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Phototropin involvement in the expression of genes encoding chlorophyll and carotenoid biosynthesis enzymes and LHC apoproteins in *Chlamydomonas reinhardtii*

Chung-Soon Im[1,*], Stephan Eberhard[2], Kaiyao Huang[3,†], Christoph F. Beck[3] and Arthur R. Grossman[1]
[1]*Department of Plant Biology, Carnegie Institution, Stanford, CA 94306, USA,*
[2]*UMR 7141 (CNRS-Université Paris VI) Laboratoire de Physiologie Moléculaire et Membranaire du Chloroplaste, Institut de Biologie Physico-Chimique F-75005 Paris, France, and*
[3]*Institute of Biology III, University of Freiburg, D-79104 Freiburg, Germany*

Received 12 December 2005; revised 18 May 2006; accepted 26 May 2006.
*For correspondence (fax +1 650 325 6857; e-mail csim@stanford.edu).
†Present address: Molecular, Cellular, and Developmental Biology Department, Yale University, New Haven, CT 06520, USA.

Summary

Phototropin (PHOT) is a photoreceptor involved in a variety of blue-light-elicited physiological processes including phototropism, chloroplast movement and stomatal opening in plants. The work presented here tests whether PHOT is involved in expression of light-regulated genes in *Chlamydomonas reinhardtii*. When *C. reinhardtii* was transferred from the dark to very low-fluence rate white light, there was a substantial increase in the level of transcripts encoding glutamate-1-semialdehyde aminotransferase (GSAT), phytoene desaturase (PDS) and light-harvesting polypeptides (e.g. LHCBM6). Increased levels of these transcripts were also elicited by low-intensity blue light, and this blue-light stimulation was suppressed in three different RNAi strains that synthesize low levels of PHOT. The levels of *GSAT* and *LHCBM6* transcripts also increased following exposure of algal cells to low-intensity red light (RL). The red-light-dependent increase in transcript abundance was not affected by the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, implying that the influence of RL on transcript accumulation was not controlled by cytoplasmic redox conditions, and that a red-light photoreceptor(s) may be involved in regulating the levels of transcripts from specific photosynthesis-related genes in *C. reinhardtii*. Interestingly, elevated *GSAT* and *LHCBM6* transcript levels in RL were significantly reduced in the PHOT RNAi strains, which raises the possibility of co-action between blue and RL signaling pathways. Microarray experiments indicated that the levels of several transcripts for photosystem (PS) I and II polypeptides were also modulated by PHOT. These data suggest that, in *C. reinhardtii*, (i) PHOT is involved in blue-light-mediated changes in transcript accumulation, (ii) synchronization of the synthesis of chlorophylls (Chl), carotenoids, Chl-binding proteins and other components of the photosynthetic apparatus is achieved, at least in part, through PHOT-mediated signaling, and (iii) a red-light photoreceptor can also influence levels of certain transcripts associated with photosynthetic function, although its action requires normal levels of PHOT.

Keywords: *Chlamydomonas reinhardtii*, phototropin, gene expression, chlorophyll, carotenoid, blue light.

Introduction

Chlorophyll (Chl) and carotenoids are the dominant pigments of the photosynthetic apparatus. These pigments are associated with specific polypeptides that are integral to light-harvesting and reaction-center complexes in photosynthetic organisms. The biosynthesis of Chl and carotenoids is sensitive to environmental conditions; stringent control of Chl biosynthesis may reflect the demand for this pigment in antennae and reaction-center complexes, but must also reflect photoreactive and regulatory features of the intermediates in this pathway (Beale, 1999). Some studies show that intermediates in Chl biosynthesis such as protoporphyrin, Mg-protoporphyrin and Mg-protoporphyrin

monomethylester may act as signaling molecules that coordinate communication between the chloroplast and the nuclear genomes (Kropat *et al.*, 1997, 2000); this communication may involve numerous signals including plastid protein synthesis, reactive oxygen species, plastid-generated hydrogen peroxide, the redox state of the photosynthetic electron transport chain, and intermediates and possibly enzymes of tetrapyrrole biosynthesis (Beck, 2005; Fey *et al.*, 2005a; Rodermel, 2001; Strand *et al.*, 2003; Surpin *et al.*, 2002). Recent studies have shown that expression of the *HEMA* gene encoding glutamyl-tRNA transferase, which catalyzes the first step in Chl biosynthesis, is controlled by heme and Mg-protoporphyrin IX (Vasileuskaya *et al.*, 2005), a downstream product in the Chl biosynthetic pathway. Evidence for a role of the redox state of photosynthetic electron transport components in the regulation of nuclear gene expression reveals the complexity of signalling between the chloroplast and nucleus (Fey *et al.*, 2005b). Carotenoids, on the other hand, play a key role in preventing photo-oxidative damage by scavenging singlet oxygen species and by transforming excess absorbed light energy into heat via the xanthophyll cycle (Niyogi, 1999; Niyogi *et al.*, 1997).

The light environment plays a major role in governing the pigment composition of pigment–protein complexes of the photosynthetic apparatus. Blue light is especially important in modulating the synthesis of Chl and carotenoids, as well as the biogenesis of the photosynthetic apparatus in alga and vascular plants. In *Chlamydomonas reinhardtii* grown under a light/dark diurnal schedule, light enhances accumulation of transcripts encoding GSAT and 5-aminolevulinic acid dehydratase (ALAD), two enzymes required for early steps in Chl biosynthesis (Matters and Beale, 1995a). Activation of a heterotrimeric G-protein and phospholipase C, an increase in cytosolic Ca^{2+} concentration, and activation of calmodulin (CaM) and a CaM-dependent kinase may be integral to the signal transduction pathway controlling light-dependent *GSAT* gene activation (Im *et al.*, 1996). Also the *HEMA*, *CPXI*, *CHLH1*, *CHLI1*, *CHLD* and *CTH1* genes, all involved in tetrapyrrole biosynthesis, were shown to be activated by light (Vasileuskaya *et al.*, 2004). Genes required for carotenoid biosynthesis, including phytoene synthase and PDS, have been shown to be controlled by blue light in *C. reinhardtii* (Bohne and Linden, 2002).

Different photoreceptors have evolved in photosynthetic organisms, enabling these organisms to perceive and react to wavelengths of light ranging from UV to far-red. Phototropin (PHOT[a]) is a photoreceptor associated with a variety of blue-light-regulated physiological processes in plants, including phototropism, chloroplast movement, and the modulation of stomatal aperture size. PHOT contains two flavin mononucleotide (FMN) binding sites, which are specialized Per Arnt Sim (PAS) domains designated LOV1 and LOV2. Blue light elicits the formation of a covalent adduct between the FMN chromophore and a conserved cysteine within the LOV domains, which results in a conformational change in PHOT structure. This conformational change is believed to activate a C-terminal serine/threonine protein kinase that autophosphorylates multiple serine residues in the N-terminal half of the protein; this in turn may cause activation of downstream components in the regulatory pathway (Christie and Briggs, 2005). Recently, it has been shown that PHOT is required for blue light regulation of sex-related processes in *C. reinhardtii*, and that *PHOT* RNAi strains of *C. reinhardtii* show impaired formation of gametes from pre-gametes, impaired reactivation of dark-inactivated gametes, and reduced levels of zygote germination (Huang and Beck, 2003).

In this paper, using RNAi-PHOT strains, we demonstrate the involvement of PHOT in the regulation of genes encoding proteins critical for photosynthetic function, including *GSAT*, *LHCBM* and *PDS* (which encodes PDS). The results suggest that the synchronized synthesis of Chl, carotenoids and Chl-binding proteins is achieved, at least in part, through PHOT-mediated blue-light signaling. Interestingly, red light (RL) also appears to function in modulating the abundance of *LHCBM* and *GSAT* transcripts. Furthermore, microarray studies indicate that PHOT can strongly influence levels of other transcripts that specifically encode proteins of the photosynthetic apparatus. Regulation of gene expression by PHOT in *C. reinhardtii* contrasts with the role of PHOT in vascular plants. In vascular plants, phot1 and phot2 are involved in relatively rapid responses that do not require changes in mRNA levels, including phototropism, stomata closure and chloroplast movement, while the cryptochrome blue-light photoreceptors play a more significant role in controlling biological processes at the level of transcription.

Results

Low-fluence-rate white-light modulation of LHCBM*,* GSAT *and* PDS *transcript abundance*

To evaluate light-regulated accumulation of transcripts encoding LHCBM6, GSAT and PDS polypeptides, *C. reinhardtii* cultures were maintained in TAP (tris-acetate-phosphate) medium in moderate light intensity (40 μmol m^{-2} sec^{-1}). Cell cultures were allowed to grow to a density of 5×10^5 cells ml^{-1} and then transferred to the dark for 24 h prior to exposing them to various light treatments, as described in the figure legends. LHCBM6 is a major LHCII polypeptide (also known as CabII-1) present in the trimeric antennae complexes of PS II. GSAT and

[a]Based on the guidelines for *Chlamydomonas* nomenclature (http://www.chlamy.org/nomenclature.html), the upper case and non-italicized PHOT has been used to denote the *Chlamydomonas* phototropin protein. However, for *Arabidopsis thaliana*, it is convention to use lower case and non-italicized phot1 and phot2 to denote the two phototropin holoproteins.

PDS are key enzymes for the synthesis of Chl and carotenoids, respectively. Light causes increased expression of *LHCBM6*, but this expression may also be regulated by the circadian clock (Jacobshagen *et al.*, 1996). In *Arabidopsis thaliana*, *Lhcb* transcripts are also destabilized by a single high-fluence pulse of blue light, and it has been shown that phot1 is required for high-fluence blue-light-mediated destabilization of *Lhcb* transcripts (Folta and Kaufman, 2003).

In the parental *C. reinhardtii* strain, CC-124, continuous illumination with 0.01 μmol m^{-2} sec^{-1} of white LED light (very-low fluence-light, VLFL) for 30 min (total fluence of 18 μmol m^{-2} sec^{-1}) was sufficient to cause increased *LHCBM6*, *GSAT* and *PDS* transcript accumulation (Figure 1). The *GSAT* transcript reached it highest level at 1 h, and then decreased by 2 h. A similar transient expression pattern of *GSAT* has been reported previously (Matters and Beale, 1994). The *PDS* and *LHCBM6* transcripts also showed some decrease in abundance at 2 h. These results confirm previous work that demonstrated light induction of these genes (Bohne and Linden, 2002; Johanningmeier and Howell, 1984; Matters and Beale, 1994, 1995b). The fluence rate effective in eliciting the increase in transcript abundance was too low to stimulate photosynthetic electron transport, suggesting that a specific photoreceptor(s) is involved in this phenomenon. The similar patterns of transcript accumulation for *LHCBM6*, *GSAT* and *PDS* genes suggest that light signals may have a congruent effect on the biosynthesis of Chl, carotenoids and light-harvesting polypeptides.

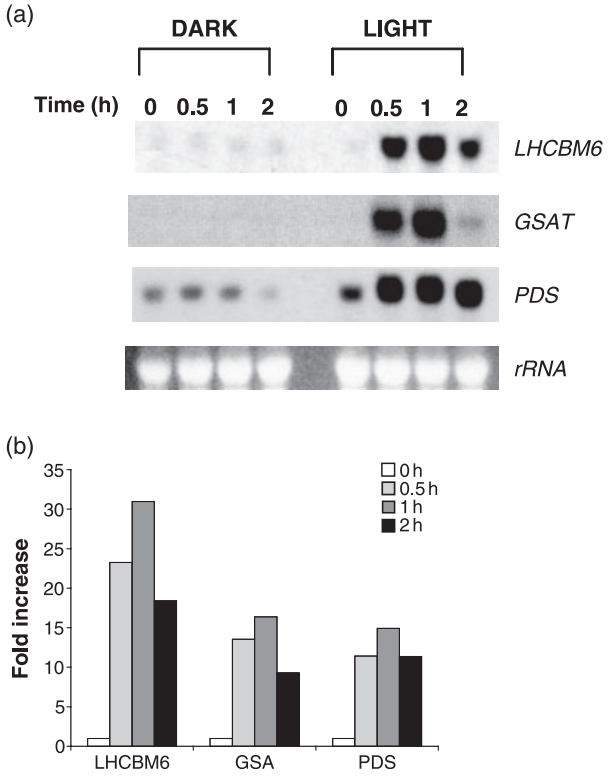

Figure 1. Effect of low-fluence white light on *LHCBM6*, glutamate-1-semialdehyde aminotransferase (*GSAT*) and phytoene desaturase (*PDS*) transcript levels in wild-type *Chlamydomonas reinhardtii* (CC-124).
Cells were grown in 40 μmol m^{-2} sec^{-1} white light to a density of 5×10^5 cells ml^{-1}, followed by 24 h of growth in the dark before exposure to white very-low fluence-light (VLFL) (0.01 μmol m^{-2} sec^{-1}) for 0, 0.5, 1 and 2 h (LIGHT).
(a) Representative Northern blot. Control cells were maintained in the dark (DARK). Total RNA was extracted, and equal RNA loadings confirmed based on 18S rRNA levels.
(b) Densitometric quantification of *LHCBM6*, *GSAT* and *PDS* transcript levels by VLFL. Values in each lane were normalized to the 18S rRNA signal and are expressed as the fold-increase compared with the control value (light, 0 h).

Low-fluence blue-light elevates levels of LHCBM6, GSAT *and* PDS *transcripts*

It has previously been reported that blue light can cause induction of the *LHCBM1*, *LHCBM3*, *GSAT* and *PDS* genes of *C. reinhardtii* and *A. thaliana* (Bohne and Linden, 2002; Gao and Kaufman, 1994; Ilag *et al.*, 1994; Johanningmeier and Howell, 1984; Matters and Beale, 1995a). We used low-fluence-rate (0.2–25 μmol m^{-2} sec^{-1}) blue irradiation from light emitting diodes with a peak emission at 471 nm to examine the effect of blue light on *LHCBM6*, *GSAT* and *PDS* transcript abundances. As shown in Figure 2, the levels of mRNAs from all three of these genes increased during exposure of cells to continuous, low-fluence-rate (0.2–1 μmol m^{-2} sec^{-1}) blue illumination; peak transcript abundance was observed after exposure to 2 h blue light. Higher blue light fluence rates (e.g. 5 and 25 μmol m^{-2} sec^{-1}; see Figure 2) did not cause a significant further increase in transcript accumulation. These results suggest that blue light is an important environmental cue for regulating genes involved in both pigment biosynthesis and the biosynthesis of light-harvesting complexes under low-light conditions. The delayed and reduced levels of transcripts induced by low blue light compared to those induced by VLFL (Figure 1) suggest a contribution from other light qualities to achieve the final transcript level. Also, some changes in the dark levels of transcripts observed in Figure 2 suggest potential circadian control of gene expression.

PHOT involvement in light regulation of the LHCBM6, GSAT *and* PDS *genes*

To examine possible PHOT photoreceptor function with respect to light-regulated expression of the *LHCBM6*, *GSAT* and *PDS* genes, we compared transcript levels for each of these genes in CC-124 and the Ri20 strains. As shown in Figure 3 (upper part), the latter strain, identical with the RNAi20 strain described previously (Huang and Beck, 2003), has <10% of the PHOT protein relative to the parental CC-124 strain. Two other PHOT RNAi strains, Bi20 and Bi30, were also examined for PHOT protein levels, as shown in Figure 3 (lower part), and were used in later experiments (Figure 9) to

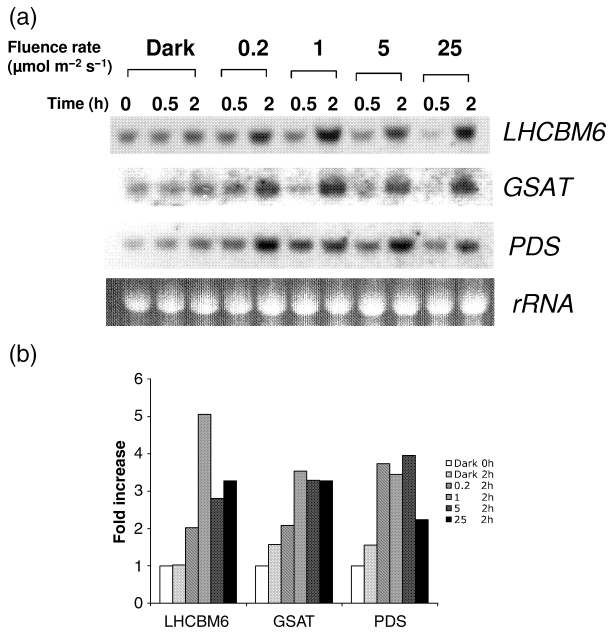

Figure 2. Effect of low-fluence blue light on accumulation of *LHCBM6*, glutamate-1-semialdehyde aminotransferase (*GSA*) and phytoene desaturase (*PDS*) transcripts in CC-124.
Cells were grown as indicated in Figure 1 before exposure to various fluences of blue LED light (0.2, 1, 5 or 25 μmol m^{-2} sec^{-1}) for 0.5 and 2 h.
(a) Representative Northern blot. Total RNA was extracted, and equal loadings were confirmed based on 18S rRNA levels.
(b) Densitometric quantification of *LHCBM6*, *GSAT* and *PDS* transcript levels under various fluence rates of blue LED light for 2 h. Values in each lane were normalized to the 18S rRNA signal and are expressed as the fold-increase compared with the control value (dark, 0 h).

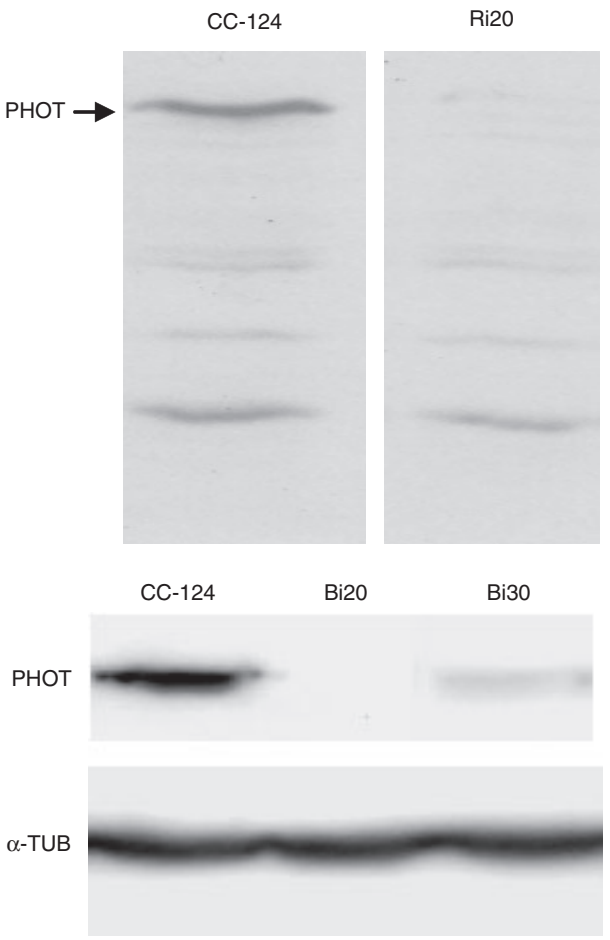

Figure 3. Phototropin (PHOT) accumulation in CC-124 and the Ri20, Bi20 and Bi30 strains.
Cells were grown as indicated in Figure 1. Protein was extracted and solubilized, and the polypeptides were resolved by SDS–PAGE, transferred to a nylon membrane and tested for interaction with a specific mono-antibody raised against the purified phototropin or α-tubulin polypeptide. Similar results were obtained with cells that were maintained in the light (not transferred to the dark for 24 h).

confirm that the phenotype of the Ri20 strain is directly linked to the levels of PHOT in the cell. Based on quantitative RT-PCR (qPCR), transcripts from *LHCBM6*, *GSAT* and *PDS* increased significantly following exposure of dark-adapted CC-124 cells to low-fluence-rate blue light (0.2–25 μmol m^{-2} sec^{-1}), as shown in Figure 4 (left panel). This increase was most apparent after a 2-h blue light treatment at 1 μmol m^{-2} sec^{-1}. However, blue-light-dependent increases in these transcripts were markedly diminished in the Ri20 strain (Figure 4, right panel); an increase was observed at 25 μmol m^{-2} sec^{-1} but not at 1 μmol m^{-2} sec^{-1}. These results suggest that PHOT is required for modulating transcript levels in the light, and that the residual PHOT (< 10% of wild-type cells) in the Ri20 strain can function to elicit changes in transcript abundance only if the alga is exposed to high enough light levels. The reduced sensitivity of the Ri20 strain to blue light that we observed is congruent with the results of Huang and Beck (2003) who showed an increase in gamete formation in the Ri20 strain only when the cells were exposed to a significantly higher fluence rate of light (> 10 μmol m^{-2} sec^{-1}) than was needed to elicit the response in wild-type cells. However, we cannot rule out the possibility of the existence of another system that senses higher-fluence-rate light.

Red light causes elevated LHCBM6 *and* GSAT*, but not* PDS *transcript levels*

In vascular plants, expression of light-harvesting polypeptides is affected by phytochrome signaling (Hamazato *et al.*, 1997). To test the possible input from other light-signaling pathways, we monitored levels of the *LHCBM6*, *GSAT* and *PDS* transcripts following exposure of CC-124 and Ri20 cells to RL. For CC-124, high-fluence-rate RL (45 μmol m^{-2} sec^{-1}) and low-fluence-rate RL (0.5 μmol m^{-2} sec^{-1}) consistently elicited elevated levels of *LHCBM6* and *GSAT*, but not *PDS* transcripts, as shown in Figures 5 and 6. An RL increase in

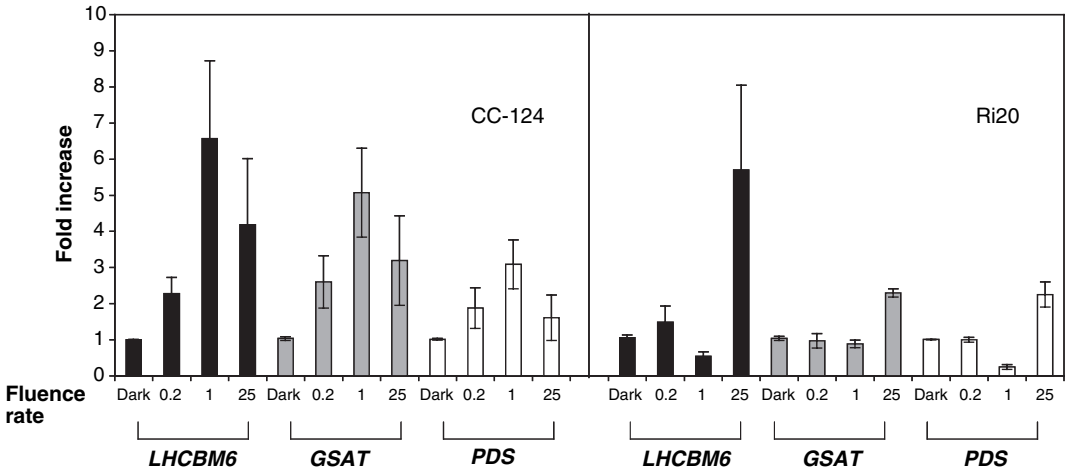

Figure 4. The effect of blue light on *LHCBM6*, glutamate-1-semialdehyde aminotransferase (*GSAT*) and phytoene desaturase (*PDS*) transcript levels in a phototropin RNAi strain (Ri20).
Both CC-124 and Ri20 cells were grown as indicated in Figure 1 before exposure to various fluence rates of blue light (0.2, 1 or 25 μmol m^{-2} sec^{-1}) for 2 h. The light was from a blue LED. Total RNA was extracted, and equal amounts of RNA were used for qPCR. The results were normalized to *CBLP* transcript levels; the transcript from this gene remained constant over the course of the experiment. The results show the mean and standard deviation for data from duplicated qPCR experiments using two different biological samples. Each qPCR experiment was performed in triplicate.

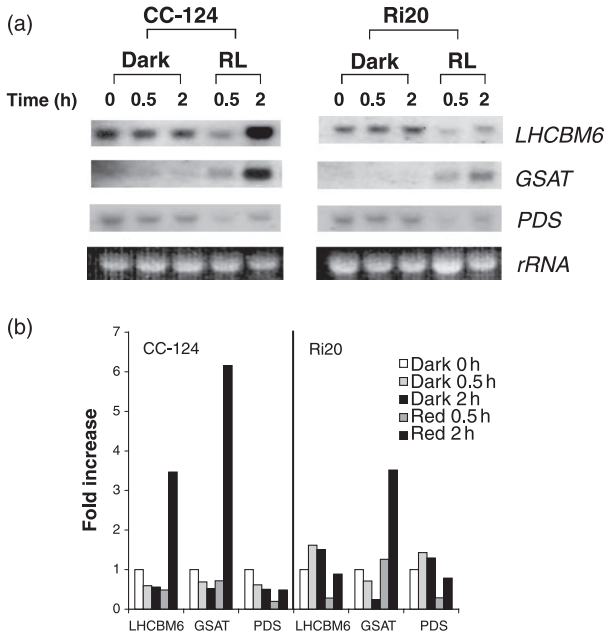

Figure 5. The effect of red light (RL) on accumulation of *LHCBM6*, *GSAT* and *PDS* transcript levels in CC-124 and the Ri20 strain.
Cells were grown as indicated in Figure 1 before exposure to 45 μmol m^{-2} sec^{-1} of RL for 0.5 and 2 h. The light was from a red LED.
(a) Representative Northern blot. Total RNA was extracted, and equal loadings were confirmed based on 18S rRNA levels.
(b) Densitometric quantification of *LHCBM6*, *GSAT* and *PDS* transcript levels by red LED light. Values in each lane were normalized to the 18S rRNA signal and are expressed as the fold-increase compared with the control value (dark, 0 h).

transcript accumulation was significantly reduced in the Ri20 strain (Figure 5a,b, left panels). These results suggest that there is more than one signaling pathway involved in light regulation of *LHCBM* and *GSAT* genes, and that there are some distinct regulatory/sensitivity features that influence the levels of transcripts encoding proteins required for carotenoid synthesis relative to those required for Chl synthesis and light-harvesting biogenesis. Similar to the results reported here, Bohne and Linden (2002) observed that RL was not effective in stimulating an increase in *PDS* mRNA in *C. reinhardtii*. The red-light-dependent change in transcript accumulation could result from changes in intracellular redox conditions or the action of a red-light photoreceptor. In addition, the results with respect to the *LHCBM6* and *GSAT* genes suggest potential interactions between blue-light- and red-light-elicited signal transduction pathways, and that PHOT is required for both responses.

Red light induction is independent of photosynthetic redox state

The qPCR data presented in Figure 6 show that elevation of the *LHCBM6* and *GSAT* transcripts can be achieved in CC-124 cells by low-fluence-rate RL (compare dark LHCBM6 with red LHCBM6 and dark GSAT with red GSAT). In these experiments, the levels of *LHCBM6* and *GSAT* transcripts increased threefold following RL treatment and five- to six-fold following blue light treatment. Although RL elicited a small change (1.5-fold) in the level of the *PDS* transcript in Figure 6, we did not observe significant RL effects on *PDS* transcript levels in an independent experiment (data not shown). The low-fluence-rate red illumination used for these experiments was unlikely to significantly affect photosynthetic activity or elicit a change in the redox state of the cell. To address this issue more directly, we investigated the

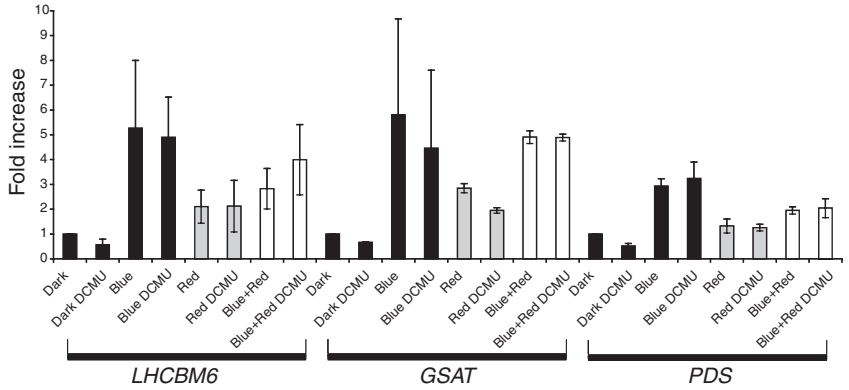

Figure 6. Effect of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on light-dependent *LHCBM6*, *GSAT* and *PDS* transcript accumulation.
CC-124 was grown as indicated in Figure 1 before exposure to blue LED light (0.5 μmol m^{-2} sec^{-1}), red LED light (0.5 μmol m^{-2} sec^{-1}), or blue and red LED light (0.5 μmol m^{-2} sec^{-1} for each light) for 2 h, with or without the addition of DCMU (1 μM). The DCMU was administered to the samples 10 min before placing the cells under the specific light conditions (which was enough time to completely block photosynthetic electron transport). Total RNA was extracted, and transcript levels were analyzed using qPCR. The changes in transcript levels following exposure of the cells to the various conditions are presented as the change (*n*-fold) relative to RNA from dark-grown cells. Expression levels were normalized to the *CBLP* gene. The results show the mean and standard deviation for data from duplicated qPCR experiments using two different biological samples. Each qPCR experiment was performed in triplicate.

effect of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks the transfer of electrons from Q_A to Q_B, on accumulation of *LHCBM6*, *GSAT* and *PDS* transcripts. Figure 6 shows that DCMU, which completely blocked photosynthetic electron transport at the concentration used in this experiment (data not shown), did not significantly alter the blue-, red-, or blue-and-red-light-dependent increase in *LHCBM6*, *GSAT* and *PDS* transcript levels. These results suggest that regulation of levels of *LHCBM6* and *GSAT* transcripts involves signaling through a red-light photoreceptor, although no red-light photoreceptor has yet been identified in *C. reinhardtii*.

To help determine if the red-light-elicited responses were a consequence of photoperception by a phytochrome-like activity, we evaluated the effect of far-RL (720 and 740 nm) on levels of *LHCBM6* and *GSAT* transcripts. Surprisingly, as shown in Figure 7, continuous far-red irradiation (0.2 μmol m^{-2} sec^{-1}) in combination with continuous red irradiation (0.2 μmol m^{-2} sec^{-1}) caused an increase in the levels of *LHCBM6* and *GSAT* mRNAs relative to red or far-RL alone. The 740-nm far-RL in conjunction with the RL resulted in the most pronounced increase in *LHCBM6* and *GSAT* transcript levels; the increase in the *LHCBM6* transcript was consistently greater than that of *GSAT*. We observed slightly higher relative *LHCBM6* and *GSAT* transcript levels after exposure of cells to 0.2 μmol m^{-2} sec^{-1} of RL (Figure 7) than to 0.5 μmol m^{-2} sec^{-1} of RL (Figure 6). This difference in the relative expression levels might be a consequence of slight differences in the physiology of cells. However, it is important to note that, while the relative levels of change may not always be identical, the relative effectiveness of RL compared to that of other light qualities is consistent. The inability to reverse the RL response with far-RL and the

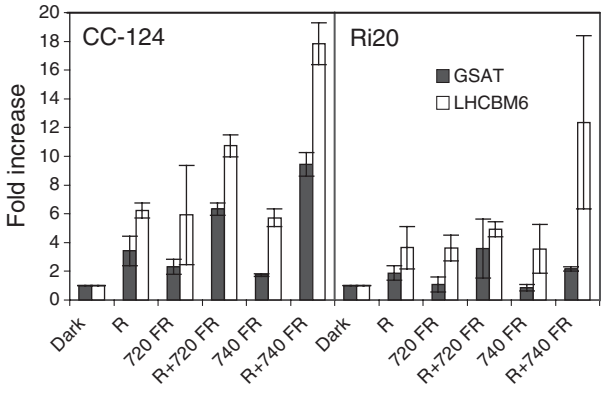

Figure 7. Effect of far-red light (RL) on accumulation of *LHCBM6* and *GSAT* transcripts.
CC-124 (left side) and the PHOT RNAi strain, Ri20 (right side), were grown as indicated in Figure 1 before exposure to RL (R, 0.2 μmol m^{-2} sec^{-1}), with or without far-RL (FR, 720 or 740 nm, 0.2 μmol m^{-2} sec^{-1}), for 2 h. The light was from red and far-red LEDs. Total RNA was extracted, and transcript levels were analyzed using qPCR. The changes in transcript levels following exposure of the cells to the various conditions are presented as the change (*n*-fold) relative to RNA from dark-grown cells. Expression levels were normalized to the *CBLP* gene. The results show the mean and standard deviation for data from duplicated qPCR experiments using two different biological samples. Each qPCR experiment was performed in triplicate.

increase in transcript levels observed in red plus far-RL relative to RL alone are similar to features of the phytochrome A (PhyA)-specific photo-induction of seed germination and *CAB* gene expression in *A. thaliana* (Hamazato *et al.*, 1997; Shinomura *et al.*, 1996). Far-red plus RL also resulted in increased *LHCBM6* and *GSAT* transcript levels in the Ri20 strain, although the increases were generally not as great as in wild-type cells. These results suggest that

signaling triggered by far-RL is either independent of or less sensitive to the level of PHOT protein than the blue light response, and it may be located genetically downstream of PHOT in the signal transduction pathway. To date, no phytochrome genes have been identified on the genome of *C. reinhardtii*. However, we cannot rule out the possibility that *C. reinhardtii* may contain a phytochrome-like photoreceptor(s) that is markedly diverged in structure from vascular plant phytochrome but has retained the functional characteristics of PhyA.

PHOT-mediated regulation of other genes encoding proteins critical for Chl and carotenoid biosynthesis

We used qPCR to examine the transcript abundance for most genes encoding proteins associated with Chl and carotenoid biosynthesis (potential regulatory targets of PHOT) in both CC-124 and the Ri20 strain (Figure 8). We also compared expression levels of genes encoding isozymes for specific steps in the Chl and carotenoid biosynthetic pathways, identified from previous comparative genomic analyses (Lohr *et al.*, 2005). VLFL (white light) was used for these experiments to eliminate the influence of changes in cellular redox that occurs as a consequence of photosynthetic electron transport. While transcript levels for a number of genes involved in Chl biosynthesis (Figure 8a) increased following the light exposure (*GSAT*, *CHLH1*, *CHLI1*, *ALAD*), they were generally higher after 2 h than after 4 h of VLFL. As expected, there was a marked increase in *GSAT* and *ALAD* transcripts (approximately 20-fold for *GSAT* and sevenfold for *ALAD*) after a 2-h exposure to VLFL, but there were also four- to sixfold increases in *UROD1*, *CHLH1* and *CHLI1* transcript levels after exposing cells to VLFL for 2 or 4 h. Among carotenoid biosynthetic genes (Figure 8b), in addition to the increase in the *PDS* transcript (3.5-fold), there were significant, but small (2.5- to threefold), increases in the levels of *HDS*, *PSY* and *ZDS* transcripts. All induction by VLFL was essentially abolished in the Ri20 strain, suggesting that expression of these genes is also controlled by PHOT.

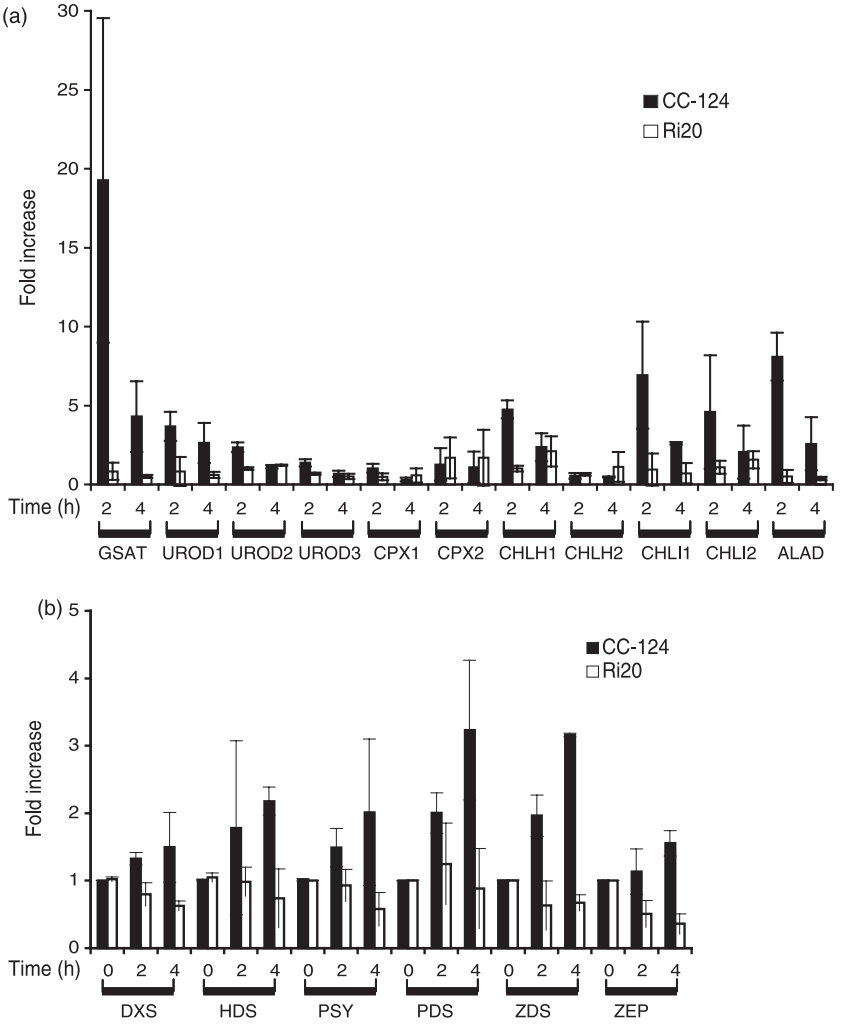

Figure 8. Changes in transcript levels for genes involved in Chl (a) and carotenoid (b) biosynthesis.

CC-124 and the Ri20 strain were grown as indicated in Figure 1 before exposure to white very-low fluence-light (VLFL) for 2 and 4 h. Total RNA was extracted and transcript levels were analyzed using qPCR. The changes in transcript levels following exposure of the cells to white VLFL (0.01 μmol m^{-2} sec^{-1}) are presented as the change (*n*-fold) relative to RNA from dark-grown cells. The relative expression level of each gene was normalized to the *CBLP* transcript level. The results show the mean and standard deviation for data from duplicated qPCR experiments using two different biological samples. Each qPCR experiment was performed in triplicate. GSAT, glutamate-1-semialdehyde aminotransferase; UROD1, UROD2 and UROD3, uroporphyrinogen III decarboxylase; CPX1 and CPX2, coproporphyrinogen III oxidase; CHLH1 and CHLH2, protoporphyrin IX Mg-chelatase subunit H; CHLI1 and CHLI2, protoporphyrin IX Mg-chelatase subunit I; ALAD, 5-aminevulinic acid dehydratase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; ZEP, zeaxanthin epoxidase.

Microarray examination

We used cDNA microarrays to identify other genes potentially controlled by PHOT. Table 1 shows a list of 44 genes whose transcripts increased significantly in CC-124, but not in Ri20, following exposure of the cells to VLFL. Surprisingly, transcripts for many genes encoding constituents of PS I, PS II and the light-harvesting complexes of both PSs increased following exposure of CC-124, but not Ri20, to VLFL. There were also increases in transcript abundance from a diverse group of genes associated with processes such as metabolite transport, sterol synthesis and cell wall structure. The identity and putative functions of genes in the miscellaneous category require more detailed examination.

To confirm microarray results that demonstrated changes in transcript abundance from genes encoding constituents of the photosynthetic apparatus following exposure of dark-adapted cells to white VLFL, we performed qPCR analysis. Genes examined encode components of LHCI (*LHCA2*, *LHCA7* and *LHCA8*), LHCII (*LHCBM1*, *LHCBM2*, *LHCBM6*, *LHCBM8* and *LIL*), the cytochrome b_6f complex (*PETO*), PS I (*PSAE*, *PSAG*, *PSAH*, *PSAL* and *PSAO*), PS II (*PSBR*, *PSBS* and *PSBW*), oxygen-evolving complex (*OEE1*, *OEE2* and *OEE3*), plastocyanin (*PC6-2*) and *S*-adenosyl-L-methionine (SAM)-related enzymes (*SAH1* and *SAS1*). Transcripts for all of these genes, except for *LHCBM8*, were elevated by VLFL in CC-124, but not in the Ri20 strain (Table 1, last two columns), suggesting that PHOT activity is required for expression of these genes. As we were unable to verify elevated *LHCBM8* transcript levels (observed in the microarray experiments) in CC-124 using a number of different specific primer pairs for the qPCR, it is likely that the microarray results were a consequence of cross-hybridization of the *LHCBM8* array element with cDNA probes derived from other *LHCBM* transcripts. These data show that PHOT activity is required for coordinated regulation of a variety of genes, including those involved in pigment biosynthesis and the biogenesis of and function of the photosynthetic electron transport chain.

Gene expression in other RNAi strains

We examined different PHOT RNAi strains to determine whether the observed phenotype of Ri20 is directly linked to PHOT levels in the cell. Both the Bi20 and Bi30 strains showed reduced levels of PHOT, but Bi30 had a slightly higher level of PHOT than Bi20, as shown in Figure 3 (lower part). Figure 9 depicts qPCR analyses of changes in the levels of the transcripts from *LHCBM6*, *GSAT*, *PDS*, *OEE1* and *LHCA2* in the RNAi transformants (compared to CC-124) following exposure of dark-adapted cells to VLFL. Both RNAi strains showed reduced transcript accumulation compared to the parental strain, and the extent of reduction was generally correlated with the level of residual PHOT in the cells (the Bi30 strain showed an intermediate response between that of CC-124 and Bi20). These results strongly suggest that the transcript levels for 'light-inducible genes' encoding proteins involved in both pigment biosynthesis and photosynthetic electron transport are reduced as a consequence of reduced levels of PHOT polypeptide in the RNAi strains.

Discussion

The Chl biosynthetic pathway is comprised of a range of complex metabolic reactions, with the probable rate-limiting step being the formation of 5-aminolevulinic acid (ALA) (Beale, 1999). ALA synthesis in plants is regulated by several different signals including photoreceptors (McCormac *et al.*, 2001), the circadian clock (Kruse *et al.*, 1997), and the developmental status of plastids (Kumar *et al.*, 1999). GSAT converts glutamate 1-semialdehyde to ALA by transamination, and the expression of *GSAT* has been shown to be regulated by light in both vascular plants and *C. reinhardtii* (Ilag *et al.*, 1994; Matters and Beale, 1995a; Sangwan and O'Brian, 1993). Carotenoid biosynthesis is also regulated by several factors (Audran *et al.*, 1998; Bouvier *et al.*, 1996; Steinbrenner and Linden, 2001), including light. In tobacco, genes for phytoene synthase and enzymes required for xanthophyll biosynthesis are activated by red or blue light, suggesting that phytochrome or cryptochrome is involved in this activation (Woitsch and Romer, 2003). Similarly, vascular plant genes encoding LHC polypeptides are controlled by photoreceptors, including PHYA, PHYB (Cerdan *et al.*, 1999; Hamazato *et al.*, 1997; Karlin-Neumann *et al.*, 1988; Reed and Chory, 1994), CRY1 and CRY2 (Mazzella *et al.*, 2001).

Despite numerous studies to define the effects of light on the biosynthesis of pigments and polypeptides of the photosynthetic apparatus, light fluence rates are often not carefully controlled; controlling the light fluence rate would help distinguish the effects of altering the cellular redox state (e.g. through changes in rates of photosynthesis) and signaling from specific photoreceptors. Figures 1 and 2 show that either 30 min of continuous VLFL (0.01 μmol m^{-2} sec^{-1}) or 2 h of continuous blue light (0.2–1 μmol m^{-2} sec^{-1}) is enough to trigger accumulation of *LHCBM6*, *GSAT* and *PDS* transcripts. Sensitivity to low-fluence-rate blue light suggests that a specific blue-light photoreceptor is involved in controlling the levels of these transcripts, at least in part. VLFL (white light) caused a more rapid and higher-amplitude increase in transcript accumulation than low-fluence-rate blue light alone, which may be partly explained by the observation that the other components, such as red and far-red irradiation, also contribute to the expression of these 'light-inducible' genes (Figure 7).

While the function of cryptochrome in *C. reinhardtii* is still not know (Reisdorph and Small, 2004), the blue-light photoreceptor PHOT is required for gamete formation and

Table 1 Relative abundance of transcripts in CC-124 compared to Ri20 after transferring cells from the dark to white very-low fluence-light, as assayed by DNA microarrays and qPCR

							Fold increase by qPCR	
Clone ID	0	30 min	2 h	4 h	Gene	Description	Wild-type, light	Ri20, light
Light-harvesting								
894078C01	1.07	1.45	2.31	3.09	LHCA2	Light-harvesting complex I	8.1	0.6
963047H05	1.03	1.72	3.52	5.03	LHCA7	Light-harvesting complex I	3.9	0.8
894033H06	1.21	1.36	2.82	3.02	LHCA8	Light-harvesting complex I	5.6	0.8
894041D11	1.28	1.33	1.59	1.93	LHCA9	Light-harvesting complex I		
894052A01	1.08	1.44	2.36	2.56	LHCB5	Light-harvesting complex II		
963069C06	0.69	1.26	2.69	4.74	LHCBM1	Light-harvesting complex II	2.8	0.4
894065B07	0.71	1.32	2.13	2.66	LHCBM2	Light-harvesting complex II	3.8	0.6
Lhcbm8	0.59	0.96	2.14	3.16	LHCBM8	Light-harvesting complex II	0.4	0.6
894005B12	1.34	1.57	2.71	1.62	LIL	40.2 kDa protein, LHC family	7.1	1.3
Photosynthetic chain								
894069E01	0.72	1.29	2.69	4.01	PC6-2	Plastocyanin	10.6	0.4
963092G08	0.75	1.00	1.50	1.84	PETO	b6f subunit	11.3	1.6
963047E03	0.77	1.23	1.84	1.81	PSAD	PS I subunit		
894083B07	0.84	1.49	2.07	2.12	PSAE	PS I subunit	4.6	1.6
894065A07	0.94	1.28	1.99	2.91	PSAG	PS I subunit	9.3	1.4
894014A05	0.80	1.11	1.67	1.96	PSAH	PS I subunit	10.7	1.2
894004A09	0.87	1.10	1.71	1.85	PSAL	PS I subunit	6.7	1.5
894019E07	0.98	1.37	2.13	2.35	PSAO	PS I subunit	5.8	0.8
894006E05	1.19	1.24	1.54	1.67	OEE2	Oxygen-evolving enhancer protein 2	4.8	1.9
SAM								
894103C12	1.31	1.83	4.45	4.04	SAH1	*S*-adenosyl homocysteine hydrolase	2.6	0.2
963047D02	0.79	1.31	2.43	2.56	SAS1	*S*-adenosylmethionine synthetase	2.7	0.2
Miscellaneous								
894037A07	1.26	1.54	1.73	1.28	ACH	Mitochondrial aconitate hydratase		
894080B03	1.23	2.43	3.35	1.84	GAP1	Glyceraldehyde-3-phosphate dehydrogenase		
894020F09	1.10	1.59	2.26	1.93	GPM	Glucose phosphomutase		
963045H04	1.51	1.41	1.68	1.74	ACS3	Putative acetyl-CoA synthase		
894006C08	1.17	1.50	2.03	1.76	GBP1P	Putative nucleic acid binding protein		
894012D09	0.96	1.73	2.93	1.68	GGR	Chloroplast geranylgeranyl hydrogenase		
894002D02	0.79	1.58	2.19	1.94	GP2	Outer cell wall protein		
894044H02	1.82	1.42	2.21	2.14	PTB2	Putative phosphate transporter B2		
963041C09	0.52	1.09	2.22	3.63	THI4	Thiazole biosyntethic enzyme	15.2	0.4
894058F11	1.20	1.38	1.89	1.64		Probable glycine-rich RNA-binding protein		
963046C03	0.92	1.28	2.08	1.94		Hypothetical luminal protein precursor		
894093H09	1.03	1.15	1.55	1.59		Cytochrome b5 domain-containing protein [*Arabidopsis thaliana*]		

Table 1 Continued

Clone ID	0	30 min	2 h	4 h	Gene	Description	Fold increase by qPCR: Wild-type, light	Fold increase by qPCR: Ri20, light
894086H03	0.95	1.35	2.80	1.59		Sterol-C-methyltransferase [*A. thaliana*]		
894065G01	1.35	1.11	1.67	1.51		Peroxiredoxin 4 [*Bos taurus*]		
894077B08	1.06	1.30	1.62	1.80		Plastidic 2-oxoglutarate/malate transporter [*Zea mays*]		
963069C02	1.31	1.20	1.70	1.69		Putative ferredoxin-dependent glutamate synthase		
894083B06	1.09	1.53	1.62	2.10		No significant blast hit		
963096G08	0.96	1.10	1.60	1.94		No significant blast hit		
					LHCBM6	Light-harvesting complex II	14.0	0.8
					GSAT	Glutamate-1-semialdehyde aminotransferase	14.3	1.9
					OEE1	Oxygen-evolving enhancer protein	12.5	1.3
					OEE3	Oxygen-evolving enhancer protein	3.1	1.1
					PSBR	PS II subunit	2.9	0.9
					PSBW	PS II subunit	2.5	1.0

RNAs for microarray analysis were from a single experiment. The arrays were analyzed as described in Experimental procedures. Transcripts that showed a > 1.5-fold difference between CC-124 and the Ri20 strain at two successive time points are presented. Selected transcripts were further analyzed by qPCR experiments using RNAs from independent biological replicates to confirm microarray results. qPCR data in bold represent additional transcripts that were not selected from the microarray results, but that encode polypeptides associated with light harvesting (LHCBM6), the oxygen-evolving complex (OEE1 and OEE3), photosystem II (PSBR and PSBW) and other process (GSAT). The changes in transcript levels following exposure of the cells to white VLFL are presented as the change (*n*-fold) relative to RNA from dark-grown cells. The relative expression level of each gene was normalized to the *CBLP* transcript level. Each qPCR experiment was performed in triplicate.

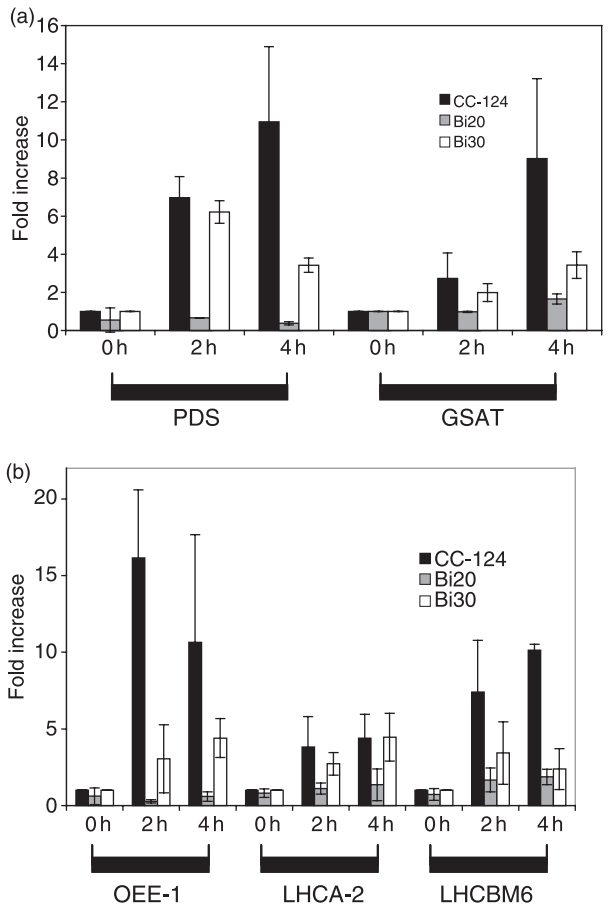

Figure 9. Changes in transcript levels for (a) *PDS* and *GSAT*, and (b) *OEE-1*, *LHCA-2* and *LHCBM6*, in strains CC-124, Bi20 and Bi30.
Cells were grown as indicated in Figure 1 before exposure to white very-low fluence-light (VLFL) (0.01 μmol m^{-2} sec^{-1}) for 2 and 4 h. Controls were maintained in the dark for 2 and 4 h. Total RNA was extracted and analyzed by qPCR. The changes in transcript levels following exposure of the cells to white VLFL are presented as the change (*n*-fold) relative to RNA from dark-grown cells. The relative expression level of each gene was normalized to the *CBLP* transcript level. The results show the mean and standard deviation for data from duplicated qPCR experiments using two different biological samples. Each qPCR experiment was performed in triplicate.

progression of the sexual cycle (Huang and Beck, 2003). In vascular plants, blue-light activation of most genes appears to be mediated by the cryptochromes. However, the data in Figure 4 show that PHOT appears to sense the light signal that modulates accumulation of *LHCBM6*, *GSAT* and *PDS* transcripts in *C. reinhardtii*. These results are supported by results using other PHOT RNAi strains, as shown in Figure 9.

GSAT expression in *C. reinhardtii* was shown to require CaM activity and an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) (Im *et al.*, 1996). It was suggested that the increase in $[Ca^{2+}]_{cyt}$ was mediated by phospholipase C-catalyzed IP_3 formation (Im and Beale, 2000). Recent work on PHOT of *A. thaliana* has also established a link between PHOT-dependent regulation and Ca^{2+} signaling. In one study, *A. thaliana* seedlings expressing the Ca^{2+}-sensitive fluorescent protein apoaequorin, exhibited a blue-light-dependent transient increase in $[Ca^{2+}]_{cyt}$ concentration, a response dramatically attenuated in a *phot1* mutant (Baum *et al.*, 1999). Another study showed that both phot1 and phot2 mediated a blue-light-dependent increase in $[Ca^{2+}]_{cyt}$ in *A. thaliana* leaves (Harada *et al.*, 2003). In that study, the authors used pharmacological agents to demonstrate that both phot1 and phot2 mediate Ca^{2+} influx from the apoplast, whereas only phot2 can mediate an increase in $[Ca^{2+}]_{cyt}$ through mobilization from internal stores, probably via a phospholipase C-dependent pathway. Furthermore, a rapid phot1-dependent increase in $[Ca^{2+}]_{cyt}$ occurred upon exposure of *A. thaliana* cotyledons and hypocotyls to blue light. This response was strongly diminished in *phot* mutants (Babourina *et al.*, 2002). Also, patch-clamp studies have shown that blue-light activation of a plasma membrane-localized Ca^{2+} channel was dramatically reduced in leaf mesophyll cells of *phot1* single mutants, and was essentially eliminated in *phot1 phot2* double mutants (Stoelzle *et al.*, 2003). Finally, blocking a phot1-mediated, transient increase of $[Ca^{2+}]_{cyt}$ by chelation prevented inhibition of hypocotyl elongation but did not affect hypocotyl phototropism (Folta *et al.*, 2003). These reports, plus data presented in this paper in conjunction with a previous study by Im *et al.* (1996), suggest that PHOT activates *GSAT* expression through Ca^{2+} signaling, and expression of *PDS* and *LHCBM* genes may share the same (or a similar) signaling pathway.

Regulation of vascular plant gene expression by inputs from multiple photoreceptors has been frequently observed. Early studies demonstrated that the action of a specific blue-light photoreceptor could modulate the responsiveness of plants towards the Pfr form of phytochrome (Elmlinger *et al.*, 1994; Oelmuller and Mohr, 1985). With respect to de-etiolation of *A. thaliana*, absorption of blue light by CRY1 modulates the ability of the plant to respond to RL pulses via the PHYB photoreceptor (Casal and Boccalandro, 1995; Janoudi and Poff, 1992). Furthermore, a pulse of RL given 2 h before unilateral blue light enhances phot1-mediated phototropism (Janoudi and Poff, 1992). This enhancement is reduced in the *phyA* and *phyB* mutants (Janoudi *et al.*, 1997a; Parks *et al.*, 1996), and absent in the *phyA phyB* double mutant (Janoudi *et al.*, 1997b). Figures 5 and 6 show that relatively low-fluence-rate RL caused elevated accumulation of *LHCBM6* and *GSAT* transcripts, and that inhibition of photosynthesis by DCMU did not significantly affect the RL response. As PHOT does not absorb light above 500 nm, it is unlikely that the red and far-RL responses are triggered by PHOT (Kottke *et al.*, 2003). Interestingly, the RL effects were reduced in the Ri20 strain, suggesting the possibility of interactions between blue- and red-light-signaling pathways. Kasahara *et al.* (2004) suggested that PHOT functions downstream of phytochrome in the signaling pathway of *Physcomitrella patens* that controls chloroplast movement;

they demonstrated that red-light-induced chloroplast movement was significantly reduced in *photA2 photB1 photB2* triple mutants. The signal transduction pathway involved in controlling *PDS* transcript abundance may be different from the pathway that controls *LHCBM* and *GSAT* transcript abundance as *PDS* expression did not appear to be responsive to RL (or the *PDS* response may be less sensitive to RL). These results suggest that both blue and RL may be important for coordinated expression of genes encoding proteins involved in Chl and carotenoid biosynthesis as well as for the synthesis of LHC polypeptides; PHOT is an integral part of the signaling pathway involved in this regulation.

The existence of phytochrome in *C. reinhardtii* has not been established. However, it has been demonstrated that far-red and blue light are most effective in eliciting increased expression of isocitrate lyase in a carotenoid-deficient mutant of *C. reinhardtii* (Petridou *et al.*, 1997). As shown in Figure 7, low-fluence-rate far-RL resulted in elevated *LHCBM6* and *GSAT* transcript levels relative to RL alone, suggesting the existence of a photoreceptor capable of absorbing far-RL. It would be premature to interpret this as support for the existence of phytochrome in *C. reinhardtii*, but the data suggest that a putative red-light/far-red-light photoreceptor(s) influences expression of *LHCBM6* and *GSAT* genes. The structure of phytochromes varies among organisms. The cyanobacterial phytochromes contain bacterial histidine kinase domains, while one phytochrome from the fern *Adiantum* (phy3) and two neochromes from the filamentous green alga *Mougeotia* are chimeric, containing both phytochrome- and PHOT-associated domains (Nozue *et al.*, 1998; Suetsugu *et al.*, 2005). The red-light photoreceptor(s) of *C. reinhardtii* may have distinctly different features from phytochromes of vascular plants, but the response characteristics observed in this paper have some similarity to PhyA-mediated responses.

In synchronized cultures of *C. reinhardtii*, the accumulation of the *GSAT* transcript was only elicited by blue or green, and not by red or orange light (Matters and Beale, 1995a). RL effects on *GSAT* expression demonstrated in this paper may be explained in the context of a PhyA-like photoreceptor. PhyA is light-labile and its accumulation in the cell requires prolonged dark incubation (> 24 h) (Shinomura *et al.*, 1996). In the studies reported here, medium-light grown cultures were incubated in the dark for 24 h prior to the light treatment. This incubation period might allow a phytochrome-like photoreceptor to accumulate to a high enough level to elicit the red/far-RL responses presented in Figure 7. In contrast, during a light/dark (12 h/12 h) diurnal cycle, previously used to synchronize *C. reinhardtii* cells, the dark incubation period may not be long enough to allow accumulation of a 'PhyA-like' photoreceptor that might be responsible for the red/far-red-light-driven increase in *GSAT* expression. Figure 6 illustrates the finding that blue light enhances red-light-elicited transcript accumulation. While the blue and RL responses do not appear to be additive, the results suggest that both blue- and red-light photoreceptors contribute independent components to the response.

Microarray and qPCR experiments, presented in Figure 8 and Table 1, suggest that PHOT activity controls the expression of a range of genes in *C. reinhardtii*. Many of these genes encode constituents of the photosynthetic apparatus. Other genes of the photosynthetic apparatus not regulated by VLFL may respond to outputs from photosynthetic electron transport. For example, the thiamine biosynthetic enzyme and the thiazole biosynthetic protein in plants appear to be regulated by post-translational modifications through the chloroplast-localized, thioredoxin system (Balmer *et al.*, 2003). SAM is an important co-factor for many cellular enzymes, including uroporphyrinogen III methyltransferase and Mg-protoporphyrin IX methyltransferase. Transcripts encoding *S*-adenosyl homocysteine hydrolase and *S*-adenosyl methionine synthetase increased to some extent following exposure of *C. reinhardtii* cells to VLFL, and they appear to be under the control of PHOT (Table 1).

The function of PHOT in *C. reinhardtii* appears to be significantly different from that of the analogous protein in vascular plants (Jiao *et al.*, 2003; Ohgishi *et al.*, 2004). Some PHOT-regulated responses in *C. reinhardtii* demonstrated in this paper have been associated with the CRY photoreceptors of plants, suggesting an increased diversification and specialization of photoreceptors in land plants. Furthermore, PHOT function appears to be linked to the activity of a putative red-light photoreceptor(s). Additional biochemical and genomic studies may help elucidate the nature of the *C. reinhardtii* red-light photoreceptor(s), and how it influences the PHOT responses.

Experimental procedures

Strains and culture conditions

The *C. reinhardtii* wild-type strain (parental strain, CC-124) and RNAi strains (Ri20, Bi20, and Bi30) that express a reduced PHOT level were used for all experiments. The Ri20 strain is identical to RNAi20, which was used to examine the importance of PHOT for gametogenesis (Huang and Beck, 2003), while the Bi20 and Bi30 strains were generated independently by the Beck group. We monitored the PHOT protein in RNAi strains over the course of these experiments to ensure that protein levels remained constant (i.e. that the RNAi phenotype was stable while performing this work). Cells were grown in TAP medium at a moderate light intensity (white light, 40 μmol m^{-2} sec^{-1}) to a density of 5×10^5 cells ml^{-1}, and then transferred to the dark for 24 h before re-exposure to the test light conditions. Light treatments were performed using LEDs for specific wavelengths (Super Bright LEDs Inc., St. Louis MO, USA and Roithner Lasertechnik GmbH, Vienna, Austria): blue (RL5-B 2430, 2400 mcd, peak at 471 nm), red (RL5-R8030, 6000 mcd, peak at 650 nm), white (RL5-W6030, 6000 mcd), and two far-red (ELD-720–524, peak at 720 nm; ELD-740–524, peak at 740 nm). An LI-1800 spectroradiometer (LI-COR Inc, Lincoln, NE, USA) was used to

measure the light fluence rate and the spectrum of light for each LED. The white-light LED (gallium nitride coated with a phosphor) showed a sharp peak of blue (475 nm) and a broad emission spectrum throughout the visible region. Measurements were made by placing the probe at the surface of the culture.

RNA isolation and RNA blot analysis

Total RNA was isolated from cells using Trisol reagent (38% phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, pH 5, 5% glycerol) plus 0.2 vol chloroform. The cells were collected by centrifugation at 5000 ***g*** for 5 min, resuspended in the Trisol-chloroform lysis reagent, and nucleic acid in the aqueous phase was precipitated by making the solution 50% isopropanol, 0.4 M sodium citrate and 0.6 M NaCl. Precipitation of the RNA was performed at 4°C for 4 h. The RNA was collected by centrifugation at 10 000 ***g*** for 30 min, washed with 70% ethanol, dried, and dissolved in sterile distilled H_2O. Total RNA isolated from cells was resolved by electrophoresis on a 1.2% formaldehyde–agarose gel, transferred to a supported nylon membrane (Schleicher & Schuell, Keene, NH, USA), and hybridized with DNA fragments labeled by the Alk-Phos direct labeling kit (Amersham Bioscience, Piscataway, NJ, USA). The hybridization signals were detected using the Gene Image CDP-Star detection kit (Amersham Bioscience).

Quantitative PCR (qPCR)

All qPCR experiments were performed in duplicate with RNAs isolated from at least two different cultures (biological replicates) except the qPCR experiment in Table 1 (this procedure was used to verify microarray experiments and the total RNAs used for these qPCR studies were from different preparations than those used for microarray analysis). Isolated total RNA was treated with RNase-free DNase I (Ambion Inc, St Austin, TX, USA) followed by phenol:chloroform extraction. For cDNA synthesis, 1 μg of DNase I-treated total RNA was reverse-transcribed and amplified using the Superscript™ III kit (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. qPCR was performed using the DyNAmo Hot Star SYBR Green qPCR kit (MJ Research Inc, Waltham, MA, USA), and analyzed by the Opticon 2 system (MJ Research Inc). Cycling conditions included an initial incubation at 95°C for 10 sec, followed by 40 cycles of 94°C for 10 sec, 55°C for 15 sec and 72°C for 10 sec. Each PCR assay was performed in triplicate. The relative transcript abundances of target genes were calculated based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The *CBLP* gene (*CBLP* transcript levels remained constant during the light/dark shifts) was used as a control gene, and each primer was designed by PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were designed to have a T_m of between 58 and 60°C with an optimal length of 20 nt. The GC content was maintained between 20% and 80% with no 3′ GC clamp. The target amplicon for each sequence was designed to be between 150 and 200 nt, with an optimal T_m of 85°C. Primer sequences for qPCR analysis of *LHCBM* transcripts were chosen based on information from previous work (Elrad *et al.*, 2002).

Western blot hybridization

Cells were grown in TAP medium (50 ml) at 40 μmol m^{-2} sec^{-1} to a concentration of 5×10^5 cells ml^{-1}, placed in the dark for 24 h (conditions that were identical to those used for culturing the strains for the light experiments) and then used for preparing protein extracts. These cultures were chilled on ice, the cells were harvested by centrifugation (5000 ***g***), pelleted cells were resuspended in 50 μl of 0.1 M DTT, 0.1 M Na_2CO_3, and aliquots of 25 μl were either stored at −80°C or used immediately for separation on SDS–PAGE. The procedures for protein extraction and Western blot analysis were essentially identical to those previously described (Huang *et al.*, 2002). Resuspended cells were solubilized by the addition of 25 μl of 2% SDS, 30% sucrose, followed by immersion into boiling water for 1 min. Cell debris was pelleted by centrifugation for 1 min at 4°C (16 000 ***g***, Eppendorf microfuge). The Chl content of the supernatant was assayed by absorbance at 680 nm, and a volume corresponding to 10 μg of Chl was loaded onto a denaturing SDS–PAGE gel (9% acrylamide, 0.1% SDS) and separated by electrophoresis. Proteins were electro-transferred for 1 h onto BioTrace PVDF filters (Pall Corp, Ann Arbor, MI, USA); the quality of the transfer was monitored by Ponceau red staining, and the resulting membranes were used for antibody detection of PHOT. Primary antibodies (used at 1:5000 dilution) were directed against the LOV1 domain of the *C. reinhardtii* PHOT protein (Huang *et al.*, 2002), and visualization of the immune complex was achieved using the ECL™ chemoluminescence kit (Amersham Bioscience).

Generation of labeled cDNA for microarray hybridization

Extracted, total RNA was treated with DNase I and purified using the RNA MinElute kit (Qiagen, Valencia, CA, USA). A 5-μg sample of cleaned RNA was mixed with 1 μg of oligo$(dT)_{12-18}$ and DEPC-treated water to a final volume of 23 μl. Samples were incubated at 70°C for 10 min and chilled on ice immediately. Denatured RNA samples were added to 17 μl of the labeling mix (1 × RT buffer, 2.5 mM DTT, 25 μM dTTP, 62.5 μM dATP, dCTP and dGTP, 400 U Superscript III reverse transcriptase) containing either 2 μl of Cy3- or Cy5-labeled dUTP (Amersham Bioscience). Labeling was performed as follows: 42°C for 30 min, 45°C for 20 min and 50°C for 30 min. An additional 200 U of Superscript III reverse transcriptase was added to the labeling reaction, and the incubation was extended for 30 min at 50°C. The labeling reaction was stopped by the addition of 2 μl of 500 mM EDTA, and the RNA template was degraded by adding 2 μl of 500 mM NaOH followed by incubation at 70°C for 10 min. Samples were then neutralized with 2 μl of 500 mM HCl and purified using the Qiaquick PCR purification kit (Qiagen). The samples, eluted from the purification column with 50 μl of pre-heated water, were dried under a vacuum and resuspended in 40 μl of hybridization solution (6 × SSC, 0.2% SDS, 0.4 μg μl^{-1} poly(A), 0.4 μg μl^{-1} yeast tRNA).

Microarray analysis

Chlamydomonas v1.1 cDNA microarrays, printed onto Corning GAPS II slides (Corning Incorporated Life Sciences, Acton, CA, USA) (Livak and Schmittgen, 2001; Zhang *et al.*, 2002, 2004), were used for all of the experiments. The printed arrays were rehydrated by holding them over a bath of boiling water for approximately 3 sec, after which they were snap-dried on a hot plate. Probe DNA was UV-crosslinked to the array slides (300 mJ). Pre-hybridization was performed in 5 × SSC, 0.1% SDS, 0.1 mg ml^{-1} BSA at 50°C for 1 h, and the arrays were then washed twice at room temperature in 0.1 × SSC, rinsed for 30 sec in water and then spin-dried (1000 ***g***, 5 min) for 10 min. Labeled samples were denatured by incubating them in boiling water for 3 min. They were then centrifuged for 1 min in a microfuge at 10 000 ***g***, and the supernatant was applied to the arrays. Incubation of the array was performed at 50°C for 20 h. After hybridization, arrays were immersed in pre-heated (50°C)

2 × SSC, 0.1% SDS, 10 mM DTT until the cover-slips fell off, and then washed in another bath containing the same solution for 5 min. Slides were then washed twice in 0.1 × SSC, 0.1% SDS, 10 mM DTT at room temperature. Two additional washes (5 min) in 0.1 × SSC, 10 mM DTT were performed before the slides were quickly rinsed in 0.01 × SSC, spin-dried (1000 ***g***, 5 min), and scanned.

Scanning and analysis of the slides

Scanning of the slides was performed using a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA). After flagging the spots (spot flags represent good, absent, empty), results were imported into GeneSpring (Agilent Technologies Inc, Palo Alto, CA, USA) and analyzed. Each slide contained four replicates of each spot. For each point in the time course (0, 30 min, 2 h and 4 h), at least three independent slides were analyzed. Only genes that had spots flagged as 'good' on at least two slides for each time point were considered for further analysis. For each time point, a Student's *t*-test was performed to evaluate the consistency of the signal for the different experimental conditions. Only genes that showed a > 1.5-fold difference between CC-124 and the Ri20 strain at two successive time points were further analyzed.

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