Defects in the Cytochrome b_6/f Complex Prevent Light-Induced Expression of Nuclear Genes Involved in Chlorophyll Biosynthesis[1]

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Mutants with defects in the cytochrome (cyt) b_6/f complex were analyzed for their effect on the expression of a subgroup of nuclear genes encoding plastid-localized enzymes participating in chlorophyll biosynthesis. Their defects ranged from complete loss of the $cytb_6/f$ complex to point mutations affecting specifically the quinone-binding Q_O site. In these seven mutants, light induction of the tetrapyrrole biosynthetic genes was either abolished or strongly reduced. In contrast, a normal induction of chlorophyll biosynthesis genes was observed in mutants with defects in photosystem II, photosystem I, or plastocyanin, or in wild-type cells treated with 3-(3′4′-dichlorophenyl)-1,1-dimethylurea or 2,5-dibromo-3-methyl-6-isopropyl benzoquinone. We conclude that the redox state of the plastoquinone pool does not control light induction of these chlorophyll biosynthetic genes. The signal that affects expression of the nuclear genes appears to solely depend on the integrity of the $cytb_6/f$ complex Q_O site. Since light induction of these genes in Chlamydomonas has recently been shown to involve the blue light receptor phototropin, the results suggest that $cytb_6/f$ activity regulates a plastid-derived factor required for their expression. This signaling pathway differs from that which regulates state transitions, since mutant *stt7*, lacking a protein kinase involved in phosphorylation of the light-harvesting complex II, was not altered in the expression of the chlorophyll biosynthetic genes.

Cells of plants and eukaryotic algae harbor, in addition to the nucleus, two DNA-containing organelles of endosymbiotic origin, the plastids, and the mitochondria. The vast majority of genetic information of these endosymbionts in the course of evolution was transferred to the nuclear genomes of their hosts (Rujan and Martin, 2001; Martin et al., 2002). However, the chloroplasts, as predicted for Arabidopsis (*Arabidopsis thaliana*), contain between 2,500 and 3,000 proteins, half of which are of cyanobacterial origin (Abdallah et al., 2000; Richly and Leister, 2004). The location of genes encoding organelle proteins in different compartments implies the existence of mechanisms that serve to integrate nuclear and organellar gene expression. The cross talk between organelles includes both anterograde (nucleus-to-organelle) and retrograde (organelle-to-nucleus) controls. Via anterograde mechanisms, nucleus-encoded proteins tightly control essential steps in gene expression within the organelles (Goldschmidt-Clermont, 1998; Leon et al., 1998; Choquet and Wollman, 2002). Retrograde signaling regulates the expression of a subset of nuclear genes (mostly those encoding organelle proteins) in response to the metabolic and developmental state of the organelle.

The first evidence for retrograde chloroplast signaling came from analysis of barley (*Hordeum vulgare*) mutants defective in plastidic ribosomes (Bradbeer and Börner, 1978). Subsequent analyses have so far provided genetic and/or molecular evidence for five chloroplast-to-nucleus signaling pathways: one that requires plastid protein synthesis, a second that is associated with chloroplast-generated singlet oxygen, a third that appears to employ hydrogen peroxide, a fourth that senses the redox poise of the plastoquinone pool, and a fifth that involves tetrapyrrole biosynthesis intermediates (for review, see Surpin et al., 2002; Gray, 2003; Gray et al., 2003; Pfannschmidt et al., 2003; Beck, 2005; Fey et al., 2005).

It is unclear, however, how these pathways interact to regulate gene expression. Photosynthetic activity appears to be an important factor in controlling gene expression, since in mutants that are deficient in the cytochrome (cyt) b_6/f complex or lacking plastidic ATP synthase, about one quarter of more than 3,000 nuclear genes analyzed were expressed at significantly different levels from wild type (Maiwald et al., 2003; Richly et al., 2003). This has led to the hypothesis that physiological effects of mutations affecting the redox state of the plastoquinone pool and/or the transmembrane H^+-gradient control nuclear gene expression. However, large differences were also observed between different mutants lacking PSI (Maiwald et al., 2003; Richly et al., 2003; Ihnatowicz et al., 2004), which

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suggests that the integrity of a photosynthetic complex, rather than its activity in electron transfer, could be the source of the signals that control nuclear gene expression. While a large collection of data is available on the genes whose expression is perturbed in response to alterations in the chloroplast (Biehl et al., 2005; Leister, 2005), the sites of signal generation within the chloroplast have not yet been identified.

In the analysis presented here, we focused on the role of the cytb$_6$/f complex in signaling toward the nucleus and present an analysis of seven mutants defective in this complex. In all of these mutants, the light induction of a subset of chlorophyll biosynthetic genes was either abolished or strongly diminished, while the expression of these genes was not affected when photosynthetic electron flow and the redox state of the plastoquinone pool were altered by inhibitors or by mutations in other photosynthetic complexes.

RESULTS

Mutants Defective in the Cytb$_6$/f Complex

The mutants analyzed and their relevant characteristics are summarized in Table I. They include two mutants isolated in a screen aimed at the identification of genes that control light sensitivity (P1-15 and P2-26). These two strains as well as five mutants (Δ*petA*, *mcd1-F16*, *tca1-693*, *petD*-PWYE, *petC-Δ1*, and *clpP1*-AUU) described before have defects in photosynthesis, i.e. they do not grow photoautotrophically and thus require acetate for growth. In addition, they are light sensitive, i.e. they grew on acetate-containing plates irradiated with a fluence rate of 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light but not when irradiated with a fluence rate of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ or 500 $\mu E\, m^{-2} \cdot s^{-1}$ in the case of the P1-15 mutant. The P1-15 and P2-26 strains displayed fluorescence induction kinetics typical for cytb$_6$/f mutants, i.e. a continued rise in fluorescence up to levels attained in the presence of 3-(3′4′-dichlorophenyl)-1,1-dimethylurea (DCMU; Fig. 1A). In P2-26, the maximum level of fluorescence obtained in the absence of DCMU was slightly higher than that seen in the presence of the herbicide, indicating a complete block in cytb$_6$/f electron transfer. The higher level in the absence of DCMU may be explained by a full oxidation of plastoquinone, acting as a quencher of fluorescence (Joliot et al., 1998). Mutant P1-15, in contrast, resembles leaky cytb$_6$/f mutants (see, for example, figure 4 in de Vitry et al., 1999), with a lower level of fluorescence in the absence

Table I. *Mutants employed and their characteristics*

L^S, No growth at 80 $\mu E\ m^{-2}\ s^{-1}$; HL^S, no growth at 500 $\mu E\ m^{-2}\ s^{-1}$; R, light resistant, i.e. growth at 500 $\mu E\ m^{-2}\ s^{-1}$; cont., continued; WT, wild type; ND, not done.

Mutant	Phenotypes			Mutational Defect	Reference
	Growth without Acetate	Light Sensitivity	Fluorescence Induction		
Δ*petA*	−	L^S	Cont. rise to F_{max}	Deletion of chloroplast *petA* gene	Kuras and Wollman (1994)
mcd1-F16	−	L^S	Cont. rise to F_{max}	Defect in a nuclear gene that controls *petD* mRNA stability	Drager et al. (1998)
tca1-693	−	L^S	Cont. rise to F_{max}	Nuclear gene; encodes a translation activator for *petA* mRNA	Wostrikoff et al. (2001)
petD-PWYE	−	L^S	Cont. rise to F_{max}	A *petD* mutation that alters the Q_O pocket of the cytb$_6$/f complex	Zito et al. (1999)
clpP1-AUU	+	R	WT	Mutation in *clpP1* start codon resulting in low ClpP protease level	Majeran et al. (2000)
petC-Δ1 clpP1-AUU	−	L^S	ND	2-bp deletion in *PETC*; devoid of Rieske protein	Majeran et al. (2000)
P1-15	−	HL^S	Cont. rise to F_{max}	Nuclear mutation	Dent et al. (2005) and this report
P2-26	−	L^S	Cont. rise to F_{max}	Nuclear mutation	This report
stt7	+	R	WT	Defect in a thylakoid-associated protein kinase involved in LHCII phosphorylation	Depège et al. (2003)
AP6	−	HL^S	ND	Mutation in nuclear gene *PCY1*	Kindle and Lawrence (1998)
Δ*psbD*	−	HL^S	ND	Deletion in chloroplast *psbD* gene	Minai et al. (2006)
Δ*psaB*	−	HL^S	ND	Deletion in chloroplast *psaB* gene	Redding et al. (1999)

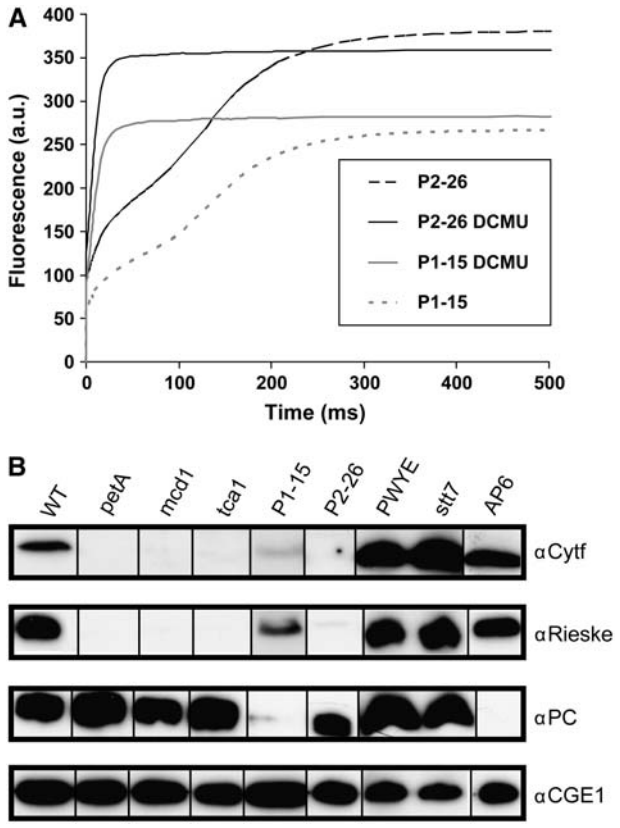


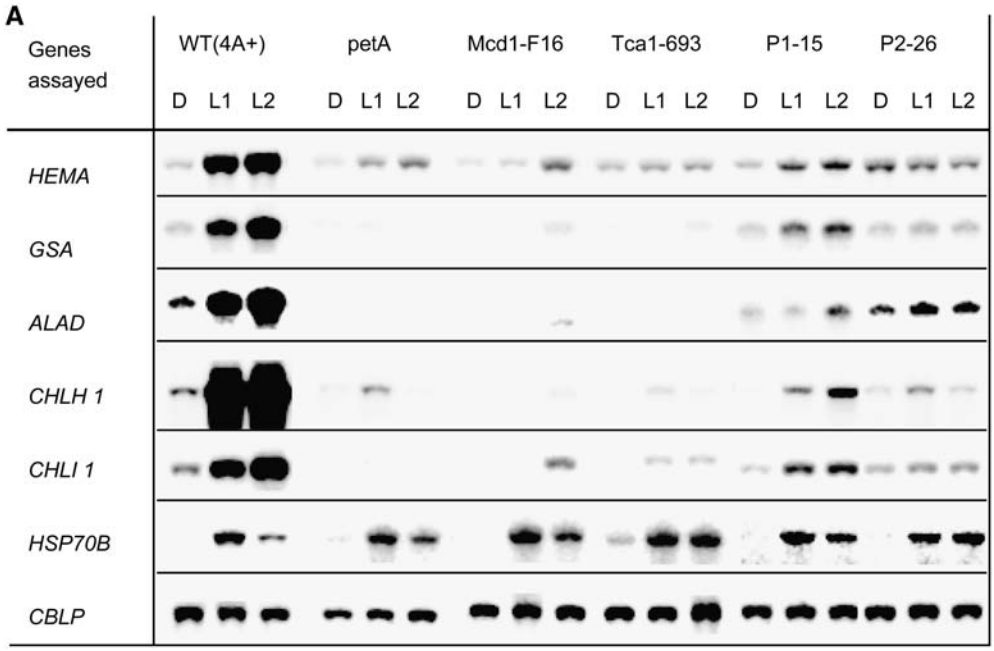
Figure 1. Analysis of mutants for fluorescence induction and two $cytb_6/f$ proteins as well as PC. A, Chlorophyll fluorescence induction curves of strains P1-15 and P2-26 measured in the absence or presence of 10 μM DCMU. Note that the F_{max} level of P1-15 in the absence of the herbicide is lower than in its presence, indicating residual electron transfer. B, Total soluble proteins (15 μg per lane) were separated by SDS-PAGE (15% polyacrylamide). After transfer to membranes, protein blots were reacted sequentially with antisera directed against cyt f, the Rieske protein, and PC as described in "Materials and Methods." Antibodies that recognized the plastidic GrpE homolog of Chlamydomonas (CGE1) served as a loading control.

of DCMU. Electron transfer through the $cytb_6/f$ complex can be revealed with high sensitivity by the slow phase of the flash-induced electrochromic shift measured at 520 nm (Joliot et al., 1998). In P1-15, a small absorption change was observed, suggesting that it is indeed capable of a slow rate of electron transfer via the $cytb_6/f$ complex (F. Rappaport, personal communication). This is consistent with a very weak signal observed in some immunoblots for cyt f and Rieske protein in extracts of the P1-15 strain (Fig. 1B).

Mutant Δ*petA* has a deletion of the plastid genes for cyt f, while *petD*-PWYE, also a plastome mutation, carries substitutions in three residues of subunit IV, inactivating the Q_O site where plastoquinol oxidation occurs. In the other three previously characterized mutants, nuclear genes are affected: *mcd1-F16* and *tca1-693* lack nuclear-encoded factors necessary for stabilization of *petD* mRNA and translation of *petA* mRNA, respectively, while *petC-Δ1* carries a short deletion in the *PETC* gene encoding the Rieske Fe-S protein, leading to a complete absence of this essential subunit. In the strain we have used, the latter mutation was combined with *clpP1*-AUU, a mutation reducing accumulation of the ClpP protease, resulting in the stabilization of a $cytb_6/f$ subcomplex lacking the Rieske protein (Majeran et al., 2000). The absence of cyt f was demonstrated by immunoblot analysis for mutant P2-26 and confirmed for mutants Δ*petA*, *mcd1-F16*, and *tca1-693* (Fig. 1B). Traces of Rieske protein could be observed in some of the mutants depending on the individual experiment, resulting from incomplete degradation when other subunits are missing (O. Vallon, unpublished data). In contrast, both cyt f and Rieske proteins accumulated normally in the *petD*-PWYE mutant, as described before (Zito et al., 1999). Also included in this analysis was the mutant *stt7*, which is defective in the protein kinase that is required for the phosphorylation of the major light-harvesting protein (LHCII), an essential requirement for state transitions to occur (Depège et al., 2003). This mutant has normal levels of $cytb_6/f$ (Fig. 1B), grows photoautotrophically, and is not light sensitive. Mutant AP6 has a defect in the *PCY* gene for plastocyanin (PC; Fig. 1B) and was used as a control. We observed a reduced level of PC in P1-15, which was shown to be caused by a second site mutation since it separated from the $cytb_6/f$ defect in crosses. This defect was not linked to the regulatory phenotype of mutant P1-15 (data not shown).

Mutants with Defects in the $Cytb_6/f$ Complex Exhibit Deregulation of Genes Involved in Chlorophyll Synthesis

A number of *Chlamydomonas reinhardtii* genes involved in chlorophyll biosynthesis (*HEMA*, *GSA*, *ALAD*, *CPXI*, *CHLD*, *CHLH1*, *CHLI1*, and *CTH1*) have been shown to be induced in dark-adapted cultures by a shift from dark to light of a moderate fluence rate (40 $\mu E \cdot m^{-2} \cdot s^{-1}$; Vasileuskaya et al., 2004). The expression of five of these nuclear genes was analyzed at the RNA level in those mutants that either lacked the $cytb_6/f$ complex or possessed strongly reduced levels. In all cases, the patterns of mRNA accumulation following the shift to light were clearly different from that of the wild type, exhibiting a lack of light-induced accumulation (Fig. 2). In some mutants, mRNA levels for genes *GSA*, *ALAD*, and *CHLI1* in the dark or after shift to light remained below the level of detection. In the P1-15 mutant, some residual light-induced mRNA accumulation was observed but this was distinctly lower than in the wild-type strain. In contrast to the five genes of tetrapyrrole biosynthesis, *HSP70B*, a nuclear gene encoding a chloroplast-localized chaperone (Schroda et al., 1999), exhibited normal light induction in the mutants. Also, expression of *CBLP*, encoding a Gβ-like protein (von Kampen et al., 1994) and used as a loading control, appeared not to be affected by defects in the $cytb_6/f$ complex. Thus, expression of a subset of nuclear genes encoding enzymes required for chlorophyll synthesis was specifically



B

	WT	petA	Mcd1-F16	Tca1-693	P1-15	P2-26
HEMA	6.2 ± 1.2	1.1 ± 0.6	1.5 ± 0.6	1.2 ± 0.2	1.6 ± 0.8	0.8 ± 0.5
GSA	7.9 ± 3.7	0.8 ± 0.6	0.9 ± 0.3	1.6 ± 0.4	1.5 ± 0.5	1.0 ± 0.3
ALAD	8.5 ± 2.0	0.8 ± 0.5	1.1 ± 0.6	1.1 ± 0.1	1.7 ± 0.5	1.2 ± 0.04
CHLH 1	27.3 ± 8.6	1.0 ± 0.7	1.4 ± 1.1	2.0 ± 0.9	3.7 ± 2.1	1.4 ± 0.2
CHLI 1	11.2 ± 4.6	1.7 ± 0.5	1.7 ± 1.1	2.3 ± 0.3	3.3 ± 0.7	1.6 ± 0.8
HSP70B	8.2 ± 3.2	6.7 ± 2.1	6.8 ± 1.4	7.2 ± 2.3	8.0 ± 2.9	7.8 ± 3.9

Figure 2. Expression of selected genes of chlorophyll biosynthesis and *HSP70B* in mutants lacking cytb$_6$/f complex components. A, RNA-blot analysis, details of which are given in "Materials and Methods." Prior to light exposure, cultures were incubated in the dark for 20 h (D). Then, cultures were shifted to light (fluence rate 40 μE m^{-2}·s^{-1}) for 1 h (L1) and 2 h (L2). At these time points samples were taken for RNA extraction. For RNA-blot analyses, 10 μg of total RNA were hybridized with probes specific for the genes indicated. The constitutively expressed *CBLP* gene, encoding a Gβ-like protein (von Kampen et al., 1994), served as a loading control. B, Quantitative evaluation of RNA-blot data from at least three experiments. The relative induction of each gene with SEM was normalized using the signal for the *CBLP* gene. The induction ratios given were determined by dividing the values of the L2 samples by those of the D samples.

deregulated in mutants defective in the cytb$_6$/f complex, i.e. showed no up-regulation in the light.

Genetic Analysis of Cytb$_6$/f Mutants

In *C. reinhardtii*, a haploid organism, second site mutations manifest themselves phenotypically and may falsely contribute to the phenotype of a first site mutation. It could be envisioned that mutations affecting gene regulation tend to accumulate over time in cytb$_6$/f mutants, provided they enhance their survival under storage conditions. To address this question, mutants with defects in the cytb$_6$/f complex were crossed with wild-type strains and random spores were picked among the progeny and scored for defective photosynthesis, light sensitivity, and light induction of chlorophyll biosynthesis genes (Table II). If the deregulation of the genes observed was due to a mutation different from that affecting the cytb$_6$/f complex, independent segregation of the phenotypes acetate requirement/light sensitivity (ac$^-$) and no light regulation of chlorophyll biosynthesis genes (lrc$^-$) would be expected (unless the two lesions were closely linked).

For the chloroplast-born mutation Δ*petA*, 93% of the progeny was acetate requiring and light sensitive, as expected from the largely uniparental transmission of chloroplast genes (Harris, 1989). Four each of the

Table II. *Analysis of progeny from crosses of mutants with wild type for cosegregation of the mutant phenotypes*

ac^-, Acetate requiring; L^S, no growth at 80 μE m^{-2} s^{-1}; HL^S, no growth at 500 μE m^{-2} s^{-1}; L^R, light resistant; lrc, light regulation of chlorophyll biosynthesis gene expression.

Crosses Performed	Phenotypes of Progeny Assayed on Plates	Regulatory Phenotypes of Eight Clones[a]
Δ*petA* (mt^+) × 4A− (mt^-)	152ac^-, L^S/12ac^+, L^R	4lrc^-, ac^-, mt^-/4lrc^+, ac^+, mt^+[a]
petD-PWYE (mt^+) × 4A− (mt^-)	94ac^-, L^S/6ac^+, L^R	4lrc^-, ac^-, mt^-/4lrc^+, ac^+, mt^+[a]
mcd1-F16 (mt^-) × 4A+ (mt^+)	23ac^-, L^S/24ac^+, L^R	4lrc^-, ac^-, L^S/4lrc^+, ac^+, L^R
tca1-693 (mt^-) × 4A+ (mt^+)	21ac^-, L^S/27ac^+, L^R	4lrc^-, ac^-, L^S/4lrc^+, ac^+, L^R
P1-15 (mt^+) × 4A− (mt^-)	24ac^-, HL^S/23ac^+, L^R	4lrc^-, ac^-, HL^S/4lrc^+, ac^+, L^R
P2-26 (mt^+) × 4A− (mt^-)	20ac^-, L^S/27ac^+, L^R	4lrc^-, ac^-, L^S/4lrc^+, ac^+, L^R

[a]To ensure the analysis of recombinants (by RNA-blot techniques) from these crosses, the lrc phenotype was assayed only in ac^- progeny that was mt^- and in ac^+ progeny that was mt^+.

ac^- mutant and ac^+ progeny were tested for their ability to induce chlorophyll biosynthesis genes upon dark-to-light shift using RNA blots. To ensure that truly recombinant progeny was tested, as opposed to unmated parental gametes that would have survived the zygote selection process, mt^+ clones were chosen for the ac+ progeny, and mt^- clones for the ac^- progeny. In the eight clones tested, strict correlation was observed between the photosynthesis defect/light sensitivity and absence of gene induction by light (Table II).

Crosses of the four nuclear mutants showed an approximately equal distribution of wild-type and ac^- phenotypes (Table II), as expected for segregation of single nuclear mutations. As above, four randomly picked clones of each phenotypic class were analyzed for the lrc phenotype and here again, perfect correlation was observed between the ac and lrc phenotypes (Table II). In spite of the small number of progeny analyzed, the fact that this result was observed for five independent mutants carrying mutations in two distinct genomes clearly shows that the defect in light induction of the chlorophyll biosynthesis genes is a direct consequence of $cytb_6/f$ deficiency.

Light Induction of Genes Involved in Chlorophyll Synthesis Is Not Affected by Other Types of Photosynthesis Mutations or by Inhibitors of Electron Transport

The deregulation phenotype of mutants defective in the $cytb_6/f$ complex raised the question whether interruption of photosynthetic electron transport by itself was sufficient to cause the alteration in gene expression observed. To address this question we made use of mutants defective in different steps of electron transport. A Δ*psbD* mutant, defective in PSII, showed a wild-type pattern of gene expression (Fig. 3). Also mutant AP6, which lacks PC (Fig. 1B) and thus, in the absence of the alternative electron carrier cyt c6 that only is present when cells are deprived of copper (Merchant and Bogorad, 1986), is unable to transport electrons from the $cytb_6/f$ complex to PSI, showed light induction of the genes assayed (Fig. 3). The slight delay in light induction in the PC mutant was not observed with other PC-defective strains (data not shown). Lastly, mutant Δ*psaB*, defective in PSI, was also not affected in the regulation of the genes assayed. We conclude that a block in photosynthetic electron transport by itself is not sufficient to prevent light induction of the nuclear genes analyzed. Importantly, while in PSII mutants the plastoquinone pool is rapidly oxidized during the dark-to-light shift, the reverse will occur in PSI and PC mutants where, just like in $cytb_6/f$ mutants, the plastoquinone pool will be overreduced. Thus, the deregulation phenotype of $cytb_6/f$ mutants cannot be attributed to an indirect effect on the redox state of the plastoquinone pool during illumination.

In accordance with these observations, the application of the PSII inhibitors DCMU and the phenolic herbicide dinoterb did not affect gene expression patterns (Fig. 3). These compounds interact with the Q_B binding site of the PSII complex and prevent reoxidation of the primary quinone acceptor by the plastoquinone pool.

More surprisingly, 2,5-dibromo-3-methyl-6-isopropyl benzoquinone (DBMIB), which inhibits the oxidation of plastoquinol at the Q_O site of the $cytb_6/f$ complex (Roberts et al., 2004), did not influence the light induction of the chlorophyll biosynthesis genes (Fig. 3). DBMIB binds at the Q_O site of $cytb_6/f$, where plastoquinol is reoxidized, suggesting that plastoquinol binding and turnover at this site is not necessary for light induction to occur. To rule out any direct effect of light on the electron transfer chain, the PSI mutant was treated with DCMU. Here again, light induction was observed both at 3 μM DCMU (Fig. 3) and 6 μM DCMU (data not shown), in spite of the fact that inactivation of both photosystems would obviously prevent any light-induced redox changes in the plastoquinone pool and $cytb_6/f$ complex.

Analysis of Mutants with Discrete Defects in $Cytb_6/f$ Subunits or in $Cytb_6/f$ Signaling

These observations suggested that the abrogation of light induction of the chlorophyll biosynthesis genes was due to the lack of accumulation of the $cytb_6/f$ complex, rather than to the absence of electron transport through the complex. We therefore analyzed mutants that accumulated the $cytb_6/f$ complex, but carried more discrete defects preventing electron transfer. The *petC-Δ1* mutant (de Vitry et al., 1999) is devoid of Rieske Fe-S protein, hence of a functional

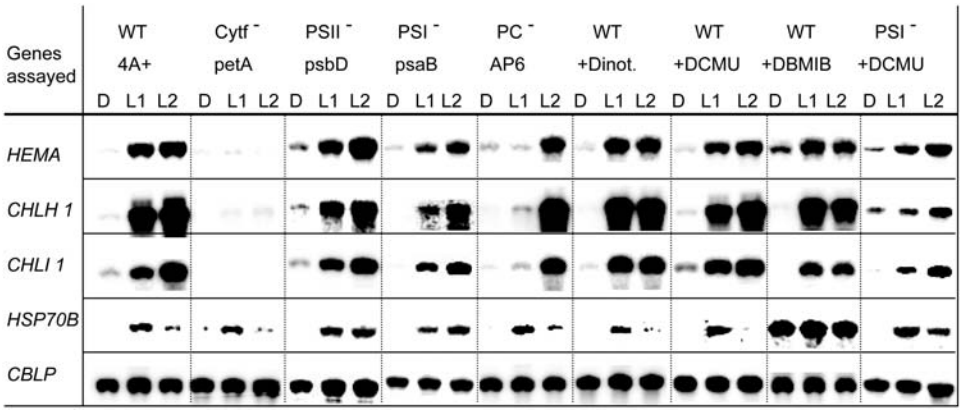


Figure 3. Effect on gene expression patterns of mutations affecting PSII, PC, PSI, and of inhibitors that block electron transfer from PSII to the cytb$_6$/f complex in wild-type cells. All cultures were incubated in the dark for 20 h (D). Cultures of the 4A+ strain and the four mutants were then exposed to white light for one (L1) and 2 h (L2). For inhibitor studies, dinoterb (final concentration 30 μM), DCMU (final concentration 3 μM), and DBMIB (final concentration 1.5 μM) were added to cultures 40 min prior to shift to light. Samples were processed as described in the legend of Figure 2. Note that DBMIB caused overaccumulation of *HSP70B* mRNA in the dark and in the light, an observation that was not pursued.

Q_O site, but accumulates almost normal levels of cyt f and other core subunits of the complex, at least when combined with a mutation that attenuates ClpP (Majeran et al., 2000). This strain clearly exhibited the same deregulated phenotype seen for the mutants that lack the cytb$_6$/f complex (Fig. 4). We also analyzed the *petD*-PWYE mutant, which carries three substitutions in subunit IV and assembles the complex (including the Rieske protein), but is unable to bind plastoquinol in the Q_O pocket and therefore to transfer electrons (Zito et al., 1999). This mutant also exhibited no light induction of the five genes analyzed (Fig. 4). The *petD*-PWYE mutant was crossed to wild type, and the phenotype of the progeny was analyzed as described above. Again, a perfect correlation was observed between the ac$^-$ and lrc$^-$ phenotypes (Table II; Fig. 5).

Together, these results indicate that normal light induction of chlorophyll biosynthesis genes not only requires the assembly of a complete cytb$_6$/f complex, but also that its Q_O site is able to bind quinones. The Q_O site is also known to regulate state transitions, activating the LHCII kinase in a redox-dependent manner. In Chlamydomonas, the Stt7 kinase has been shown to be required for this process (Depège et al., 2003). In Arabidopsis, mutation of the orthologous gene *Stn7* (Bellaflore et al., 2005) not only impairs state transitions, but also affects expression of nuclear genes, as does inactivation of the paralogous gene *Stn8* (Bonardi et al., 2005). We therefore analyzed the Chlamydomonas *stt7* mutant and found that it exhibited a pattern of gene expression similar to that of the wild-type strain (Fig. 4), indicating that the Stt7 kinase is not involved in light induction of chlorophyll biosynthesis genes.

DISCUSSION

The light induction of the five chlorophyll biosynthesis genes analyzed here has recently been shown to be mediated via the blue light receptor phototropin (Im et al., 2006). The fact that induction of these genes observed after a shift from dark to light is markedly diminished in strains with reduced levels of phototropin implies an essential role of this photoreceptor in the regulation of these genes. This signaling pathway is envisioned to act at the level of activation of transcription or, alternatively, via stabilization of the transcripts. For one gene (*GSA*), a partial characterization of the blue light signaling pathway has suggested the participation of a heterotrimeric G-protein and phospholipase C, an increase in the cytosolic Ca^{2+} concentration, and activation of calmodulin as well as of a calmodulin-dependent

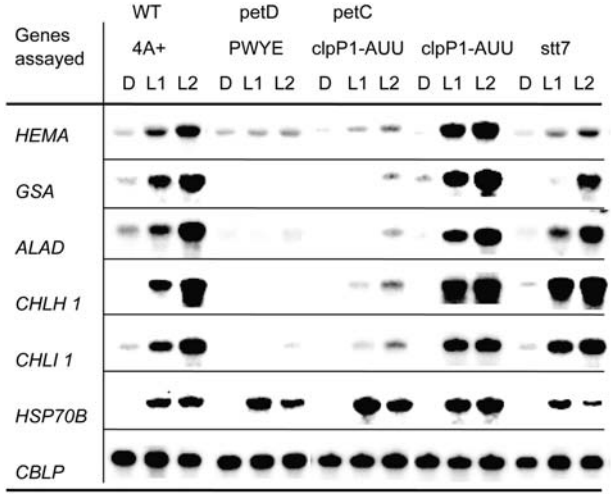


Figure 4. Expression of genes in mutants with defects in cytb$_6$/f subunits or in state transitions. Mutant cultures, after incubation for 20 h in the dark (D), were shifted to light (40 μE m^{-2} s^{-1}) for one (L1) and 2 h (L2). Samples were processed as described in the legend of Figure 2. Strain *clpP1*-AUU is a parent of the strain *petC-Δ1 clpP1*-AUU, and is used to show that the attenuation of ClpP is not responsible for an impairment in light induction.

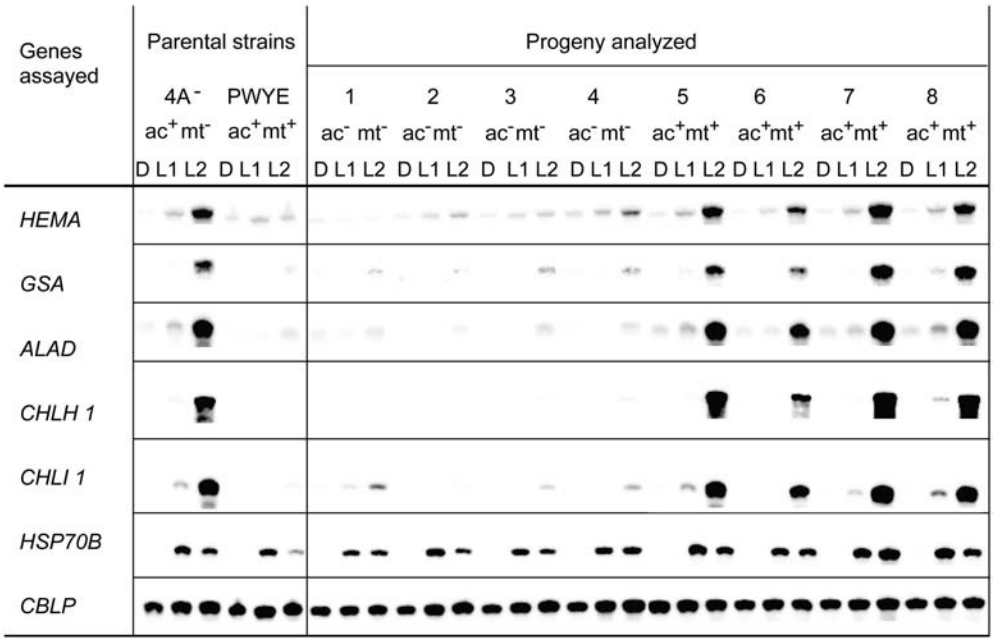


Figure 5. Analysis of lrc phenotypes among progeny from a cross between the *petD*-PWYE mutant and wild type. The data represent one example for the analyses performed with progeny from all crosses listed in Table II. The analyses were done as described in the legend of Figure 2. Above the columns, the phenotype of the strain analyzed with respect to acetate requirement and mating type is listed.

kinase (Im et al., 1996). Here we show that mutants lacking a functional cytb_6/f complex are unable to carry out this light-induction process. This suggests that the light activation of these nuclear genes is in part controlled by signals originating from the chloroplast, where the gene products reside. However, none of the mechanisms hitherto described as mediating retrograde chloroplast-to-nucleus signaling (Beck, 2005; Nott et al., 2006) appear able to explain our results.

Intermediates of the tetrapyrrole biosynthetic pathway are known to participate in the regulation of chlorophyll biosynthesis genes in particular at the level of transcript accumulation. In Arabidopsis, Mg-Protoporphyrin IX accumulates in photodamaged chloroplasts, leading to repression of *LHCB1* expression (Strand et al., 2003). The light-mediated activation of *HEMA1* and, to a minor degree that of *GSA*, was impaired by chloroplast-damaging treatments such as the application of the phytoene desaturase-inhibitor norflurazon, by far-red light pretreatment, or by inhibitors of plastid protein synthesis (Kumar et al., 1996; McCormac and Terry, 2004). In contrast, the *C. reinhardtii HEMA* gene is induced by the feeding of Mg-Protoporphyrin IX (Vasileuskaya et al., 2005), which also activates expression of three nuclear *HSP70* genes (Kropat et al., 1997; Vasileuskaya et al., 2004). It could be hypothesized that lack of light induction in cytb_6/f mutants is related to a defect in the accumulation of Mg-protoporphyrin IX, or its release from the chloroplast. However, no link can be made at present between cytb_6/f activity and the metabolism of tetrapyrroles. We note that *HSP70B* is normally induced in cytb_6/f mutants, which suggests that the mechanism of tetrapyrrole signaling is not impaired.

We have also examined the possibility that reactive oxygen species, produced in the cytb_6/f mutants upon exposure to light, would prevent the induction of the chlorophyll biosynthetic genes. Exogenous addition of hydrogen peroxide (2 mM) did not prevent the light induction of the genes assayed; neither did singlet oxygen generated by treatment with methylene blue in the light (Anthony et al., 2005), suggesting that reactive oxygen species are unlikely to play a role in this deregulation (data not shown).

Redox control has been invoked as a major player in the field of chloroplast-to-nucleus signaling in higher plants (for review, see Fey et al., 2005) as well as in green algae (Escoubas et al., 1995; Maxwell et al., 1995; Durnford and Falkowski, 1997). This is based mostly on experiments where the redox state of the plastoquinone pool was manipulated by illumination in the presence of DCMU or DBMIB, as well as by changes in the light regime. For a number of nuclear genes, the transcript levels have been shown to vary in a manner consistent with long-term regulation of photosystem stoichiometry and light-harvesting capacity. However, the chlorophyll biosynthesis genes that we have studied have not been identified as redox controlled in these studies. Here, we show that treatment with DCMU and DBMIB do not prevent Chlamydomonas wild-type cells from inducing the chlorophyll biosynthesis genes (Fig. 3), in spite of the fact that they will change drastically, and in opposite ways, the redox state of the plastoquinone pool (fully oxidized with DCMU, fully reduced with DBMIB).

Moreover, PSII mutants on the one hand, and PSI as well as PC mutants on the other hand, do undergo changes in the redox state of the plastoquinone pool and of the high potential chain of the cytb$_6$/f complex (cyt f and Rieske protein) when illuminated, but in opposite directions. Still, all showed normal light induction of the chlorophyll biosynthesis genes (Fig. 3). It could be argued that cyclic electron transport, which is still carried out in the presence of DCMU or in PSII mutants (Finazzi et al., 1999), can lead to reduction of the plastoquinone pool in response to light. But we have observed light induction in a DCMU-treated PSI mutant, where no redox change can be brought about by illumination (Fig. 3). Thus, we conclude that changes in the redox state of the plastoquinone pool play no role in the light induction of the chlorophyll biosynthesis genes. In this respect, the signaling we describe differs markedly both from the putative redox control of nuclear genes mentioned above, and from state transitions.

State transitions govern light-energy distribution between photosystems and the balance between linear and cyclic electron transport, and are redox controlled (for review, see Wollman, 2001). Cytb$_6$/f plays a central role in this process: cytb$_6$/f mutants are unable to perform this reorganization because they fail to activate the protein kinase responsible for LHCII phosphorylation. The kinase has been suggested to be physically associated with the cytb$_6$/f complex. The complete loss of state transitions in the *petD*-PWYE mutant clearly demonstrated the involvement of the Q_O site in kinase activation (Zito et al., 1999). Based on inhibitor studies (Finazzi et al., 2001), a dynamic model has been proposed where activation of the kinase requires oscillation of the Rieske protein between its proximal and distal positions. The protein kinase Stt7 is required for LHCII phosphorylation since strains with defects in the *STT7* gene are unable to undergo state transitions (Fleischmann et al., 1999). Here we show that the Stt7 kinase is not involved in the control of the chlorophyll biosynthesis genes, since an *stt7* mutant exhibited a wild-type pattern of gene expression (Fig. 4). This does not necessarily mean that the signaling process under study has no component in common with state transitions, because the Stt7 kinase could simply be a downstream component of a cascade. However, the fact that the control of signaling is different, redox in one case, light in the other, argues for distinct mechanisms.

Still, the fact that mutants with defects in the Q_O site (*petD*-PWYE and *petC-Δ1*) are completely unable to induce the chlorophyll biosynthesis genes (Fig. 4), in spite of having an assembled (or partially assembled) cytb$_6$/f complex, indicates a requirement for a functional Q_O site. How do we account for a light-signaling process that is at the same time dependent on the presence of a functional Q_O site, and independent on changes in its occupancy?

We examined the possibility that the cytb$_6$/f complex itself senses light. A chlorophyll molecule is present in the cytb$_6$/f complex (Stroebel et al., 2003), which in principle could act as a light sensor if its excited state could be transduced into a stable modification/conformational change of the complex. Its phytyl chain lies in the vicinity of the Q_O site, which led us to consider the possibility that its conformation or presence was affected in Q_O site mutants. However, a series of *petD* mutants lacking this chlorophyll molecule (kindly provided by F. Zito) still showed induction of the chlorophyll biosynthesis genes by light, ruling out this hypothesis. In contrast, a mutant carrying a *petD* deletion mutation was defective in induction (data not shown). No mutants are available that affect the other pigment of the complex, β-carotene, but the fact that its excited states are extremely short lived does not make it an attractive candidate for a signaling role.

We thus would like to propose another type of mechanism whereby an essential component of a light-dependent signal-generating system would interact with the cytb$_6$/f complex and be stabilized/activated by this interaction. Light could act directly on the associated signaling component, if it was to harbor a chromophore. Alternatively, and possibly more likely, it could just be part of a light-signaling cascade in which phototropin serves as a light receptor. Not much is known about how phototropin regulates gene expression (Huang and Beck, 2003; Im et al., 2006). It can be hypothesized that the pathway that leads to activation of the chlorophyll biosynthesis genes has a branch located in the thylakoid membrane, which could act as a sensor of the state of the photosynthetic apparatus.

Our results are not in line with the assumption that physiological states conditioned by photosynthetic mutations represent the principal trigger for different modes of plastid signaling and nuclear response (Maiwald et al., 2003; Ihnatowicz et al., 2004; Biehl et al., 2005). Rather, the identification of a role for the cytb$_6$/f complex in plastid signaling suggests that components of the photosynthetic machinery themselves may be involved. The results thus provide new routes to analyze signaling by the chloroplast. One of the principal questions to be addressed will have to focus on the molecular nature of the signal generated and its subsequent transduction to the cytosol/nucleus.

MATERIALS AND METHODS

Algal Strains

Chlamydomonas reinhardtii strain 4A+ (*mt+*) and mutant *stt7* were obtained from J.-D. Rochaix (University of Geneva). Strain 4A− (*mt*$^-$), a wild-type strain that is near isogenic to 4A+, was generated by backcrossing to 4A+ (Dent et al., 2005). Mutants P1-15 (CAL007.01.20) and P2-26 (CAL011.01.03) were generated in the 4A+ strain as described previously (Dent et al., 2005). In these two mutants, the phenotypes acetate requirement/light sensitivity were not linked to zeocin resistance, suggesting that these phenotypes were caused by separate mutational events (Dent et al., 2005; data not shown). Mutants defective in PSI (*ΔpsaB*), PC (AP6), PSII (*ΔpsbD*), cytb$_6$/f (*ΔpetA*, *mcd1-F16*, *tca1-693*, *petD*- PWYE, and *petC-Δ1clpP*-AUU), or state transitions (*stt7*), have been described before (Table I).

Culture Conditions

Strains were grown heterotrophically or photoheterotrophically in Tris-acetate phosphate (TAP) media or photoautotrophically in minimal media

(Harris, 1989). Mutant stocks were maintained on TAP agar medium at a very low light intensity of 5 μE m^{-2} s^{-1} at 23°C. Liquid cultures of wild-type cells were grown with continuous irradiation (40 μE m^{-2} s^{-1}) at 23°C to a density of 3 to 5 $\times$ 10^6 cells per mL. For light induction, the cultures were then divided into subcultures of 50 mL each, and incubation continued in the dark. After 20 h of dark incubation, these cultures were exposed to white light (40 μE m^{-2} s^{-1}) and samples were taken for RNA isolation (von Gromoff et al., 1989). Mutant cultures, due to their light sensitivity, were grown at very low light intensity and treated like wild-type cells after reaching a density of 3 to 5 $\times$ 10^6 cells per mL. For testing light-sensitivity phenotypes, mutants and wild type were incubated at a light intensity of 80 μE m^{-2} s^{-1} or 500 μE m^{-2} s^{-1} on TAP plates using a high light incubator with a cooling system.

For measurements of fluorescence induction at room temperature, dark-adapted cells were transferred to a 1 mL cuvette placed in a home-built fluorimeter (Joliot et al., 1998), in the absence or presence of DCMU (10 μM), and fluorescence induction was recorded over 1.5 s of illumination.

Genetic Analyses

The mutants were crossed with wild-type strains following standard protocols (Harris, 1989). For gamete generation, vegetative cells were resuspended in nitrogen-free (TAP-N) medium at a density of 1 $\times$ 10^7 cells/mL and incubated for 16 h in the light. Gametes of mt^+ and mt^- were mixed, incubated for 1 h, and then plated on TAP agar (4%) medium. These plates were incubated in the light for 24 h and then in the dark for at least 4 d. Zygotes adhering to the agar after nonmated cells had been scraped off were germinated by irradiation and random spores were spread on TAP agar plates (1.5%). For testing acetate requirement and light sensitivity, the progeny of random spores was irradiated with 80 μE m^{-2} s^{-1} or 500 μE m^{-2} s^{-1} on TAP or photoautotrophically in minimal plates. All plates were scored for cell growth and color after 10 to 14 d of incubation.

Immunoblot Analyses

Cells were sedimented by centrifugation (3,000*g* for 5 min) and resuspended in 0.1 M dithiothreitol/0.1 M Na_2CO_3. Then, 0.66 volumes of 5% SDS/30% Suc were added. In cases where the lysates were too viscous, samples were sonicated. Homogenization of the suspensions was achieved by rapid shaking at room temperature for 20 min. The protein concentration was determined by staining with amido black, using bovine serum albumin as a standard (Popov et al., 1975). Total soluble protein (15 μg) was separated on 15% polyacrylamide gels under denaturing (0.1% SDS) conditions. The proteins were transferred from the gels to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences). Protein blots were reacted sequentially with antisera directed against cyt f, the Rieske Fe-S protein, PC (Li et al., 1996), and CGE1 (chloroplast GrpE homolog of Chlamydomonas; Schroda et al., 2001).

Peroxidase-conjugated anti-rabbit serum (Sigma-Aldrich) was used to detect the primary antibodies. For signal detection we used the enhanced chemiluminescence system (Amersham Biosciences).

RNA Gel-Blot Analyses

RNA extraction, electrophoretic separation of RNA, and hybridizations were performed as described previously (von Gromoff et al., 1989). Ten micrograms of total RNA per lane were separated on formaldehyde-containing agarose gels and transferred to nylon membranes (Hybond-N; Amersham). Prehybridization (2 h) and hybridization (18 h) were performed at 65°C in 0.1 M NaCl, 50 mM Tris HCl (pH 7.5), 0.1% sodium pyrophosphate, 10$\times$ Denhardt solution, 1% SDS, 10% dextran sulfate, 60% formamide, and 100 μg/mL of sheared, denatured herring sperm DNA. The probes were labeled with [α-^{32}P]dCTP by the random priming protocol. After hybridization the membranes were washed twice in 2$\times$ standard saline citrate (SSC) at room temperature, once in 2$\times$ SSC, 1% SDS for 30 min at 65°C, and once in 0.2$\times$ SSC at room temperature.

Probes for Hybridization

The probes used for detection of *HSP70B* and *HEMA* transcripts have been described previously (Schroda et al., 2001; Vasileuskaya et al., 2004). Probes used to detect mRNAs of the other genes were a 1.6 kb *Eco*RI, *Sma*I cDNA fragment for *GSA* (Matters and Beale, 1994), a 1.7 kb cDNA fragment for *ALAD* (Matters and Beale, 1995), a 1.4 kb cDNA fragment for *CHLH1* (H subunit of Mg-chelatase; Chekounova et al., 2001), and a 459 bp PCR fragment from genomic DNA for *CHLI1* (I subunit of Mg-chelatase). For a loading control, *CBLP* encoding a Gβ-like protein (von Kampen et al., 1994) was used as a probe.

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