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Biosynthesis of the Chloroplast Cytochrome b_6f Complex: Studies in a Photosynthetic Mutant of *Lemna*

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The biosynthesis of the cytochrome b_6f complex has been studied in a mutant, no. 1073, of *Lemna perpusilla* that contained less than 1% of the four protein subunits when compared with a wild-type strain. RNA gel blot analyses of the mutant indicated that the chloroplast genes for cytochrome *f*, cytochrome b_6, and subunit IV (*petA*, *petB*, and *petD*, respectively) are transcribed and that the *petB* and *petD* transcripts undergo their normal processing. Analysis of polysomal polyA$^+$ RNA indicated that the level of translationally active mRNA for the nuclear-encoded Rieske Fe-S protein (*petC*) was reduced by >100-fold in the mutant. Immunoprecipitation of in vivo labeled proteins indicated that both cytochrome *f* and subunit IV are synthesized and that subunit IV has a 10-fold higher rate of protein turnover in the mutant. These results are discussed in terms of the assembly of the cytochrome complex and the key role of the Rieske Fe-S protein in this process.

INTRODUCTION

All organisms capable of photosynthetic autotrophic metabolism or heterotrophic respiratory metabolism share the ability to couple electron transport to proton translocation across a membrane. In all cases, this coupled electron transport is carried out in distinct multisubunit membrane complexes. The most widespread and well studied of these complexes is the cytochrome bc_1 (complex III) and b_6f class of electron transport complexes. These complexes function as quinol/cytochrome *c* (plastocyanin) oxidoreductases. In all known organisms, this complex contains a *b*-type cytochrome containing two hemes of different potentials, a *c*-type cytochrome, and a high potential 2Fe-2S center bound by a protein known as a Rieske Fe-S protein (Malkin, 1988). In chloroplasts, there is an additional subunit (subunit IV) that has been shown to bind plastoquinone (Doyle et al., 1989). In mitochondria and purple bacteria, the protein sequence homologous to subunit IV has become fused with the cytochrome *b* protein, yielding a larger protein with a possible added role in quinone binding (Hauska et al., 1988).

Although extensive studies have been carried out on the mechanism of electron transfer and proton translocation catalyzed by cytochrome bc_1/b_6f complexes (Rich, 1986), the mechanism by which these complexes are assembled has only started to receive attention. Recent studies have begun to investigate the questions of how these proteins are inserted into a membrane, when and where prosthetic groups are attached, and what directs the assembly of the individual subunits into a functional complex.

The present work considers the mechanism of assembly of the chloroplast cytochrome b_6f complex using a photosynthetic mutant of the higher plant *Lemna perpusilla* no. 1073. This mutant was originally isolated as a flowering mutant, and subsequent biochemical characterization indicated that it was a photosynthetic mutant lacking the thylakoid membrane cytochrome b_6f complex (Shahak et al., 1976; Malkin and Posner, 1978; Lam and Malkin, 1985). However, this mutant was not defective in heme biosynthesis or in heme insertion because its thylakoids showed normal amounts of another chloroplast cytochrome, cytochrome *b*-559. Lam and Malkin (1985) also used protein gel blotting to show that not only were the prosthetic groups lacking in the mutant but that the apoproteins for cytochrome *f*, cytochrome b_6, Rieske Fe-S protein, and subunit IV were absent.

The present work considers the mRNA levels for the cytochrome complex subunits, the transient synthesis of the corresponding polypeptides, and the fate of these proteins in the thylakoid membrane of *L. perpusilla* no. 1073. These results are considered in relation to the mechanism of assembly of this multimeric complex in thylakoids.

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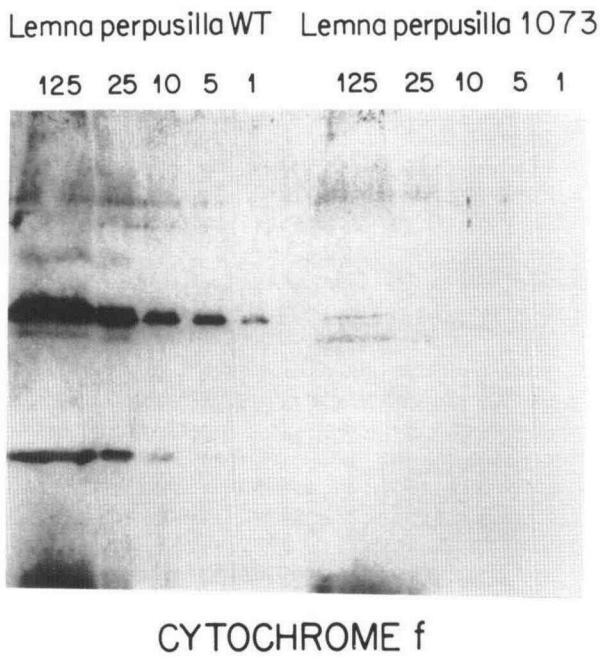

Figure 1. Quantitative Protein Blot Analysis of *L. perpusilla* Wild-Type (WT) and Mutant (1073) Thylakoids for Cytochrome *f*.

Proteins were resolved by SDS-PAGE and electroblotted onto nitrocellulose. The blot was probed with antiserum raised against spinach cytochrome *f*. The numbers represent the amount of chlorophyll in micrograms loaded in each lane. The antibody was visualized with goat anti-rabbit horseradish peroxidase.

RESULTS

Protein Gel Blot Analysis of Thylakoid Proteins in *Lemna*

To determine accurately the level of the subunits of the cytochrome complex in *L. perpusilla* no. 1073 versus the wild-type *L. perpusilla*, quantitative protein gel blotting was done. By varying the amount of protein loaded onto the gel before blotting, it was possible to compare the signals of the wild type and mutant and, thus, provide an estimate on the relative amounts of protein that accumulated in the respective thylakoids. These blots were normalized on the basis of the amount of chlorophyll loaded. Figure 1 shows a quantitative protein gel blot using a cytochrome *f* antibody from spinach. By comparing the signal intensity from the 1 μg lane of the wild type versus the 125 μg lane for the mutant, it was possible to conclude that cytochrome *f* accumulated in the mutant thylakoids to <1% of the level that it occurred in the wild-type thylakoids. Extending this type of analysis to the other subunits of the cytochrome complex, as shown in Figure 2, indicated that in the mutant, cytochrome b_6 accumulated to <1%, subunit IV accumulated to <3%, and the Rieske protein accumulated to <2% of the levels of these subunits relative to wild-type membranes. The amount of another photosynthetic membrane protein, light-harvesting chlorophyll-protein complex II (LHCP II), that accumulated in wild-type and mutant thylakoids was identical (data not shown), although there was a slight decrease in the amount of subunit II of photosystem I (the *psa*D gene product) in the mutant (see Figure 2).

RNA Gel Blot Analysis of the Cytochrome b_6f Genes

To determine whether the lack of protein accumulation in the mutant membranes was due to a failure in mRNA accumulation, RNA gel blot analyses were done on total RNA and a polysomal polyA$^+$ RNA fraction. Figure 3 shows the results of an analysis of the chloroplast-encoded subunits of the cytochrome b_6f complex. This blot contained equal amounts of total RNA that was ethanol precipitated before loading onto the gel. Therefore, the slight differences in intensity cannot necessarily be interpreted as a difference in the absolute RNA levels. It is clear from this figure that all plants, including the closely related *L. gibba*, accumulated a single predominant mRNA transcript of approximately 4.1 kb for *pet*A (cytochrome *f*).

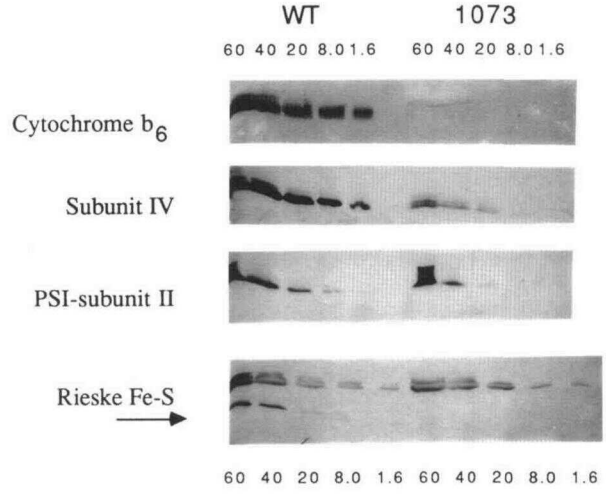

Figure 2. Quantitative Protein Blot Analysis of *L. perpusilla* Wild-Type (WT) and Mutant (1073) Thylakoids for Subunits of the Cytochrome b_6f Complex.

Analyses were carried out as in Figure 1 using spinach antibodies raised against cytochrome b_6, subunit IV, the Rieske Fe-S protein, and, as a control, subunit II (the *psa*D gene product) of spinach chloroplast photosystem I (PSI). Because of contamination by nonspecific reactions with the Rieske antibody, the presence of the cross-reacting product is indicated by the arrow in the bottom panel.

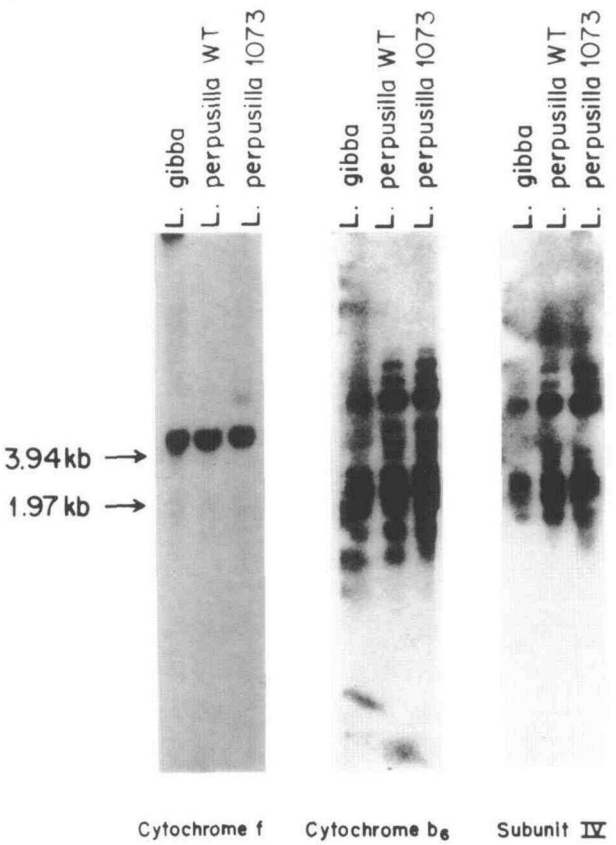

Figure 3. RNA Gel Blot Analysis of Chloroplast-Encoded Cytochrome b_6f Components.

Whole *Lemna* tissue was frozen in liquid nitrogen before RNA isolation. Total RNA was isolated using a guanidinium/phenol procedure. Twenty micrograms of total RNA was denatured and subjected to formaldehyde agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized with one of the following ^{32}P hexamer-labeled cDNA clones: a 400-bp fragment encoding pea cytochrome *f* (courtesy of Dr. A. Barkan), a 300-bp fragment encoding spinach cytochrome b_6 (courtesy of Dr. H. Bohnert), and a 420-bp fragment encoding pea subunit subunit IV (courtesy of Dr. A. Barkan). Hybridization was at 42°C in 5 × SSC, 40% formamide for 48 hr. mRNA sizes are based on isolated avocado ribosomal RNA standards (courtesy of Dr. M. Tucker). WT, wild type; 1073, mutant.

There was, in addition, a second, larger minor band found in the mutant. Whether this band represents a second, larger *pet*A transcript is not clear. From the intensity of the 4.1-kb band, it would appear that the *pet*A transcript accumulated to the same level in all three plants.

Equivalent blots were probed with clones that are specific for the coding region for *pet*B (cytochrome b_6) and *pet*D (subunit IV). Both of these probes detected at least 12 different transcripts in all three organisms. The large number of different sized transcripts for both of these genes was a result of their cotranscription into a single large polycistronic transcript that subsequently underwent a complex set of post-transcription processing and splicing steps. This cotranscription and post-transcriptional processing has been observed in maize, spinach, and pea (Heinemeyer et al., 1984; Berends et al., 1986; Rock et al., 1987). It is interesting to note that the mRNA accumulation for both of these genes in the mutant appears to be about twice that of the wild type. Whether this is due to an increase in transcriptional activity or mRNA stability is not known. From these studies, it can be concluded that transcripts for all three chloroplast cytochrome complex genes accumulate in the *L. perpusilla* mutant.

Although there was accumulation of mRNAs for the chloroplast genes in the preceding experiment, it was still possible that the mutant was defective in the transcription or accumulation of the mRNA for the nuclear-encoded subunit, the Rieske Fe-S protein. In fact, RNA gel blot analysis of total RNA for the Rieske Fe-S transcript indicated that the mRNA level in the mutant was <10% of that found in the wild type (data not shown). However, this signal was quite low and required hybridizations under low-stringency conditions.

To increase the hybridization signal, polysomal polyA$^+$ RNA was isolated from the mutant and wild type. The result of this RNA gel blot analysis is shown in Figure 4. Equal loadings of RNA were used to allow for direct comparison between transcript accumulation in the wild type and mutant. After a 1-day exposure, a single transcript was seen in the wild type, but only a very faint band was observed in the mutant. After a 5-day exposure, a signal was observed in the mutant with the same molecular weight as in the wild type. Scanning densitometry of the 1-day exposure indicated that the transcript in the mutant only accumulated to 0.8% of the level in the wild type. RNA dot blots gave a value of about 1% for the mRNA accumulation in the mutant (data not shown). Whether the lack of mRNA accumulation was due to an effect on transcription activity or on the stability of the mRNA is not known because run-on transcription analysis has not been done. However, RNA gel blot analysis of two other nuclear photosynthetic genes, *cab* and *rbc*S, encoding LHCP II and the small subunit of ribulose-1,5-bisphosphate carboxylase, showed no change in the mRNA level between the mutant and wild-type strains (data not shown). Therefore, it can be concluded that the lesion in this mutant does not affect the accumulation of mRNA for all nuclear-photosynthetic genes.

Immunoprecipitation of Thylakoid Proteins

Because RNA gel blot analyses indicated that mRNAs for the chloroplast subunits of the cytochrome complex accumulated in the *L. perpusilla* mutant, it was of interest to determine whether these transcripts were translationally

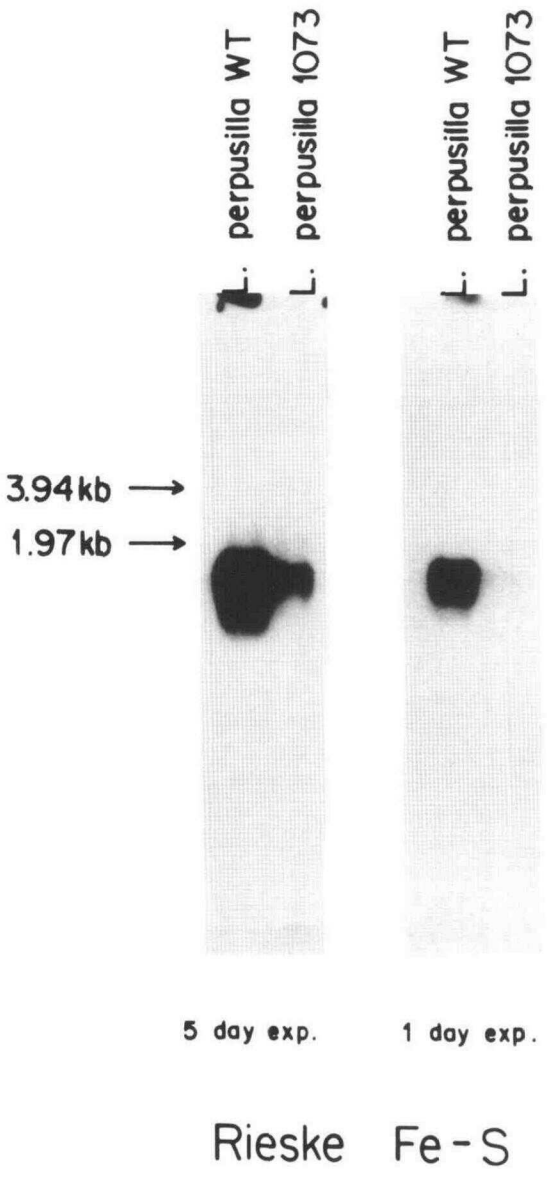

Figure 4. RNA Gel Blot Analysis of the Rieske Fe-S Protein.

RNA gel blot analyses were carried out as in Figure 3 using polysomal polyA$^+$ RNA, isolated using the procedure of Lincoln et al. (1987). This blot was probed with an 850-bp EcoRI fragment encoding pea Rieske Fe-S protein (courtesy of Dr. J. Gray). WT, wild type; 1073, mutant.

active. To study this, we developed a procedure to immunoprecipitate a specific protein from entire in vivo labeled plants. This procedure is very sensitive and can detect proteins that are made in very small amounts and/or are very rapidly turned over. As shown in Figure 5, by measuring the levels of immunoprecipitated proteins using polyclonal antibodies raised against spinach proteins, we were able to show that the mutant is active in the synthesis of cytochrome *f* and subunit IV of the cytochrome complex (molecular masses of 33 kD and 17 kD, respectively), although in the case of cytochrome *f*, the level of this protein accumulated to a lower level than that observed in the wild-type strain. This result may be related to the more rapid rate of turnover of the subunits observed in the mutant strain (see below). As a control in these experiments, the syntheses of LHCP II (molecular mass of 28 kD) and plastocyanin (data not shown) were measured by the immunoprecipitation procedure, and the results shown in Figure 5 indicate that LHCP II is synthesized at comparable levels in both strains. The complex blotting pattern seen with the LHCP II antisera, such as the diffuse band at 33 kD, is probably a contamination of D-1 and D-2 from photosystem II that cross-reacts with the antisera.

When similar experiments were done using polyclonal antisera raised against spinach cytochrome b_6 or Rieske

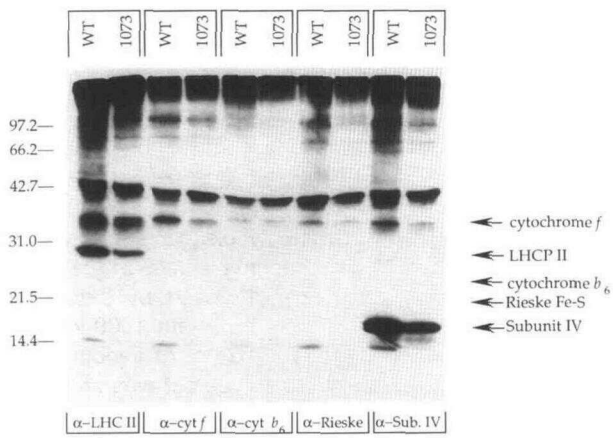

Figure 5. Synthesis of Chloroplast Membrane Proteins by *L. perpusilla*.

Intact *L. perpusilla* wild-type (WT) and mutant no. 1073 (1073) plants (five to 10 individual plantlets) were labeled for 2 hr in low light with 1 mC of ^{35}S-TransLabel (ICN, Costa Mesa, CA). Whole plants were homogenized and total proteins were acetone precipitated. This total plant protein fraction was resuspended and denatured with 2% SDS. This total protein suspension was precleared with 80 μL of preimmune serum and 100 μL of protein A-agarose beads (Bio-Rad). Specific proteins were immunoprecipitated by the addition of polyclonal antisera raised against spinach proteins designated LHCP II (10 μL), cytochrome *f* (25 μL), cytochrome b_6 (60 μL), Rieske (60 μL), and 100 μL of protein A-agarose beads. This immunoprecipitate was denatured and resolved by SDS-PAGE and visualized by fluorography. Each lane represents an immunoprecipitation from the same amount of plant tissue based on chlorophyll content. The band seen in all the lanes at 43 kD corresponds to the leading edge of the very large amount (>250 μg/lane) of the heavy chain of IgG used in the immunoprecipitation.

Fe-S protein, it was not possible to detect any synthesis of these proteins in either the wild type or mutant. The lack of Rieske Fe-S protein synthesis was expected because very little mRNA accumulated for this protein. However, in the case of cytochrome b_6, the lack of detectable protein could be due to low affinity of the heterologous antibody used or could reflect a low level of protein synthesis. The latter explanation is supported by our results that suggest that post-transcriptional regulation of the synthesis or assembly of cytochrome b_6 may occur. In our studies, we have found that in vitro labeling of isolated spinach chloroplasts with ^{35}S-TransLabel and subsequent isolation of the cytochrome complex did not result in the insertion of labeled cytochrome b_6 into the resolved complex even though subunit IV and cytochrome *f* were shown by this procedure to be labeled and inserted into the complex. This result suggests that the rate of turnover of the cytochrome b_6 subunit is much lower than that of the other subunits and that the complex is able to undergo subunit exchange without requiring de novo synthesis of the entire complex.

Pulse-Chase Determination of Protein Turnover

To determine why the chloroplast-encoded cytochrome subunits fail to accumulate despite having normal levels of mRNA and comparable levels of protein synthesis, the rate of turnover of several proteins in whole plants was measured. This was done by giving entire plants a 1-hr radiolabeling pulse of ^{35}S-TransLabel, followed by extensive washing and chasing with an approximate 1000-fold increase in cold methionine and cysteine. At various time points in the chase, the protein of interest was immunoprecipitated from a solution containing total plant proteins. By this procedure, the turnover rate for subunit IV in the mutant and the wild type was measured. As shown in Figure 6, subunit IV in the wild type could be detected up to 17 hr after the chase addition, whereas in the mutant this subunit disappeared after approximately 1 hr to 3 hr. Approximate half-times for turnover of subunit IV in the mutant and wild type were 2 hr and 24 hr, respectively. Thus, this subunit turns over more rapidly (10 times) in the mutant as compared with the wild-type strain.

To verify that the mutant was not simply more active in protein turnover or contained an elevated level of proteases, we also measured the turnover rate for plastocyanin, as shown in Figure 7. Plastocyanin showed no significant difference in protein turnover in the mutant when compared with the wild type and could be detected up to 24 hr after the chase addition in both strains. We also studied whether this turnover was specific for proteins destined for insertion into the thylakoid membrane by measuring the turnover of LHCP II (data not shown). This result indicated that there was no increase in the rate of turnover of LHCP II in the mutant as compared with the wild type. We conclude from these results that the chloroplast-encoded subunits of the cytochrome complex are synthesized at the same rate in the mutant and wild type and that turnover is accelerated in the mutant. The fact that the pattern of protein degradation for subunit IV seen in Figure 6 is different between the wild type and mutant suggests that accessibility to proteases is different in the mutant.

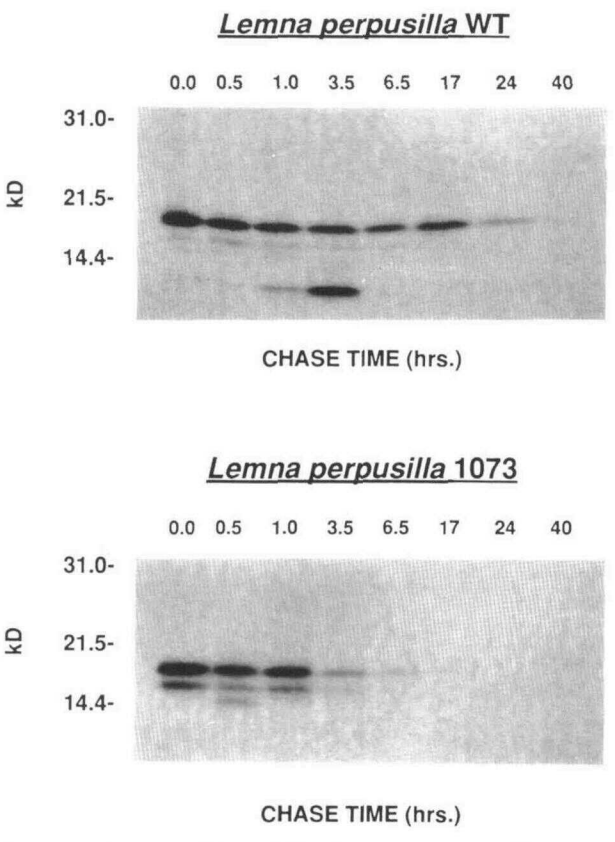

Figure 6. Turnover of Subunit IV of the Cytochrome b_6f Complex in *L. perpusilla*.

Intact *L. perpusilla* wild-type (WT) and mutant no. 1073 (1073) plants (five to 10 individual plantlets) were labeled for 1 hr in low light with 1 mC of ^{35}S-TransLabel. After residual label was removed by washing, cold methionine and cysteine were added to 20 mM. At the time point indicated, whole plants were homogenized and total proteins were acetone precipitated. This total plant protein fraction was resuspended and denatured with 2% SDS. Subunit IV was immunoprecipitated by the addition of polyclonal antisera raised against spinach subunit IV and protein A-agarose beads. This immunoprecipitate was denatured and resolved by SDS-PAGE and visualized by fluorography. Each lane represents an immunoprecipitation from the same amount of plant tissue based on chlorophyll content.

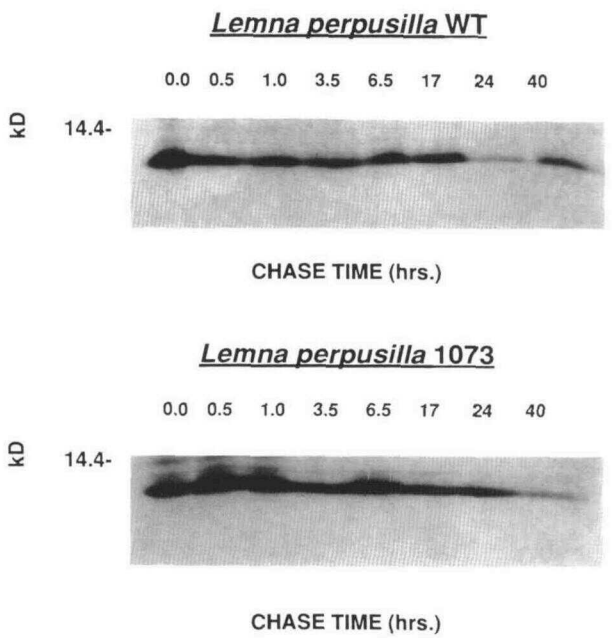

Figure 7. Turnover of Plastocyanin in *L. perpusilla*.

Turnover of plastocyanin in wild-type (WT) and mutant (1073) *L. perpusilla* plantlets was measured as in Figure 6 using an antibody raised against spinach plastocyanin.

Subunit Membrane Association

To determine whether subunit IV is associated with the thylakoid, it was necessary to in vivo label whole plants and to isolate thylakoid membranes before immunoprecipitation. To test further the association with thylakoids, the membranes were treated with the chaotropic agent NaBr. Washing thylakoid membranes with NaBr removes peripheral proteins, such as the CF1 complex (Kamienietzky and Nelson, 1975). Figure 8 shows the results of immunoprecipitation of in vivo labeled subunit IV and LHCP II from NaBr-washed thylakoids. It is clear that both proteins are associated with the thylakoid membrane because NaBr washing did not lead to their removal. The fact that there was less labeled subunit IV in the mutant thylakoids is to be expected because this protein is more rapidly turned over and would not, therefore, accumulate to the same level as in the wild type. (See, for example, the 6.5 hr time point in the pulse-chase experiment shown in Figure 6.) LHCP II, another known integral membrane protein, was immunoprecipitated equivalently from the thylakoids isolated from the mutant and wild type because the rate of turnover for this protein was the same in both plants.

DISCUSSION

The results from the present study of the photosynthetic mutant *L. perpusilla* no. 1073 indicate that the mutation in

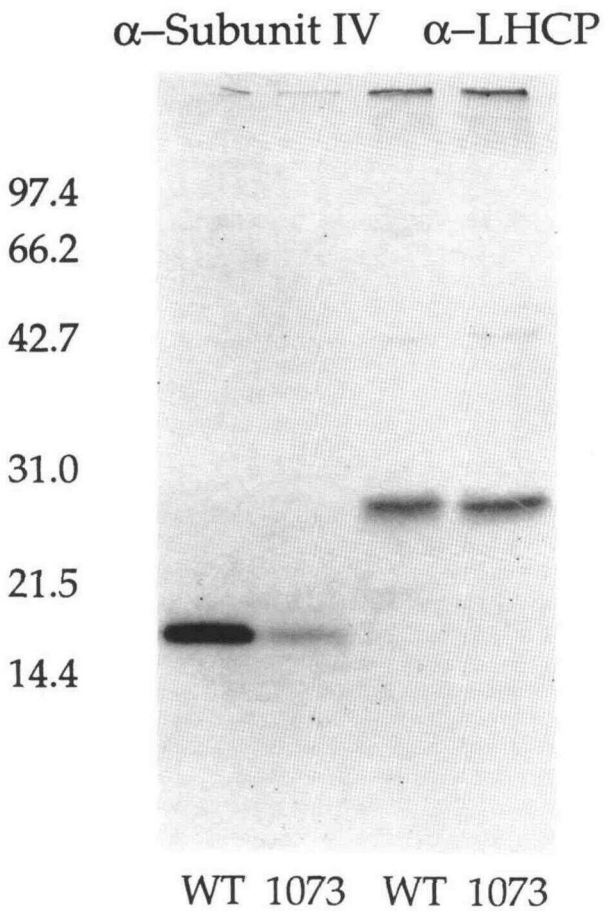

Figure 8. Association of Subunit IV and LHCP II with Thylakoid Membranes in *L. perpusilla*.

Intact *L. perpusilla* wild-type (WT) and mutant no. 1073 (1073) plants (five to 10 individual plantlets) were labeled for 5 hr in low light with 1 mC of ^{35}S-TransLabel. Intact chloroplasts were isolated from the labeled plants and were washed twice to remove any residual label. These chloroplasts were lysed and the thylakoids were collected by centrifugation. The thylakoid membranes were washed twice with the chaotropic agent NaBr at 3 M. After the NaBr was removed by washing, total thylakoid proteins were acetone precipitated. This total thylakoid protein fraction was resuspended and denatured with 2% SDS. Specific proteins were immunoprecipitated by addition of polyclonal antisera and protein A-agarose beads. This immunoprecipitate was denatured and resolved by SDS-PAGE and visualized by fluorography. Each lane represents an immunoprecipitation from the same amount of plant tissue based on chlorophyll content.

this organism affects the transcription and/or mRNA accumulation of the *petC* gene encoding the Rieske Fe-S protein of the cytochrome b_6f complex. A possible explanation involving a second mutation that affects the accumulation of chloroplast-encoded subunits cannot be excluded by this work but appears less likely because this mutation has been induced by x-rays that presumably generate nuclear mutations. The three chloroplast-encoded subunits, cytochrome *f*, cytochrome b_6, and subunit IV, appear normal in relation to their transcription, mRNA accumulation, and protein synthesis; in the case of subunit IV, it was also possible to document an association of this subunit with the thylakoid membrane. The fact that these three subunits failed to accumulate to a detectable level in the mutant is most likely a result of their accelerated rate of turnover in the thylakoids, as documented by a 10-fold greater rate of turnover of subunit IV in the mutant as compared with the wild type.

A preliminary observation of interest in relation to the turnover of the constituents of the cytochrome complex was the finding that the rate of turnover of individual protein components of the complex was not identical because the rate of turnover of cytochrome b_6 appeared to be considerably lower than that of other protein subunits. This implies that a mechanism must exist for the degradation of specific components within the complex without a degradation of the entire complex. This situation is similar in some respects to observations relating to the turnover of photosystem II components in the photosynthetic membrane, where the turnover of the integral membrane protein D1 is considerably greater than the rate of the other protein components in this complex (Mattoo et al., 1981; Ohad et al., 1985). The mechanism by which a single subunit in a multisubunit complex is specifically degraded, resynthesized, and reinserted into a complex is not known at the present time, but our findings on the cytochrome b_6f complex, taken in conjunction with the previous work on the photosystem II complex, would suggest that this is a general property of thylakoid membrane complexes.

The finding that the nuclear-encoded subunit of the cytochrome b_6f complex is essential for the stable assembly of this complex in thylakoids is consistent with a similar concept first presented by Miles (1982) after characterization of maize mutants that lacked the cytochrome complex. Biochemical (Miles, 1982; Metz et al., 1983) and molecular biological (Barkan et al., 1986) studies led to the conclusion that the entire cytochrome complex failed to assemble in the mutants and that a "key assembly protein" of nuclear origin was required for complete assembly of this complex. Subsequent work by Herrmann's group with *Oenothera* (Stubbe and Herrmann, 1982) has supported this view, and Willey and Gray (1988) extended the proposal to specify that the Rieske Fe-S protein may function as this key nuclear protein. The present results with the *L. perpusilla* mutant identify the Rieske protein as essential for the stable assembly of the thylakoid cytochrome complex and argue against a concept of a stable complex that lacks any one component of the cytochrome complex. Assembly of the chloroplast cytochrome complex appears to operate by an "all-or-none" mechanism whereby all components must be present for the stabilization of the complex.

In addition to the few cytochrome b_6f complex mutants in higher plants, several photosynthetic mutants have been isolated from the green alga *Chlamydomonas reinhardtii* that are defective in the cytochrome b_6f complex. The most well-characterized mutants include the UV-irradiated nuclear mutant *ac21* (Levine and Goodenough, 1970), the chloroplast mutants FuD2, FuD4, FuD6, and FuD8 generated by mutagenesis with 5-fluorodeoxyuridine (Bennoun et al., 1978), and the nuclear mutant F18 generated with 5-fluorouracyl (Girard et al., 1980). These mutants have been characterized by Bennoun and coworkers (Bendall et al., 1986; Lemaire et al., 1986, 1987). In general, many of these mutants are similar to those detected with higher plants in that they fail to accumulate any of the cytochrome b_6f subunits. However, in a few algal strains, some of the chloroplast-encoded subunits were present in the thylakoid membrane even though the nuclear-encoded subunit, the Rieske Fe-S protein, was absent. These mutants appear to be substantially different from other cytochrome b_6f mutants, and further study at the molecular genetic level might prove instructive in terms of the assembly of the complex.

In yeast, a number of studies using respiratory mutants have led to the conclusion that there are two different but coexistent pathways for the transport, prosthetic group attachment, and assembly of the cytochrome bc_1 subunits (Crivellone et al., 1988). One pathway that includes only the nuclear-encoded cytochrome c_1 subunit is insensitive to mutations affecting the accumulation of cytochrome *b*. Cytochrome c_1 is able to be transported into the mitochondria, undergo heme attachment, and to be inserted stably into the membrane, independent of the accumulation of cytochrome *b*. In the other assembly pathway, subunits such as the Rieske Fe-S protein, core I, core II, and the low molecular weight subunits are strongly affected by the status of cytochrome *b*. When cytochrome *b* is missing, these proteins show lower levels of transport and a lack of prosthetic group attachment and fail to insert stably into the membrane. In a recent study using gene disruption mutants in yeast, Crivellone et al. (1988) concluded that only the Rieske Fe-S protein had no effect on the assembly of the other subunits. A number of studies have shown that it is possible to assemble "partial" cytochrome bc_1 complexes in yeast (Capeillere-Blandin and Ohnishi, 1982; Japa and Beattie, 1988; Gatti et al., 1989) and that the tight assembly of the complex that is observed in the case of the cytochrome b_6f complex does not occur. It is not yet clear why organisms that require a functional cytochrome complex in either their respiratory or photo-

synthetic membranes have apparently evolved different mechanisms for the assembly of these complexes, particularly when all of these organisms must assemble this complex from a number of different nuclear-encoded and organelle-encoded subunits.

METHODS

Growth of Lemna gibba and L. perpusilla Wild Type and *L. perpusilla* No. 1073

Lemna fronds were grown photoheterotrophically under aseptic conditions on 0.5% glucose in E medium (Tobin, 1978) at 25°C using heat-filtered incandescent lights (50 $\mu E/m^2/sec$ to 80 $\mu E/m^2/sec$). Fernbach flasks containing 1000 mL of medium were inoculated with one to 10 individual plants and allowed to reach a density of two to three plants deep over a period of 4 weeks to 5 weeks.

Isolation of Total RNA

Whole plants were frozen in liquid nitrogen and pulverized with a mortar and pestle. This frozen slurry was then mixed and further pulverized with guanidinium thiocyanate reagent (5 M guanidine thiocyanate, 25 mM Tris, pH 8.0, 2% Sarkosyl, 10 mM EDTA, 0.1 M β-mercaptoethanol, and 4 mg/mL diethyldithiocarbamate) at 2 mL/g, wet tissue weight. This suspension was then centrifuged at 8000 rpm for 20 min in an HB4 rotor (Sorvall) to pellet the cell debris. After discarding the pellet, the nucleic acids were precipitated from the supernatant by addition of 0.03 volumes of 3 M sodium acetate and 0.75 volume of ethanol, and the suspension was placed at −20°C overnight. The pellet from this precipitation was resuspended in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.2% SDS, and 3 mg/mL diethyldithiocarbamate at the level of 1 mL/g of starting tissue. This solution was extracted twice with 2 volumes of phenol/chloroform/isoamyl alcohol (25:25:1) and then twice with 2 volumes of chloroform/isoamyl alcohol (24:1). The pH of the final aqueous phase was adjusted to 5.0 by the addition of glacial acetic acid. Nucleic acids were precipitated from this solution by addition of NaCl to 200 mM and 0.6 volume of isopropyl alcohol, and the suspension was placed at −20°C overnight. The precipitate was collected by centrifugation at 8000 rpm for 20 min in an HB4 rotor. The pellet was resuspended in 0.1% SDS to the level of 1 mL/g of starting tissue. The RNA was precipitated selectively from this solution by the addition of $MgCl_2$ to 1 mM and 0.25 volume of 8 M LiCl. The RNA was allowed to precipitate overnight at 4°C. The pellet was collected by centrifugation, as above, and resuspended in 10 mM Tris, pH 8.0, 0.1% SDS, 1.0 mM EDTA to the level of 1 mg/mL of starting tissue. RNA was precipitated as above after addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. This final pellet was resuspended in 10 mM Tris, pH 8.0, 1.0 mM EDTA to the level of 0.1 mL/g of starting tissue and stored at −70°C until needed.

Isolation of Polysomal PolyA$^+$ RNA

Entire plants were frozen in liquid nitrogen and stored at −80°C. Polysomal polyA$^+$ mRNA was isolated using the procedure of Lincoln et al. (1987).

RNA Gel Blot Analysis

After ethanol precipitation, RNA was resuspended and an aliquot was quantitated by UV absorbance. All lanes were loaded with equal amounts of resuspended RNA. With RNA gel blots using polyA$^+$ RNA, 2 μg of RNA were loaded per lane. RNA gel blots using total RNA were loaded with 10 μg of RNA per lane. RNA was denatured with formaldehyde and formamide at 50°C for 30 min. RNA was separated using a denaturing formaldehyde agarose gel electrophoresis system (Sambrook et al., 1989). RNA was transferred to either nitrocellulose or nylon membrane by capillary blotting overnight.

Preparation and Labeling of cDNA Radiolabeled Probes

Isolated DNA clones were generously donated as follows: a 400-bp fragment encoding pea cytochrome *f* and a 420-bp fragment encoding pea subunit IV from Dr. A. Barkan, a 500-bp fragment encoding spinach Rieske Fe-S protein from Dr. R. Herrmann, an 850-bp fragment encoding pea Rieske Fe-S protein from Dr. J. Gray, a 300-bp fragment encoding spinach cytochrome b_6 from Dr. H. Bohnert, and a 920-bp fragment encoding maize LHCP II and a 900-bp fragment encoding maize ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit from Dr. W. Taylor.

The DNA inserts were cut out of the plasmid by the appropriate restriction enzymes and separated by agarose gel electrophoresis. These inserts were visualized by ethidium bromide staining and excised and recovered as directed in the commercial product Gene Clean (Bio-101, La Jolla, CA). Isolated insert DNA was quantified by optical absorbance at 260 nm.

Isolated DNA inserts were radiolabeled with ^{32}P by using either a commercial nick-translation kit (Amersham) or by hexamer labeling using a commercial random primer kit. Specific activity for the probes was at least 10^8 cpm/μg of DNA.

Immunoprecipitation from Whole *Lemna* Plants

Whole plant tissue (about 10 intact *Lemna* plants containing about 30 μg of chlorophyll) was placed in a small (0.5-mL) hand-held ground-glass homogenizer and 320 μL of a cold solution containing 0.3 M sucrose, 50 mM Tris-HCl buffer, pH 7.8, 10 mM NaCl, and 5 mM $MgCl_2$ was added and homogenized to completion on ice. Protease inhibitors (1 mM EDTA, 1 mM EGTA, 20 μM α-aminocaproic acid, 20 μM benzamidine, 10 μg/mL aprotinin, 200 μg/mL trypsin inhibitor, 10μg/mL pepstatin, and 20 mM phenylmethylsulfonyl fluoride) were added before homogenization. This homogenate was transferred to a 1.8-mL microcentrifuge tube and 1.3 mL of cold acetone was added; the proteins were allowed to precipitate for 1 hr at −20°C. After the precipitation, the microcentrifuge tube was centrifuged at 4°C in a

microcentrifuge for 10 min, the supernatant was decanted carefully, and the pellet was allowed to air dry.

This pellet was resuspended with a microcentrifuge homogenizer in 200 μL of 2% SDS and heated to 55°C for 10 min. The denatured protein was centrifuged for 20 min at top speed in a microcentrifuge at room temperature. The supernatant was removed and placed in a 5-mL polypropylene tube. The pellet was washed with 1 mL of 4 × immunoprecipitation buffer (4% Triton X-100, 0.8% SDS, 20 mM EDTA, 800 mM NaCl, and 50 mM Tris-Cl, pH 7.5). The suspension was centrifuged at top speed in a microcentrifuge and the supernatant was carefully combined with the first supernatant. The volume was brought up to 4 mL with distilled water diluting the SDS concentration to about 0.1%. This total protein extract (TPE) was first clarified using preimmune serum. Preimmune serum (50 μL) was added to the TPE and the solution was rocked overnight at 4°C.

Bio-Rad protein A-agarose beads were added to the TPE (twice the volume of preimmune serum), and the suspension was incubated for 1 hr to 2 hr while rocking at 4°C. The beads were collected by centrifugation at 6500 rpm for 10 min in an HB4 rotor (Sorvall). TPE supernatant was removed, leaving behind the protein A-agarose bead pellet. This clarified TPE was transferred to a 5-mL tube, and 25 μL of the specific immunized serum was added and the solution rocked overnight at 4°C. The specific antigen-antibody complex was precipitated by incubation with Bio-Rad protein A-agarose beads (PAB) and subsequent centrifugation.

After centrifugation, the TPE supernatant was removed from the pellet of protein A-agarose beads, and the pellet was washed with 1 mL of immunoprecipitation wash buffer: 0.15% Triton X-100, 0.03% SDS, 5 mM EDTA, 300 mM NaCl, and 50 mM Tris-Cl, pH 7.5. This wash step was repeated in the following order: 3 × 1 mL of wash buffer, 1 × 1 mL of double-distilled H_2O, 3 × 1 mL of wash buffer, and 2 × 1 mL of double-distilled H_2O. In the final two washes, it was important to remove all liquid from the PAB pellet with a 25-μL Hamilton syringe.

This final "dry" PAB pellet was resuspended in twice the pellet volume of 2 × sample buffer and heated at 55°C for 20 min. This denatured sample was then run on standard gel electrophoresis.

Pulse-Chase Labeling of Intact *Lemna* Plants

Very high specific activity of labeling was accomplished by labeling entire plants by floating them directly in a 1:1 mix of fresh growth media (minus sucrose) and ^{35}S-TransLabel (>1000 Ci/mmol, ICN, Costa Mesa, CA). A small number (30 to 50) of individual plants were washed and placed in a 2-mL beaker. A custom-designed plunger was placed on top of the plants to submerge them completely in labeling media. The total volume of about 100 μL was just sufficient to submerge the plants completely. The plants were illuminated from beneath with heat-filtered incandescent light at a level of 50 $\mu E/m^2/sec$ for 1 hr. At the end of labeling, the plants were washed twice with double-distilled H_2O and twice with growth media and returned to a fresh 2-mL beaker containing fresh growth media containing 20 mM cysteine and 20 mM methionine and allowed to grow as described above. At each time point, five to 10 plants were removed and washed several times, and the total proteins were extracted as described below in an immunoprecipitation procedure.

SDS-PAGE

Analytical SDS-PAGE was carried out at 23°C with a slightly modified Laemmli buffer system (Laemmli, 1970) using a 1.5-mm slab gel with a 4% stacking gel and a 13% to 21% gradient resolving gel. Before electrophoresis, samples were solubilized with 100 mM DTT, 1% SDS, 10 mM Tris-HCl buffer, pH 8.3, at 25°C for 3 hr or 50°C for 15 min. Electrophoresis was carried out at 25°C with a constant current of 15 mA for 12 hr to 14 hr. After electrophoresis, the gel was either fixed and stained with Coomassie Brilliant Blue or treated with En^{3}Hance and dried on Whatman No. 3MM paper using a Hoefer gel dryer for fluorography.

Immunoblotting

Protein gel blotting was carried out as described in the Bio-Rad alkaline phosphatase instruction manual with the following modifications: 0.1-μm nitrocellulose paper (Schleicher & Schuell) was used, 0.5% SDS was included in the transfer buffer, Carnation nonfat dry milk (3%) was used as a blocking reagent, and ^{125}I-protein A (ICN, Costa Mesa, CA) and autoradiography were used as the visualization process.

Other Methods

Chlorophyll determinations were made using 80% acetone with the extinction coefficients of Arnon (1949).

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REFERENCES

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. **14,** 1–15.

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

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

Bennoun, P.A., Masson, A., Piccioni, R., and Chua, N.-H. (1978). Uniparental mutants of *Chlamydomonas reinhardtii* defective in photosynthesis. In Chloroplast Development, G. Akoyunoglou

and J.H. Argyroudi-Akoyunoglou, eds (New York: Elsevier/North-Holland), pp. 721–726.

Berends, T., Kubicek, Q., and Mullet, J. (1986). Localization of the genes coding for the 51 kDa PSII chlorophyll apoprotein, apocytochrome b_6, the 65–70 kDa PSI chlorophyll apoproteins and the 44 kDa PSII chlorophyll apoprotein in pea chloroplast DNA. Plant Mol. Biol. **6,** 125–134.

Capeillere-Blandin, C., and Ohnishi, T. (1982). Investigation of the iron-sulfur clusters in some mitochondrial mutants of *Saccharomyces cerevisiae*. Eur. J. Biochem. **122,** 403–413.

Crivellone, M.D., Wu, M., and Tzagoloff, A. (1988). Assembly of the mitochondrial membrane system. Analysis of structural mutants of the yeast QH_2-cytochrome *c* reductase. J. Biol. Chem. **263,** 14323–14333.

Doyle, M.P., Li, L.-B., Yu, L., and Yu, C.-A. (1989). Identification of a M_r = 17,000 protein as the plastoquinone-binding protein in the cytochrome b_6f complex from spinach chloroplasts. J. Biol. Chem. **264,** 1387–1392.

Gatti, D.L., Meinhardt, S.W., Ohnishi, T., and Tzagoloff, A. (1989). Structure and function of the mitochondrial bc1 complex—A mutational analysis of the yeast Rieske iron-sulfur protein. J. Mol. Biol. **205,** 421–435.

Girard, J., Chua, N.-H., Bennoun, P., Schmidt, G., and Delosme, M. (1980). Studies on mutants deficient in the photosystem I reaction centers in *Chlamydomonas reinhardtii*. Curr. Genet. **2,** 215–221.

Hauska, G., Nitschke, W., and Herrmann, R.G. (1988). Amino acid identities in the three redox center-carrying polypeptides of the cytochrome bc_1/b_6f complexes. J. Bioenerg. Biomembr. **20,** 211–228.

Heinemeyer, W.J., Alt, J., and Herrmann, R.G. (1984). Nucleotide sequence of the clustered genes for apocytochrome b_6 and subunit 4 of the cytochrome *b/f* complex in the spinach plastid chromosome. Curr. Genet. **8,** 543–549.

Japa, S., and Beattie, D.S. (1988). Cytochrome *b* is necessary for the assembly of subunit VII in the cytochrome b-c_1 complex of yeast mitochondria. Arch. Biochem. Biophys. **264,** 231–237.

Kamienietzky, A., and Nelson, N. (1975). Preparation and properties of chloroplasts depleted of chloroplast coupling factor 1 by sodium bromide treatment. Plant Physiol. **55,** 282–287.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. Nature **227,** 680–685.

Lam, E., and Malkin, R. (1985). Characterization of a photosynthetic mutant of *Lemna* lacking the cytochrome b_6f complex. Biochim. Biophys. Acta **810,** 106–109.

Lemaire, C.J., Girard-Bascou, J., Wollman, F.-A., and Bennoun, P. (1986). Studies on the cytochrome b_6f complex. I. Characterization of the complex subunits in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta **851,** 229–238.

Lemaire, C.J., Girard-Bascou, J., and Wollman, F.A. (1987). Characterization of the b_6/f complex subunits and studies on the LHC-kinase in *Chlamydomonas reinhardtii* using mutant strains altered in the b_6/f complex. In Progress in Photosynthesis Research, Vol. IV, J. Biggins, ed (The Hague: Martinus Nijhoff/Dr. W. Junk Publishers), pp. 655–658.

Levine, R.P., and Goodenough, U.W. (1970). The genetics of photosynthesis and of the chloroplast in *Chlamydomonas reinhardtii*. Annu. Rev. Genet. **4,** 397–408.

Lincoln, J.E., Cordes, S., Read, E., and Fischer, R.L. (1987). Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. Proc. Natl. Acad. Sci. USA **84,** 2793–2797.

Malkin, R. (1988). Structure-function studies of photosynthetic cytochrome bc_1 and b_6f complexes. ISI Atlas of Science: Biochemistry **1,** 57–64.

Malkin, R., and Posner, H.B. (1978). On the site of function of the Rieske iron-sulfur center in the chloroplast electron transport chain. Biochim. Biophys. Acta **501,** 552–554.

Mattoo, A.K., Pick, U., Hoffman-Falk, H., and Edelman, M. (1981). The rapidly metabolized 32,000-dalton polypeptide of the chloroplast is the "proteinaceous shield" regulating photosystem II electron transport and mediating diuron herbicide sensitivity. Proc. Natl. Acad. Sci. USA **78,** 1572–1576.

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

Miles, D. (1982). The use of mutations to probe photosynthesis in higher plants. In Methods in Chloroplast Molecular Biology, M. Edelman, R.B. Hallick, and N.-H. Chua, eds (Amsterdam: Elsevier Biomedical Press), pp. 75–107.

Ohad, I., Kyle, D.J., and Hirschberg, J. (1985). Light-dependent degradation of the Qb-protein in isolated pea thylakoids. EMBO J. **4,** 1655–1659.

Rich, P.R. (1986). A perspective on Q-cycles. J. Bioenerg. Biomembr. **18,** 145–156.

Rock, C.D., Barkan, A., and Taylor, W.C. (1987). The maize plastid *psb*B-*psb*F-*pet*B-*pet*D gene cluster: Spliced and unspliced *pet*B and *pet*C RNAs encode alternative products. Curr. Genet. **12,** 69–77.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Shahak, Y., Posner, H.B., and Avron, M. (1976). Evidence for a block between plastoquinone and cytochrome *f* in a photosynthetic mutant of *Lemna* with abnormal flowering behavior. Plant Physiol. **57,** 577–579.

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

Tobin, E.M. (1978). Light regulation of specific mRNA species in *Lemna gibba* L. G-3. Proc. Natl. Acad. Sci. USA **75,** 4749–4753.

Willey, D.L., and Gray, J.C. (1988). Synthesis and assembly of the cytochrome *b-f* complex in higher plants. Photosynth. Res. **17,** 125–144.