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Electron Transport Regulates Cellular Differentiation in the Filamentous Cyanobacterium *Calothrix*

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Differentiation of the filamentous cyanobacteria *Calothrix* sp strains PCC 7601 and PCC 7504 is regulated by light spectral quality. Vegetative filaments differentiate motile, gas-vacuolated hormogonia after transfer to fresh medium and incubation under red light. Hormogonia are transient and give rise to vegetative filaments, or to heterocystous filaments if fixed nitrogen is lacking. If incubated under green light after transfer to fresh medium, vegetative filaments do not differentiate hormogonia but may produce heterocysts directly, even in the presence of combined nitrogen. We used inhibitors of thylakoid electron transport (3-[3,4-dichlorophenyl]-1,1-dimethylurea and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) to show that the opposing effects of red and green light on cell differentiation arise through differential excitations of photosystems I and II. Red light excitation of photosystem I oxidizes the plastoquinone pool, stimulating differentiation of hormogonia and inhibiting heterocyst differentiation. Conversely, net reduction of plastoquinone by green light excitation of photosystem II inhibits differentiation of hormogonia and stimulates heterocyst differentiation. This photoperception mechanism is distinct from the light regulation of complementary chromatic adaptation of phycobilisome constituents. Although complementary chromatic adaptation operates independently of the photocontrol of cellular differentiation, these two regulatory processes are linked, because the general expression of phycobiliprotein genes is transiently repressed during hormogonium differentiation. In addition, absorbance by phycobilisomes largely determines the light wavelengths that excite photosystem II, and thus the wavelengths that can imbalance electron transport.

INTRODUCTION

The cyanobacteria are an ancient group of oxygenic photoautotrophic prokaryotes (Stanier and Cohen-Bazire, 1977). Many filamentous cyanobacteria show a complexity of cellular differentiation unmatched among prokaryotes and are true multicellular organisms (Adams, 1992; Tandeau de Marsac and Houmard, 1993). Cyanobacterial physiology and differentiation are strongly influenced by light spectral quality and intensity because their growth depends on the quanta absorbed by phycobilisomes and chlorophyll *a* protein complexes.

The phycobilisomes are ordered arrays of pigment-bearing phycobiliproteins and linker polypeptides associated primarily with photosystem II, but extrinsic to the thylakoid membrane (Bryant, 1987). The specific composition and absorbance spectra of the phycobilisomes vary with light and nutrient status (Tandeau de Marsac and Houmard, 1993), and thus the wavelengths that specifically excite photosystem II also change according to growth conditions.

The absorbed quanta drive photosynthetic electron transport, with electrons transferred from water to ferredoxin by the sequential action of two photosystems and an electron transport chain contained in the thylakoid membranes. Electron transport is susceptible to specific inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks the transfer of electrons from the photosystem II complex to plastoquinone (Trebst, 1980), and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which prevents the oxidation of plastoquinone by the cytochrome b_6f complex (Rich et al., 1991).

Some or all of respiratory electron transport from reserve carbohydrates to oxygen is via the same electron transport components that connect photosystem II to photosystem I (Peschek, 1980, 1987; Peschek and Schmetterer, 1982). Hence, in cyanobacteria, the transfer of energy between the two photosystems, which is regulated by the redox state of electron transport components (Allen, 1992), can be influenced by respiratory electron transport as well as by imbalanced excitation of the two photosystems (Mullineaux and Allen, 1986; Vernotte et al., 1990).

Calothrix sp strain PCC 7601 (*Fremyella diplosiphon* UTEX 481) and the closely related *Calothrix* sp strain PCC 7504 (Lachance, 1981) are fresh water, filamentous cyanobacteria. Light spectral quality helps to regulate three processes of acclimation in these strains.

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First, the cells control the expression of a suite of phycobiliprotein genes such that the absorbance of the light-harvesting phycobilisomes complements the spectral quality of the incident light. This regulation is termed complementary chromatic adaptation (CCA) (Bennett and Bogorad, 1973; Tandeau de Marsac, 1983, 1991; Grossman et al., 1988). Physiological evidence indicates that CCA is under the control of a red/green light photoreversible pigment(s), because brief pulses of either light followed by darkness are sufficient to cause CCA (Fujita and Hattori, 1960; Ohki and Gantt, 1983). As yet, the molecular identity of the putative photoreversible pigment(s) is unknown, although many aspects of the gene regulation involved in CCA have been studied (Grossman, 1990; Tandeau de Marsac, 1991).

Second, vegetative filaments of the *Calothrix* spp strains may differentiate into short, gas-vacuolated filaments termed hormogonia, which are motile and buoyant (Herdman and Rippka, 1988; Tandeau de Marsac et al., 1988). Induction of hormogonia can reach 100% of filaments if a culture in mid-exponential phase growth is transferred to fresh medium and incubated for 12 to 24 hr under continuous red light; green light generally inhibits the induction. However, the light effects largely depend on the light regime of the preculture; precultures grown under green or white fluorescent light are very susceptible to induction by a red light incubation, but a preculture grown under red light is less susceptible to induction by red light (Damerval et al., 1991). The differentiation involves a coordinated repression of phycobiliprotein expression, induction of gas vesicle and pili synthesis, and fragmentation of filaments (Csiszàr et al., 1987; Damerval et al., 1987, 1991). Hormogonia are transient, and within 48 hr under favorable growth conditions they give rise to vegetative filaments or to heterocystous filaments in the absence of fixed nitrogen.

Third, many filamentous cyanobacteria can produce nitrogen-fixing heterocyst cells. The regulation of heterocyst differentiation by nitrogen compounds has been intensively investigated (Mulligan and Haselkorn, 1989; Buikema and Haselkorn, 1991; Haselkorn et al., 1991) particularly in *Anabaena* sp strain PCC 7120, which differentiates heterocysts every 10 cells along a filament. However, unlike *Anabaena*, *Calothrix* sp PCC 7601 and 7504 generally differentiate only a single terminal heterocyst per filament. Green light promotes heterocyst differentiation in *Calothrix* sp PCC 7601 and 7504 (T. Damerval and G. Guglielmi, unpublished results) and *A. azollae* (Wu et al., 1982; Wyman and Fay, 1987), whereas red light incubation lowers heterocyst differentiation in *Calothrix*. In *Calothrix* sp PCC 7601, heterocyst development is blocked at the late proheterocyst stage and, hence, the filaments require fixed nitrogen for growth, but *Calothrix* sp PCC 7504 differentiates fully competent heterocysts.

Our goal was to identify the mechanism of the red/green photoperception system(s) that influences hormogonium and heterocyst differentiation in *Calothrix* sp PCC 7601 and 7504, and to determine if this photoperception system is distinct from that which regulates CCA. We describe the patterns of cellular differentiation and complementary chromatic adaptation under a series of light and inhibitor treatments that served to alter the oxidation state of electron transport components. The expression of genes involved in hormogonium differentiation, CCA, and vegetative growth was measured under various treatments to determine some of the regulatory interactions between these processes. We propose that the regulation of hormogonium and heterocyst differentiation by red and green light is via photosynthetic electron transport and is distinct from the regulation of CCA.

RESULTS

Cellular Absorbance Spectra and Fluorescence Analysis

Figure 1 shows the whole cell absorbance spectra of *Calothrix* sp PCC 7601 grown under red or green light. The process of CCA greatly changes the absorbance of the phycobilisomes, with high levels of phycoerythrin in cells grown under green light and increased levels of phycocyanin but no phycoerythrin in cells grown under red light. Hence, the excitation spectrum of photosystem II is very different under the two light regimes. In cells grown under green light, there is relatively little absorbance of red light by the phycobilisomes, and, thus, if green light–grown cells are shifted to red light, they suffer a preferential excitation of photosystem I via chlorophyll *a*. This was verified by using chlorophyll fluorescence analysis to determine the relative oxidation of the photosystem II Q_A centers

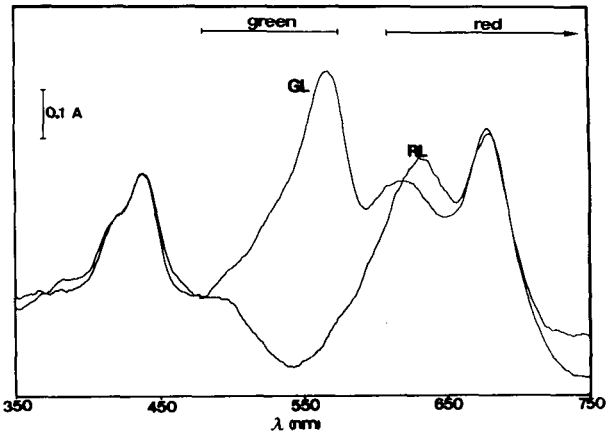

Figure 1. Spectral Absorbance of *Calothrix* sp PCC 7601 Grown under Red or Green Light.

Horizontal bars give the wavelength range where light transmission was greater than 25% with the green (GL) and red (RL) filters used to provide growth and treatment light. Major absorbing species are as follows: chlorophyll *a*, 440 and 682 nm; phycocyanin, 618 nm; allophycocyanin, 650 nm; phycoerythrin, 565 nm; and several carotenoids between 450 and 520 nm. Spectra were corrected for light scattering by subtraction of a scattering baseline and normalized to the 680 nm chlorophyll *a* peak.

during steady state photosynthesis. Q_A is the primary stable quinone electron acceptor of photosystem II, whose oxidation state varies depending on the relative excitation of photosystems I and II. A culture grown under red light was compared to one grown under green light. Under red light excitation, photosystem II was more oxidized (82%) in the green light–grown culture than in the red light–grown culture (69%) (difference significant at $P = 0.025$; see Methods for calculation of Q_A oxidation). This indicates that in cells grown under green light, a red light treatment preferentially excites photosystem I, with concomitant net oxidation of the electron transport chain. Most of the following experiments were performed using cultures grown under green light. In contrast, in cells grown under red light, red light excites both photosystems I and II via chlorophyll a and phycocyanin, respectively (Figure 1). Therefore, red light does not imbalance electron transport in these cells.

Induction of Differentiation by Electron Transport Inhibitors

Figure 2 shows micrographs of *Calothrix* sp PCC 7601 vegetative filaments grown under green light with nitrate as nitrogen source, and filaments of the same culture 24 hr after transfer to fresh media, again with nitrate. Transfer of the green light–grown culture to fresh medium and 24 hr of red light incubation resulted in complete differentiation of gas-vacuolated hormogonia. Conversely, transfer to fresh medium followed by green light incubation resulted in no hormogonia. However, extensive regions of cell division without elongation were present under the green light treatment.

Hormogonium differentiation under red light proceeded in the presence of 10 μM DCMU. This inhibitor concentration was sufficient to lower linear electron transport to 10% of control levels, as measured by oxygen evolution and presented in Figure 3. Hence, as previously observed (Damerval et al., 1991), hormogonium differentiation does not depend on a complete linear photosynthetic electron transport. The effect of 10 μM DCMU under green light was more dramatic, because many filaments differentiated into hormogonia in the presence of the inhibitor, contrasting sharply with the lack of hormogonia in the culture incubated under green light alone. Therefore, DCMU altered the light control of differentiation, such that green light plus DCMU promoted differentiation as did red light plus or minus DCMU. Light was required nevertheless, because hormogonia did not differentiate in the dark in the presence of DCMU (data not presented). In the hormogonia differentiated in the presence of DCMU, gas vacuolation and cell divisions without elongation were less extensive than under red light without DCMU (Figure 2).

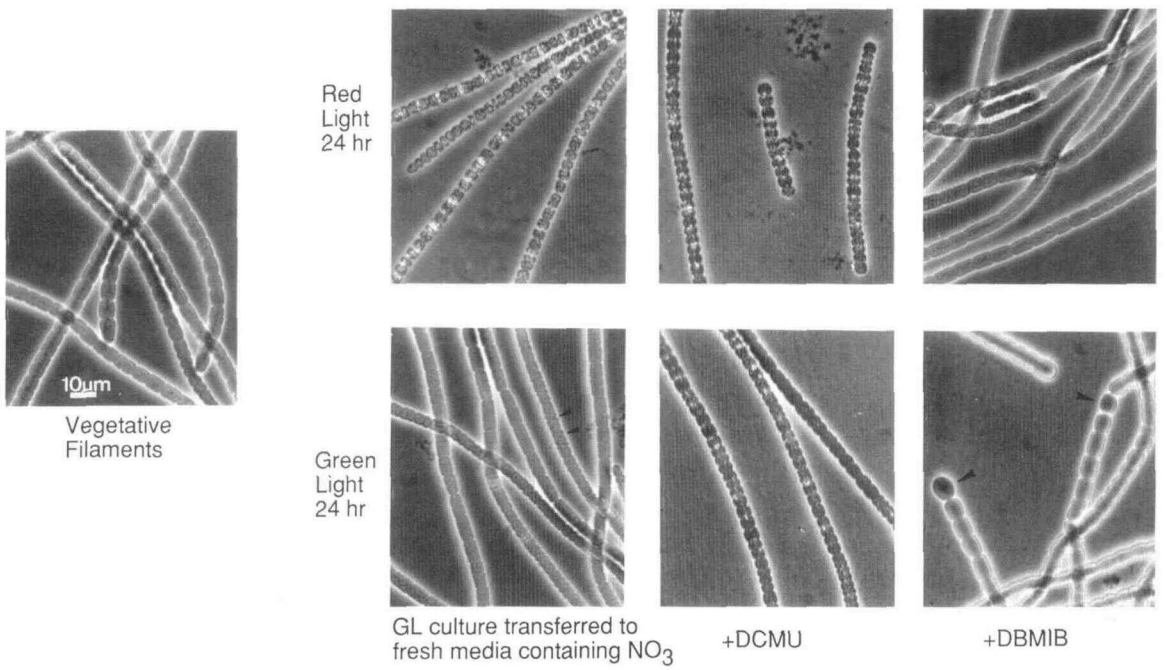

Figure 2. Differentiation of *Calothrix* sp PCC 7601 under Light and Inhibitor Treatments.

A green light (GL) culture was transferred to fresh media containing nitrate and photographed after 24 hr under red or green light, with (+) or without DCMU (10 μM) or DBMIB (5 μM). Arrows show regions of cell division without elongation that are common after media transfer (GL) or developing proheterocysts (GL + DBMIB). Bar = 10 μm; all micrographs are at the same magnification.

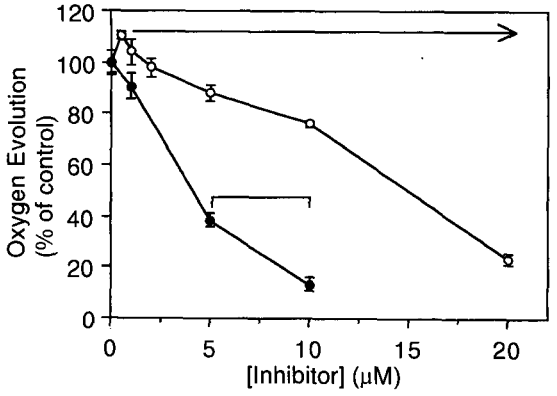

Figure 3. Oxygen Evolution following Inhibitor Treatments.

Oxygen production expressed as percentage of the control rate without inhibitor, measured at a constant light of 60 μmol of photons m^{-2} sec^{-1}, with cells similar to those used for differentiation experiments. DBMIB, open circles; DCMU, filled circles. The mean of four to eight replicates is presented with SE as error bars. The horizontal arrow shows the range of DBMIB concentrations effective in altering light regulation of cellular differentiation; the horizontal bracket shows the effective concentration range for DCMU.

In contrast, DBMIB at 5 μM strongly inhibited hormogonium differentiation under red light and promoted the differentiation of proheterocysts under green light, even though fixed nitrogen was present in the media and heterocyst differentiation would normally be repressed. The apparent intercalary proheterocysts visible in Figure 2 are transient, and filament fragmentation adjacent to the nascent proheterocyst soon regenerates the typical *Calothrix* pattern of single terminal heterocysts. DBMIB at 5 μM allowed linear electron transport to proceed at 90% of control rates (Figure 3).

Table 1 summarizes several replications of the experiment presented in Figure 2, with mid-exponential phase cultures grown under green light and transferred to fresh media containing nitrate. Differentiation was scored following 24 hr of incubation after transfer, in the presence or absence of the electron transport inhibitors. The induction of hormogonia under green light and DCMU ranged from 0 to 50% and required at least 15 μmol of photons m^{-2} sec^{-1}, whereas red light promoted nearly complete differentiation at 7 to 8 μmol of photons m^{-2} sec^{-1}. Green or white light alone gave no hormogonia, but white light and DCMU stimulated differentiation similar to the green light/DCMU treatment. At 5 μM, DBMIB consistently inhibited the differentiation of hormogonia and also promoted differentiation of proheterocysts, although the promotion of proheterocysts was variable depending on the preculture and the light treatment.

Figure 4 presents a parallel experiment, with all treatments the same as those of Figure 2, except that the green light culture was transferred from medium containing nitrate to fresh medium lacking fixed nitrogen. The results of these treatments confirm those of Figure 2. Twenty-four hours of red light resulted in 100% differentiation of hormogonia, whereas green light resulted in no hormogonia, although divisions without elongation were extensive. Once again, 10 μM DCMU promoted the differentiation of hormogonia under green light. The electron transport inhibitors atrazine and metribuzine, which, like DCMU, block electron transfer from photosystem II to plastoquinone, had the same effects on differentiation as DCMU (data not presented). Conversely, 5 μM DBMIB blocked hormogonium differentiation and promoted the differentiation of proheterocysts under both red and green light (Figure 4). A double treatment with both DCMU and DBMIB was similar to DBMIB alone, with inhibition of hormogonium differentiation and stimulation of heterocyst differentiation (data not presented).

Table 2 summarizes the replications of the experiment presented in Figure 4, with green light cultures in mid-exponential phase transferred to medium lacking fixed nitrogen, followed by 24 hr of incubation with or without inhibitors. Proheterocysts sometimes developed under both green and red light treatments in the absence of DBMIB, although the average frequency of proheterocysts increased in the presence of DBMIB under both light treatments. DCMU generally inhibited the differentiation of proheterocysts under all light treatments.

The data presented in Tables 1 and 2 were subjected to statistical analysis using the Instat package (GraphPad Software, San Diego, CA). The effects of the light and inhibitor

Table 1. Effect of Inhibitors on Differentiation of *Calothrix* sp PCC 7601 Grown under Green Light and Transferred to Fresh Medium Containing Nitrate

Treatment	Hormogonia (%)	Divisions (%)	Heterocystous Filaments (%)
Control (0 hr)	0.3 ± 0.3	3 ± 1	3 ± 2
RL	87 ± 10	15 ± 10	None
RL, DCMU	80 ± 16	18 ± 6	None
RL, DBMIB	2 ± 2	11 ± 7	4 ± 4
GL	3 ± 3	31 ± 16	6 ± 3
GL, DCMU	38 ± 14	15 ± 8	2 ± 2
GL, DBMIB	None	22 ± 20	7 ± 5
WL	None	10 ± 10	1 ± 1
WL, DCMU	50 ± 22.2	29 ± 16	1 ± 1

Hormogonia were identified as filaments containing significant regions of gas vacuolation and cell divisions without elongation; if gas vacuoles were lacking, the filaments were scored under divisions. Heterocystous filaments contained at least one proheterocyst. RL, red light (8 to 10 μmol of photons m^{-2} sec^{-1}); GL, green light (15 μmol of photons m^{-2} sec^{-1}); WL, white fluorescent light (15 μmol of photons m^{-2} sec^{-1}), all for 24 hr. DCMU (10 μM) or DBMIB (5 μM) was present in media during 24-hr treatment. Differentiation was scored as the percentage of total filaments under each treatment; the mean of three to 12 replicates ± SE is presented.

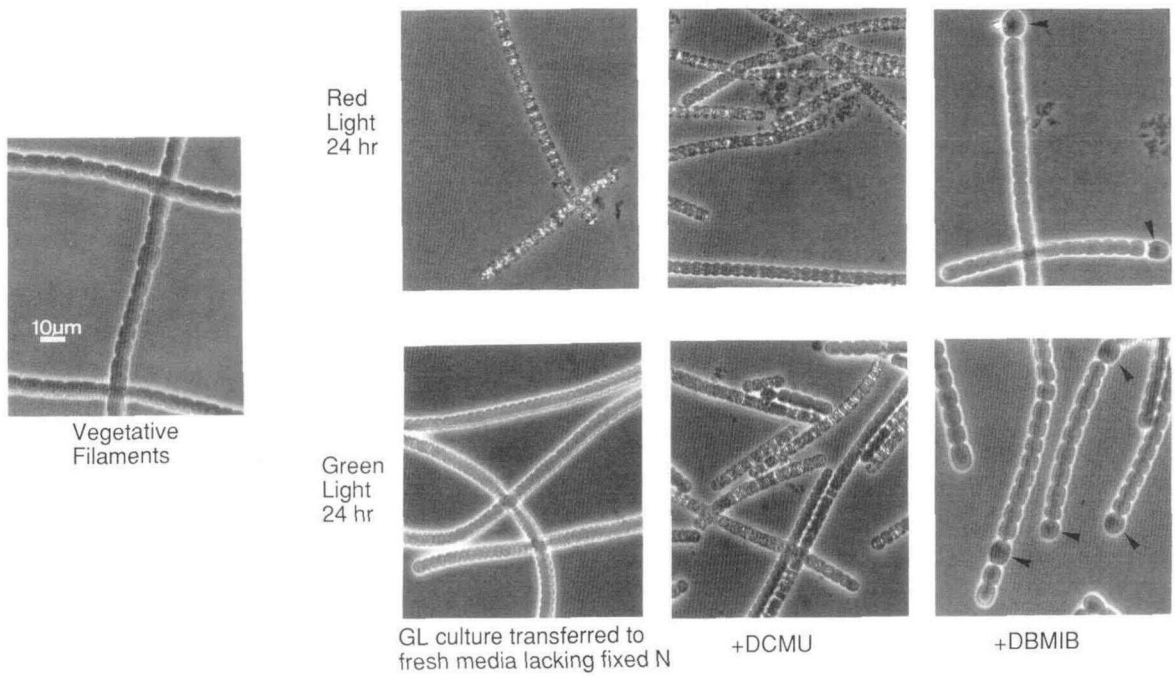

Figure 4. Differentiation of *Calothrix* sp PCC 7601 under Light and Inhibitor Treatments during Nitrogen Starvation.

A green light (GL) culture was transferred to fresh media lacking fixed nitrogen and photographed after 24 hr under red or green light, with (+) or without DCMU (10 μM) or DBMIB (5 μM). Arrows show proheterocysts. Note cell division without elongation in green light treatment. Bar = 10 μm; all micrographs are at the same magnification.

treatments were assessed by repeated measures analysis of variance, followed by Bonferroni multiple comparison tests. This method measures the specific effects of each treatment, without interference from culture-to-culture variation. DCMU significantly increased differentiation of hormogonia under green and white light, with $P < 0.01$, and did not alter hormogonium differentiation under red light. The presence of DCMU also inhibited the differentiation of heterocysts in comparison with green light or green light/DBMIB treatments, with $P < 0.05$. DBMIB significantly lowered hormogonium differentiation under red light, with $P < 0.001$, and promoted heterocyst differentiation under all light treatments, with $P < 0.05$.

For the cellular divisions without elongation, no statistically significant changes in frequency were found among the treatments. The divisions sometimes occurred under all treatments, with large variation between replications.

Table 3 presents a further test of the effect of DCMU on hormogonium differentiation. *Calothrix* sp PCC 7601 cultures were grown under red light and transferred to fresh medium, then incubated under red light in the presence or absence of DCMU. The red light culture was somewhat resistant to the induction of hormogonia by incubation under red light. However, the addition of DCMU induced a large differentiation of hormogonia.

Table 2. Effect of Inhibitors on Differentiation of *Calothrix* sp PCC 7601 Grown under Green Light and Transferred to Fresh Medium Lacking Combined Nitrogen

Treatment	Hormogonia (%)	Divisions (%)	Heterocystous Filaments (%)
Control (0 hr)	None	None	7
RL	97	2	6
RL, DCMU	97	3	None
RL, DBMIB	14.5	None	42
GL	None	51	25
GL, DCMU	57	5	15
GL, DBMIB	None	None	50

Hormogonia were identified as filaments containing significant regions of gas vacuolation and cell divisions without elongation; if gas vacuoles were lacking, the filaments were scored under divisions. Heterocystous filaments contained at least one proheterocyst. RL, red light (8 to 10 μmol of photons m^{-2} sec^{-1}); GL, green light (15 μmol of photons m^{-2} sec^{-1}); WL, white fluorescent light (15 μmol of photons m^{-2} sec^{-1}), all for 24 hr. DCMU (10 μM) or DBMIB (5 μM) was present in media during 24-hr treatment. Differentiation was scored as the percentage of total filaments under each treatment; the mean of two to three replicates is presented. Variation was similar to that given in Table 1.

Table 3. Effect of DCMU on Differentiation of *Calothrix* sp PCC 7601 Grown under Red Light and Transferred to Fresh Medium Containing Nitrate

Treatment	Hormogonia (%)	Divisions (%)	Heterocystous Filaments (%)
Control (0 hr)	None	2	None
RL	4	31	None
RL, DCMU	90	8	None

RL, red light (8 to 10 μmol of photons m^{-2} sec^{-1}) for 24 hr. DCMU (10 μM) present in media during 24-hr treatment. Differentiation was scored as the percentage of total filaments under each treatment.

Thus, the presence of DCMU and light of any spectral quality stimulates the differentiation of hormogonia, no matter what the light regime of the preculture (Tables 1 to 3). Conversely, in the absence of DCMU, hormogonia are highly induced only by red light incubation of cultures previously grown under green or white light (Tables 1 and 2).

The related strain *Calothrix* sp PCC 7504 displays much the same pattern of light and inhibitor regulation of hormogonium induction, as shown in Table 4. DCMU promoted hormogonium differentiation under green light. The strain grows in aggregated clumps in liquid culture, unlike *Calothrix* sp PCC 7601, which grows as an even suspension. Hence, it is difficult to obtain even exposure of cells to treatments, and hormogonium differentiation is seldom driven to 100%. Heterocyst frequency was low in the trials with this strain, but a DCMU inhibition of heterocyst differentiation was still apparent under green light.

In summary, the two inhibitors of electron transport altered the light regulation of differentiation, but in opposing directions. DCMU promotes hormogonium differentiation and inhibits heterocyst differentiation, whereas DBMIB blocks hormogonium differentiation and promotes heterocyst differentiation.

Inhibitors and Complementary Chromatic Adaptation

We followed the course of CCA under the various light and inhibitor treatments described above. Transfer of a green light culture to red light results in a shift in the absorbance profile of the cells because phycoerythrin synthesis stops and phycocyanin-2 synthesis commences (Gendel et al., 1979; Ohki and Gantt, 1983; Oelmüller et al., 1988a, 1988b). Phycoerythrin is eliminated from the cells by dilution with each cell division; therefore, several generations after the transfer from green to red light are required to lose all phycoerythrin and accumulate maximal levels of phycocyanin (Figure 1). Nevertheless, the color change from reddish brown to green is noticeable within 24 hr after transfer of a green light culture to red light, and very marked within 72 hr (four to five generations). Figure 5 compares the absorbance spectra of cells maintained under green light and cells transferred to red light for 72 hr, with or without 5 μM DBMIB. The culture transferred to red light underwent 100% differentiation of hormogonia, which subsequently grew into vegetative filaments. These filaments underwent CCA, as marked by the increase in phycocyanin, reflecting new synthesis, and the decline in phycoerythrin from dilution. The culture transferred to red light and DBMIB did not differentiate any hormogonia, but did undergo CCA, with an increase in phycocyanin and some decline in phycoerythrin; the smaller decline in phycoerythrin possibly reflects a lower dilution rate than under red light alone. Hence, the DBMIB treatment completely blocked red light promotion of hormogonia, but did not block red light regulation of CCA. DCMU does not appear to alter the process of CCA per se. However, the dose of DCMU required to induce hormogonium differentiation under green light also greatly inhibits growth because photosynthetic energy is not available. Therefore, CCA could not be measured spectrally under the DCMU treatments.

The light regulation of hormogonium differentiation and of CCA was further examined by the analysis of mRNA abundance, as presented in Figure 6. Total RNA was isolated from a green light–grown culture at time 0, and then after 8 or 24 hr of incubation under red light, with or without DCMU and DBMIB, or under continued green light incubation. Under the red light and red light/DCMU treatments, gas-vacuolated hormogonia differentiated. Under the red light/DBMIB and the green light treatments, hormogonia did not differentiate.

During hormogonium differentiation in the red light and red light/DCMU treatments, the gas vesicle genes are transiently expressed, while the expression of all phycobiliprotein genes is repressed (8-hr samples). As differentiation is completed, gas vesicle synthesis stops and phycobiliprotein gene expression recommences (24-hr samples). This pattern was very similar in both the red light and red light/DCMU treatments. Expression of the allophycocyanin and red light–specific phycocyanin-2 genes was low in the 8-hr samples due to the general repression of phycobiliprotein genes during hormogonium differentiation, but increased in the 24-hr samples. The gas vesicle transcripts disappeared from the red light/DCMU

Table 4. Effect of DCMU on Differentiation of *Calothrix* sp PCC 7504 Grown under Green Light and Transferred to Fresh Medium Lacking Combined Nitrogen

Treatment	Hormogonia (%)	Divisions (%)	Heterocystous Filaments (%)
Control (0 hr)	None	None	None
RL	37	25	4
RL, DCMU	79	10	8
GL	None	30	15
GL, DCMU	30	11	2

RL, red light (8 to 10 μmol of photons m^{-2} sec^{-1}); GL, green light (15 μmol of photons m^{-2} sec^{-1}), for 24 hr. DCMU (10 μM) present in media during 24-hr treatment. Differentiation was scored as the percentage of total filaments under each treatment.

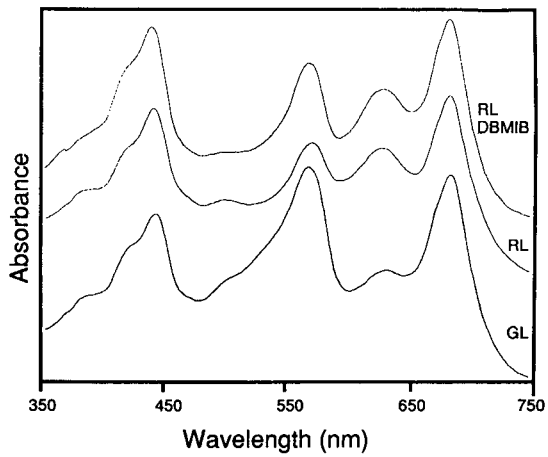

Figure 5. Spectral Absorbance of *Calothrix* sp PCC 7601 after Light and DBMIB Treatments.

GL denotes the spectrum of culture maintained under green light; RL, the spectrum of a green light culture 72 hr after transfer to red light; RL + DBMIB, as given for RL but with 5 μM DBMIB. The 680-nm chlorophyll *a* peak was 1 absorbance unit in all cases; spectra were offset to facilitate comparison.

samples after 24 hr, whereas some remained in the red light samples after 24 hr. Furthermore, both the allophycocyanin and phycocyanin-2 messages were somewhat more abundant in the presence of DCMU in the 8- and 24-hr samples. This may reflect a faster differentiation under DCMU treatment, causing earlier loss of gas vesicle transcripts and earlier resumption of phycobiliprotein synthesis. Very little expression of the green light–specific phycoerythrin gene was detected in either the red light– or the red light/DCMU–treated samples at 8 or 24 hr.

The red light/DBMIB treatment blocked hormogonium differentiation as well as the expression of gas vesicle genes. However, phycobiliprotein gene expression was strong and reflected regulation by CCA. The red light–specific phycocyanin-2 was expressed in the 8- and 24-hr samples, as was the constitutive allophycocyanin; both were more abundant under red light and DBMIB than under red light alone. Very little green light–specific phycoerythrin was detected.

Under green light, hormogonia did not differentiate and, hence, no gas vesicle messages were detected. Phycobiliprotein gene expression included the green light–specific phycoerythrin gene and the constitutive allophycocyanin genes, but not the red light–specific phycocyanin-2 genes. This qualitative pattern of phycobiliprotein gene expression under green light was not altered by the addition of DCMU or DBMIB (data not presented).

In summary, the analysis of mRNA accumulation shows that CCA continued in the presence of both inhibitors. The red light–specific phycocyanin-2 message was present under all the red light treatments, whereas the phycoerythrin message was only weakly expressed, except under green light. All phycobiliprotein gene expression was transiently repressed in the red light and red light/DCMU treatments that differentiated hormogonia, but recommenced in the 24-hr samples. The presence of the inhibitors did not qualitatively alter the pattern of CCA, although DBMIB in particular, and possibly DCMU as well, augmented the accumulation of both allophycocyanin and phycocyanin-2 messages.

DISCUSSION

Hormogonium Differentiation and Electron Transport

A model of the regulation of hormogonium differentiation in *Calothrix* spp strains must account for the opposing effects of red and green light, the requirement for continuous red light for maximal differentiation, the sensitivity of the response to culture growth light and growth phase, and the opposing actions of the inhibitors DCMU and DBMIB (this paper; Csiszàr et al., 1987; Damerval et al., 1987, 1991). Regulation by alterations in the redox state of an electron transport component can explain these experimental results in the following way.

Green light–grown cultures normally differentiate hormogonia after transfer to red light because red light preferentially excites photosystem I, causing net oxidation of the electron transport chain. Green light generally inhibits differentiation because it preferentially excites photosystem II, reducing the electron transport chain. The effect of DCMU provides a test of this mechanism. DCMU blocks photosystem II electron donation to plastoquinone, resulting in oxidation of the electron transport chain under light of any color, because electrons are removed by the action of photosystem I, but are not replaced by photosystem II. As expected, DCMU partially overcomes green light inhibition, and some hormogonia differentiation proceeds under light of any color. However, even in the presence of DCMU, a higher intensity of green than red light is required to promote differentiation because green light excites photosystem I with low efficiency in comparison with red light.

Red light–grown cultures are less susceptible to the red light induction of hormogonia because red light gives a balanced excitation of photosystems I and II in these cells. DCMU renders these cells more susceptible to red light induction of hormogonia by preventing photosystem II electron donation.

At a whole cell level, DCMU and DBMIB have similar effects; by blocking linear photosynthetic electron transport, they lower cellular supplies of ATP and NADPH. However, the action sites of the inhibitors are one step apart along the electron transport chain; DCMU blocks reduction of plastoquinone by photosystem II, while DBMIB blocks the oxidation of plastoquinone by the cytochrome b_6f complex. The opposing actions of DCMU and DBMIB on differentiation help to define the electron transport component involved in the regulation. DBMIB partially mimics green light excitation of photosystem II by causing net reduction of the plastoquinone pool no matter what the light treatment. Thus, hormogonia are probably induced

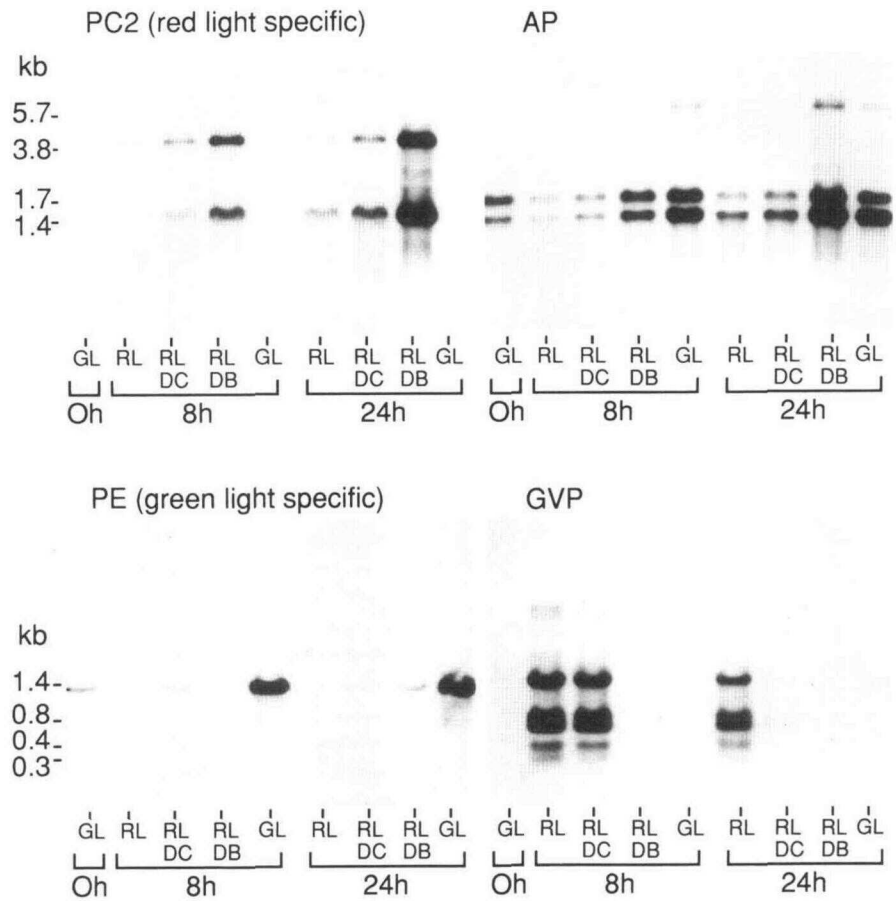

Figure 6. CCA and Hormogonia Differentiation under Light and Inhibitor Treatments.

Cells grown under green light (GL) were transferred to fresh medium and incubated under red light (RL) with or without DCMU (DC) or DBMIB (DB), or under continuous green light. Total RNA was extracted after 0, 8, or 24 hr, separated electrophoretically, and blotted to Hybond-N membranes before hybridization with DNA probes. Hormogonia differentiated under the red light and red light plus DCMU treatments, but not under the red light plus DBMIB or green light treatments. The probes are described in Methods. AP, allophycocyanin, constitutively expressed in vegetative cells; PE, phycoerythrin (green light inducible); PC2, phycocyanin-2 (red light inducible); GVP, gas vesicle protein (expressed during hormogonia differentiation). Expression of phycobiliproteins is repressed during hormogonium differentiation (red light and red light plus DCMU treatments, 8-hr samples).

by net oxidation of the plastoquinone pool, as under red light or DCMU treatment. The differentiation is inhibited by net reduction of the plastoquinone pool under green light or DBMIB treatment. An alternate possibility is that the redox sensor is a component of the cytochrome b_6f complex, whose oxidation state is coupled to that of the plastoquinone pool. There are several redox active moieties of the cytochrome b_6f complex of uncertain function that are potential candidates (Rich et al., 1991; Hope et al., 1992).

DBMIB inhibited hormogonium differentiation at concentrations greater than 1 μM, but electron transport was not significantly inhibited until above 5 μM DBMIB (Figure 3). However, the concentration of DCMU (5 to 10 μM) required to stimulate hormogonium differentiation resulted in strong inhibition of electron transport (Figure 3). DCMU eliminates photosystem II electron donation, but the subsequent oxidation of plastoquinone is dependent on sufficient excitation of photosystem I to withdraw electrons. In contrast, DBMIB can slow the oxidation of plastoquinone while permitting nearly control rates of net electron transport. This distinction between rates of net electron transport under the two inhibitor treatments, together with the opposing actions of DCMU and DBMIB, indicate that the effects on differentiation result from a specific alteration in the balance of oxidation and reduction of plastoquinone or a related electron transport component, and not from a general shortage of ATP or reducing equivalents.

The variability of hormogonium differentiation under red light in cultures at different growth states and the differing susceptibility of cultures to hormogonium induction by DCMU demonstrate that factors other than electron transport status

influence hormogonium differentiation. Several known factors contribute to the variability, including the partial toxicity of the DCMU treatment, poor differentiation in cultures with low levels of carbohydrate reserves (data not presented), and possibly the precise growth state and filament length of cultures. Hence, the electron transport signal is a major component of the regulatory network, but is not always sufficient for induction.

The shared electron transport chain for respiratory and photosynthetic electron flow means that changes in the redox state of plastoquinone can potentially sense not only imbalanced photosystem excitation, but also imbalanced respiratory electron transport. A precedent for this notion is the finding that respiratory electron flow into the plastoquinone pool can drive reorganizations of light energy transfer between the two photosystems (state transitions) in cyanobacteria (Mullineaux and Allen, 1986; Vernotte et al., 1990). Thus, an oxidized plastoquinone pool could indicate a general imbalance between the supply and demand of electrons. Hence, light regulation of hormogonium differentiation via electron transport may represent a special case of a more general regulatory mechanism.

A fraction of filaments were observed undergoing cell division without elongation under all treatment conditions (Tables 1 to 3). Damerval et al. (1991) considered these divisions as the initial stage of hormogonium differentiation, and indeed several cycles of cell division precede gas vacuolation and full hormogonium differentiation. In some cases (red light, red light plus DCMU, and green light plus DCMU), these filaments were most probably in the process of hormogonium differentiation, particularly in the presence of DCMU because the differentiation was sometimes incomplete. However, cell division without elongation also occurred in cultures that did not differentiate hormogonia (Figure 4, green light treatment, and Tables 1 to 3). Thus, these divisions are not confined specifically to hormogonium differentiation. Various stresses may induce these divisions, with the ongoing nature of the stress giving signals, such as electron transport redox state, which determine the further path of differentiation.

Heterocyst Differentiation

DCMU or red light inhibited heterocyst differentiation in *Calothrix* sp PCC 7601, whereas DBMIB and/or green light stimulated heterocyst differentiation. Thus, the actions of light and inhibitors on heterocyst differentiation are the reverse of those on hormogonium differentiation, indicating that hormogonium and heterocyst differentiation are opposing processes in *Calothrix* sp. Under red light, hormogonia differentiate prior to heterocysts upon transfer to media lacking fixed nitrogen. Thus, the redox-driven hormogonium differentiation can temporarily override heterocyst differentiation. Under green light or DBMIB treatment, the redox signal augments the number of heterocysts differentiated in response to nitrogen shortage (Table 2) and drives heterocyst differentiation even in the presence of nitrate in some cultures (Figure 2). As demonstrated in other systems, nitrate apparently only partially inhibits the differentiation of heterocysts (Fay, 1973; Almon and Boger, 1988), although in *Calothrix* sp PCC 7601, ammonia completely inhibits the differentiation, no matter what the light or inhibitor treatment (data not presented). Thus, at some level, nitrogen can dominate the redox regulation of heterocysts. A possible connection between the regulation of nitrogen metabolism and electron transport is the bacterial P_{II} regulatory protein (*glnB* gene product), which was recently isolated from a cyanobacterium and shown to respond to both nitrogen sources and imbalanced electron transport (Tsinoremas et al., 1991). We are currently investigating the implications of the opposing regulation of hormogonia and heterocysts.

Light Regulation of Cellular Differentiation and Complementary Chromatic Adaptation

The regulation of cellular differentiation by the redox state of electron transport is distinct from the regulation of phycobiliprotein expression by light spectral quality. The DBMIB treatment completely blocked red light induction of hormogonia, while permitting the cells to grow and respond appropriately to red light by altering their phycobiliprotein content. Conversely, DCMU stimulated hormogonium induction even under green light, but did not qualitatively change phycobiliprotein gene expression. Thus, *Calothrix* sp PCC 7601 responds to light spectral quality both specifically, possibly through the action of a photoreversible pigment(s), and more generally by sensing imbalances in electron transport. Electron transport can be altered through inhibitor treatments, whereas the photoreversible regulation of CCA is independent of such artificial physiological manipulations.

Nevertheless, the two systems are clearly linked. The light spectral qualities that cause imbalanced or balanced electron transport are determined by phycobilisome absorbance, which is defined by the CCA process. Conversely, electron transport status influences phycobilisome number and/or composition in species with and without CCA (Öquist, 1974; Lönneborg et al., 1985; Kalla et al., 1986; Fujita and Murakami, 1987; Fujita et al., 1987; Tandeau de Marsac and Houmard, 1993). Such a regulatory linkage between electron transport and total phycobilisome synthesis explains the quantitative, general increase in phycobiliprotein gene expression in the presence of the inhibitors (Figure 6), even though the qualitative green/red light regulation of phycoerythrin and phycocyanin-2 was not altered by the inhibitors. Chromatic adaptation and cellular differentiation are further linked at the level of gene regulation, because hormogonium differentiation involves a transient general repression of phycobiliprotein gene expression (Figure 6; Damerval et al., 1991) and hence temporarily interrupts the process of CCA that accompanies a shift in light regime.

Figure 7 outlines our current understanding of the light regulation of cellular differentiation in *Calothrix* spp strains. We hypothesize that under stress, an unknown sensory system stimulates cell division without elongation and puts the cells in a state of competence for differentiation. Hormogonium

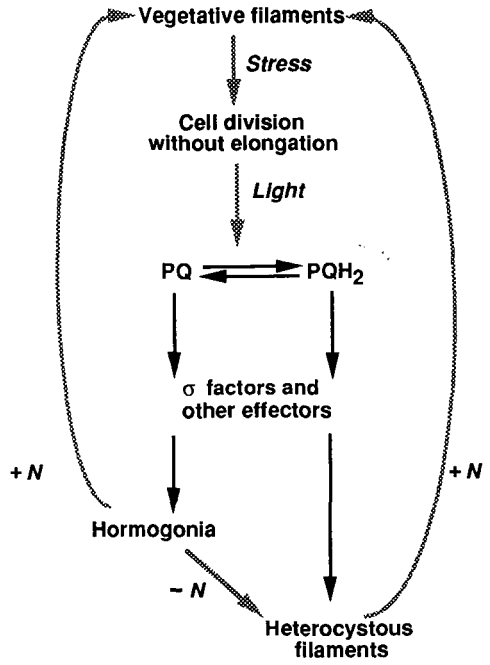

Figure 7. Light Regulation of Cellular Differentiation in *Calothrix* spp Strains.

This figure summarizes the sequence of differentiation when *Calothrix* spp strains are transferred to fresh media or subjected to other stresses. N refers to presence (+) or absence (−) of combined nitrogen in the medium. Environmental factors are in italics; differentiation events are shown with light arrows, whereas regulatory events are in dark arrows.

differentiation is promoted if plastoquinone is oxidized by red light excitation of photosystem I or by other treatments. Hormogonium differentiation temporarily overrides heterocyst differentiation, even under nitrogen starvation. If plastoquinone is in a reduced state, heterocyst differentiation is stimulated. This stimulation is synergistic with the induction of heterocysts by nitrogen starvation, because more heterocysts differentiate under a combined nitrogen starvation and green light or DBMIB treatment than under either treatment alone. CCA regulates the expression of phycoerythrin and phycocyanin-2 independently of the redox signal; however, electron transport status influences the general level of phycobilisome gene expression.

Calothrix spp strains thus control hormogonium and heterocyst differentiation, as well as phycobilisome composition, by integrating diverse environmental and physiological information through regulatory networks, which are connected both biochemically and genetically. Our results demonstrate that photosynthetic electron transport is a major element in the regulation of cellular differentiation in *Calothrix* sp strains. Our current work aims to elucidate further elements of these interacting regulatory systems.

METHODS

Materials

The inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) were purchased from Sigma, and prepared freshly as stock solutions of 2 and 100 mM in water and ethanol, respectively.

Culture Conditions

The cyanobacteria *Calothrix* sp strain PCC 7601 (*Fremyella diplosiphon* UTEX 481) and *Calothrix* sp strain PCC 7504 (a very closely related strain that differentiates functional heterocysts) were grown in BG-11 medium (Rippka et al., 1979) at 27 to 30°C. Cultures were continuously bubbled with 1% CO_2/99% air (v/v) and illuminated with cool-white fluorescent tubes with cellulose acetate filters interposed between the culture vessel and the fluorescent tubes. Photosynthetically active radiation between 400 and 700 nm was measured with a LI-COR LI-185B meter equipped with a quantum sensor, with photosynthetic photon flux density expressed as micromoles of photons per square meter per second. For induction of cellular differentiation, cells were harvested by filtration onto glass microfiber filters (Whatman GF/A) and resuspended to the initial volume in fresh BG-11 medium or in BG-11o medium, which lacks combined nitrogen. Inhibitor treatments were with final concentrations of 10 μM DCMU or 5 μM DBMIB, unless otherwise noted.

Oxygen evolution was measured with an oxygen monitor (model 5300; Yellow Springs Instruments, Yellow Springs, OH). Samples were dark adapted for 3 min prior to measurements. The data presented in Figure 3 were obtained by measuring oxygen evolution within 2 hr after the addition of inhibitors to the culture flask; however, similar results were obtained with measurements taken at the end of the typical 24-hr differentiation treatments.

Estimation of Hormogonium and Heterocyst Differentiation

Twenty-four hours after transfer of the cultures to fresh media, 1- to 2-mL samples were fixed with formaldehyde (1% v/v final). Four to eight fields of each culture were photographed at a magnification of 200. Filaments were scored for the presence of division without elongation in the absence of gas vacuolation, hormogonium differentiation (gas-vacuolated filaments), and proheterocysts (large rounded cells, which are dark under phase-contrast and light under bright-field microscopy). The results are expressed as a percentage of total filaments because the number of cells per filament changes rapidly during the cellular divisions without elongation, which often precede hormogonium or heterocyst differentiation. In addition, hormogonium differentiation proceeds on an all-or-nothing basis for each filament. Hence, the presence of significant gas vacuolation in a filament indicates that most cells of the filament will soon differentiate to the hormogonial state, even if some vegetative cells remain in the filament at the moment of sampling. The differentiation of hormogonia after 24 hr of red light incubation

was used as a positive control, and cultures that failed to differentiate after that treatment (~10% of the total tested) were excluded from the compilation of Tables 1 and 2.

Chlorophyll Fluorescence Induction Analysis

Chlorophyll fluorescence induction was measured with a chlorophyll fluorometer system (Walz PAM; Effeltrich, Germany) with a stirrer-equipped cuvette (model KS-101), using red (650 ± 12.5 nm) excitation light. Samples were dark adapted for 3 min and then fluorescence parameters were determined as follows. Minimum fluorescence (F_0) was measured by illumination with a measuring beam (0.01 μmol of photons m^{-2} sec^{-1}) and remained stable for several minutes (no evidence of variable fluorescence induction). Variable fluorescence (F_v) was induced by a 12 μmol of photons m^{-2} sec^{-1} red light excitation beam. Minimum fluorescence in the light-adapted state ($F_{0'}$) was measured by briefly (~1 sec) interrupting the excitation light after fluorescence had reached steady state, ~300 sec after induction began. Maximal fluorescence in the light-adapted state ($F_{m'}$) was measured by a 800-msec flash of white light (15,000 μmol of photons m^{-2} sec^{-1}) after fluorescence reached steady state (F_s), whereas true maximal fluorescence (F_m) was measured by the addition of DCMU (10 μM final) to the sample. F_v/F_m values were similar in both red light and green light cultures (0.63 ± 0.01; 0.64 ± 0.01), as were $F_{v'}/F_{m'}$ (0.53 ± 0.02; 0.58 ± 0.02) (Genty et al., 1989), indicating the cultures were in similar physiological states. The oxidation of photosystem II Q_A centers (the primary stable quinone acceptor of photosystem II) was calculated using the equation of van Kooten and Snel (1990) for photochemical quenching, $Qp = (F_{m'} - F_s)/(F_{m'} - F_{0'})$.

RNA Preparation and Hybridization with ^{32}P-Labeled Probes

Total RNA was prepared as described by Glatron and Rapoport (1972). Samples containing 20 μg of total RNA, as determined by absorbance at 260 nm, were electrophoresed for 10 to 11 hr at 40 to 50 V through 1.2% (w/v) agarose gels containing 16% (v/v) formaldehyde. The buffer was 0.5 M *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), pH 7.8, 10 mM EDTA. Equal loads of total RNA in each lane were verified by the equivalent ethidium bromide fluorescence from the rRNA bands under UV light. The RNA was transferred to Hybond N by capillary blotting and fixed to the membrane by 2 min of UV light and 2 hr at 80°C. The DNA probes are as follows: allophycocyanin, a 1.1-kb DraI fragment encoding allophycocyanin subunits α and β (*apcA1B1*) (Houmard et al., 1988); phycocyanin-2, a 0.4-kb SspI fragment located just upstream from the ATG codon and carrying the transcribed but untranslated region of the phycocyanin-2 operon (*cpcB2A2*) (Capuano et al., 1988); phycoerythrin, a 1.1-kb XbaI-HincII fragment encoding part of the β and α phycoerythrin subunits (*cpeB* and *cpeA*) (Mazel et al., 1986); gas vesicle gene, a 0.24-kb HindIII-HincII fragment of the gene for the gas vesicle structural protein GvpA (*gvpA*) (Damerval et al., 1987); all were derived from *Calothrix* sp PCC 7601. DNA probes were labeled using the Megaprime random priming kit and ^{32}P-dCTP (3000 Ci/mmol) (Amersham). Hybridizations were for 12 hr at 45°C in 50% (v/v) formamide containing 1 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (w/v) SDS, 7 μg/mL sonicated DNA, and 100 mM NaH_2PO_4. Membranes were washed in 2 × and 0.1 × SSC, both containing 0.1% (w/v) SDS, and then exposed to autoradiography film for 24 hr.

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