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# Mutants of *Chlamydomonas*: Tools to study thylakoid membrane structure, function and biogenesis

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**Abstract** — The unicellular green alga *Chlamydomonas reinhardtii* is a model system for the study of photosynthesis and chloroplast biogenesis. *C. reinhardtii* has a photosynthesis apparatus similar to that of higher plants and it grows at rapid rate (generation time about 8 h). It is a facultative phototroph, which allows the isolation of mutants unable to perform photosynthesis and its sexual cycle allows a variety of genetic studies. Transformation of the nucleus and chloroplast genomes is easily performed. Gene transformation occurs mainly by homologous recombination in the chloroplast and heterologous recombination in the nucleus. Mutants are precious tools for studies of thylakoid membrane structure, photosynthetic function and assembly. Photosynthesis mutants affected in the biogenesis of a subunit of a protein complex usually lack the entire complex; this pleiotropic effect has been used in the identification of the other subunits, in the attribution of spectroscopic signals and also as a 'genetic cleaning' process which facilitates both protein complex purification, absorption spectroscopy studies or freeze-fracture analysis. The cytochrome *b6 f* complex is not required for the growth of *C. reinhardtii*, unlike the case of photosynthetic prokaryotes in which the cytochrome complex is also part of the respiratory chain, and can be uniquely studied in *Chlamydomonas* by genetic approaches. We describe in greater detail the use of *Chlamydomonas* mutants in the study of this complex. © Société française de biochimie et biologie moléculaire / Elsevier, Paris

***Chlamydomonas* / thylakoid / membrane proteins / cytochrome $b_6f$**

## 1. Introduction

The primary reactions of oxygenic photosynthesis take place in an integrated multi-enzymatic system, the thylakoid membrane [1]. Thylakoids are closed flattened vesicles found in the stroma of chloroplasts and in the cytoplasm of cyanobacteria. The main functions of this system are to capture light energy and to use it to produce reducing compounds and ATP *(figure 1)*. Our understanding of the way this complex system operates has come from a combination of different approaches: biophysical at first, then increasingly biochemical. In recent years, genetics and molecular genetics have yielded a considerable amount of information not only on the mechanisms of photosynthesis, but also on the biogenesis of the thylakoid membrane. A large number of these studies have used the unicellular green alga *Chlamydomonas reinhardtii* as a model system. In this review, we will try to give an overview of the ways photosynthesis mutants of *C. reinhardtii* can increase our knowledge of thylakoid membrane structure, function and biogenesis.

The first reaction of photosynthesis is absorption of light by specialized pigments (chlorophylls and carotenoids in plants). The energy is transferred between pigments, ultimately to a specialized chlorophyll *a* located in a reaction center. Two types of reaction centers are found, photosystem I (PSI) and photosystem II (PSII), each connected to a specific light harvesting complex (LHCI or LHCII). Upon excitation by light, an electron is abstracted from the photochemical chlorophyll, giving rise to a strong oxidant (the cationic chlorophyll) and a strong reductant. This starts a series of redox reactions known as the photosynthetic electron transfer chain *(figure 1)*, where the two photosystems operate in series. The ultimate electron donor to PSII is water, whose oxidation yields oxygen and protons. On the reducing side, electrons are conveyed via a lipophilic carrier, plastoquinone (PQ), to the cytochrome $b_6f$ complex, which ultimately reduces PSI via the soluble carrier platocyanin (PC). PSI donates electrons to ferredoxin (Fd), which reduces $NADP^+$ into NADPH. The operation of this electron transfer chain generates a transmembrane electro-chemical gradient which is utilized by a $H^+$-ATPsynthase to generate ATP.

Several mutants deficient in photosynthesis have been isolated from higher plants, mostly in barley [2] and maize [3]. However, work with these mutants is difficult, in particular because they have to be maintained as heterozygous diploids. In the early 1960s, Paul Levine recognized the immense potential of *C. reinhardtii* as a model system to study photosynthesis [4]. While its photosynthesis apparatus seemed very similar to that of higher plants, it had several important advantages as a genetic

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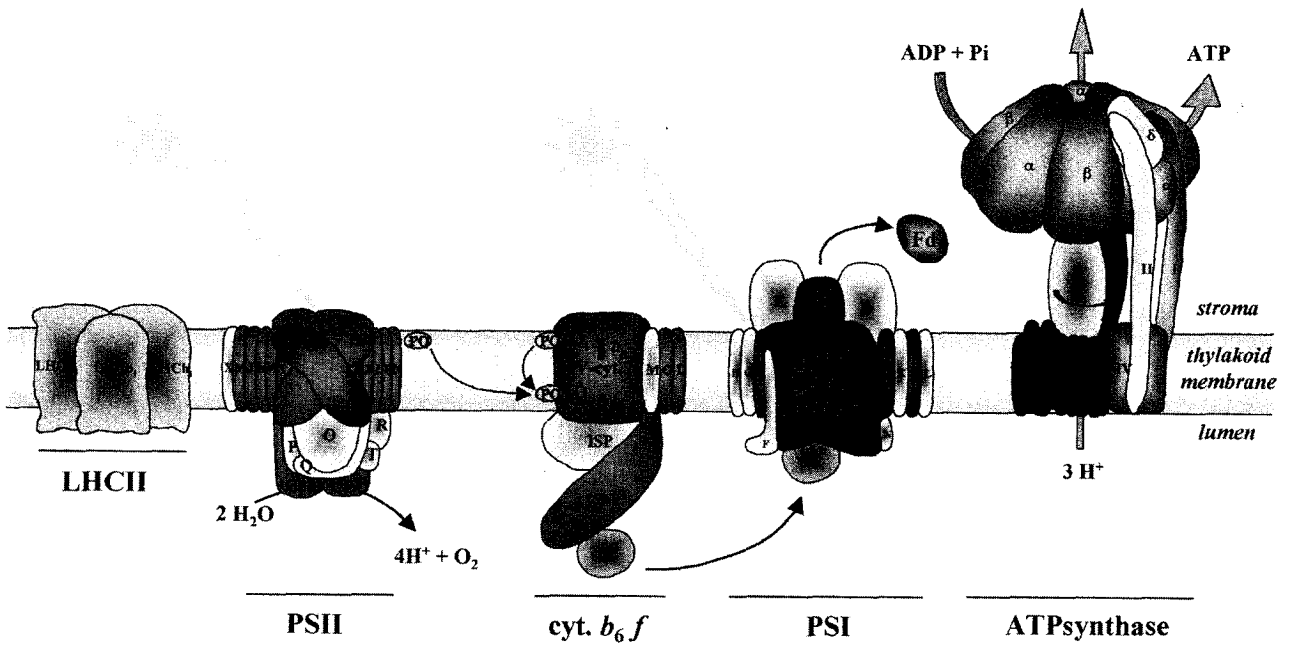


**Figure 1.** Schematic representation of the thylakoid membrane. Subunits drawn in light colors are coded by the nuclear genome and imported in the chloroplast; subunits drawn in dark colors are coded by the chloroplast genome. There are numerous antenna complexes. To simplify only LHCII is indicated. Adapted from Choquet and Minai.

system (see [5-7]). *C. reinhardtii* is a facultative phototroph, i.e., it can grow in the absence of light when provided with acetate as a source of reduced carbon. This allows the isolation of mutants unable to perform photosynthesis. In particular, *C. reinhardtii* offers an interesting situation for the study of the cytochrome $b_6f$ complex because it is dispensable for growth (unlike another major model organism, the cyanobacterium *Synechocystis*, in which cytochrome $b_6f$ is also part of the respiratory chain). *C. reinhardtii* is a unicellular eukaryote (about 10 μm long) with a short doubling time (7–8 h) and is amenable to cloning on a Petri dish, which facilitates the isolation of mutants. *C. reinhardtii* has one large single chloroplast which occupies about 40% of the cell volume. Its sexual reproduction *(figure 2)* is easily controlled in the laboratory, and genetic analysis is facilitated by the ability to dissect tetrads directly after meiosis. Today, several hundred genetic loci have been identified in *C. reinhardtii* (640 are listed in the *Chlamydomonas* Database available at http://probe.nalusda.gov:8300/cgi-bin/browse/chlamydb), of which more than 200 affect photosynthesis. Genetic and molecular maps are available for the nuclear genome, as well as tools for positional and insertional cloning. The mitochondrial genome is entirely sequenced [8, 9] and the chloroplast genome is in way of completion. Transformation of the nucleus [10] and chloroplast [11] genomes is easily performed, allowing site-directed mutagenesis in both compartments. As a justification of the insight of the early *Chlamydomonas* geneticists, it is now by far the most advanced model system for chloroplast molecular biology [12]. Mechanisms discovered in this alga are beginning to be found operating in higher plants as well (e.g., the CES mechanism, see below, reviewed in [13]), and strategies developed for this alga have been used in higher plants (e.g., the plastid transformation selection scheme using antibiotic resistance carried by the *aadA* gene, see below [14]). An extensive description of the properties of *C. reinhardtii* can be found in two books devoted to the organism [5, 12].

## 2. Tools for genetic analysis in Chlamydomonas

The cell cycle of *C. reinhardtii (figure 2)* is haplobiontic, i.e., dominated by a haploid phase in which recessive mutations are readily expressed. Nuclear mutations are preferentially induced by ultraviolet light, while chloroplast or mitochondrial mutations are better obtained by fluorodeoxyuridin or $Mn^{2+}$ treatment, respectively. Haploid cells exist under two mating types (+) and (−) which can propagate vegetatively. Gametogenesis is induced by nitrogen starvation which derepresses sexual competence. After agglutination, two gametes of opposite mating types fuse to produce a zygote. A small portion of these zygotes

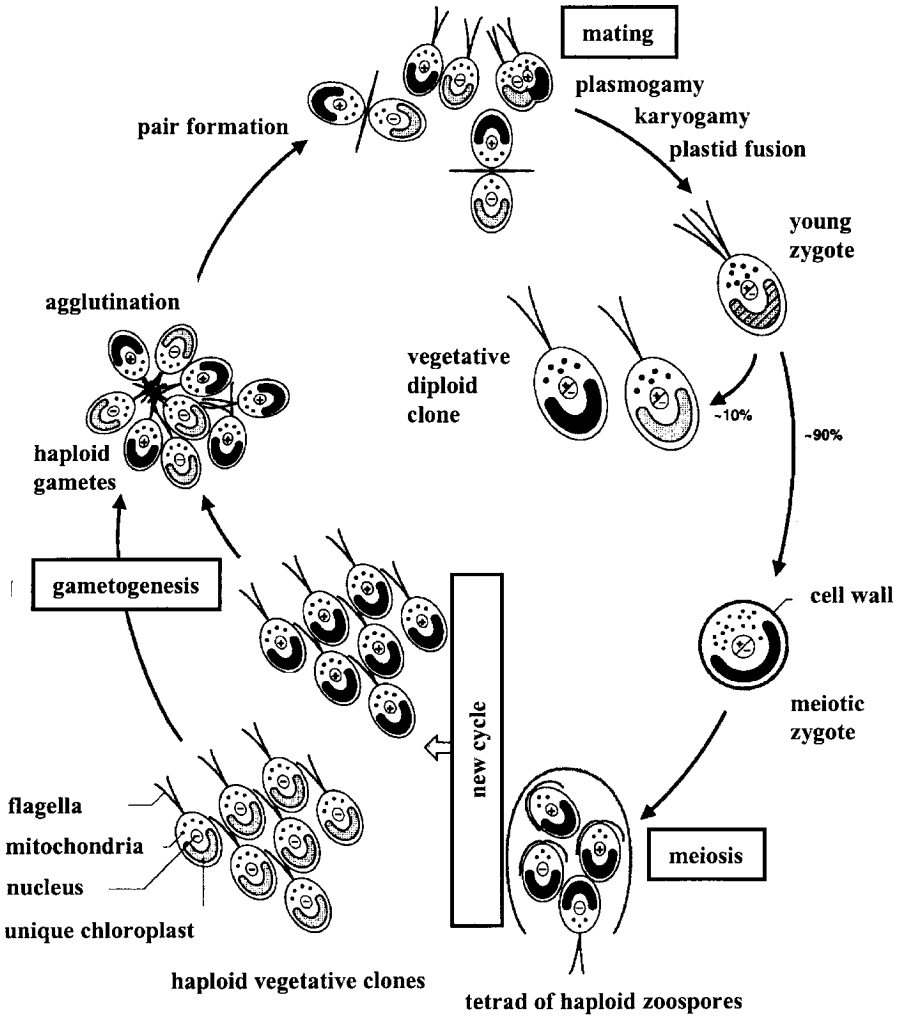


**Figure 2.** Sexual cycle of *C. reinhardtii*. Adapted from [7].

will propagate as vegetative diploids, in which genetic complementation can be studied [15]. Complementation can also be studied in agglutinated zygotes in meiotic diploids [16, 17]. The majority, however, will undergo meiosis and produce a tetrad of four haploid progeny which can be separated and analyzed. Nuclear, chloroplast and mitochondrial mutations are distinguished on the basis of their meiotic segregation pattern. Nuclear mutations show biparental heredity following the laws of Mendel (each tetrade yielding two wild-type colonies and two mutant colonies). In contrast, chloroplast and mitochondrial mutations are generally uniparentaly transmitted, respectively by the mating type (+) and (−). The occurrence of rare recombination events between the paternal and maternal chloroplast genomes has been used to study linkage between chloroplast markers [18].

The nuclear genome is estimated to be 100 Mbp, with approximately 10 000 to 20 000 genes [5]. More than 200 genetic markers of the nuclear genome are distributed on 17 linkage groups. A molecular map is being constructed by C. Silflow (http://www.botany.duke.edu/DCMB/ChlamyGen/maps.html) [12], which lists an additional linkage group. As this new map develops, it is expected to allow positional cloning of nuclear genes, in combination with a recently constructed BAC library (P. Lefebvre, personal communication). In the meantime, gene cloning

will mostly use either insertional mutagenesis ('gene tagging') or complementation by indexed libraries. Both techniques rely on transformation of the nuclear genome, which is easily achieved by vortexing wall-less cells in the presence of DNA and glass beads [10]. Recently, higher transformation efficiencies have been obtained by electroporation [19]. Transformants are selected using either complementation of an auxotrophy (the *ARG7* gene for arginosuccinate lyase [20], or the *NIT1* gene for nitrate reductase [21]) or resistance to an antibiotic (the *CRY1* gene for emetine [22], or the *ble* gene for phleomycin [23]). Since the introduced DNA integrates non-homologously, transformation with these markers generates a variety of 'tagged' mutants. After screening for the appropriate phenotype and genetic analysis to insure linkage of the mutation with the introduced DNA, phage libraries or plasmid rescue can be used to pull out the flanking DNA and go back to the WT version of the gene of interest. Several photosynthesis genes have been cloned that way (Rochaix and Goldschmidt-Clermont, personal communication), and by transformation with an indexed library [24] which appears as more straightforward, since it can allow direct access to the piece of DNA that complements a given mutation (e.g. [25]). Taken together, these techniques offer a vast array of possibilities for gene cloning.

Site directed mutagenesis of a nuclear gene is a difficult task. In the absence of an efficient screen for homologous recombinants, it still relies on the prior generation of a null mutant, into which mutated versions of the gene can be introduced by co-transformation with a selectable marker (e.g. [26]). Recently, one of us (C.V.) has been able to generate site-directed mutants of the Rieske $Fe_2S_2$ protein of the cytochrome $b_6f$ complex [27]. In a series of cytochrome $b_6f$ mutants obtained by UV mutagenesis, those deficient in the Rieske protein were screened on the basis of the phenotype of the previously characterized ac21 mutation [28]: accumulation of 50 to 75% of the other subunits in a cytochrome $b_6f$ subcomplex. Three such mutations were found to lie in the *PetC* gene, based on recombination and complementation tests and on complementation by the WT *PetC* gene. One of these mutants had a small deletion in the coding region, and completely lacked Rieske protein, and was used as recipient for site-directed mutagenesis. Other approaches to the manipulation of nuclear genes, such as over-expression and antisense are feasible (Schroda et al., unpublished), but a major caveat lies in the variable and generally low level of expression of the transgenes [29].

In contrast, the chloroplast genome appears more amenable to genetic manipulation. It is formed by circular molecules of 196 kbp *(figure 3)*, present at approximately 80 copies per cell. It contains about 30 genes coding for subunits of photosynthesis complexes, 18 tRNA genes, 14 genes coding for ribosomal subunits, five rRNA genes, six genes coding for an RNA polymerase, three genes implicated in chlorophyll synthesis and one gene coding for the protease subunit ClpP. Genes coding for subunits of the same photosynthesis complex are not organized in operons, but generally distributed all over the chloroplast genome; see PSII (*psb* genes), PSI (*psa* genes), $b_6f$ (*pet* genes) or ATPase (*atp* genes). Several transcription units have been described, generally grouping genes for different complexes ([30], reviewed by Stern and Drager [12]). Transformation can be achieved by 'biolistics' [11], whereby DNA-coated tungsten particles are introduced by particle bombardment into the chloroplast. The most widely used selection scheme uses resistance to spectinomycin and streptomycin carried by the *aadA* gene, which has been engineered into a convenient cassette by Goldschmidt-Clermont [31]. The cassette, flanked by *C. reinhardtii* chloroplast DNA, can be introduced at any site, to either interrupt or disrupt a gene (e.g. [32]), or to introduce a site-directed mutation (e.g. [33]). This successful strategy has been later used for chloroplast transformation in higher plants [14]. Restoration of phototrophy in a non-photosynthetic mutant has also been used, for example using the *atpB* deletion mutant *ac-u-c-2-21* [11].

## 3. Isolation of photosynthesis mutants: the use of fluorescence induction kinetics

The first photosynthesis mutants were isolated in Levine's laboratory on the basis of their inability to grow in the absence of acetate and to fix $^{14}CO_2$ [4], but strategies more specific for mutants in the light reactions were needed. Enrichment for such mutants could be achieved by growing the cells in the presence of metronidazole, a dye that becomes toxic when reduced by the electron transfer chain [34]. The most important technical breakthrough, however, was the introduction of chlorophyll fluorescence as a screen. Initially, mutants were detected simply on the basis of their high fluorescence yield [35]. Later, Bennoun and co-workers used the kinetics of fluorescence induction as a tool for rapidly characterizing such mutants; the most recent development of this technique allows simultaneous screening of hundreds of colonies on a Petri dish by digital fluorescence imaging (reviewed by Rochaix et al. [12]).

Only a portion of the light energy absorbed by photosynthesis pigments is used for photochemistry. The rest is emitted either as heat, or as fluorescence. The fluorescence yield rises when photochemistry is impaired, for example when no oxidized acceptor is available. Most of the fluorescence is emitted by antenna pigments associated to PSII, because closed PSI centers act as strong fluorescence quenchers. These characteristics make fluorescence induction a remarkable non-invasive tool to study the state of the electron transfer chain *(figure 4)*. When dark-adapted WT cells are submitted to continuous actinic light, the fluorescence rises from its initial level (F0) up to a

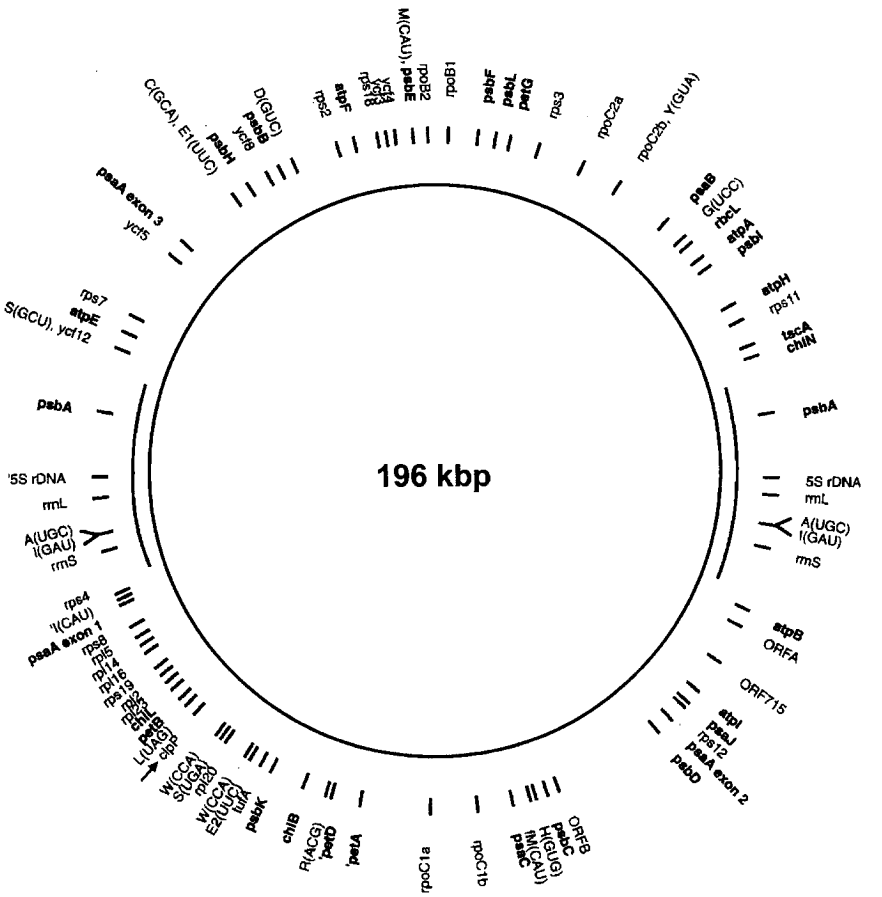


**Figure 3**. Map of the genome of the chloroplast of *C. reinhardtii*. Adapted from [75].

maximum, then declines to its stationary level (Fs). The rise, which has a complex structure, is due to the progressive closing of PSII centers as the PQ pool becomes reduced. Subsequently, the activation of PSI and cytochrome $b_6f$ leads to reoxidation of the PQ pool, reactivating PSII photochemistry and lowering the fluorescence yield. In WT under moderate light, PQ is never completely reduced, so that the highest point in the curve is still below the intrinsic maximum fluorescence (Fm). Fm can be obtained by treating the cells with DCMU, an inhibitor that blocks the acceptor side of PSII.

When the electron transfer chain is blocked at any step beyond PSII, as in mutants lacking cytochrome $b_6f$, PC or PSI, the fluorescence decline is not observed, and fluorescence rises continuously up to a maximum *(figure 4)*. This maximum is even higher than Fm because of chemical quenching effect of oxidized PQ is absent in these mutants. Distinction between cytochrome $b_6f$ and PSI

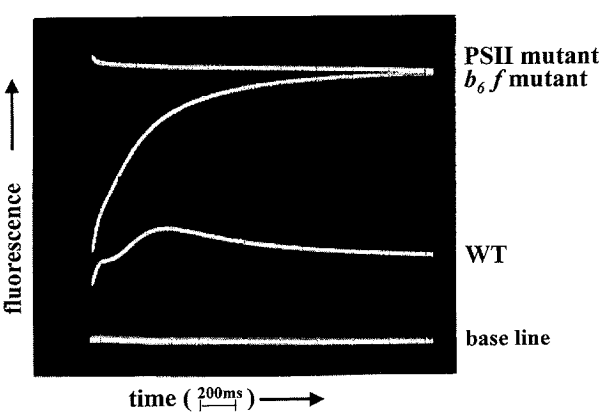


**Figure 4**. Kinetic of fluorescence emission of *C. reinhardtii* wild-type (WT), PSII deficient mutant (PSII), $b_6f$ deficient mutant. Illumination time, 2 s.

mutants is made possible by the fact that the latter have a higher fluorescence level, owing to the loss of PSI fluorescence quenchers. In a mutant lacking PSII reaction centers, the high fluorescence state is obtained already at the onset of illumination. The slight decline observed afterwards is due to the quenching effect of oxidized PQ, and is not observed in double mutants that also lack cytochrome $b_6f$ or PSI. Low fluorescence mutants usually harbour a LHCII deficiency or donor side mutants of PSII.

## 4. Mutants as tools in biochemical, structural and functional analysis

A major challenge faced by early researchers in photosynthesis was to correlate the variety of light-induced signals that they were discovering (such as 'photoact II' or 'photoact I') with a well-defined biochemical entity. Purification schemes were devised to isolate those enzymes in a test tube, and it was recognized that they were in fact made up of several polypeptides associated with cofactors in a supramolecular complex. Mutants of *C. reinhardtii* have provided another valuable source of information, since it was recognized that different mutants that specifically lacked one type of thylakoid membrane complex (say, PSII or PSI, or ATPase) were deficient in a particular subset of polypeptides in their thylakoid membrane [36-38]. Such a comparison is shown in *figure 5*, which illustrates the remarkable specificity of these deficiencies: in a given mutant, only the bands corresponding to the missing enzyme are affected, the others retain normal staining intensity. In addition, these mutants provide a remarkable starting material for biochemical purification: for example, pure PSII preparations are easily obtained from a *C. reinhardtii* double mutant lacking PSI and ATPase [39]. This 'genetic cleaning' is also useful in biophysical studies [40-42].

In combination with the biochemical purification of complexes, the genetic approach has allowed functional identification of all the major bands resolved by SDS-PAGE. In addition, sequencing of the chloroplast genome has revealed numerous new open reading frames, whose function can be studied by reverse genetics (i.e., disruption of the gene, generation of antibodies to the over-expressed protein or sequence-based peptides etc.). This is for example how PetL has been identified as a bona fide subunit of cytochrome $b_6f$ [43], or Ycf3 and Ycf4 as co-factors for PSI assembly [44]. At present, the number of polypeptides attributed to each of the thylakoid membrane complexes is 21 for PSII, 13 for PSI, three for LHCII, four for LHCI, three single subunit minor antenna complexes, seven for cytochrome $b_6f$ and nine for the ATPase.

Correlated with these biochemical changes, the lack of a photosynthesis complex also affects the ultrastructural organization of the membrane, as revealed by freeze fracture electron microscopy. This technique splits biological membrane at the junction of the two lipid layers, thus allowing visualization of the hydrophobic core of the

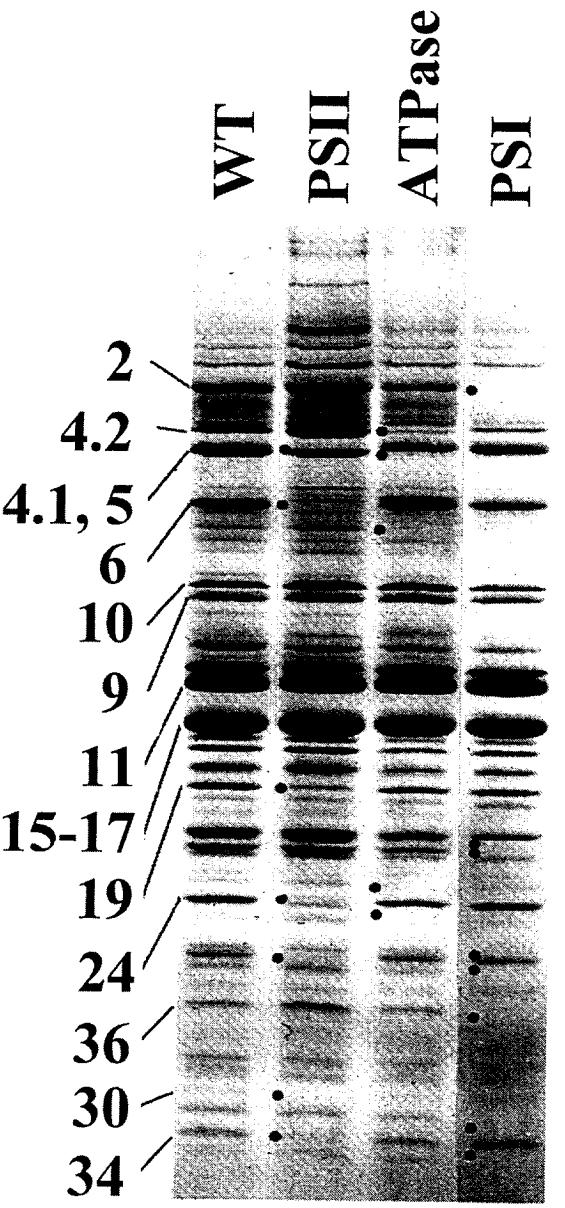


**Figure 5.** Comparison of thylakoid membrane polypeptides of *C. reinhardtii* after urea/SDS-PAGE and Coomassie blue staining. WT, wild-type strain; PSII, PSII-deficient mutant; ATPase, ATPase-deficient mutant; PSI, PSI-deficient mutant. Polypeptides are numbered according to the nomenclature of [36]. Courtesy of P. Delepelaire.

membrane. Fracture faces carry a distinctive array of intramembrane particles, characterized by their size and density. *Figure 6* shows the comparison of WT and a mutant lacking PSII. On the hemi-membrane that is in contact with the thylakoid lumen (EF), large particles appear in the WT. These particles are missing in the mutant and are therefore believed to contain the PSII reaction center [45]. Similar studies with LHC, PSI and cytochrome $b_6f$ mutants and with double and triple mutants have shown how each complex participates to the formation of specific types of freeze fracture particles (reviewed in [12]). Interestingly, different types of complexes often associate within the same particle, which may have important functional implications. In *C. reinhardtii* as in higher plants, thylakoids are appressed on part of their length, even though they do not form regular grana stacks. On each fracture face, stacked and unstacked regions can be recognized by their different particle sizes and densities (not visible on *figure 6* which shows only stacked regions). The comparison of WT and mutants therefore also reveals the distribution of the complexes between the two domains. This has largely contributed to our current knowledge of thylakoid membrane lateral heterogeneity, where PSII is located mostly in the stacked regions, PSI exclusively in the unstacked regions, and cytochrome $b_6f$ in both. Another approach to this question is immuno-electron microscopy, where *C. reinhardtii* mutants have provided excellent negative controls [12].

When the mutation affects the properties of the complex without compromising its stability, it provides a new and invaluable material for functional analysis. This is how the D1 polypeptide has been identified as the target of PSII-directed herbicides, and hence a possible reaction center component [46]. *C. reinhardtii* mutants have greatly contributed to the analysis of electron transfer mechanisms in PSII and in PSI, respectively reviewed by Ruffle and Sayre and by Webber and Bingham [12] and in cytochrome $b_6f$ complex [27, 43, 47, 48].

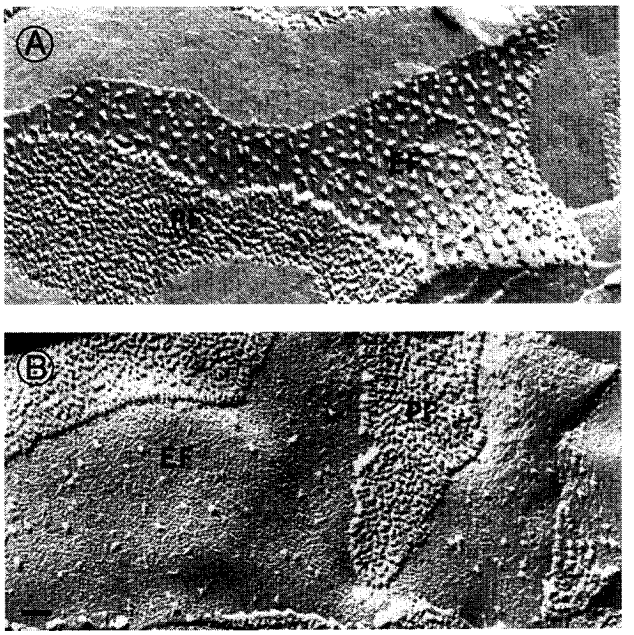


**Figure 6**. Freeze-fracture thylakoid membrane micrographs of WT (**A**) and the F34 mutant lacking PSII (**B**). Note the reduced particle density and size on the exoplasmic (EF) face of the latter, while the periplasmic face (PF) is unaltered. Bar is 50 nm. Courtesy of J. Olive.

## 5. Interplay between chloroplast and nucleus: structural and expression genes

Based on the nature of the molecular lesion, mutations impairing photosynthesis can be grouped in two categories: some lie in the genes (in the chloroplast or the nucleus) that encode the structural components of the photosynthesis complexes, while others affect genes (mostly nuclear) specifically involved in the expression of the structural genes. As mentioned above, more than 30 chloroplast genes encode subunits of photosynthesis complexes. Since the first characterization of a uniparental mutant of *C. reinhardtii* lacking PSI [49], many photosynthesis mutants have been found to lie in the coding regions of chloroplast genes [47, 50, 51]. Others are located in the 5' region of photosynthesis genes, thus interfering with translation [52] or with transcription or mRNA processing [53]. Some of the components of the photosynthesis apparatus are encoded in the nuclear genome (see *figure 1*), and not surprisingly, several photosynthesis mutations have been described in those genes [54-56]. However, the majority of nuclear photosynthesis mutations lie in genes involved in the expression of chloroplast structural genes or in the assembly of the complexes [6]. For example, at least 14 genes concur to the correct *trans*-splicing of the chloroplast *psaA* mRNA [17, 57], and three to translation initiation on the *psbC* message [52, 58]. A three-letter nomenclature has been devised for nuclear genes involved in expression of chloroplast genes: the first letter denotes the type of defect (*M* for mRNA production, processing or stabilization; *T* for translation; *C* for co-factor synthesis or assembly), the second letter identifies the complex concerned (*A* for PSI, *B* for PSII, *C* for cytochrome $b_6f$, *D* for ATPase) and the third the particular subunit involved (*A* for *psaA* or *psbA* or *petA* or *atpA* gene product, etc.). Thus, the F34 mutation which prevents translation of CP43, the *psbC* gene product, will be the *tbc-1-1* allele of the *TBC1* locus [58].

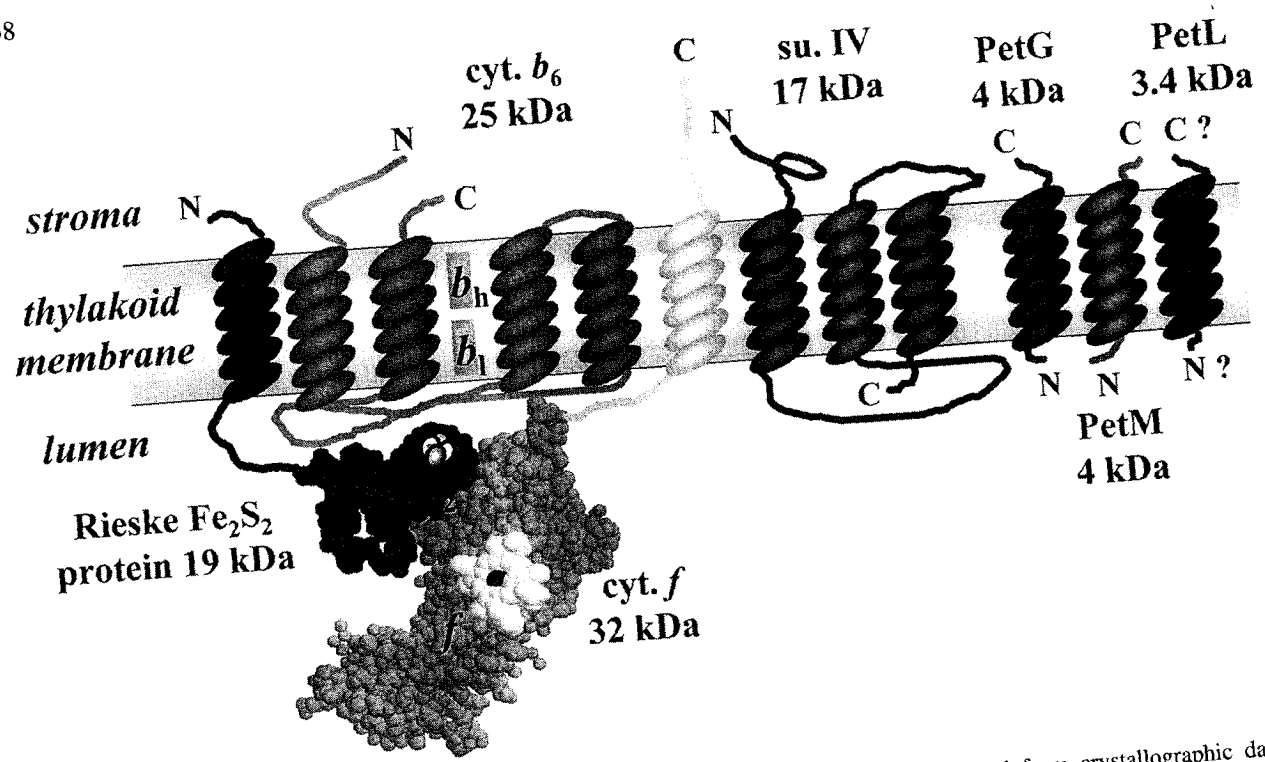


**Figure 7**. Topology of the cytochrome $b_6f$ subunits. Cyt. $f$ and Rieske protein are reproduced from crystallographic data on homologous proteins [76, 77].

## 6. Concerted accumulation of subunits in photosynthesis complexes: the case of cytochrome $b_6f$

An important characteristic of most photosynthesis mutations, obvious on *figure 5*, is their pleiotropy: several polypeptides are missing in a given category of mutant, even though each mutation is likely to act primarily at the level of only one polypeptide. In other words, the various subunits of a complex accumulate in a concerted manner. Detailed studies of this phenomenon have been carried out on PSII [59, 60], PSI and the ATPase, respectively reviewed by Webber and Bingham and by Strotman et al. [12]. Here, we will focus on the case of cytochrome $b_6f$. *C. reinhardtii* mutants have allowed unique studies of this complex because it is not required for growth, which is not the case for most prokaryotes in which it is also part of the respiratory chain. The cytochrome $b_6f$ complex plays a central role in the photosynthetic electron transfer chain between photosystem II and I. It couples translocation of protons across the membrane to the oxidation of the lipophilic electron carrier plastoquinol and reduction of the small hydrophilic protein plastocyanin (replaced by cytochrome $c_6$ when Cu is missing, (reviewed in [12]). Upon plastoquinol oxidation, one electron reduces cytochrome $b_6$, while the other reduces plastocyanin via a high potential chain comprising the Rieske $Fe_2S_2$ protein and cytochrome $f$.

### 6.1. Characterization of the subunits

The cytochrome $b_6f$ complex of *Chlamydomonas* comprises seven subunits (*figure 7*) (*table I*). The four large subunits have been shown to be in a 1:1:1:1 ratio. Cytochrome $f$, cytochrome $b_6$ and subunit IV are encoded by chloroplast genes [61], while the Rieske iron-sulfur protein is encoded by a nuclear gene [62]. In addition, the purified complex [63] contains three transmembrane subunits of low molecular mass, the products of the chloroplast genes *petG* [64] and *petL* [52] and of the nuclear gene *PetM* [65]. These polypeptides co-migrate as a 4 kDa band in SDS-PAGE.

### 6.2. Cooperation of the nuclear and chloroplast genome

Although the cytochrome $b_6f$ complex comprises only two nucleus-encoded subunits (see above), at least 14 nuclear loci contribute to the expression of the complex. These nuclear loci affect expression of a single chloroplast *pet* gene at the post-transcriptional level (*table II*). Five of them are implicated in the binding of the c-type heme to apo-cytochrome $f$ and apo-cytochrome $c_6$. Covalent binding occurs by thioether linkage between the sulfhydryl groups of two cysteine residues of the protein and the vinyl groups of the tetrapyrrole ring of the heme. These *CCS* genes, together with the chloroplast *ccsA* gene,

**Table I.** Cytochrome $b_6f$ subunits in *C. reinhardtii*. Gene encoded by the chloroplast (C) or the nucleus (N). Molecular mass (M) of the mature subunit calculated from the sequence without cofactors. Name refers to the polypeptide migration pattern of thylakoid membranes after SDS/PAGE electrophoresis [36] and/or pulse-labeling in the presence of an inhibitor of cytoplasmic translation [71]. TMH, number of transmembrane α-helices. The N-terminal helix of the Rieske protein of the *C. reinhardtii* cytochrome $b_6f$ complex is perhaps placed at the membrane surface [72], but is transmembrane in the fine structural characterization of the cytochrome $bc_1$ complex [69, 70].

| *Subunit* | *Gene* | *C/N* | $M_r$ ($10^{3\ kDa}$) | *Name* | *TMH* | *Function* |
|---|---|---|---|---|---|---|
| Cytochrome *f* | *petA* | C | 31.2 | 8.2 | 1 | Binds 1 *c*-heme, reduces PC |
| Cytochrome $b_6$ | *petB* | C | 24.2 | L1 | 4 | Binds 2 *b*-hemes, quinones at $Q_O$ and $Q_I$ sites |
| Rieske $Fe_2S_2$ protein | *PetC* | N | 18.4 | 19.1 | 1? | Binds 1 $Fe_2S_2$, contributes to $Q_O$ site |
| Subunit IV | *petD* | C | 17.4 | L4, 24' | 3 | Contributes to quinone binding |
| PetG | *petG* | C | 4.0 | | 1 | Necessary for complex formation |
| PetL | *petL* | C | 3.4 | | 1 | Complex stability, binding of Rieske protein |
| PetM | *PetM* | N | 4.0 | | 1 | ? |

define a novel pathway for heme *c* binding, distinct from those of mitochondria and bacteria (reviewed in [66]).

In contrast, the two *b*-hemes of cytochrome $b_6$ are considered as non-covalently bound. Still, heme-binding to cytochrome $b_6$ is partly resistant to an acetone acid treatment which is classically used to extract *b*-hemes [5], suggesting that one of two *b*-hemes of holocytochrome $b_6$ is tightly bound to the polypeptide. We exploited this property to define a pathway for the conversion of apo- to holo-cytochrome $b_6$, and to identify mutants that are blocked at one step of this pathway.

*C. reinhardtii* strains carrying substitutions in either one of the four histidines which coordinate the $b_h$ or $b_l$ hemes to the apoprotein were created. These mutations resulted in the appearance of distinct immunoreactive species of cytochrome $b_6$ *(figure 8)*, which allowed us to specifically identify cytochrome *b*6 with altered $b_h$ or $b_l$ ligation. In gabaculine-treated wild type and site-directed mutant strains (i.e., heme-depleted, gabaculine is an inhibitor of the tetrapyrrole biosynthetic pathway), we established that: i) the single immunoreactive band, observed in strains carrying the $b_l$-site directed mutations, corresponds to apocytochrome $b_6$; and ii) the additional band present in strains carrying *b*h-site directed mutations corresponds to a $b_l$-heme-dependent intermediate in the formation of holocytochrome $b_6$.

Five nuclear mutants (*ccb* strains) which are defective in holocytochrome $b_6f$ formation display a phenotype which is indistinguishable from that of strains carrying site-directed $b_h$-ligand mutants. The defect is specific for cytochrome $b_6$ assembly because the *ccb* strains can synthesize other *b*-cytochromes and all *c*-type cytochromes. The *ccb* strains, which define four nuclear loci (*CCB1*, *CCB2*, *CCB3*, and *CCB4*), provide the first evidence that a *b*-type cytochrome requires trans-acting factors for its heme-association.

The concerted accumulation of cytochrome $b_6f$ subunits is shown by the immunoblot of *figure 9A*, where a *petB* deletion mutant is compared to WT. Only trace amounts of Rieske protein, subunit IV and PetG are detectable. The autoradiogram of *figure 9B* shows that synthesis of subunit IV is normal, and its rapid degradation has been followed in chase experiments [32]. The case of cytochrome *f* is different: chase experiments show that this polypeptide is stable in the *petB* mutant, and that its reduced accumulation is due to a reduction in its synthesis rate. Studies with *petA* mutants and chimeric constructs harbouring the *petA* 5' untranslated region have led to a model of translational control [13], called control by epistasy of synthesis (CES). The translation initiation rate on the *petA* mRNA is regulated by an epistatic factor, which senses the presence of unassembled cytochrome *f*

**Table II.** Nuclear loci participating in the expression of the chloroplast *pet* gene (reviewed in [66, 73, 74]).

| *Nuclear loci (number of alleles)* | *Function* |
|---|---|
| *CCS1* (6), *CCS2* (5), *CCS3* (1), *CCS4* (1), *CCS5* (1) | *c*-heme attachment to cytochromes *f* and $c_6$ |
| *CCB1* (1), *CCB2* (1), *CCB3* (1), *CCB4* (3) | *b*-heme attachment to cytochrome $b_6$ |
| *MCA1* (5) | Stability/maturation of *petA* mRNA |
| *MCB1* (1) | Stability/maturation of *petB* mRNA |
| *MCD1* (2) | Stability/maturation of *petD* mRNA |
| *MCG1* (1) | Stability/maturation of *petG* mRNA |
| *TCA1* (7) | Translation of *petA* mRNA |

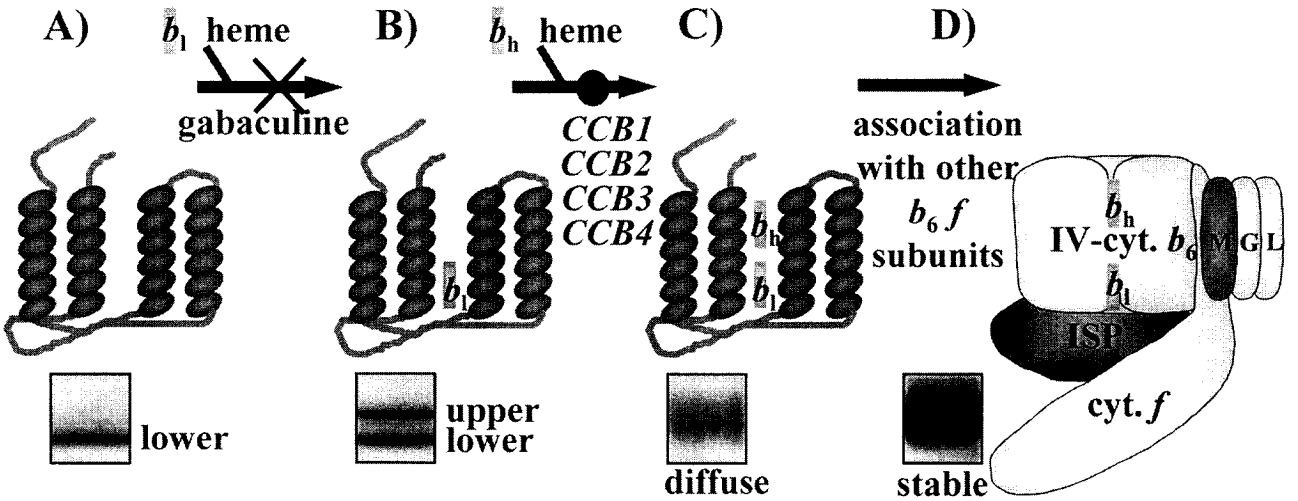


**Figure 8**. Schematic pathway of the conversion of apo- to holo-cytochrome $b_6$, and cytochrome $b_6$ patterns after urea/SDS-PAGE. **A.** Membrane insertion occurs even in the absence of heme binding. **B.** Formation of a $b_l$-heme-dependent intermediate ('upper') is prevented by gabaculine or $b_l$-site mutations. **C.** Formation of $b_h$- and $b_l$-binding holocytochrome $b_6$ (diffuse) is prevented by *ccb* or $b_h$-site mutations. **D.** Holocytochrome $b_6$ accumulates in a concerted process with other $b_6f$ subunits.

via its stroma-exposed C-terminal domain. This negative feedback ensures that the protease-stable cytochrome *f* is not made in excess of its assembly partners cytochrome $b_6$ and subunit IV. A similar CES mechanism probably operates in PSII (where it may explain the reduced synthesis of D1 in a D2 mutant [59], and of CP43 in a D1 mutant [50]) and in the ATPase [67]. Similar observations have been reported in higher plant chloroplasts and in mitochondria (reviewed in [13]), which suggests that CES may represent a major regulatory mechanism in organelles.

Some of the cytochrome $b_6f$ subunits are not strictly required for complex assembly. While the deletion of *petG* drastically destabilized the complex in *Chlamydomonas* [64], allowing only a few percent of accumulation of the other $b_6f$ subunits, that of *petL* exerted an effect only when the culture entered stationary phase [43]. Mutants in the *PetC* gene also assemble a relatively stable subcomplex [27], as do several *petB* or *petD* mutants affecting the $Q_0$ site [47, 48]. Deletion of PetM has been achieved only in *Synechocystis* and did not affect the complex [68]. In some mutants, the complex is assembled and accumulated but some subunits are less tightly bound; for example, a loosened biochemical interaction of the Rieske protein with the rest of the cytochrome $b_6f$ complex was observed in the *petL* deletion mutant [43] and in the Fud2 mutant altered in the $Q_O$ plastoquinol binding site of cytochrome $b_6$ due to a 12 amino acid duplication in the lumenal CD loop connecting helices C and D [47].

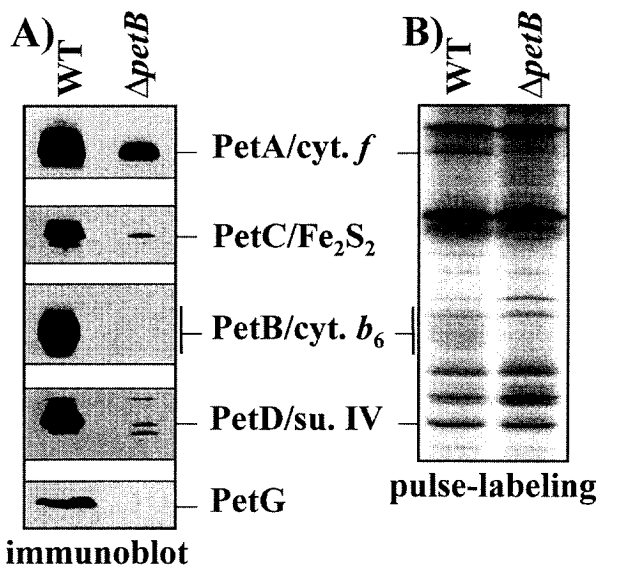


**Figure 9**. Accumulation and synthesis of cytochrome $b_6f$ subunits in whole cells wild-type (WT) and mutant strain deleted in the *petB* gene (Δ*petB*). **A.** Content of cytochrome $b_6f$ subunits analyzed by immunoblotting experiments. **B.** Autoradiogram of a urea/SDS-PAGE gel showing chloroplast-encoded proteins radiolabeled for 5 min in the presence of $^{14}$C-acetate and an inhibitor of cytoplasmic translation. Note normal synthesis of subunit IV, but reduced synthesis of cytochrome *f*.

## 7. Conclusion

In conclusion, the study of the thylakoid membrane is an example of multidisciplinarity. *C. reinhardtii* allows the complementary development of genetic, biophysical and biochimical approaches. The example of the cytochrome $b_6f$ complex illustrates the contribution of *C. reinhardtii* to the understanding of gene expression and function in the chloroplast. Some in vitro studies can be difficult with *C. reinhardtii,* such as those implicating isolation of intact chloroplasts and mitochondria. In contrast, *C. reinhardtii* is well suited to in vivo studies. The fine structural characterization of the photosynthetic and respiratory complexes is in progress (the tridimensional structures of LHCII, PSI and the soluble domains of cytochrome *f* and Rieske protein have already been determined) and has led to new concepts, such as the electron transfer by domain movement proposed for the Rieske protein [69, 70]. The ability to test models by structure/function correlations is precious. Growing questions concern the mechanisms of assembly and degradation of these complexes, their interactions in the membrane and interactions between the chloroplast and other cell compartments.

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