorylate for 20 min in the dark (NaF was not included in this sample). Lane 4 was a non-illuminated WT sample run to demonstrate the requirement for light-activation of the protein kinase. Arrows indicate the major phosphoprotein species at 45, 32, 28, 27 and 10 kd, which were present in both WT and mutant membranes.

Structural Analysis of Thylakoid Membranes

When membrane preparations were examined by freeze-fracture electron microscopy, marked differences were evident in membrane sub-structure. Figure 9a and 9b show, respectively, Stage II lut-1 and WT membranes. Although grana stacks were evident, the density of EF_s particles, considered to be PSII core complexes plus bound light-harvesting chl-protein complexes (148), decreased dramatically in mutant lamellae during chloroplast maturation. These results were consistent with our interpretation of biochemical and biophysical data suggesting a reduction in PSII complexes.

Polypeptide Content of Thylakoids

PSII-associated chl-protein complexes were gently extracted by solubilization of isolated mutant and WT thylakoids with octyl-glucopyranoside (29). These complexes were then subjected to LDS-PAGE. The two PSII RC chl-protein complexes, CP47 and CP43 (the apoproteins of which migrated at 51 and 45 kd, respectively, in our gel system), were clearly present in Stage I lut-1 chloroplasts. (Figure 10a). Their migration was identical to the PSII complexes of WT membranes (data not shown). This was additional evidence indicating the structural integrity of PSII centers in young mutant chloroplasts. CP47 and CP43 were not observed when late stage mutant membranes were similarly solubilized with octyl-glucopyranoside (data not shown).

The polypeptide profiles of WT and mutant membranes were determined by SDS-PAGE in the presence of 4 M urea (Figure 10b).

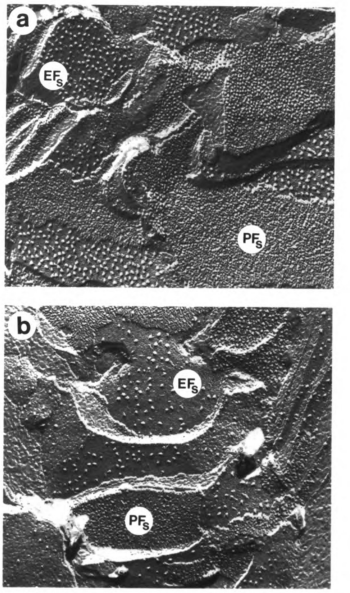

Figure 9 Freeze-fracture micrograph of (a) WT and (b) Stage II lut-1 thylakoids. The density of the particles on the EF_S fracture face was reduced in mutant membranes in comparison to WT. x 100,000

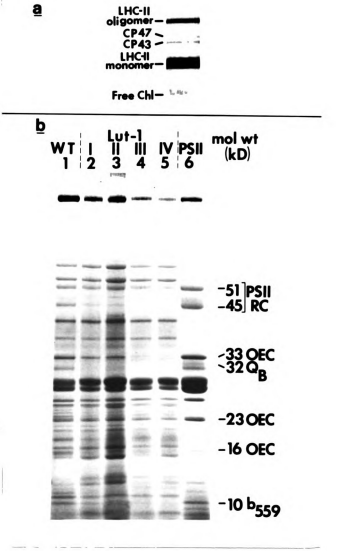

Figure 10 PAGE of: a) PSII chl-protein complexes extracted with octyl-glucopyranoside (29) from Stage I, lut-1 thylakoids and run on an LDS polyacrylamide gel under conditions in which chl remained stably bound to the polypeptides. Both RC chl-protein complexes, CP47 and CP43, were present in young mutant chloroplast membranes, indicating proper co-factor and complex associations; b) separation of thylakoids on an SDS gel containing 4 M urea. WT, the four mutant Stages and a PSII-enriched grana fraction from WT are shown in lanes 1, 2 to 5 and 6, respectively. The PSII enriched fraction was isolated as described in ref. 17. As judged by coomassie blue staining intensity, PSII-associated proteins at 45, 33, 32, and 10 kd were lost from mutant membranes with increasing age. Additional immunoblotting analyses (data not shown) indicated the loss of polypeptide species at 23 and 16 kd which are part of the OEC (7).

Whereas the WT (lane 1) and Stage I mutant (lane 2) protein species were qualitatively identical, later stages in mutant development (lanes 4-6) were progressively depleted of polypeptides at 45, 32-34 and approximately 10 kd. A polypeptide of at 51 kd decreased only slightly in staining intensity in the late stage mutant membranes.

Two lines of investigation were used to show that the polypeptides which were lost during mutant thylakoid maturation were associated with PSII. First, an isolated grana fraction from WT thylakoids which almost exclusively contained PSII (prepared by the method of Berthold et al. (17); lane 6 of Figure 10b) contained proteins co-migrating with the polypeptides which were depleted over time in lut-1. Second, protein blots probed with antibodies raised against authentic PSII polypeptides from maize demonstrated the identity of the depleted proteins (Figure 11).

Antisera raised against the 51 kd protein (thought to be the P680-binding RC protein (see ref. 43,125) reacted with two closely migrating species on protein blots of mutant membranes isolated from young, intermediate and mature lut-1 leaves (corresponding to Stages I, II and IV, respectively; lanes 2, 3 and 4 in Figure 11a). The intensity of the antibody reaction decreased slightly with increasing Stage (lanes were loaded on an equal protein basis) which indicated a slight reduction in the levels of these species. The doublet signal was not observed on blots in which samples were heated and/or boiled before loading on gels. These harsher denaturing conditions generally yielded a smeared and diffuse staining region. We consider the two reacting species to be forms of the same protein. Possibly because of residual chl (not removed by the gentle solubilization procedure

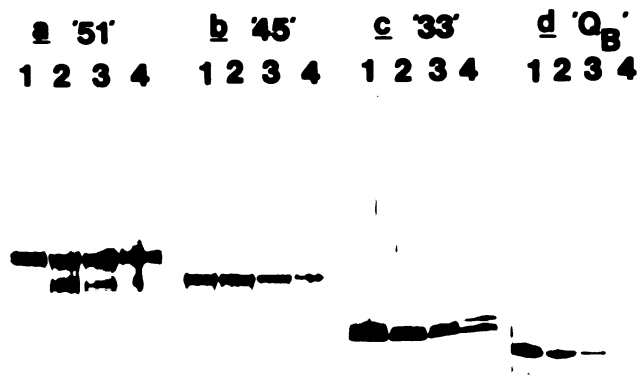

Figure 11 Protein blots of SDS gels probed with monospecific polyclonal antisera reactive against the PSII polypeptides at 51 kd (panel a), 45 kd (panel b), 33 kd (panel c) and 32 kd (panel d). In all panels: lane 1, WT thylakoids; lanes 2 to 4, lut-1 thylakoids corresponding to Stges I, II and IV. Lanes were loaded on an equal protein basis. In all cases, except for the 51 kd species, there was a significant reduction of the target protein with increasing mutant Stage. Inclusion of urea in the SDS gels for samples in panels c and d aided in resolving the two proteins migrating in the 32 to 34 kd range.

required for sharper banding patterns), the 51 kd polypeptide migrated as two distinct populations which could be detected by the more sensitive immunoblotting methods. There was a broad. diffuse immunologically reactive polypeptide of approximately 42 kd in the mutant samples (Figure 11a, lanes 2, 3 and 4). We interpret these to be degradation products of the 51 kd protein, but cannot determine whether they formed during sample preparation or in vivo.

In contrast to the 51 kd polypeptide, results using the antisera raised against the 45 kd protein indicated a more significant depletion of this chl a-binding RC protein with increasing mutant Stage (Figure 11b, lanes 2, 3 and 4). Patterns for the 33 kd polypeptide, a nuclear-encoded component of the water oxidizing complex (3), and the 32 kd Q^B-binding protein paralleled that of the 45 kd protein, confirming the loss of other PSII constituents in mutant chloroplasts during lut-1 leaf expansion (Figure 11c, 11d). In the late stages of lut-1 membrane ontogeny, the anti-33 antisera reacted with a larger size polypeptide (approximately 34 kd) (Figure 11c, lane 4). We believe this is a partially processed form of the mature 33 kd protein; this concept will be discussed in greater detail in Chapters 4 and 5.

Discussion

This study has shown that lut-1 is a developmentally expressed plastome mutant. The biochemical phenotype is a loss of PSII function due to a selective reduction in the concentration of PSII core complexes in thylakoids. This phenomenon only occurred as leaves (plastids) matured. We did not observe this alteration in PSII stoichiometry in chloroplasts isolated from WT leaves during maturation and do not consider this mutant behavoir to be mimicking a senescence process.

PSII Function

In young leaves of lut-1, PSII activity was nearly normal, based upon in vivo fluorescence transients (Figure 5) and the fluorescence and partial reactions measured with isolated thylakoids (Tables 4,5). Chl was properly associated with RC proteins as indicated by the presence of CP43 and CP47 in early stage lut-1 membranes (Figure 10a). When O_2 flash yield measurements were used to study early-stage chloroplasts, the oscillatory pattern of O_2 evolution was identical to that of WT, and only small changes in the kinetic parameters of this process were detected (Figure 7). The most significant difference was the reduced steady-state O_2 flash yield in the mutant thylakoids which simply indicates loss of PSII centers. Lut-1 was similar in this way to a nuclear-encoded Chlamydomonas mutant, F34SU2, which has a reduced concentration of PSII complexes in its thylakoids (83).

Since the evolution of O_2 results from the integration of many reaction steps, the O_2 flash yield experiments strongly indicate that the lut-1 chloroplasts have the capacity to produce a normal PSII

center (i.e., the genes for PSII proteins are transcribed and translated in early stage tissue and functionally normal proteins are synthesized and assembled in mutant thylakoids).

The early stage membranes also carried out light-dependent phosphorylation of PSII-associated polypeptides (Figure 8). This process has previously been shown to be a regulatory step controlling light harvesting (3). An active protein phosphorylation in lut-1 suggests that overall membrane processes coupling electron flow to energy distribution in the chloroplast function properly in early stage lut-1 chloroplasts.

We are left with the conclusion that PSII centers in lut-1 initially assemble and function properly, but then are selectively lost during chloroplast maturation. Since the loss of functional activity was specific for PSII (PSI and light harvesting chl a/b pigment-proteins were retained; Tables 4, 5 and Figure 10b), we sought evidence to explain why PSII function degenerated over during leaf maturation in lut-1.

Evidence for Physical Loss of PSII Centers

The loss of PSII activity in lut-1 membranes corresponded directly with the physical depletion of the PSII core complexes from the thylakoids. This conclusion is based upon three lines of evidence. 1) A comparison of lut-1 and WT thylakoid ultrastructure by freeze-fracture analysis showed a reduced number of particles on the EF_s fracture face (Figure 9); these particles have been shown to be PSII core complexes and associated light-harvesting pigment proteins (reviewed in ref. 148); 2) the polypeptide profile of lut-1

thylakoids isolated from increasingly older leaf tissue showed a progressive depletion of coomassie blue staining polypeptides typically ascribed to the PSII RC core complex (Figure 10b) (except for the putative RC apoprotein at 51 kd); 3) immunoblots of polypeptides separated by PAGE confirmed the depletion of proteins at 45 kd and 33 kd as well as the diffusely staining 32 kd Q_B-binding polypeptide considered the apoprotein of the secondary electron acceptor in the PSII RC (93).

The physical absence of PSII structural units is consistent with the alterations in the chl fluorescence properties and diminished photosynthetic electron transport activities, over time, of mutant chloroplasts. The changes in the 77 K fluorescence spectra of chloroplasts from the different mutant leaf stages included a blue-shift of the short-wavelength emission peak from 685 nm (Stage I) to 682 nm (Stage IV) (Figure 6a,b). This is characteristic of membranes in which the light-harvesting complexes are functionally disconnected from PSII RC complexes (102). The resulting pool of "free" light harvesting complex has an increased chl fluorescence emission yield (Figure 6b) at 77 K and at room temperature (see high F_o and F_m values for in vitro chl fluorescence in Table II).

A reduced number of PSII centers per unit membrane was also indicated by the faster half-rise times to F_m in the younger lut-1 membranes, compared to WT; i.e., there was more light-harvesting chl sensitizing the PSII RC in the mutant than in WT.

In summary, we conclude that lut-1 displayed a novel phenotype resulting from the premature and selective loss of those proteins which comprise the PSII core complex. This was not due to defective PSII complexes, but rather a dysfunction in the stability of this lipoprotein complex. The depletion of PSII-associated proteins from lut-1 thylakoids may be due to one of two processes. One possible explanation would be a reduction in the rate of synthesis, either at the level of transcription or translation, of one or more plastome-encoded proteins which are part of PSII. Accordingly, the absence of PSII component(s) would alter the normal assembly of the membrane protein complex. This explanation would require that the mutation be in a regulatory process controlling protein synthesis, and that the regulation be specific for PSII proteins. A second equally plausible explanation which would account for the reduction in PSII complexes would be the presence of a faulty enzyme which is involved in protein processing needed for assembly and stabilization of the PSII complex or for its normal turnover. If some required enzyme-mediated protein-protein associations were not correctly formed, we would expect these PSII units to be unstable, resulting in their deterioration over time. If degradation of membrane proteins was involved, it was a relatively efficient and rapid process since breakdown products of most PSII polypeptides were not observed on immunoblots.

The next chapter describes studies undertaken to distinguish between these two different mechanisms by which premature PSII degradation could occur. Preliminary data indicate that the mutation does not affect the synthesis of PSII proteins, and that abnormal

processing of nuclear-encoded polypeptides associated with the water oxidation process occurs in lut-1 plastids. These data are presented in Chapters 4 and 5.

CHAPTER 4

Tobacco Plastome Mutant Lacks Stable PSII Protein Complexes

Abstract

Tobacco mutant lutescens-1 is a chloroplast-encoded mutant which exhibits a developmentally expressed-phenotype. The core complex of PSII, an integral membrane protein complex, is specifically and selectively lost from mutant chloroplast thylakoids during leaf expansion. In this study several possible molecular mechanisms were examined which might explain the disruption of the normally well-regulated levels of this chloroplast lipoprotein complex. In comparisons of lut-1 and wild type tobacco, no differences were found in chloroplast DNA restriction fragment patterns or in mRNA transcript sizes and levels corresponding to chloroplast genes encoding PSII polypeptides between lutescens-1 and wild type tobacco. In addition, mutant chloroplast protein synthesis was functional. Thus, neither transcriptional nor translational processes appeared to be defective in lut-1 chloroplasts. Instead, the inability to accumulate wild type levels of PSII polypeptides indicated an aberrant post-translational process, which caused selective turnover of the PSII complexes after their insertion into thylakoids. The removal of PSII centers also inhibited the normal processing of two nuclear-encoded PSII constituents considered essential for water oxidation.

Introduction

Thylakoid membranes of chloroplasts contain protein complexes (with bound pigments and redox reaction cofactors) which catalyze the conversion of absorbed radiant energy into stable chemical intermediates. The biogenesis of these energy-coupling membranes requires proteins derived from the genomes of both the nucleus and chloroplast (plastome). Of particular interest to our laboratory are regulatory mechanisms involved in maintaining the optimal stoichiometries of the various lipoprotein complexes comprising the photosynthetic apparatus under varying environmental conditions. Changes in the relative number of the photosystem reaction centers and light-harvesting pigment-protein complexes in higher plant chloroplasts occur as a result of a changes in light intensity and quality (101,111). These adaptive alterations in the protein composition of membranes require the coordinated regulation of gene expression between the nuclear and plastid genomes. We have chosen to use chloroplast-encoded mutants of Nicotiana tabacum to examine the genetic control of PSII assembly and maintenance. One mutant, lut-1, was described in detail in Chapter 3. It has an unusual developmental phenotype in which PSII complexes, having functional properties nearly identical to those of WT, are lost prematurely from mutant thylakoid membranes during leaf maturation. PSI remains largely unaffected. The depletion of lut-1 PSII complexes is pleiotropic: both nuclear- and chloroplast-encoded subunits are quantitatively reduced. In successively older mutant leaves, this results in a decreasing ratio of PSII to PSI.

Because lut-1 is a plastome mutant, our search for the lesion responsible for the physical reduction of PSII units focused on the status of mutant cpDNA and mutant chloroplast protein synthesis. Overall, our results indicated that mutant chloroplast biosynthetic properties were functionally intact. We suggest that a post-translational process, probably involved in the assembly and maintenance of either PSII core complexes or an individual subunit of the complex, was altered in lut-1.

Materials and Methods

Plant Material

Growth conditions and maintenance of WT and mutant lut-1 tobacco (Nicotiana tabacum var L. C. from the Connecticut Agricultural Experiment Station) plants were described in Chapter 3. Intact chloroplasts (for DNA and RNA isolation) and thylakoid membranes (for SDS-PAGE analysis) were isolated from WT and mutant lut-1 leaves of the following ages: young, 7-9 days; middle, 15-17 days; old, 24-27 days after appearance of the visible emerging leaf from axillary buds.

Isolation of Nucleic Acids

Operations were carried out at 0-4 ^{o}C unless otherwise noted. cpDNA was isolated following the general guidelines of (71). Deveined leaves were ground in 400 mM sorbitol, 100 mM Tricine-NaOH, pH 7.8, 40 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 % (w/v) bovine serum albumin and 0.2 % (w/v) polyvinylpyrrolidone. The homogenate was passed through two and then four layers of pre-moistened Miracloth (Calbiochem, San Diego, CA). Chloroplasts were pelleted by centrifuging at 3000g for 10 min and then suspended in grinding medium. This preparation was loaded onto discontinuous sucrose gradients, 10 ml each of 25, 34 and 51 % (w/w) (46), and centrifuged at 65000g for 1 h. Intact chloroplasts were collected at the 34 and 51 % interface and diluted 1:1 into 400 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, and 20 mM EDTA. Chloroplasts were pelleted by centrifuging at 2600g for 10 min using a swinging bucket rotor (Sorvall HB-4) and washed once with the same buffer. They were then lysed in this buffer containing 2 % (w/v) sarkosyl and 200 ug/ml

proteinase K by incubating at 37 ^{o}C for 30 min. Protein was extracted with buffer-saturated, re-distilled phenol; residual phenol was removed with ether. DNA was precipitated overnight at -20 ^{o}C by adding 2.5 vol cold ethanol after the solution was brought up to 200 mM sodium acetate (pH 6.0). Precipitated DNA was dissolved in 10 mM Tricine-NaOH, pH 7.8, 10 mM KCl and 0.2 mM EDTA. The solution was adjusted to ρ= 1.58 with CsCl and 400 ug/ml ethidium bromide. After centrifugation at 200,000g for 20 h at 15 ^{o}C in a VTi50 rotor (Beckman, Palo Alto, CA), the DNA band was collected and ethidium bromide was extracted with isoamyl alcohol. The DNA solution was dialyzed extensively against 10 mM Tricine-NaOH, pH 7.8, 10 mM KCl and 0.2 mM EDTA.

For cpRNA isolation, glassware was baked, and water for solutions was autoclaved with 0.1 % (v/v) diethylpyrocarbonate. RNA was prepared from intact chloroplasts by collecting the plastids at the 34-51 % sucrose gradient interface described above, and slowly diluting them with the same volume of 2X extraction buffer (150 mM NaCl, 100 mM Tricine-NaOH, pH 7.8, 2.5 mM $MgCl_2$). After centrifuging at 2600g for 10 min in the HB-4 rotor, the chloroplasts were lysed by adding 1X extraction buffer containing 1 % (w/v) sarkosyl and 1 % (w/v) SDS. The solution was gently mixed for 30 min at 4 ^{o}C. Protein was extracted and RNA was precipitated as described (118).

The concentration of nucleic acid preparations was determined by A260 readings using the formulas (118): 1 A260 unit = 40 ug RNA or 1 A260 unit = 50 ug DNA.

Restriction enzymes were used according to instructions provided by suppliers. Agarose gels (0.7 % w/v) in 40 mM Tris, pH 7.8, 5 mM

sodium acetate, 0.5 mM EDTA and 0.5 ug/ml ethidium bromide were run at 50 V for 5 to 6 h.

Northern Blot Analysis

Formaldehyde gels (0.9 % w/v) were run according to protocols described in ref. 157. Five to ten ug of cpRNA were loaded per lane. As described in the Results section, loading levels were normalized to the hybridization signal obtained with the psbB gene probe. Escherichia coli 16S (1541 bp) and 23S (2904 bp) rRNA were used as size markers. After electrophoresis, marker lanes were stained in 1 ug/ml ethidium bromide, 20 mM ammonium acetate and photographed. The remainder of the gel was transferred to nitrocellulose (0.45 um pore size) according to procedures described in ref. 157.

Plasmids used to provide specific probes for chloroplast genes were: pAH484 containing psbA, in a 3.68 kbp EcoRI fragment (75), which codes for the 32 kd quinone-binding polypeptide which is considered to be the apoprotein of the PSII secondary electron acceptor, Q_B (93); pWHsp207/E3 containing the 3' end of psbB, in 1.44 kbp Sal I/Eco RI fragment (121), which codes for a 51 kD PSII chl a-binding reaction center polypeptide; and pWHsp408b containing both psbC and psbD, in a 4.4 kbp Bam HI fragment (4). psbC codes for a 44 kd polypeptide considered to be an internal PSII antennae. psbD codes for a 32 kd polypeptide similar in sequence to the Q_B-binding protein. We have conformed to the recommended chloroplast gene nomenclature of ref. 64 in this work. Probes were labeled by nick translation, with a BRL Nick Translation Kit (Gaithersburg, MD; Cat. No. 8160 SB) using

[α-^{32}P]dATP or [α-^{32}P]dCTP, to a specific activity of 10^6 to 10^7 cpm/ug DNA.

RNA blots were pre-hybridized for a minimum of 4 h and then hybridized for 18 h with labeled probe as described by (165) except that dextran sulfate was omitted from the hybridization solution. After washing 3 x 5 min with 2X SSC, 0.1 % (w/v) SDS at room temperature and 2 x 15 min with 0.1X SSC, 0.1 % (w/v) SDS at 50 °C, blots were air dried and autoradiographed using DuPont Cronex intensifying screens.

In Vivo [^{35}S]Met Labeling

In vivo protein synthesis was monitored by feeding [^{35}S]Met (>800 mCi/mmol) to excised leaves (103). After a 4-h labeling interval, chloroplast thylakoids were isolated and membrane proteins were analyzed by SDS-PAGE using the discontinuous buffer system of Laemmli (97) as described in Chapter 2. Molecular weight markers were: bovine serum albumin, 68 kD; ovalbumin, 44 kD; carbonic anhydrase, 29 kd; and cyt c, 12.4 kd. Incorporation of label was determined by autoradiography of dried gels.

Protein Detection by Immunoblotting

Monospecific polyclonal antisera prepared against 16, 23 and 33 kd spinach polypeptides were generously provided by Dr. C. Jansson, MSU/DOE Plant Research Lab., Michigan State University. Extraction of WT OEC polypeptides was performed as described previously (124).

Immunoblotting protocols were described in Chapter 3.

Results

CpDNA Restriction Patterns

Since lut-1 is a plastome mutant, detection of an alteration in the organization of its plastid DNA could possibly identify the molecular lesion. To check the integrity of lut-1 cpDNA, restriction fragment patterns of WT and mutant cpDNA were compared. DNA was extracted from intact WT and mutant chloroplasts and digested with restriction endonucleases. No distinctive differences were observed when using enzymes typically capable of generating 40 to 60 fragments from tobacco cpDNA were used (73). The enzymes Eco RI, Hind III and Bgl II, each used alone, and a double digest using Eco RI and Hind III, are shown in Figure 12. DNA was also treated with the enzymes Bam HI and Xba I; again no differences were seen between WT and lut-1 cpDNA fragment patterns (data not shown). We conclude that because mutant and WT cpDNA restriction fragment patterns were identical, lut-1 cpDNA had no major rearrangements or gross insertions or deletions.

Northern Blot Analysis

The simplest explanation which would explain the decreased levels of PSII polypeptides in lut-1 membranes was that chloroplast protein synthesis was defective in mutant chloroplasts. A defect in protein synthesis would result if a component of the transcriptional or translational machinery becomes inactivated over time as lut-1 leaves expand and mature, with a specific effect on PSII protein synthesis. We tested the idea that plastid protein synthesis was reduced or lacking in mutant leaves by taking two approaches. The first was to

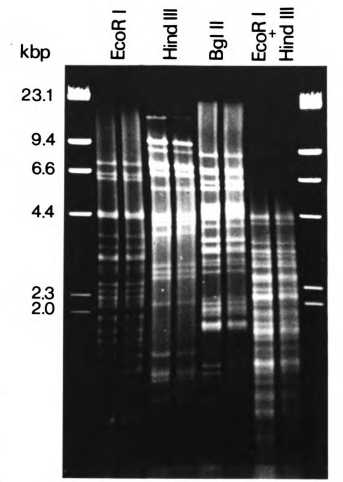

Figure 12 Restriction fragment pattern of WT and lut-1 cpDNA digested with enzymes indicated. Each pair of lanes, labeled with the restriction enzyme(s) used, consists of digested WT cpDNA on the left, and lut-1 cpDNA on the right. No differences were observed between WT and mutant fragment patterns. Molecular size markers were Hind III fragments of lambda DNA.

examine the transcript levels of chloroplast mRNA's of PSII polypeptides by Northern blot analysis using DNA probes specific for plastome genes. A reduction in transcription of a chloroplast-encoded PSII polypeptide could effectively limit the amount of that subunit. Sub-stoichiometric production of a complex component would then limit the number of PSII complexes which could be assembled.

By Northern blot analysis, transcript levels of chloroplast-encoded PSII polypeptides of WT and mutant chloroplasts were compared. Preparations of cpRNA from young and old leaves of both WT and mutant were blotted onto nitrocellulose and probed usng specific clones of PSII plastome genes. These were psbA, psbB, psbC and psbD, which code for, respectively, the Q_B-binding, 51, 44 and D2 (a PSII-associated protein whose function is not known; 93) polypeptides.

The amount of RNA of the different WT and mutant preparations loaded on gels was normalized to yield a similar hybridization signal with the psbB probe (pWHsp207/E3) which contains the 3' end of the gene coding for a 51 kd chl a binding apoprotein of the PSII reaction center (121) (Figure 13a). This protein was found to be synthesized and stably inserted throughout the lifetime of the mutant chloroplasts (Chapter 3) and thus served as a reference for the Northern blot analyses. Relative to this transcript, levels of the other PSII gene transcripts were unchanged among the cpRNA preparations of young and old mutant tissue (Figure 13b,c). In addition, the hybridization signals of mutant preparations were nearly equivalent to those obtained for WT cpRNA (where the same internal standard, the psbB transcript, was used) derived from leaf tissue of comparable age to

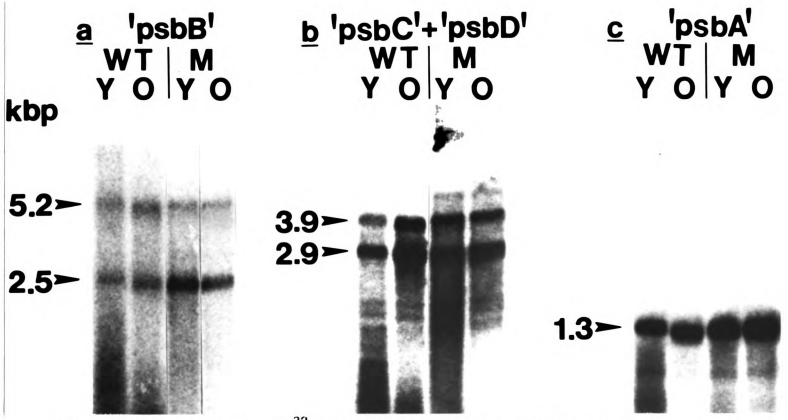

Figure 13 Hybridization of ^{32}P-labeled plastome-encoded PSII gene probes to WT and lut-1 (M) cpRNA preparations run on formaldehyde agarose gels and blotted onto nitrocellulose filters. The cloned (a) psbB, (b) psbC, psbD and (c) psbA genes code for the 51, 44, D2 ("32-like") and 32 kd (Q_B-binding protein) PSII polypeptides, respectively. The sizes of major RNA transcripts detected by each probe is indicated in kilobase pairs (kbp). In chloroplasts from young (Y) and old (O) leaves of WT and and lut-1, transcript levels and sizes were the same. This indicated that altered mutant transcription was not responsible for the depletion of PSII polypeptides in lut-1 chloroplasts.

the mutant samples (Figure 13a,b,c). There were minor differences in the intensity of major hybridization signals among the different cpDNA preparations. These differences were due to degradation of the RNA transcripts within each preparation. The extent of this degradation was variable as judged by the level of diffuse hybridization signals at lower molecular weight regions of the blots. The similarity in degree of hybridization of different PSII gene probes to cpRNA preparations from fully green WT tobacco tissue and from the increasingly chl deficient lut-1 tissue, indicated that the stoichiometry of PSII chloroplast-encoded transcripts, relative to each other, remained constant.

Transcript sizes of both mutant and WT cpRNA samples were comparable to values reported in the literature. In kilobase pairs, they were: 1.3, psbA (154); 2.5 and 5.2, psbB (121); 2.9 and 3.9, psbC and psbD (4). In the latter case, the two genes were on the same DNA probe, pWHsp408b (4), so a unique assignment of transcript size to gene was not possible. We did not observe the additional larger and smaller transcripts, reported by Morris and Herrmann with the psbB gene (121), or by Alt et. al with the psbC and psbD genes (4). Breakdown of larger transcripts in the cpRNA preparations and the use of heterologous probes may explain why fewer hybridization signals were detected.

We conclude that mutant chloroplast transcripts of PSII polypeptides were neither diminished nor altered. Since faulty plastid transcription was apparently not responsible for the loss of PSII proteins from lut-1 membranes, the next step was to check for functional protein synthesis.

Chloroplast Protein Synthesis

To test for chloroplast protein synthesis in vivo, L-[^{35}S]Met was introduced into the transpiration stream of excised lut-1 and WT leaves. Thylakoid membranes were subsequently isolated and labeled proteins were analyzed by SDS-PAGE and autoradiography. This allowed us to check for proper translation of chloroplast mRNA.

The results of this in vivo labeling experiment indicated that mutant chloroplasts, which were not accumulating PSII proteins, were still capable of synthesizing these proteins. Labeled polypeptides of WT and lut-1 membranes (derived from 15 to 17 day old leaves, comprising the middle-age category) were separated by PAGE and are shown in Figure 14a). Samples were loaded on the basis of equal amounts of thylakoid protein. PSII-associated polypeptides were identified in two ways. The first was by co-migration of coomassie-blue staining bands in the membrane preparations with those bands in the PSII-enriched (grana) fraction prepared from WT chloroplasts and the second method was by immunoblotting (both described in Chapter 3).

The mutant membranes had reduced levels, as judged by coomassie-blue staining intensity, of the PSII reaction center polypeptides at M_r 51 and 44 kd, and of a polypeptide at 33 kd, which has been implicated in the water oxidation process (7). The 51 kd polypeptide was reduced in amount to a lesser degree than the other PSII proteins, even in fully expanded mutant leaves (as previously shown in Chapter 3). The quinone binding protein (i.e., the apoprotein for the secondary electron acceptor, Q_B; ref. 93), migrated as a diffuse band at approximately 34 kd in this PAGE system.

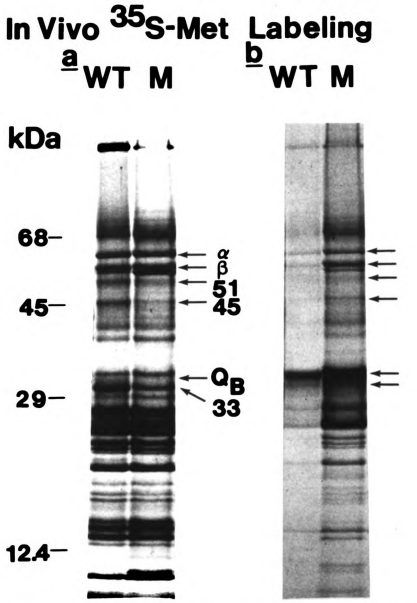

Figure 14 (a) Coomassie-stained gel and (b) autoradiogram of WT and lut-1 (M) thylakoid membrane proteins, isolated from middle-aged leaves, labeled in vivo with [^{35}S]Met. The alpha and beta subunits of ATP synthase, and the 51, 44 and 32 kd (Q_B-binding protein) polypeptides are plastome-encoded species which were synthesized by lut-1 chloroplasts (panel b, lane M). However, the PSII associated 44 and 32 kd species did not accumulate in membranes, compared to WT levels (panel a, lanes WT and M). The 33 kd polypeptide is one of several nuclear-encoded PSII-associated polypeptides, the levels of which also decreased in mutant thylakoids.

The autoradiogram of these lanes (Figure 14b) showed co-migration of radioactivity with both the 44 and 51 kd polypeptides, indicating that both were labeled, i.e., synthesized, by lut-1 chloroplasts. WT membranes incorporated less label into these two proteins (note that samples were compared on an equal protein basis). Since all leaf samples were treated in an identical fashion, this implies a faster rate of PSII protein synthesis in the mutant chloroplasts. We cannot, however, rule out differences in methionine uptake or pool sizes, which would confound this analysis. The 32 kd Q_B-binding protein was highly labeled in both WT and mutant chloroplasts, as expected for this rapidly turned-over protein (93). Other chloroplast-encoded proteins, such as the alpha and beta subunits of the ATP synthase complex (170) were synthesized and membrane-associated in mutant chloroplasts, which again indicated active protein synthesis in lut-1 chloroplasts. The synthesis of the nuclear-encoded 33 kd protein was not easily monitored since it did not accumulate the same proportion of label (with respect to the level of stained proteins in the thylakoids) as chloroplast-encoded proteins. As in WT membranes, it was only slightly labeled in mutant membranes, in comparison to the 51, 44 and 32 kd (Q_B-binding) proteins.

We conclude that lut-1 chloroplasts were capable of synthesizing plastome-encoded polypeptides that function in PSII. The apparently higher than WT labeling rate of mutant chloroplast-encoded proteins without their net accumulation suggested a rapid turnover of these proteins (except for the 51 kd reaction center species) during leaf maturation.

These results, indicating that protein synthesis is functional in lut-1 chloroplasts, are consistent with the Northern blot analyses. The mRNA of plastome-encoded PSII genes was present throughout mutant leaf development, and this mRNA was used for protein synthesis. Lut-1 chloroplasts were capable of both transcription and translation of genes encoding PSII proteins.

Altered Size of Nuclear-Encoded Polypeptides

Three nuclear-encoded proteins, migrating at relative molecular masses of 16, 23 and 33 kd, are known to be involved in forming the OEC of PSII in spinach chloroplasts (7). The presence of analogous proteins in tobacco membranes was examined by analyusis of immunoblots to learn the fate of these cytoplasmically-synthesized proteins during the loss of the chloroplast-encoded PSII constituents from lut-1 membranes. Protein blots of WT and mutant thylakoid membranes were probed with monospecific polyclonal antisera raised against the spinach polypeptides (8). This allowed identification of the OEC proteins within a complex polypeptide profile in the lower molecular weight regions of our gels.

Both WT and mutant tobacco thylakoids had three polypeptides in the 21-23 kd size range reacting with antibodies which were monospecific to the spinach 23 kd OEC protein (Figure 15a,b). These multiple bands were not observed when blots were tested with pre-immune sera (data not shown). The presence of multiple bands for this protein was likely to be the result of the N. tabacum genotype. Having arisen from a cross between N. tomentosiformis (2n = 24) and N. sylvestris (2n = 24), N. tabacum is an allotetraploid (with an

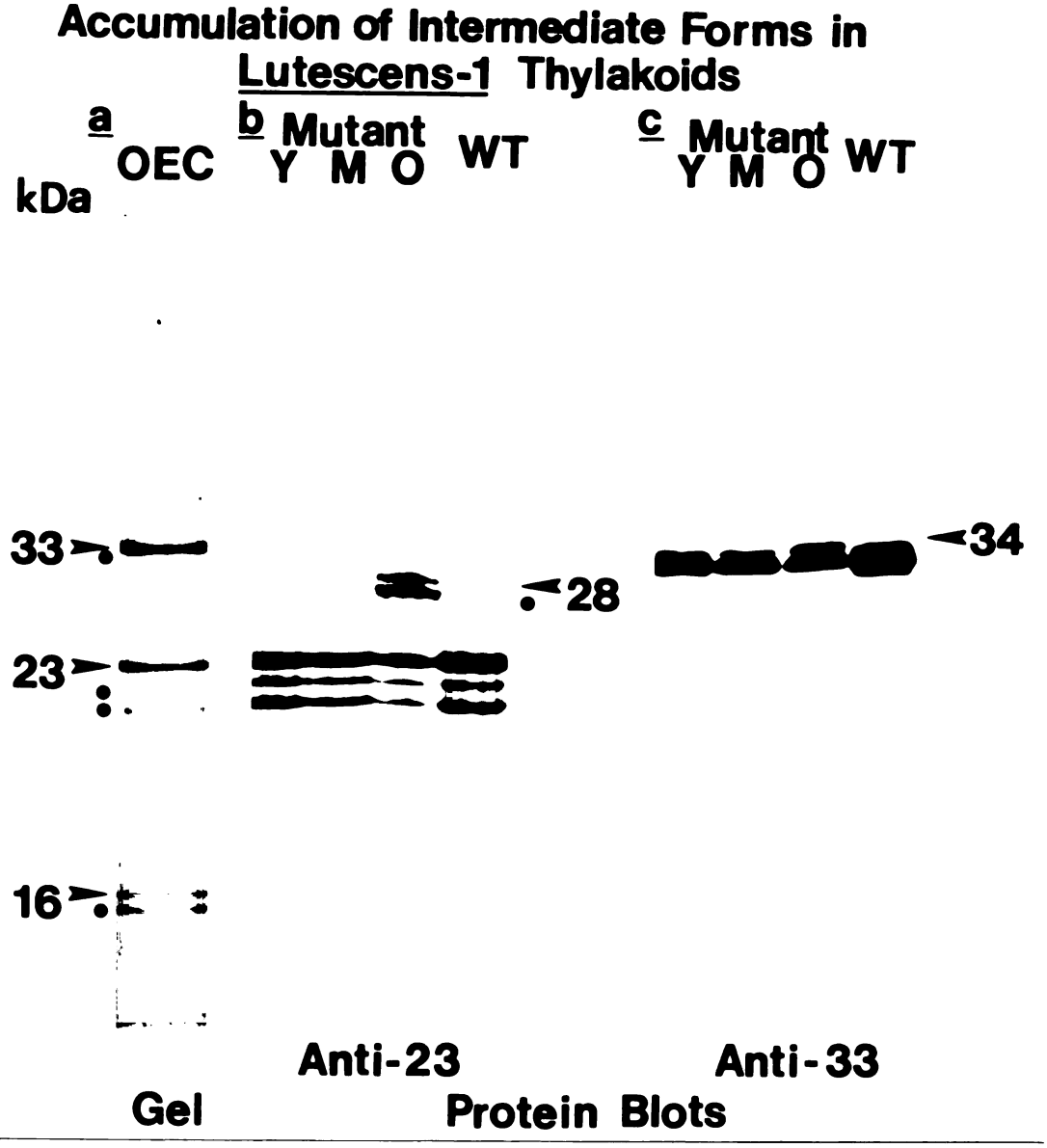

Figure 15 (a) SDS-PAGE of the OEC from tobacco WT stained with coomassie blue and extracted as described (124). Species indicated with a closed circle (●) are allozymic forms found in N. tabacum (see Results for details); and (b, c) protein blots of lut-1 and WT thylakoid membranes probed with polyclonal antisera reactive against either the 23 kd or the 33 kd polypeptides. These immunoblots revealed higher molecular weight forms of these two nuclear-encoded PSII constituents. While mature forms of the 23 and 33 kd polypeptides were depleted from lut-1 thylakoids, higher molecular weight species of these proteins began to accumulate at 28 (a doublet) and 34 kd respectively. Y, M and O correspond to the age of the lut-1 leaves from which thylakoids were isolated. Y: young (7 days); M: middle (20 days); O: old (35 days).

N. sylvestris cytoplasm), having a chromosome number (2n) of 48 (62). Potentially each parental genome may contribute an allele for the 23 kd protein so that two genes are expressed and observed on protein blots. Polyacrylamide gel analysis of purified O_2-evolving PSII preparations from each of the parental lines and from N. tabacum confirmed the presence of different size class isozymes for the 16, 23 and 33 kd proteins in the two parental lines, with N. tabacum showing a combination of the variants (allozymes) (J. Duesing, P. Yang, and C. Arntzen, unpublished observations). This appearance of allozymes is similar to the appearance of allozymes observed for the small subunit of ribulose-1,5-bisphosphate carboxylase in N. tabacum (170). Observation of a third "23 kd" band in N. tabacum, rather than the anticipated two allozymic forms, was possibly due to proteolysis or to a post-translational modification of one of the 23 kd polypeptides to yield a species with a slightly different molecular mass.

Although a very closely migrating doublet at the mature molecular mass of 33 kd could be discerned when extracted N. tabacum OEC was analyzed by SDS-PAGE and coomassie-blue stained (Figure 15a), multiple mature 33 kd size species were not observed on protein blots. The reaction used to detect the primary antibody involves generation of a colored reagent and its deposition at the site of the immune complex. The blots in Figure 15 were overdeveloped in order to observe a 34 kd higher molecular weight form (described below). This overdevelopment hindered resolution of the 33 kd allozymes on immunoblots.

For the 23 and 33 kd polypeptides, increasing amounts of higher molecular weight species were detected as the mature forms were depleted from mutant membranes (Figure 15b,c). A doublet at 28 kd

(possibly allozymic forms) reacted with the anti-23 kd serum whereas the anti-33 kd serum recognized a 34 kd species. The 28 kd dimer and 34 kd higher molecular weight forms were not observed when blots were probed with pre-immune serum or when blots of WT membranes of increasingly older leaves were probed with specific antisera. Under our conditions, the 16 kd protein was depleted over time without detection of larger cross-reacting species (data not shown).

Discussion

The biogenesis and composition of the photosynthetic membrane system are affected by environmental factors such as light intensity and quality (56,159). It is our long-range goal to determine the different regulatory mechanisms involved in the structural and functional adaptation of chloroplasts in response to changes in the environment. One step in this direction is to define genetic determinants which affect stoichiometry of thylakoid components.

Studies of the biochemical phenotype of the lut-1 plastome mutant revealed that it involves a developmentally expressed, physical depletion of PSII-associated polypeptides (Chapter 3). The molecular analysis of the structural defect in lut-1, presented in this chapter, points to the existence of an aberrant post-translational process which impairs the assembly of PSII and/or alters the stability of this complex in thylakoid membranes. As a result, the normal regulation of a stoichiometric ratio of PSII to PSI centers is disrupted; i.e., the chloroplast is not able to regulate properly the relative amounts of these two complexes. It is likely that the membrane protein turnover process which caused the selective loss of PSII is related to the abnormal activity of a usual chloroplast component. We conclude that turnover of the PSII complex was accelerated in lut-1, or effectively became more efficient because degradative aspects outpaced the usual rate of maintenance protein synthesis. The reasoning behind these conclusions follows.

The cpDNA restriction fragment digest patterns (Figure 12) indicated that there were no detectable alterations in mutant plastid genome organization. Northern blot analysis was used to check whether

the transcription of chloroplast-encoded PSII proteins was defective (Figure 13). The intensity of the hybridization signal of the probe for the 51 kd PSII reaction center polypeptide (gene psbB) was used as a reference point (since the 51 kd polypeptide was present in all membrane preparations). Compared to the signal level of this probe, the signal levels of other PSII gene transcripts (which were detected by specific gene probes) were found to be the same in both young and old lut-1 leaves. These levels were comparable to the levels observed in WT cpRNA preparations derived from similarly aged WT leaf tissue. Sizes of major mutant transcripts matched the size of major WT transcripts.

We have also shown that chloroplast proteins, including those PSII polypeptides which are depleted, are synthesized by lut-1 chloroplasts. Mutant chloroplasts were able to incorporate exogenously supplied [^{35}S]Met into plastome-encoded polypeptides (Figure 14b). However, mutant membranes do not accumulate most PSII polypeptides as judged by the lack of stained polypeptides (Figure 14a), even though protein synthesis was detected. That is, there was no net accumulation, specifically of those polypeptides identified as PSII constituents, in older lut-1 chloroplasts (except for the 51 kd polypeptide identified as a PSII reaction center protein).

Together with plastome-encoded PSII proteins, the mature forms of nuclear-encoded PSII polypeptides are depleted from lut-1 membranes (Figure 15). The appearance of larger size-class polypeptides reacting with the anti-23 and anti-33 sera in later stage mutant membranes was evidence for abnormal protein processing in the mutant plastids. It is known that both of these proteins are synthesized in

the cytoplasm as larger size-class precursors (166). We conclude that these unprocessed or partially processed proteins accumulate in the mutant plastids. Chapter 5 is devoted to the characterization of these apparent "intermediate products". The presence of these putative precursors may be a clue to lut-1's dysfunction. Whether this pool of unprocessed proteins was the result of a primary lesion in protein maturation which caused the depletion of PSII complexes cannot be determined at this time.

Transcriptional control of plastid genes (for example, photogene expression) is one was of controlling the level of chloroplast gene products (139). This manuscript and other recent related studies point to another level of regulation. Leto and co-workers (103), using a nuclear mutant in maize, found unstable PSII complexes the constituents of which (both nuclear and plastome-encoded) appeared to turn over more rapidly than their WT counterparts. They ruled out transcriptional and translational defects by examining mRNA levels and protein synthesis in experiments similar to the ones described in this report. They concluded that the nucleus plays a role in stabilizing PSII complexes. An example of a post-transcriptional defect in a plastome mutant has been described by Sears and Herrmann (143). They described an Oenothera hookeri mutant in which the beta and epsilon subunits of the chloroplast ATP synthase were fused into a single protein. Because in vitro translation of mutant cpRNA yielded properly-sized peptides, they concluded that the defect was in a process following normal transcription of the plastid genes. A recent report by Inamine et. al (80) noted that the relatively small change in carboxylase mRNA levels observed upon illumination of pea plants

cannot account for the large increase in the specific activity of the enzyme. These investigators suggest that the light regulated synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase is at the level of translation rather than at the level of transcription.

In conclusion, this study has demonstrated the possibility of regulation of stoichiometry of chloroplast membrane components by a post-translational process involving protein degradation. As discussed above, there is increasing evidence for other forms of chloroplast developmental control besides transcriptional regulation. The identification and characterization of post-translational processes may provide an understanding of when they are physiologically important, such as in periods of environmental stress which lead to changes in chloroplast development and activity.

CHAPTER 5

Evidence for Two-Step Processing of Nuclear-Encoded Chloroplast Proteins During Membrane Assembly

Abstract

The tobacco plastome mutant, lut-1 displays abnormal degradation of the chloroplast-encoded polypeptides which form the core complex of PSII. Two nuclear-encoded proteins (present in multiple allozymic forms), which normally function in the water oxidation process of PSII, accumulate as larger size-class polypeptides in the mutant membranes. These accumulated proteins are intermediate in size between the full-length primary protein synthesized in the cytoplasm and the proteolytically processed mature polypeptides. Trypsin treatment of unstacked mutant thylakoids and of inside-out vesicle (PSII-enriched) vesicle preparations indicated that the intermediate size forms were correctly localized on the inner surface of the thylakoid membrane, but not surface-exposed in the same way as the mature proteins. Only one of the intermediate size-class proteins could be extracted by salt washes.

We interpret these data as evidence for two-step processing of imported proteins which function in the water oxidation step of photosynthesis, and which are located in the lumen (the space within the thylakoid vesicles). The second step in proteolytic processing may be related to transport through a second membrane (the first

transport step through the chloroplast envelope having been completed); this step may be arrested in the mutant due to the absence of the PSII core complex.

Introduction

The biogenesis of the photosynthetic apparatus in higher plants requires a interplay between the nuclear and chloroplast genomes. The mechanism(s) by which nuclear-encoded, cytoplasmically synthesized proteins are targeted and properly sorted to their correct cellular location are not yet fully understood. In this process, proteins destined to be chloroplast membrane components must be transported across the chloroplast envelope, proteolytically processed to their mature size, and finally assembled with other subunits either in the stroma or thylakoid membranes (50).

Three nuclear-encoded polypeptides of 16, 23 and 33 kd are known to be involved in photosynthetic oxygen evolution (7). These proteins are associated with the pigmented core complex of PSII, but are extrinsically bound at the inner thylakoid surface. They have been shown to be synthesized in the cytoplasm as larger, soluble precursors and post-translationally imported into chloroplasts (168). The maturation of these proteins is particularly intriguing because their destination in the thylakoid lumen requires translocation across both the chloroplast envelope and thylakoid membranes.

Studies of the chloroplast-encoded photosynthetic tobacco mutant, lut-1, afforded an opportunity to examine the processing of intermediates of two of the OEC polypeptides (the 23 and 33 kd species). The phenotype of this mutant is a developmentally expressed loss of PSII activity. Lut-1 PSII centers in immature leaves are functionally equivalent to those in wild type chloroplasts; they have the capacity to carry out the process of oxygen evolution normally (Chapter 3). During the course of normal plastid (leaf) ageing,

however, lut-1 chloroplasts undergo a specific and progressive loss of PSII polypeptides. Analyses of transcript levels of lut-1 chloroplast-encoded PSII genes and chloroplast protein synthesis indicated that reduced transcriptional and translational activities are not responsible for the physical depletion of PSII constituents. Instead, the defect resulted from an inability of lut-1 chloroplasts to retain adequate (WT) levels of PSII complexes, due to selective turnover of most of the protein components of the PSII core complex. This chapter will describe the fate of the nuclear-encoded OEC proteins in membranes depleted of the PSII core complex.

Materials and Methods

Plant Material

WT and mutant lut-1 tobacco (Nicotiana tabacum var L.C. from the Connecticut Agricultural Experiment Station) plants were grown as described in Chapters 2 and 3. Mutant plants were maintained as variegated sectorial and/or periclinal chimeras, and pruned to force growth of mutant axillary buds which gave rise to completely mutant shoots suitable for chloroplast analysis.

Isolation of Chloroplast Membranes (Thylakoids) and PSII Particles

Thylakoid membranes were prepared as described in Chapter 3 except that NaCl, $MgCl_2$ and protease inhibitors were omitted from all buffers; the isolation medium was 400 mM sorbitol, 100 mM Tricine-NaOH, pH 7.8, 5 mM sodium ascorbate, pH 7.8 and 0.3% (w/v) polyvinylpyrrolidone. Chloroplasts were washed twice in 10 mM Tricine-NaOH, pH 7.8 to ensure complete unstacking of the thylakoids. Chl was determined by the method of Mackinney (107).

PSII particles were prepared as previously described (17) using the following modifications (124). Thylakoids were resuspended in MES buffer (20 mM MES-NaOH, pH 6.5, 10 mM NaCl and 5 mM $MgCl_2$) at 2 mg chl/ml and incubated with Triton X-100 at a ratio of 1 to 20 (w/w; chl to detergent) on ice, in the dark, with stirring for 30 min. The sample was then centrifuged at 40,000 x g for 30 min. The resultant PSII pellet was resuspended in MES buffer and either used for trypsin experiments (described below) or treated with Tris/NaCl to remove the OEC polypeptides. The latter procedure involved dilution of the PSII particles to 1 mg chl/ml, 1 M NaCl (with 4 M NaCl) and 50 mM Tris, pH

9.0, stirring for 15 min in the dark at 20 ^{o}C, and centrifugation at 40,000 x g for 20 min. The supernatant is referred to as the Tris/NaCl wash or extract, and contained the 16, 23 and 33 kd polypeptides comprising the OEC (92).

Trypsin Treatments

Trypsin incubations were performed in the dark at room temperature with shaking on a rotary platform shaker (50 rpm). Thylakoid membranes were diluted in MES buffer to a final concentration of 150 ug chl/ml. Trypsin (Type III, from bovine pancreas, 12,000 units/mg protein; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 37.5 ug/ml (final ratio: 150 ug trypsin/ug chl). PSII particles were subjected to trypsin digestion in an analogous manner except that the final trypsin concentration was 12.5 mg/ml, yielding a trypsin to chl ratio of 50 ug trypsin/mg chl. At times indicated in the figures, samples which were in the presence of trypsin were transferred into microfuge tubes and quickly centrifuged (13,000 x g, 15 s). The supernatant was then removed by aspiration, 3X SDS sample buffer was added, the sample was quickly vortexed, and heated at 95 ^{o}C for 1 min before SDS-PAGE.

SDS-PAGE and Protein Detection by Immunoblotting

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (19) as described in Chapter 2. Gradient (10-17.5%) polyacrylamide SDS slab gels were run at a constant current of 15 mA. Molecular weight markers were: bovine serum albumin, 68 kd; ovalbumin, 45kd; carbonic anhydrase, 29 kd; and cyt c, 12.4 kd.

Monospecific polyclonal antisera prepared against the purified 16, 23 and 33 kd spinach polypeptides, as previously described (2), were generously provided by Dr. C. Jansson, MSU/DOE Plant Research Lab., Michigan State University.

Protein detection by immunoblotting was performed as described in Chapter 3.

Isolation of Poly A^+-RNA, *In Vitro* Translation, and Immunoprecipitation

Cytoplasmic RNA was isolated from WT tobacco and fractionated on a poly U sepharose column following the procedure of Cashmore (31). Cell-free protein synthesis was performed with nuclease-treated rabbit reticulocyte lysates (Promega Biotec, Madison, WI) following supplier instructions (except volumes were increased five to eight-fold) using either [^{35}S]Met or [U-^{14}C]Leu (Amersham Corp., Arlington Heights, IL).

Immunoprecipitation of translation products was carried out using modified procedures of Rochaix and Malnoe (138) and Schmidt et al. (141). After incubation of the translation mixture, 1 volume of NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.5% (w/v) Triton X-100) was added to two volumes of lysate. To this mixture, one-tenth volume of pre-immune serum was added and the solution was shaken on a rotary platform shaker (50 rpm) for 1 h at room temperature. One-tenth volume of a slurry of Protein A-Sepharose beads (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) washed twice in 100 mM phosphate buffer, pH 7.0 and resuspended (125 mg/ml) in 25 mM Tris-HCl, pH 7.5, was added, and the mixture was shaken at room temperature for 1 h. Beads were separated from the mixture by

centrifuging samples for 1 min. in a microfuge and discarded. One-twentieth volume of immune serume was added to the mixture which was then shaken either overnight at 4 ^{o}C or at 37 ^{o}C for 4 h. A volume of fresh Protein A beads equal to the immune serum volume was added and the mixture was again shaken either overnight at 4 ^{o}C or at 37 ^{o}C for 4 h before beads were collected by centrifugation. Pelleted beads were washed twice with 500 ul NET and then twice with 750 ul of wash buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.5 and 1% (v/v) 2-mercaptoethanol). Elution of the immune complex was achieved by boiling beads for 1 min in 40 ul of 3X SDS sample buffer. Samples were analyzed by SDS-PAGE.

Results

Polypeptides of the OEC

The OEC has been biochemically characterized in several plant species, but most extensively in spinach where is it comprised of three proteins of 33, 23, and 16 kd. The procedures developed to isolate the OEC from spinach are generally applicable for chloroplasts of other plant species, although the apparent molecular weights of the isolated proteins sometimes vary (in maize, for example, the three proteins are 14, 25, 32 kd; ref. 24).

Preparation of the OEC from N. tabacum by the high pH-high salt wash procedure (2,91) resulted in a preparation containing seven polypeptides (Figure 16, lane 1) rather than three as in spinach. These seven proteins fell in three size-classes: a very closely spaced doublet of approximately 33 kd, three proteins of 21, 22 and 23 kd, and a doublet of 15.5 and 16 kd. Polyclonal antibodies prepared against the spinach OEC reacted with this complex of seven proteins.

N. tabacum is an allotetraploid (2n = 48) which is the result of a hybridization of N. sylvestris (2n = 24) with N. tomentosiformis (2n = 24) (62). For comparison to N. tabacum, the OEC was prepared from the two parental lines (unpublished data of J.H. Duesing, P. Yang, C.J Arntzen). Each putative parental species contained only three proteins in the OEC complex when these were compared to the N. tabacum OEC by SDS-PAGE; the "16" and "33" kd doublets of N. tabacum corresponded directly to the similarly sized proteins in the parental strains. As discussed in Chapter 4, we interpret this polymorphism to indicate that the N. sylvestris and N. tomentosiformis genomes each contained an allele for these polypeptides which was expressed in the

N. tabacum OEC. These gene products or allozymes were of slightly different mature size. In the case of the 23 kd size-class, two of the N. tabacum OEC polypeptides corresponded to proteins isolated from the parental lines. Presumably these also represented products of separate alleles in the combined genome. The third polypeptide in this size class was also recognized by an antibody to the spinach 23 kd protein. We suggest this species is either a degradation product or an altered processing product of one of the two 23 kd size-class proteins in the OEC.

In summary, N. tobacum contains seven polypeptides in its OEC. These fall into three size classes (approximately 16, 23, and 33 kd), each member of which reacts with monospecific antisera elicited against the spinach 16, 23, or 33 kd proteins, respectively. For the remainder of this Chapter we shall consider the polymorphic forms within each size class to be related (since all members of a group showed parallel behavior), and simply refer to the "16", "23", or "33" kd proteins.

Primary Product Size

In comparison to chloroplasts from WT tobacco, chloroplasts from fully expanded leaves of lut-1 are depleted in nuclear- and chloroplast-encoded PSII polypeptides (Chapter 3). In examining the levels of the 23 and 33 kd OEC proteins by immunoblotting, higher molecular weight species at 28 (a doublet probably due to two allozymes) and 34 kd, respectively, were observed to accumulate in thylakoid membranes from these mutant chloroplasts concomitant with a

reduction in the level of the mature protein species (Chapter 4, Figure 15b,c, page 102).

Both the 23 and 33 kd polypeptides of the OEC are synthesized in the cytoplasm as larger (by 10 and 6 kd, respectively) precursor molecules in spinach (168). To verify that the tobacco cytosolic precursors were also made as larger size polypeptides, poly A^+-RNA was isolated from WT tobacco and translated in a rabbit reticulocyte cell-free system. The presence of the precursors to the 23 and 33 kd proteins was checked by immunoprecipitation with monospecific antisera The primary translation product reacting with the 23 kd antisera migrated at 33 kd (Figure 16, lane 2). A doublet was observed for this immunoprecipitated product. The two species are possibly derived from the different mRNA's each from the two different parental alleles although proteolysis and/or early termination of translation cannot be ruled out.

The primary translation product reacting with the 33 kd antisera migrated at 38 kd. In addition, a smaller 35-kd protein, was observed (Figure 16, lane 3). This 38 kd labeled species was larger than the membrane-bound precursor observed on immunoblots (see Chapter 4, Figure 15b,c, page 102) and may have been the result of early termination of translation in the rabbit reticulocyte system.

These results show that the membrane protein preparations, in which the 28 and 34 kd polypeptides were detected, were not contaminated with cytoplasmically synthesized precursor proteins. The 28 and 34 kd polypeptides were considered to be intermediate proteins because they were intermediate in size between the primary protein

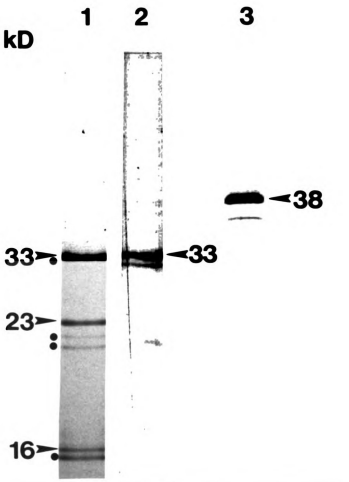

Figure 16 Mature and primary product size of OEC polypeptides. Lane 1: Tris/NaCl extract containing WT OEC polypeptides separated by SDS-PAGE and stained with coomassie blue. The multiple bands for each species (indicated with closed circles (●) were polymorphic forms (see Results). Lanes 2 and 3: Autoradiograms of immunoprecipitated primary products synthesized in vitro using poly A^+-RNA, isolated from WT tobacco leaves, in a rabbit reticulocyte cell-free translation system. Lane 2: anti-23 kd serum immunoprecipitated a major band at 33 kd (arrow) and a minor band at 32 kd; lane 3: anti-33 kd serum immunoprecipitated a major band at 38 kd (arrow) and a minor band at 35 kd.

products and the mature, processed products. Investigations to determine the location of these intermediates are described below.

Topology

Several tests were carried out to determine whether the intermediate forms of the OEC proteins were in their proper location on the lumenal side of thylakoid membranes. The first approach was to proteolytically digest well-washed, unstacked mutant thylakoid membranes and look for removal of the higher molecular species. Under the conditions used, surface-exposed membrane proteins such as the LHC-II (150) were partially digested (data not presented). Under the trypsin digestion conditions (150 ug trypsin/mg chl) neither the 34 nor the 28 kd intermediate forms were altered in size (Fig 17a, b). Higher trypsin to chl ratios were effective in cleaving the intermediates, but these conditions also digested the mature forms, indicating penetration of the protease through the membranes (data not shown). From these experiments, we concluded that the intermediate forms of the OEC were either inserted into the thylakoid membranes or translocated across them, and not merely adhering to the thylakoid surface.

The experiments on intact membranes, which are right-side-out, were complemented by analogous experiments with detergent-prepared PSII particles. These particles are grana-enriched thylakoid fragments which are inside-out vesicles, and consist primarily of unsealed pairs of membranes stabilized at the edges by detergent (47). In these PSII preparations the inner thylakoid surface is exposed to added proteolytic enzymes. The PSII particles prepared from lut-1

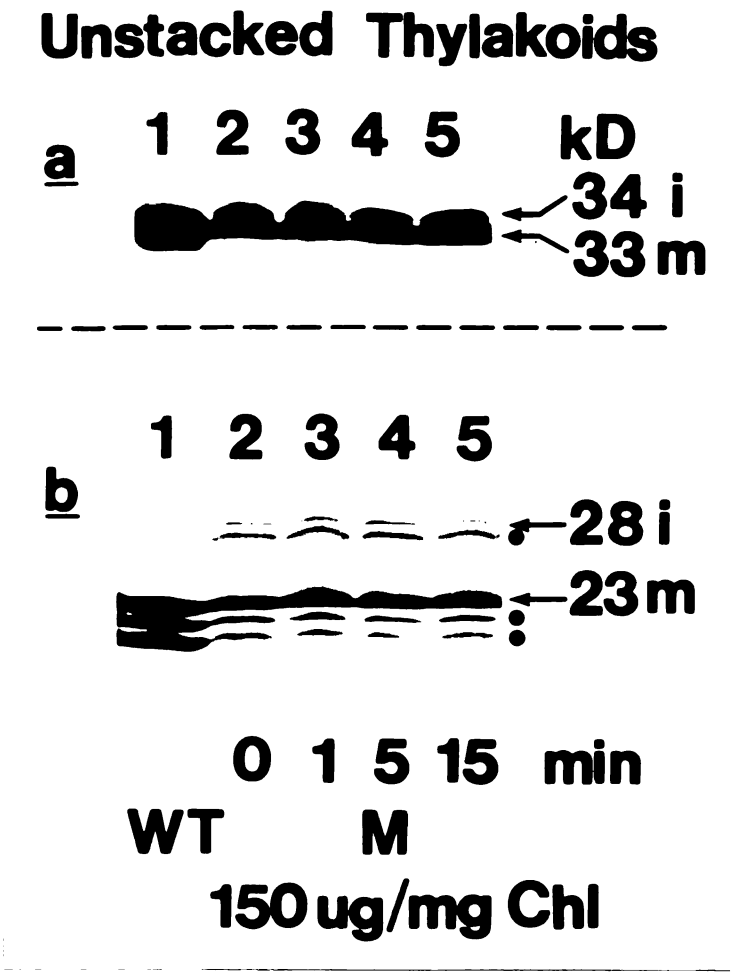

Figure 17 Protein blots of WT thylakoids and trypsin-treated (50 ug trypsin/mg chl; incubation times are indicated below the figure) unstacked mutant thylakoid membranes (M) probed with antisera reactive against either the (a) 33 kd or (b) 23 kd polypeptides. Arrows indicate the mature (m) and intermediate (i) forms of the two OEC polypeptides. Polymorphic forms are indicated by closed circles (●). Both the intermediate and mature forms of the OEC polypeptides were trypsin-insensitive. This indicated that they were not exposed to the stromal phase.

membranes were subjected to a time course of trypsin digestion (50 ug trypsin/mg chl). The immunoblots are shown in Figure 18. Under the conditions used, the mature 23 kd polypeptide was quickly digested away, whereas the precursor form was not (Figure 18a). Neither the mature 33 kd nor intermediate 34 kd forms were removed by this trypsin treatment (Figure 18b). At higher trypsin to chl ratios, both intermediate and mature forms of the 23 and 33 kd proteins could be removed, indicating that all species were accessible to the protease although penetration of the enzyme cannot be ruled out (data not shown). An experiment identical to that shown in Figure 18a was performed with WT thylakoids. The results were the same: the mature 23 kd protein was digested more rapidly that the mature 33 (data not shown).

These data are consistent with the postulated topography of the polypeptides comprising the OEC presented in (7) and the results of others (60). The 33 kd protein is closely associated with the core complex of PSII whereas the 23 kd protein is more extrinsically located with greater exposure to the lumen. The 23 kd protein is therefore more susceptible than the 33 kd protein to trypsin cleavage. In contrast to the mature 23 protein, the 28 kd intermediate was partially protected from proteolytic digestion, which suggests that the latter form was not in its proper membrane location (Figure 18b). Conceivably, the 28 kd intermediate was at least partially sequestered in the thylakoid membrane itself, rendering it inaccessible to proteolytic degradation. There was no differential degradation of the 33 and 34 kd forms under the conditions described here or under harsher trypsin treatments (data not shown).

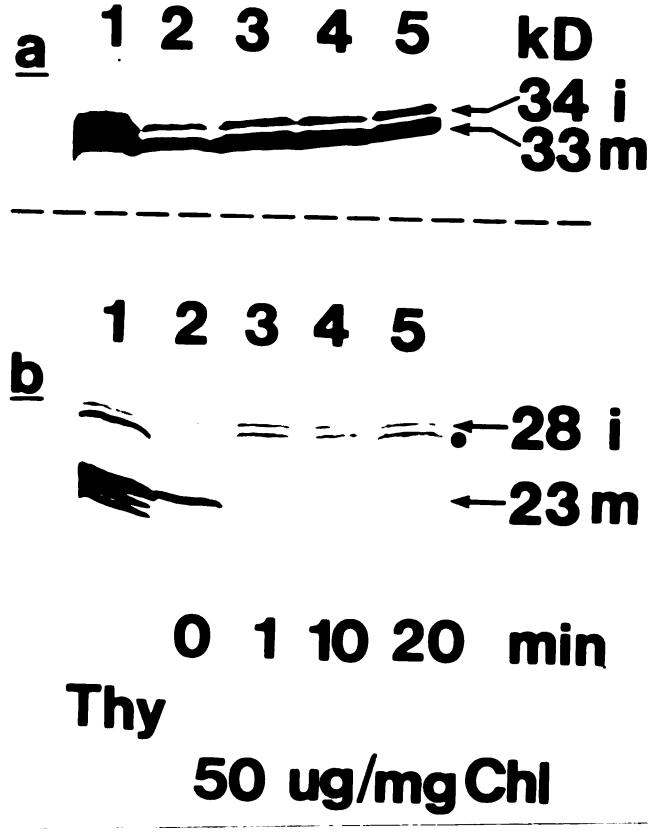

Figure 18 Protein blots of lut-1 thylakoids (THY) and trypsin-treated (50 ug trypsin/mg chl; incubation times are indicated below the figure) inside-out vesicles (PSII-enriched preparations) derived from mutant thylakoids (see Materials and Methods) probed with antisera reactive against either the (a) 33 kd or (b) 23 kd polypeptides. Arrows indicate the mature (m) and intermediate (i) forms of the two OEC polypeptides. Under these conditions, neither the intermediate 34 kd nor mature 33 kd species was digested. Higher ratios of trypsin to chl achieved proteolysis of both forms. The mature 23 kd polypeptide, but not the intermediate 28 kd form was removed by this trypsin treatment.

A third test devised to examine the position of the intermediate sized OEC proteins was to remove the OEC polypeptides from PSII particles with an alkaline pH-high salt (NaCl) wash. In previous studies with spinach PSII preparations, it was shown that the 16, 23 and 33 kd proteins may be extracted from inside-out vesicles with Tris-HCl, pH 9.3 (92), whereas the 23 and 16 kd proteins but not the 33 kd species are removed with a 1 M NaCl wash (2). Inside-out vesicles were prepared from mutant chloroplasts and subjected to the OEC extraction conditions followed by immunoblotting analysis. The results are shown in Figure 19. In panel a, protein blots were probed with anti-23 kd serum; in panel b, blots were probed with anti-33 kd sera. Mutant PSII particles prepared from lut-1 thylakoids are shown in lane 2. The yield of PSII was low but both the mature 23 and its 28 kd intermediate were present in this preparation. Lane 5 is the PSII particle depleted of the mature OEC polypeptides; lane 6 is the Tris/NaCl extract. It can be seen that the 28 kd intermediate species was not extracted by the alkaline pH-high salt wash. It remained in the depleted PSII fraction. The alkaline pH-high salt wash contained the mature 23, but lacked the 28 kd form. In contrast, both the 34 kd intermediate and the mature 33 were extracted from the mutant PSII complex (Figure 19b, lane 5). Although the extraction was not exhaustive, it is clear that the 34 kd intermediate responded to extraction in a fashion like that of the 33 kd protein and was removed under the conditions which removed the mature form.

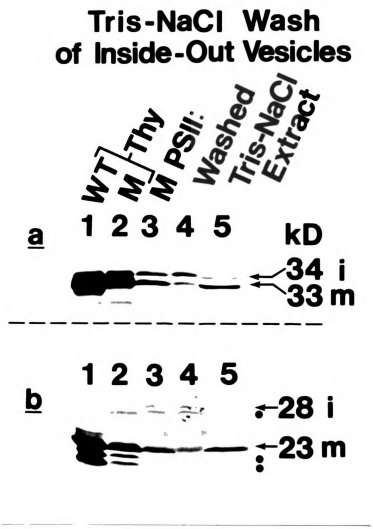

Figure 19 Protein blots probed with antisera prepared against the (a) 33 kd and (b) 23 kd polypeptides. Lanes are: (1) WT thylakoids, (2) lut-1 thylakoids, (3) a lut-1 PSII-enriched preparation, (4) the lut-1 PSII-enriched preparation remaining after extraction with Tris/NaCl, and (5) Tris/NaCl extract of a lut-1 PSII-enriched preparation. The intermediate (i) 34 kd, mature (m) 33 and 23 kd forms were extracted by the Tris/NaCl treatment (lane 5). The intermediate 28 kd species was not extracted and remained with the membrane fraction (lane 4). Polymorphic forms of the mature 23 and intermediate 28 kd polypeptides are indicated by the closed circles (●).

Discussion

Extensive previous research has shown that three polypeptides of 16, 23 and 33 kd are involved in oxygen evolution reactions in spinach chloroplasts (7). These proteins are extrinsically localized on the inner thylakoid surface (8), and can be extracted from inside-out thylakoid vesicles by high pH-high salt washes (91,92).

The results obtained in this study show that the OEC of N. tabacum chloroplasts contains seven polypeptide species which fall in three size classes near 16, 23 and 33 kd (Figure 16, lane 1). The use of monospecific antibodies prepared against each of the individual spinach proteins provided evidence that each spinach polypeptide corresponds to a doublet or triplet set of proteins in the tobacco chloroplast. We conclude that two or more allozymes of each OEC protein are present in tobacco due to its genetic composition (a tetraploid nuclear genome (60); see results section).

Chloroplasts in early stages of leaf expansion in the tobacco plastome mutant, lut-1, contain an active PSII (7), including OEC polypeptides of the mature size-classes (Chapter 4, Figure 15b,c, page 102). In fully expanded leaves, however, the PSII core complex proteins are absent from lut-1 thylakoids (Chapter 3), and monospecific antibodies to the 33 and 23 kd proteins recognize larger size-class polypeptides of approximately 34 and 28 kd, respectively (Figure 15b,c). The size of the primary protein products synthesized in cell-free translation assays were 38 and 33 kd; the 34 and 28 kd polypeptides detected in the mutant membranes are therefore "intermediate-size proteins".

The 28 and 34 kd intermediate size OEC polypeptides were thylakoid associated and not subject to digestion by trypsin addition to right-side-out thylakoids (Figure 17). The 34 kd protein was present and subsequently removed from the inner thylakoid surface with a high pH-high salt wash in tandem with the 33 kd mature form (Figure 19). In contrast, the 28 kd species were removed neither by trypsin nor by the Tris/NaCl wash from inside-out vesicles (PSII particles), unlike the mature 23 kd form. This indicated that the attachment of the 28 kd intermediate form to membranes was more hydrophobic in nature, and possibly lacked surface-exposed domains.

We have previously concluded that PSII core complexes are prematurely removed from lut-1 mutant thylakoids due to an accelerated turnover of the core polypeptides (Chapter 4). The current study has shown that intermediate size-class OEC proteins accumulate under these conditions. We suggest that this accumulation is due to the inability of the thylakoid system to complete the last step in a two-step processing of the OEC proteins due to an absence of their membrane binding site (the PSII core complex).

In the mutant membranes which accumulate the 28 and 34 kd intermediates, other protein processing steps appear to occur normally. The third nuclear-encoded (16 kd) OEC polypeptide whose primary translation product is approximately 26 kd (168), and the 32 kd chloroplast-encoded protein which is synthesized as a 34.5 kd primary product (63) are both processed normally; we did not observe higher molecular weight forms of these proteins by immunoblotting analysis (data not shown). Protein blots were also probed with a monoclonal antibody against the major nuclear-encoded light-harvesting

chlorophyll apoprotein, and no higher molecular weight forms were observed (data not shown). The 23 and 33 kd polypeptides of the OEC appeared to be the only species of the PSII complex to have accumulated as intermediate forms.

There are now several lines of evidence for two-step processing of proteins imported into organelles. Two-step processing of yeast mitochondrial proteins was delineated when required co-factors were absent or when a needed transmembrane potential was artificially collapsed in yeast mitochondria (54,55). Processing intermediates have been shown for the small subunit of ribulose-bisphosphate carboxylase (Rubisco) in pea (119,135) and for the L-18 chloroplast ribosomal protein from Chlamydomonas (141). The Rubisco small subunit intermediate was observed only in a heterologous system and in vitro; the L-18 intermediate was observed in vivo.

In conclusion, we have provided evidence for two-step processing of OEC polypeptides which normally reside in the chloroplast lumen. One of these (the 28 kd intermediate form of the 23 kd mature protein) does not reach its proper site of localization. We hypothesize that the final stage of processing of these proteins requires their site of binding--the PSII core complex--and perhaps the integration of co-factors such as manganese, chloride and/or calcium ions which are known to be involved in the water oxidation process (60).

CHAPTER 6

Summary and Hypothesis

A. Summary

The research reported in this thesis began with a survey of fifteen chl-deficient mutants of Nicotiana tabacum. Genetic crosses demonstrated that the mutations were maternally inherited. This indicated that the lesions were cytoplasmically located and thus chloroplast-encoded.

Chapter 2 described the studies undertaken to characterize the collection of tobacco plastome mutants. In vivo chl fluorescence properties of intact leaf tissue from each mutant were used to classify thirteen of the fifteen isolates into four separate categories. The remaining two mutants exhibited chl fluorescence properties which changed with leaf age. These two developmental mutants were each placed into their own separate category. Subsequent analyses of the photosynthetic properties of all the mutants showed the limitations of in vivo chl fluorescence in defining the complex biochemical phenotypes of the mutations.

All of the mutants were impaired in photosynthetic function as determined by electron transport assays, in vitro room temperature chl fluorescence properties and liquid nitrogen chl fluorescence emission spectroscopy of isolated chloroplast thylakoid membranes. Analysis of

the ultrastructure of chloroplasts by electron microscopy revealed alterations in the structural organization of mutant plastids. Analysis by polyacrylamide gel electrophoresis of the polypeptide profile of mutant thylakoids revealed the disruption of entire protein complexes. Typically all the constituents of a complex were depleted from the thylakoid membranes. The PSII complex was the most frequently affected complex, possibly because all six of the PSII reaction center polypeptides are chloroplast-encoded. A major finding of this set of studies is that, rather than causing discrete defects in the photosynthetic apparatus, mutations in the plastome cause extensive pleiotropic alterations in chloroplast structure. Thus, the chloroplast genome contributes important information for the structural and molecular organization of plastids.

The work on lut-1, described in Chapters 3 through 5, illustrated the utility of chloroplast-encoded photosynthetic mutants for studying the assembly and maintenance of the PSII complex in thylakoid membranes. In Chapter 3, studies characterizing lut-1, one of the plastome mutants having leaves which displayed changing chl fluorescence properties as a function of age, demonstrated the developmental phenotype of this mutant. Electron transport assays of isolated thylakoids revealed a progressive loss of PSII activity which occurred during maturation of mutant leaves. Examination of the polypeptide profile of mutant membranes showed that the loss of PSII function was due to a depletion of both nuclear- and chloroplast-encoded proteins identified as constituents of PSII. Because of the pleiotropic effect of the mutation, the component or

process which was primarily responsible fo the observed phenotype could not be easily defined.

A possible reason for the premature depletion of PSII complexes in lut-1 thylakoids was that a co-factor, such as chl, was not stably associated with the reaction center polypeptides. Another reason considered was that one of the polypeptide subunits was structurally altered such that the configuration of the PSII complex was unstable in the membrane bilayer. These ideas, which implied that mutant PSII complexes were inherently unstable, were tested by comparing properties of PSII complexes in thylakoids from young lut-1 leaves to the properties of PSII complexes in thylakoids from WT leaves. Three traits specific to PSII centers were examined. PSII reaction center chl-protein complexes, oxygen evolution patterns during flashing-light illumination, and the ability of PSII to undergo light-induced protein phosphorylation were evaluated. Using these three properties as criteria for properly functioning PSII centers, it was concluded that PSII complexes in lut-1 thylakoids were functionally equivalent to those PSII complexes of WT thylakoids.

Chapter 4 described a second approach taken to address the question of why PSII proteins were being prematurely lost from mutant thylakoids. This was an examination of the ability of mutant chloroplasts to synthesize PSII polypeptides. Two components of the protein synthesis apparatus of mutant chloroplasts were compared to those of WT. First, the structural organization of mutant cpDNA was compared to WT cpDNA by restriction fragment pattern analysis. No changes in the organization of mutant cpDNA were detected. Second, the major transcripts of chloroplast-encoded PSII polypeptides in mutant

and WT cpRNA preparations were found to be similar between mutant and WT chloroplasts. On the basis of these results, it was concluded that protein synthesis at the level of transcription was functional in lut-1 chloroplasts.

The ability of mutant chloroplasts to translate mRNA coding for the structural proteins of PSII was examined by providing [^{35}S]Methionine to mutant chloroplasts through the petiole of excised leaves. Chloroplast-encoded polypeptides which comprise the PSII core complex were radiolabeled; i.e., protein synthesis in mutant chloroplasts was functional.

Although PSII polypeptides were synthesized in lut-1 chloroplasts, they did not accumulate in thylakoid membranes. In contrast, proteins of other complexes in mutant thylakoids did accumulate to WT levels. This finding led to my major conclusion that PSII polypeptides in lut-1 thylakoids were degraded more rapidly than they were synthesized. Judging by the large number of chloroplast-encoded polypeptides observed to be labeled, in mutant thylakoids of fully-expanded leaves, it appeared that at that stage of leaf development, the rate of mutant chloroplast protein synthesis was as high or higher than that of WT. This conclusion is tentative, however, since it was not possible to measure the absolute rates of protein synthesis in mutant and WT chloroplasts.

In Chapter 5, evidence was presented which indicated that the 23 and 33 kd nuclear-encoded polypeptides of the OEC undergo two-step processing. When protein blots of thylakoid membranes isolated from mature lut-1 leaves were probed with polyclonal antisera specific for the 23 kd polypeptide, a 28 kd species was detected although the

mature 23 kd species was depleted. A similar analysis using antisera specific for the 33 kd polypeptide revealed a 34 kd species in mutant membranes depleted of the the mature 33 kd polypeptide. The two higher molecular weight species, at 28 and 34 kd, were larger than the fully processed mature proteins, but smaller than their cytoplasmically synthesized precursors. These intermediate forms were located close to the inner thylakoid surface, which is where the mature 23 and 33 kd polypeptides are found in WT chloroplasts. Based on its trypsin insensitivity and resistance to extraction by a high pH-high salt wash, the 28 kd intermediate was concluded to have more hydrophobic character than its mature 23 kd counterpart. The 34 kd intermediate behaved like its mature 33 kd counterpart.

The first processing step for nuclear-encoded polypeptides probably occurs after translocation of their precursor molecules through the chloroplast envelope. Based on the results reported in Chapter 5, I conclude that the 23 and 33 kd polypeptides undergo a second processing step which occurs after translocation through the thylakoid membrane and during assembly with the PSII core complex. Furthermore, I suggest that the 28 and 34 kd intermediates accumulated in lut-1 thylakoids because there were not enough PSII core centers present with which to associate.

B. Hypothesis

A mechanism for the lut-1 mutation must address three major questions. A) Why was PSII specifically affected? B) Why was the mutation developmentally expressed? and C) Why did extensive chlorosis occur? I offer the following answers to these questions.

PSII was prematurely degraded because a protease specific for a PSII component was effectively more active in mutant chloroplasts. This increased activity arose from either an alteration in the catalytic properties of the enzyme (e.g., a higher affinity for its substrate), an increased amount of enzyme, or a change in level or activity of a secondary molecule that regulated the activity of the protease. Because lut-1 is a plastome mutant, it follows that either this over-active protease is chloroplast-encoded, or the synthesis of the protease-regulating molecule is under control of the chloroplast genome. This concept of a PSII-specific protease leads to the idea that the turnover of PSII complexes is controlled independently of the other membrane protein complexes. It suggests a previously unrecognized means by which levels of PSII complexes could be modulated during changes in growth conditions (e.g., changes in light quality) or degraded during chloroplast senescence. It also points out that abnormal proteolytic activity in lut-1 may have been simply an aberrant maintenance process which was effectively accelerated.

The lut-1 mutation appeared in a developmental fashion because of the gradual reduction of PSII polypeptides. This temporal aspect of the mutation may be explained by recognizing that the net accumulation of proteins is the balance of two processes: synthesis and degradation. During rapid leaf expansion, the rate of protein

synthesis is high. This rate falls after leaves (and organelles) reach their mature size. Since the lut-1 mutation was responsible for a high level of proteolytic activity, the high degradation rate of PSII polypeptides outpaced and eventually surpassed the (unaffected) biosynthetic rate. Thus what appeared to be an inability to synthesize PSII polypeptides was actually an increase in their removal.

It is possible that the biosynthetic machinery of mutant chloroplasts sensed the imbalance in the synthesis and degradation of the PSII core complex because the rate of protein synthesis appeared to be greater in mutant chloroplasts than WT chloroplasts (this point was discussed in the summary section) in order to compensate for the proteins lost. The loss of PSII proteins also appeared to exert some control over the protein synthesis in the cytoplasm. The nuclear-encoded precursors of the 23 and 33 kd polypeptides continued to be synthesized and imported into mutant chloroplasts. However, as suggested, in the absence of the PSII core complex, the precursors underwent only one of their two processing steps. This resulted in the accumulation of their intermediate forms in mutant thylakoids.

PSII reaction center chl-protein complexes account for roughly 15 to 20 % of the total chl in the thylakoid membrane (84). If there were a depletion of only PSII complexes from thylakoids of mature lut-1 leaves, the reduction in chl content of mutant leaves would be expected to be only 20 %. However, mature mutant leaves contained less than 5 % of the chl in WT leaves of similar age. I suggest the severe chlorosis was likely caused by the photooxidation of chl. The majority of the antennae pigment-protein complexes serve to funnel

excitation energy to PSII (28). The normal transfer of excitation energy from the light-harvesting complexes to PSII centers was blocked in lut-1 thylakoids because PSII core complexes were depleted. This functional disconnection of antennae from PSII traps was shown by the room temperature and 77 K chl fluorescence data presented in Chapter 3. Incoming light energy absorbed by antennae chl molecules in mutant thylakoids might cause phootoxidation (i.e., damage to both proteins and lipids) because a large fraction of the light energy was not converted into photochemical energy, but dissipated within the chl pigment bed. The excited chl could sensitize photooxidation by activating molecular oxygen and transforming it into the highly reactive superoxide form. Carotenoids normally serve to protect thylakoids from chl-sensitized oxidation reactions. However, I conclude that the absence of PSII centers results in an excessive rate of chl deactivation in the antenna bed, and this was not adequately quenched by the available carotenoids.

It has not been possible to identify the exact moleuclar nature of the lut-1 mutation because of its pleiotropic biochemical effects and apparent developmental progression. However, the analysis of lut-1 is a strong example of how plastome mutants can elucidate the contribution made by the chloroplast genome to the ontogeny of the photosynthetic apparatus, and reveal processes that potentially regulate the stability and stoichiometry of chloroplast thylakoid components.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Ahrens WH, CJ Arntzen, EW Stoller 1981 Chlorophyll fluorescence assay for the determination of triazine resistance. Weed Sci 29:316-322

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

3. Allen JF, J Bennett, KE Steinback, CJ Arntzen 1981 Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation between photosystems. Nature 291:25-29

4. Alt J, J Morris, P Westhoff, RG Herrmann 1984 Nucleotide sequence of the clustered genes for the 44 kd chlorophyll a apoprotein and the "32" kd-like protein of the photosystem II reaction center in the spinach plastid chromosome. Curr Genet 8:597-606

5. Anderson JM 1982 The role of chlorophyll-protein complexes in the function and structure of chloroplast thylakoids. Mol Cell Biochem 46:161-172

6. Anderson JM, A Melis 1983 Localization of different photosystems in separate regions of chloroplast membranes. Proc Natl Acad Sci USA 80:745-749

7. Andersson B, C Jansson, U Ljungberg, H-E Akerlund 1985 Polypeptides in water oxidation. In KE Steinback, S Bonitz, CJ Arntzen, L Bogorad, eds, Molecular Biology of the Photosynthetic Apparatus. Cold Spring Harbor Laboratory, New York (in press)

8. Andersson B, C Larsson, C Jansson, U Ljungberg, H-E Akerlund 1984 Immunological studies on the organization of proteins in photosynthetic oxygen evolution. Biochim Biophys Acta 766:21-28

9. Arntzen, CJ 1978 Dynamic structural features of chloroplast lamellae. In L Vernon, R Sanadi, eds, Current Topics in Bioenergetics, Vol 8. Academic Press, New York, pp 111-160

10. Babcock, GT, WR Widger, WA Cramer, WA Oertling, JG Metz 1985 The axial ligands of chloroplast cytochrome b-559: Identification and requirement for a heme-crosslinked polypeptide structure. Biochem 24:3638-36

11. Baker NR 1984 Development of chloroplast photochemical functions. In NR Baker, J Barber, eds, Chloroplast Biogenesis. Elsevier, Amsterdam, pp 207-252

12. Barber J (ed) 1977 Primary Processes of Photosynthesis. Elsevier, Amsterdam

13. Barber J 1982 Influence of surface charges on thylakoid structure and function. Ann Rev Plant Physiol 33:261-295

14. Bedbrook JR, G Link, DM Coen, L Bogorad, A Rich 1978 Maize plastid gene expressed during photoregulated development. Proc Natl Acad Sci USA 75:3060-3064

15. Bengis C, N Nelson 1977 Subunit structure of chloroplast photosystem I reaction center. Biol Chem 252:4564-4572

16. Bennoun P, BA Diner, F-A Wollman, G Schmidt, N-H Chua 1981 Thylakoid polypeptides associated with photosystem II in Chlamydomonas reinhardtii: comparison of system II mutants and particles. In G Akoyunoglou, ed, Photosynthesis III. Structure and Molecular Organization of the Photosynthetic Apparatus. Balaban International Science Services, Philadelphia, pp 839-849

17. Berthold DA, GT Babcock, CF Yocum 1981 A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. EPR and electron-transport properties. FEBS Lett 134:231-234

18. Binder A, A Jagendorf, E Ngo 1978 Isolation and composition of the subunits of spinach chloroplast coupling factor protein. J Biol Chem 253:3094-3100

19. Bishop NI 1971 Preparation and properties of mutants: Scenedesmus. Methods Enzym 23:130-143

20. Bishop NI 1982 Isolation of mutants of Scenedesmus obliquus defective in photosynthesis. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 51-63

21. Borner T, Sears BB 1985 Plastome Mutants. Curr Genet (in press)

22. Bose S 1982 Chlorophyll fluorescence in green plants and energy transfer pathways in photosynthesis. Photochem Photobiol 36:725-731

23. Bottomley W, HJ Bohnert 1982 The biosynthesis of chloroplast proteins. In B Parthier, D Boulter, eds, Encyclopedia of Plant Physiology, 14B. Springer-Verlag, New York, pp 531-596

24. Bricker TM, JG Metz, D Miles, LA Sherman 1983 Biochemical characterization of a highly active O_2-evolving photosystem II preparation from maize. Biochim Biophys Acta 724:447-455

25. Bricker TM, HB Pakrasi, LA Sherman 1985 Characterization of a spinach photosystem II core preparation isolated by a simplified method. Arch Biochem Biophys 237:170-176

26. Burke JJ, KE Steinback, I Ohad, CJ Arntzen 1978 Control of photosynthetic Competence in the Y-1 mutant of Chlamydomonas rinhardi. In G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds, Chloroplast Development. Elsevier, Amsterdam, pp 413-418

27. Butler W 1977 Chlorophyll fluorescence: a probe for electron transfer and energy transfer. In A Trebst, M Avron, eds, Encyclopedia of Plant Physiology, Vol 5. Springer-Verlag, Berlin, pp 148-167

28. Butler, WL 1978 Energy distribution in the photochemical apparatus of photosynthesis. Ann Rev Plant Physiol 29:345-378

29. Camm EL, BR Green 1980 Fractionation of thylakoid membranes with the nonionic detergent octyl-β-glucopyranoside. Plant Physiol 66:428-432

30. Casadoro G, G Hoyer-Hansen, CG Kannangara, S Gogh 1983 An analysis of temperature and light sensitiviy in tigrina mutants of barley. Carlsberg Res Comm 48:95-129

31. Cashmore AR 1982 The isolation of poly-A^+ RNA from higher plants. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 387-392

32. Chua N-H, P Bennoun 1975 Thylakoid membrane polypeptides of Chlamydomonas reinhardtii: wild type and mutant strains deficient in the photosystem II reaction center. Proc Natl Acad Sci USA 72:2175-2179

33. Chua N-H, F Blomberg 1979 Immunochemical studies of thylakoid membrane polypeptides from spinach and Chlamydomonas reinhardtii. J Biol Chem 254:215-223

34. Chua N-H, N Gillham 1977 The sites of synthesis of the principal thylakoid membrane polypeptides in Chlamydomonas reinhardtii. J Cell Biol 74:441-452

35. Chua N-H, K Matlin, P Bennoun 1975 A chlorophyll-protein complex lacking in photosystem I mutants of Chlamydomonas reinhardtii. J Cell Biol 67:361-377

36. Cramer WA, AR Crofts 1982 Electron and proton transport. In Govindjee, ed, Photosynthesis. Energy Conversion by Plants and Bacteria. Academic Press, New York, pp 387-467

37. Crofts AR, CA Wraight 1983 The electrochemical domain of photosynthesis. Biochim Biophys Acta 726:149-185

38. Crouse EJ, JM Schmitt, H-J Bohnert 1985 Chloroplast and cyanobacterial genomes, genes and RNAs: a compilation. Plant Mol Biol Report 3:43-89

39. Darr SC, VS Machado, CJ Arntzen 1981 Uniparental inheritance of a chloroplast photosystem II polypeptide controlling herbicide binding. Biochim Biophys Acta 634:219-228

40. Dekker JP, HJ Van Gorkom, M Brok, L Ouwehand 1984 Optical characterization of photosystem II electron donors. Biochim Biophys Acta 764:301-309

41. Delepelaire P 1984 Partial characterization of the biosynthesis and integration of the photosystem II reaction centers in the thylakoid membranes of Chlamydomonas reinhardtii. EMBO J 3:701-706

42. Delepelaire P, N-H Chua 1979 Lithium dodecyl sulfate-polyacrylamide gel electrophoresis of thylakoid membranes. Proc Natl Acad Sci USA 72:2175-2179

43. de Vitry C, F-A Wollman, P Delepelaire 1984 Function of the polypeptides of the photosystem II reaction center in Chlamydomonas reinhardtii. Localization of the primary reactants. Biochim Biophys Acta 767:415-422.

44. Diner BA 1985 The reaction center of photosystem II. In LA Staehelin, CJ Arntzen, eds, Encyclopedia of Plant Physiology, Photosynthesis III. Springer-Verlag, Berlin (in press)

45. Driesel AJ, J Speirs, HJ Bohnert 1980 Spinach chloroplast mRNA for a 32000 dalton polypeptide. Size and location on the physical map of the chloroplast DNA. Biochim Biophys Acta 610:297-310

46. Douce R, J Joyard 1982 Purification of the chloroplast envelope. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 239-256

47. Dunahay TG, LA Staehelin, LA, M Siebert, PD Ogilvie, S Berg 1984 Structure, biochemical, and biophysical characterization of four oxygen-evolving photosystem II preparations from spinach. Biochim Biophys Acta 764:179-193

48. Dyer T 1984 The chloroplast genome: its nature and role in development. In NR Baker, J Barber, eds, Chloroplast Biogenesis. Elsevier, Amsterdam, pp 24-69

49. Eaglesham AR, RJ Ellis 1974 Protein synthesis in chloroplasts II. Light-driven synthesis of membrane proteins by isolated pea chloroplasts. Biochim Biophys Acta 335:396-407

50. Ellis RJ 1983 Chloroplast protein synthesis: principles and problems. In DB Roody, ed, Subcellular Biochemistry. 9:237-261

51. Erickson JM, M Rahire, P Bennoun, P Delepelaire, B Diner, J-D Rochaix 1984 Herbicide resistance in Chlamydomonas reinhardtii results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II. Proc Natl Acad Sci USA 81:3617-3621

52. Erickson JM, M Rahire, J-D Rochaix 1985 Herbicide resistance and cross-resistance: changes at three distinct sites in the herbicide-binding protein. Science 228:204-207

53. Forbush B, B Kok, M McGloin 1971 Cooperation of charges in photosynthetic O_2 evolution--II Damping of flash yield oscillation, deactivation. Photochem Photobiol 14:307-321

54. Gasser SM, G Daum, G Schatz 1982 Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J Biol Chem 257:13034-13041

55. Gasser SM, A Ohashi, G Daum, PC Bohni, J Gibson, CA 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

56. Glick RE, SW McCauley, A Melis 1985 Effect of light quality on chloroplast membrane organization and function in pea. Planta 164:487-494

57. Golden SS, R Haselkorn 1985 Mutation to herbicide resistance maps within the psbA gene of Anacystis nidulans R2. Science 229:1104-1107

58. Goloubinoff P, M Edelman, RB Hallick 1984 Chloroplast-coded atrazine resistance in Solanum nigrum: psbA loci from susceptible and resistant biotypes are isogenic except for a single codon change. Nucl Acid Res 12:9489-9496

59. Govindjee (ed) 1982 Photosynthesis. Energy Conversion by Plants and Bacteria, Vol 1. Academic Press, New York

60. Govindjee, T Kambara, W Coleman 1985 The electron donor side of photosystem II: the oxygen evolving complex. Photochem Photobiol 42:187-210

61. Govindjee, G Papageorgiou 1971 Chlorophyll fluorescence and photosynthesis: fluorescence transients. In AC Giese, ed, Photophysiology, Vol 6. Academic Press, New York, pp 1-46

62. Gray JC, SD Kung, SG Wildman, SJ Sheen 1974 Origin of Nicotiana tabacum detected by polypeptide composition of fraction I protein. Nature 252:226-227

63. Grebanier AE, DM Coen, A Rich, L Bogorad 1978 Membrane proteins synthesized but not processed by isolated maize chloroplasts. J Cell Biol 78:734-746

64. Hallick RB, W Bottomley 1983 Proposals for the naming of chloroplast genes. Plant Mol Biol Reporter 1:38-43

65. Hallier MW, UW Heber 1977 Cytochrome f deficient plastome mutants of Oenothera. Plant Cell Physiol 3:257-273

66. Haehnel W 1984 Photosynthetic electron transport in higher plants. Ann Rev Plant Physiol 35:659-693

67. Haworth P, JL Watson, CJ Arntzen 1983 The detection, isolation and characterization of a light-harvesting complex which is specifically associated with photosystem I. Biochim Biophys Acta 724:151-158

68. Henningsen KW, BM Stummann 1982 Use of mutants in the study of chloroplast biogenesis. In B Parthier, D Boulter, eds, Encyl Plant Physiol, New Series Vol 14B. Springer-Verlag, New York, pp 597-644

69. Herrmann FH, D Matorin, K Timofeev, T Borner, AB Rubin, R Hagemann 1974 Studies on primary reactions of photosynthesis in plastome mutants of Antirrhinum majus and Pelargonium zonale having impaired photosynthesis. Biochem Physiol Pflanz 165:393-400

70. Herrmann FH, B Schumann, T Borner, R Knoth 1976 Struktur and Funktion der genetischen Information in der Plastiden. Photosynthetica 10:1654-1671

71. Herrmann RG 1982 The Preparation of circular DNA from plastids. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 259-280

72. Herrmann RG, J Alt, B Schiller, WR Widger and WA Cramer 1984 Nucleotide sequence of the gene for apocytochrome b-559 on the spinach plastid chromosome: implications for the structure of the membrane protein. FEBS Lett 176:239-244

73. Herrmann RG, PR Whitfeld 1982 Restriction mapping of chloroplast DNA using low-melting-temperature agarose. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 451-468

74. Hiller RG, BL Moller, G Hoyer-Hansen 1980 Characterization of six putative photosystem I barley mutants. Carlsberg Res Comm 45:315-328

75. Hirschberg J, L McIntosh 1983 Molecular basis of herbicide resistance in Amaranthus hybridus. Science 222:1346-1349

76. Hirschberg J, A Bleecker, DJ Kyle, L McIntosh, CJ Arntzen 1983 Molecular basis of triazine-resistance in higher-plant chloroplasts. Z Naturforsch 39c:412-420

77. Holschuh K, W Bottomley, PR Whitfeld 1984 Structure of the spinach chloroplast genes for the D2 and 44 kd reaction-centre proteins of photosystem II and for $tRNA^{Ser}$ (UGA). Nucl Acids Res 12:8819-8834

78. Horton P 1983 Relations between electron transport and carbon assimilation; simultaneous measurement of chlorophyll fluorescence, transthylakoid pH gradient and O_2 evolution in isolated chloroplasts. Proc R Soc Lond (B) 217:405-416

79. Hurt E, G Hauska 1981 A cytochrome f/b_6 complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chlroplasts. Eur J Biochem 117:591-599

80. Inamine G, B Nash, H Weisbach, N Brot 1985 Light regulation of the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in peas: evidence for translational control. Proc Natl Acad Sci USA 82:5690-5694

81. Izawa S 1980 Acceptors and donors for chloroplast electron transport. Methods Enzymol 69:413-433

82. Izawa S, RL Pan 1978 Photosystem I electron transport and phosphorylation supported by electron donation to the plastoquinone region. Biochem Biophys Res Comm 83:1171-1177

83. Joliot P, P Bennoun, A Joliot 1973 New evidence supporting energy transfer between photosynthetic units. Biochim Biophys Acta 305:317-328

84. Kaplan S, CJ Arntzen 1982 Photosynthetic membrane structure and function. In Govindjee, ed, Photosynthesis. Energy conversion by plants and bacteria, Vol. 1. Academic Press, New York, pp 65-151

85. Karlson U, RL Schultz 1965 Fixation of central nervous system for electron microscopy by aldehyde perfusion. I. Preservation with aldehyde perfusates versus direct perfusion with osmium tetroxide with special reference to membranes and extracellular space. J Ultra Res 12:160-186

86. Kirk JTO 1978 Genetic control and plastid biochemistry. In JTO Kirk, RAE Tilney-Bassett, eds, The Plastids. Elsevier, Amsterdam, pp 560-614

87. Koivuniemi P, NE Tolbert, PS Carlson 1981 Characterization of the thylakoid membranes of the tobacco aurea mutant Su/su and of three green revertant plants. Planta 151:40-47

88. Kok B, B Forbush, M McGloin 1970 Cooperation of charges in photosynthetic O_2 evolution. A linear four step mechanism. Photochem Photobiol 11:457-475

89. Krause GH, J-M Briantais, C Vernotte 1983 Characterization of chlorophyll fluorescence quenching in chloroplasts by fluorescence spectroscopy at 77 K I. pH-dependent quenching. Biochim Biophys Acta 723:169-175

90. Kung SD, JP Thornber, SG Wildman 1972 Nuclear DNA codes for the photosystem II chlorophyll-protein of chloroplast membranes. FEBS Lett 24:185-188

91. Kuwabara T, N Murata 1982 Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the photosystem II particles of spinach chloroplasts. Plant Cell Physiol 23:533-539

92. Kuwabara T, N Murata 1983 Quantitative analysis of the inactivation of photosynthetic oxygen evolution and the release of polypeptides and manganese in the photosystem II particles of spinach chloropalsts. Plant Cell Physiol 24:741-747

93. Kyle DJ 1985 The 32000 dalton Q_B protein of photosystem II. Photochem Photobiol 41:107-116

94. Kyle DJ, P Haworth, CJ Arntzen 1982 Thylakoid membrane protein phosphorylation leads to a decrease in connectivity between photosystem II reaction centers. Biochim Biophys Acta 680:336-342

95. Kyle DJ, LA Staehelin, CJ Arntzen 1983 Lateral mobility of the light-harvesting complex in chloroplast membranes controls excitation energy distribution in higher plants. Arch Biochim Biophys 222:527-541

96. Kyle DJ, S Zalik 1982 Development of photochemical activity in relation to pigment and membrane accumulation in chloroplasts of barley and its virescens mutant. Plant Physiol 69:1392-1400

97. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

98. Lam E, W Ortiz, R Malkin 1984 Chlorophyll a/b proteins of photosystem I. FEBS Lett 168:10-14

99. Lavorel J, AL Etienne 1977 In vivo chlorophyll fluorescence. In J Barber, ed, Primary Processes of Photosynthesis. Elsevier, Amsterdam, pp 203-268

100. Leary JJ, DJ Brigati, DC Ward 1983 Rapid and sensitive colorimetric method for visualizing biotin labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc Natl Acad Sci 80:4045-4049

101. Leong T-Y, JM Anderson 1983 Changes in composition and function of thylakoid membranes as a result of photosynthetic adaptation of chloroplasts from pea plants grown under different light conditions. Biochim Biophys Acta 723:391-399

102. Leto K, CJ Arntzen 1981 Cation-mediated regulation of excitation energy distribution in chloroplasts lacking organized photosystem II complexes. Biochim Biophys Acta 637:107-117

103. Leto KJ, E Bell, L McIntosh 1985 Nuclear mutation leads to an accelerated turnover of chloroplast-encoded 48 kd and 34.5 kd polypeptides in thylakoids lacking photosystem II. EMBO J 4:1645-1653

104. Leto KJ, A Keresztes, CJ Arntzen 1982 Nuclear Involvement in the appearance of a chloroplast-encoded 32,000 dalton thylakoid membrane polypeptide integral to the photosystem II complex. Plant Physiol 69:1450-1458

105. Levine 1971 Preparation and properties of mutant strains of Chlamydomonas reinhardi. Methods Enzymol 23:119-129

106. Ljungberg U, H-E Akerlund, C Larsson, B Andersson 1984 Identification of polypeptides associated with the 23 and 33 kDa proteins of photosynthetic oxygen evolution. Biochim Biophys Acta 767:145-152

107. MacKinney G 1941 Absorption of light by chlorophyll solutions. J Biol Chem 140:315-322

108. Maniatis T, EF Fritsch, J Sambrook 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York

109. Markwell MAK, SM Haas, LL Bieber, NE Tolbert 1978 A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206-210

110. Mattoo AK, H Hoffman-Falk, JB Marder, M Edelman 1984 Regulation of protein metabolism: coupling of photosynthetic electron transport to in vivo degradation of the rapidly-metabolized 32,000-dalton protein of the chloroplast membranes. Proc Natl Acad Sci USA 81:1380-1384

111. Melis A 1984 Light regulation of photosynthetic membrane structure, organization, and function. J Cell Biochem 24:271-285

112. Melis A 1985 Functional properties of photosystem II_β in spinach chloroplasts. Biochim Biophys Acta 808:334-342

113. Melis A, APGM Thielen 1980 The relative cross-section of photosystem I and photosystem II in chloroplasts from three types of Nicotiana tabacum. Biochim Biophys Acta 589:275-286

114. Metz JG, D Miles 1982 Use of a nuclear mutant of maize to identify components of photosystem II. Biochim Biophys Acta 681:95-102

115. Metz JG, J Wong, NI Bishop 1980 Changes in the electrophoretic mobility of a chloroplast membrane polypeptide associated with the loss of the oxidizing side of photosystem II in low fluorescent mutants of Scenedesmus. FEBS Lett 114:61-66

116. Miles D 1980 Mutants of higher plants: maize. Methods Enzymol 69:3-23

117. Miles D 1982 The use of mutations to probe photosynthesis in higher plants. In M Edelman, RB Hallick,N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 75-107

118. Miller KR, RA Cushman 1979 A chloroplast membrane lacking photosystem II. Thylakoid stacking in the absence of the photosystem II particle. Biochim Biophys Acta 546:481-497

119. Mishkind ML, SR Wessler, GW Schmidt 1985 Functional determinants in transit sequences: import and partial maturation by vascular plant chloroplasts of the ribulose-1,5-bisphosphate carboxylase small subunit of Chlamydomonas. J Cell Biol 100:226-234

120. Mishkind JE, GW Schmidt 1983 Postranscriptional regulation of ribulose 1,5-bisphosphate carboxylase small subunit accumulation in Chlamydomonas reinhardtii. Plant Physiol 72:847-854

121. Morris J, RG Herrmann 1984 Nucleotide sequence of the gene for the P680 chlorophyll a apoprotein of the photosystem II reaction center from spinach. Nucl Acids Res 12:2837-2850

122. Mullet JE, JJ Burke, CJ Arntzen 1980 Chlorophyll-proteins of photosystem I. Plant Physiol 65:814-822

123. Mullet JE, AR Grossman, N-H Chua 1982 Synthesis and assembly of the polypeptide subunits of photosystem I. Cold Spring Harbor Symp Quant Biol 46:979-984

124. Nakatani HY 1984 Photosynthetic oxygen evolution does not require the participation of polypeptides of 16 and 24 kilodaltons. Biochem Biophys Res Commun 120:299-304

125. Nakatani HY, B Ke, E Dolan, CJ Arntzen 1984 Identity of the photosystem II reaction center complex polypeptide. Biochim Biophys Acta 765:347-352

126. Ohad I, G Drews 1982 Biogenesis of the photosynthetic apparatus in prokaryotes and eukaryotes. In Govindjee, ed, Photosynthesis II. Academic Press, New York, pp 89-140

127. Ohad I, DJ Kyle, CJ Arntzen 1984 Membrane protein damage and repair: removal and replacement of inactivated 32 kilodalton polypeptides in chloroplast membranes. J Cell Biol 99:481-485

128. O'Malley PJ, GT Babcock, RC Prince 1984 The cationic plastoquinone radical of the chloroplast water-splitting complex. Hyperfine splitting from a single methyl group determines the EPR spectral shape of signal II. Biochim Biophys Acta 765:283-288

129. Olive J, F-A Wollman, P Bennoun, M Recouvreur 1979 Ultrastructure-function relationships in Chlamydomonas reinhardtii thylakoids, by means of a comparison between the wild type and the F34 mutant which lacks the photosystem II reaction center. Molec Biol Rep 5:139-143

130. Papageorgiou G 1975 Chlorophyll fluorescence: an intrinsic probe of photosythesis. In Govindjee, ed, Bioenergetics of photosythesis. Academic Press, New York, pp 319-371

131. Paterson DR, CJ Arntzen 1982 Detection of altered inhibition of photosytem II reactions in herbicide-resistant plants. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology, Elsevier, Amsterdam, pp 109-118

132. Powles SB 1984 Photoinhibition of photosynthesis induced by visible light. Ann Rev Plant Physiol 35:15-44

133. Rasmussen OF, G Bookjans, BM Stumann, KW Henningsen 1984 Localization and nucleotide sequence of the gene for the membrane polypeptide D2 from pea chloroplast DNA. Plant Mol Biol 3:191-199

134. Robertson DS, IC Anderson, MD Bachman 1978 Pigment deficient mutants: genetic, biochemical, and developmental studies. In DB Walden, ed, Maize breeding and genetics. John Wiley & Sons, New York, pp 461-494

135. Robinson C, RJ Ellis 1984 Transport of proteins into chloroplasts. The precursor of small subunit of ribulose bisphosphate carboxylase is processed to the mature size in two steps. Eur J Biochem 142:343-346

136. Rochaix J-D 1981 Organization, function and expression of the chloroplast DNA of Chlamydomonas reinhardtii. Experientia 37:323-332

137. Rochaix J-D, M Dron, M Rahire, P Malnoe 1984 Sequence homology between the 32K dalton and the D2 chloroplast membrane polypeptide of Chlamydomonas reinhartdii. Plant Mol Biol 3:363-370

138. Rochaix J-D, P Malnoe 1982 Use of DNA-RNA hybridizations for locating chloroplast genes and for estimating the size and abundance of chloroplast DNA transcripts. In M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier, Amsterdam, pp 477-490

139. Rodermel SR, L Bogorad 1985 Maize plastid photogenes: mapping and photoregulation of transcript levels during light-induced development. J Cell Biol 100:463-376

140. Satoh K, HY Nakatani, KE Steinback, JL Watson, CJ Arntzen 1983 Polypeptide composition of a photosystem II core complex: presence of a herbicide binding protein. Biochim Biophys Acta 724:142-150

141. Schmidt RJ, NW Gillham, JE 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 protein made in the chloroplast. Mol Cell Biol 5:1093-1099

142. Schmidt RJ, AM Myers, NW Gillham, JE Boynton 1984 Chloroplast ribosomal proteins of Chlamydomonas synthesized in the cytoplasm are made as precursors. J Cell Biol 98:2011-2018

143. Sears BB, Herrmann 1985 Plastome mutation affecting the chloroplast ATP synthase involves a post-transcriptional defect. Curr Genet 9:521-528

144. Sjostrand FS 1967 The preparation of specimens by chemical fixation. In Electron Microscopy of Cells and Tissues, Instrumentation and Techniques. Academic Press, New York, pp 145-

145. Somerville CR 1986 Analysis of photosynthesis with mutants of higher plants and algae. Ann Rev Plant Physiol (in press)

146. Staehelin LA, PA Armond, KR Miller 1976 Chloroplast membrane organization at the supramolecular level and its functional implications. Brookhavem Symp Biol 28:316-317

147. Staehelin LA, CJ Arntzen (eds) 1985 Photosynthesis III. Encyclopedia of Plant Physiology. Springer-Verlag, Berlin

148. Staehelin LA, CJ Arntzen 1983 Regulation of chloroplast membrane function: protein phosphorylation changes the spatial organization of membrane components. J Cell Biol 97:1327-1337

149. Steinback KE, S Bose, DJ Kyle 1982 Phosphorylation of the light-harvesting chlorophyll-protein regulates excitation energy distribution between photosystem II and photosystem I. Arch Biochem Biophys 216:356-361

150. Steinback KE, JJ Burke, CJ 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 195:546-557

151. Steinback KE, L McIntosh, L Bogorad, CJ Arntzen 1981 Identification of the triazine receptor protein as a chloroplast gene product. Proc Natl Acad Sci USA 7463-7467

152. Strobaek S, GC Gibbons, B Haslett, D Boulter, SG Wildman 1976 On the nature of the polymorphism of the small subunit of ribulose-1,5-diphosphate carboxylase in the amphidiploid Nicotiana tabacum. Carlsberg Res Comm 41:335-343

153. Strotmann H, S Bickel-Sandkotter 1984 Structure, function, and regulation of chloroplast ATPase. Ann Rev Plant Physiol 35:97-120

154. Sugita M, M Sugiura 1984 Nucleotide sequence and transcription of the gene for the 32,000 dalton thylakoid membrane protein from Nicotiana tabacum. Mol Gen Genet 195:308-313

155. Thielen APGM, HJ van Gorkom 1981 Redox potentials of electron acceptors in Photosystem II_{α} and II_{β}. FEBS Lett 129:205-209

156. Thomas PE, D Ryan, W Levin 1976 An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal Biochem 75:186-176

157. Thomas PS 1980 Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201-5205

158. Tilney-Bassett RAE 1978 The inheritance and genetic behavoir of plastids. In JTO Kirk, RAE Tilney-Bassett, eds, The Plastids, Their Chemistry, Structure, Growth and Inheritance. Elsevier, Amsterdam, pp 251-524

159. Tobin EM, J Silverthorne 1985 Light regulation of gene expression in higher plants. Ann Rev Plant Physiol 36:569-593

160. 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

161. Trebst A 1972 Measurement of Hill reactions and photoreduction. Methods Enzymol 24:146-165

162. Vermaas WFJ, CJ Arntzen 1983 Synthetic quinones influencing herbicide binding and photosystem II electron transport. The effects of triazine-resistance on quinone binding properties in thylakoid membranes. Biochim Biophys Acta 725:483-491

163. Vermaas WFJ, CJ Arntzen, L-Q Gu, C-A Yu 1983 Interactions of herbicides and azidoquinone at a photosystem II binding site in the thylakoid membrane. Biochim Biophys Acta 723:266-275

163a. Vermaas, WFJ, G Renger, G Dohnt 1984 The reduction of the oxygen-evolving system in chloroplasts by thylakoid components. Biochim Biophys Acta 764:194-202

164. von Wettstein D, BL Moller, G Hoyer-Hansen, D Simpson 1982 Mutants in the analysis of the photosynthetic membrane polypeptides. In JA Schiff, ed, On the Origins of Chloroplasts. Elsevier, Amsterdam, pp 263-273

165. Wahl GM, M Stern, R Stark 1979 Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc Natl Acad Sci USA 76:3683-3687

166. Westhoff P, J Alt, RG Herrmann 1983 Localization of the genes for the two chlorophyll a-conjugated polypeptides (mol wt 51 and 44 kd) of the photosystem II reaction center on the spinach plastid chromosome. EMBO J: 2229-2237

167. Westhoff P, J Alt, WR Widger, WA Cramer, RG Herrmann 1985 Localization of the gene for apocytochrome b-559 on the plastid chromosome of spinach. Plant Mol Biol 4:103-110

168. Westhoff P, C Jansson, L Kelin-Hitpaß, R Berzborn, C Larsson, SG Bartlett 1985 Intracellular coding sites of polypeptides associated with photosynthetic oxygen evolution of photosystem II. Plant Mol Biol 4:137-146

169. Westhoff P, N Nelson, H Bunemann, RG Herrmann 1981 Localization of genes for coupling factor subunits on the spinach plastid chromosome. Curr Genet 4:109-120

170. Whitfeld PR, W Bottomley 1983 Organization and structure of chloroplast genes. Ann Rev Plant Physiol 34:279-310

171. Widger WR, WA Cramer, M Hermodson, D Meyer, M Guillifor 1984 Purification and partial amino acid sequence of the chloroplast cytochrome b-559. J Biol Chem 259:3870-3876

172. Williams JC, LA Steiner, G Feher, MI Simon 1984 Primary structure of the L subunit of the reaction center from Rhodopseudomonas sphaeroides. Proc Natl Acad Sci USA 81:7303-7307

173. Williams JC, LA Steiner, RC Ogden, MI Simon, G Feher 1983 Primary structure of the M subunit of the reaction center from Rhodopseudomonas sphaeroides. Proc Natl Acad Sci USA 80:6505-6509

174. Wollman F-A, J Olive, P Bennoun, M Recouvreur 1980 Organization of the photosystem II centers and their associated antennae in thylakoid membranes: a comparative ultrastructural, biochemical, and biophysical study of Chlamydomonas reinhardtii wild type and mutants lacking in photosystem II reaction centers. J Cell Biol 87:728-735

175. Wydrzynski TJ 1982 Oxygen evolution in photosynthesis. In Govindjee, ed, Photosynthesis. Energy conversion by plants and bacteria. Academic Press, New York, pp 469-506

176. Yamagishi A, S Katoh 1984 A photoactive photosystem-II reaction center complex lacking a chlorophyll-binding 40 kilodalton subunit from the thermophilic cyanobacterium Synechococcus sp. Biochim Biophys Acta 765:118-124

177. Yamagishi A, S Katoh 1985 Further characterization of the two photosystem II reaction center complex preparations from the thermophilic cyanobacterium Synechococcus sp. Biochim Biophys Acta 807:74-80

178. Youvan DC, EJ Bylina, M Alberti, H Begusch, JE Hearst 1984 Nucleotide and deduced amino acid polypeptide sequences of the photosynthetic-reaction, B870 antenna, and flanking polypeptides from Rhodopseudomonas capsulata. Cell 37:949-957

179. Zielinski RE, Price CA 1980 Synthesis of thylakoid membrane proteins by chloroplasts isolated from spinach: cytochrome b-559 and P700-chlorophyll a-protein. J Cell Biol 85:435-445

180 Zurawski G, HJ Bohnert, PR Whitfeld, W Bottomley 1982 Nucleotide sequence of the gene for the M_r 32,000 thylakoid membrane protein from Spinacia oleracea and Nicotiana debneyi predicts a totally conserved primary translation product of M_r 38,950. Proc Natl Acad Sci USA 79:7699-7703