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Characterization of Complementary Chromatic Adaptation in *Gloeotrichia* UTEX 583 and Identification of a Transposon-like Insertion in the *cpeBA* Operon

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Many cyanobacteria are able to alter the pigment composition of the phycobilisome in a process called complementary chromatic adaptation (CCA). The regulatory mechanisms of CCA have been identified in *Fremyella diplosiphon*, which regulates both phycoerythrin and phycocyanin levels, and *Nostoc punctiforme*, which regulates only phycoerythrin production. Recent studies show that these species use different regulatory proteins for CCA. We chose to study the CCA response of *Gloeotrichia* UTEX 583 in an effort to expand our knowledge about CCA and its regulation. We found that *Gloeotrichia* 583 has a CCA pigment response more similar to that of *N. punctiforme* rather than *F. diplosiphon* and exhibits none of the CCA-regulated morphological responses seen in *F. diplosiphon*. Preliminary experiments suggest that *Gloeotrichia* 583 contains a homolog to the CCA photoreceptor from *N. punctiforme* but not the CCA photoreceptor from *F. diplosiphon*. Additionally, two spontaneous mutants lacking phycoerythrin production were identified. Analysis has shown that these mutants contain a transposon-like insertion in the *cpeA* gene, which encodes the α subunit of phycoerythrin. These results suggest that CCA in *Gloeotrichia* UTEX 583 is more similar to that of *N. punctiforme* than it is to *F. diplosiphon*, a closely related species.

Keywords: complementary chromatic adaptation • cyanobacteria • *Gloeotrichia* UTEX 583.

Abbreviations: AP, allophycocyanin; CCA, complementary chromatic adaptation; GL, green light; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; PEC, phycoerythrocyanin; RL, red light

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers *Gloeotrichia* UTEX 583 *cpeBA* (HM751857), hypothetical protein G1 (HM751858) and *cpcBA* partial sequence (HM852510).

Introduction

The need to optimize light harvesting is universal in photosynthetic organisms. Many plants alter chloroplast placement and number in response to changes in the light environment. Among prokaryotes, diversity in bacteriochlorophyll types likely reduces direct competition among anoxygenic photosynthetic bacteria (Chew and Bryant 2007) whereas cyanobacteria, oxygenic photosynthetic bacteria, build light-harvesting antennae capable of capturing wavelengths that are poorly absorbed by chlorophyll (Glazer 1982). These light-harvesting antennae are called phycobilisomes (PBSs), and much of the diversity in cyanobacteria is found in their light-harvesting pigments and light-harvesting strategies (Gantt 1981, Glazer 1982, Grossman 2003). PBSs are structured with allophycocyanin (AP, maximum absorbance ~650 nm) at their core and rod-like fans of phycocyanin (PC, maximum absorbance ~620 nm), phycoerythrocyanin (PEC, maximum absorbance ~540 nm) or phycoerythrin (PE, maximum absorbance ~566 nm) radiate away from the core (Bogorad 1975, Gantt 1981, Glazer 1982, Sidler 1994). Complementary chromatic adaption (CCA), a strategy to maximize light-harvesting efficiency in fluctuating light environments, has evolved within cyanobacteria that contain AP, PC and PE (Bennett and Bogorad 1973, Haury and Bogorad 1977, Vogelmann and Scheibe 1978, Kehoe and Gutu 2006). During CCA, cells sense the relative abundance of red light (RL) and green light (GL) and regulate transcription of the PBS-encoding genes to build light-harvesting antennae best suited to absorb the available wavelengths of light (Kehoe and Gutu 2006).

Not all species of cyanobacteria capable of CCA respond to changes in the light environment in the same manner: CCA-capable species have been classified into three groups based on how they structure the PBS under specific light environments. Group I species contain both PE and PC but do not alter their PC or PE composition in response to changes in the abundance of specific light wavelengths. Group II species

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initiate or increase the production of PE in GL but do not alter production of PC in response to changes in light conditions. Finally, group III species alter the PBS by inhibiting production of PE and increasing production of PC in RL while dramatically decreasing production of PC and simultaneously initiating production of PE in GL (Tandeau de Marsac 1977). The ability to alter the pigment composition in the PBS allows group II and group III species to fine tune this structure to suit the prevailing wavelengths of light and may allow these species to inhabit environments uninhabitable to species restricted to using RL for photosynthesis. It is also possible that CCA allows group II and group III species to coexist with non-adapting species that are restricted to absorbing RL via PC and/or chlorophyll by regulating PBS pigments to absorb available GL (Six et al. 2007, Stomp et al. 2004, 2007, 2008). Specifically, it was shown that when an adapting strain was co-cultured in white light with a RL-absorbing *Synechococcus* strain the adapting strain altered its PBS to absorb GL. Also, when the same strain was co-cultured in white light with a GL-absorbing *Synechococcus* strain, the adapting strain altered its PBS to absorb RL (Stomp et al. 2004, 2008). Thus the ability to chromatically adapt can allow an organism to inhabit a niche where competition, not physical attributes of the niche, limits wavelength availability.

Most studies on CCA have used *Fremyella diplosiphon*, a freshwater, filamentous group III cyanobacteria as a model for all CCA species. *F. diplosiphon* regulates production of PC and PE and undergoes morphological changes in cell shape and filament length during CCA (Bennett and Bogorad 1973, Haury and Bogorad 1977, Vogelmann and Scheibe 1978). The regulation of CCA in *F. diplosiphon* involves at least two photosensory systems, regulator for complementary chromatic adaptation (Rca) and control of green-light induction (Cgi) (Seib and Kehoe 2002, Alvey et al. 2003, 2007, Terauchi et al. 2004, Kehoe and Gutu 2006, Li et al. 2008). The Rca system is a complex phospho-relay consisting of RcaE, a phytochrome-class sensor kinase and two response regulators, RcaF and RcaC (Chiang et al. 1992, Kehoe and Grossman 1996, 1997, Li et al. 2008). The Rca system is required for transcriptional regulation of PBS-related genes in RL and GL, specifically the GL induction of *cpeCDESTR* (containing PE linker and regulatory proteins) and the RL induction of *cpcB2A2* (the red-light-inducible form of PC) and *pcyA* (required for phycocyanobilin synthesis) (Seib and Kehoe 2002, Alvey et al. 2003, 2007). RcaC acts by binding a DNA sequence, the L-box, which is found in the promoters of *cpcB2A2*, *pcyA* and (in reverse orientation) *cpeCDE*. When bound to the L-box, RcaC promotes transcription of *cpcB2A2* and *pcyA* and represses transcription of *cpeCDE* (Alvey et al. 2003, Li et al. 2008). Recent work by Bordowitz and Montgomery (2008) has shown that cell shape and filament-length differences between cultures grown in RL and GL are regulated by RcaE, though possibly through signaling intermediates other than RcaF and RcaC (Whitaker et al. 2009, Borodwitz et al. 2010).

The Cgi system works with the Rca system and regulates GL induction of PE synthesis genes *cpeBA* and *cpeCDESTR* but has not been characterized in detail (Kehoe and Gutu 2006). At this time, no components of the Cgi system have been reported in *F. diplosiphon* and it is unknown whether or not the Cgi system affects other morphological changes associated with CCA. An additional morphological change linked to CCA by Bennet and Bogorad (1973) was hormogonia production; this change was later shown to be regulated by addition to the oxidation state of the electron transport chain and not one of the known CCA regulatory systems (Campbell et al. 1993).

Recent genomic work has revealed potential homologs to RcaE in at least two species (Karniol et al. 2005, Li et al. 2008). The marine *Synechococcus* sp. PCC 7335 has a group III chromatic response, possesses homologs to RcaE, RcaF and RcaC and contains a regulatory L-box in the promoters of some chromatically regulated genes (Li et al. 2008). However, this *Synechococcus* L-box is not located at all the same sites as in *F. diplosiphon*. Therefore, species that have similar CCA responses at the PBS measurement level may have different regulatory mechanisms. CcaS and CcaR, a two-component sensor kinase and a response regulator, respectively, constitute a separate regulatory system recently shown to control the group II chromatic adaptation response of *Nostoc punctiforme* (Hirose et al. 2010). CcaS is a RL/GL-absorbing cyanobacteriochrome with a histidine-kinase domain. Unlike RcaE, which experiences RL-induced phosphorylation, CcaS becomes phosphorylated in GL and subsequently phosphorylates the response regulator, CcaR. The phosphorylated form of CcaR binds a promoter element called the G-box inducing GL transcription of the *cpeC-cpcG2-cpeR1* operon. CpeR1 then promotes a higher rate of transcription of the *cpeBA* operon. Interestingly, the CcaS/CcaR system has not been identified in group III species, and is therefore unlikely to be the Cgi system (Hirose et al. 2010). To determine how widespread the Rca and Cca regulatory pathways are, we began a study of additional chromatically adapting cyanobacteria that were originally identified in the work of Tandeau de Marsac (1977). We have focused on *Gloeotrichia* UTEX 583 because it was easily accessible and synonymous to a strain used in Tandeau de Marsac's study (*Calothrix* sp. PCC 7103). In this study, we characterize the chromatic adaptation response of *Gloeotrichia* UTEX 583, identify and characterize lesions in two green mutants that lack the ability to synthesize PE in any light condition and present evidence that indicates that *Gloeotrichia* 583 has a CCA response more similar to group II adapters than group III and also contains a potential homolog to CcaS but not RcaE.

Results

Pigment production in *Gloeotrichia* UTEX 583 and *Fremyella diplosiphon*

Both *F. diplosiphon* (also known as *Calothrix* sp. or *Tolypothrix* sp. PCC 7601) and *Gloeotrichia* UTEX 583 (here after called

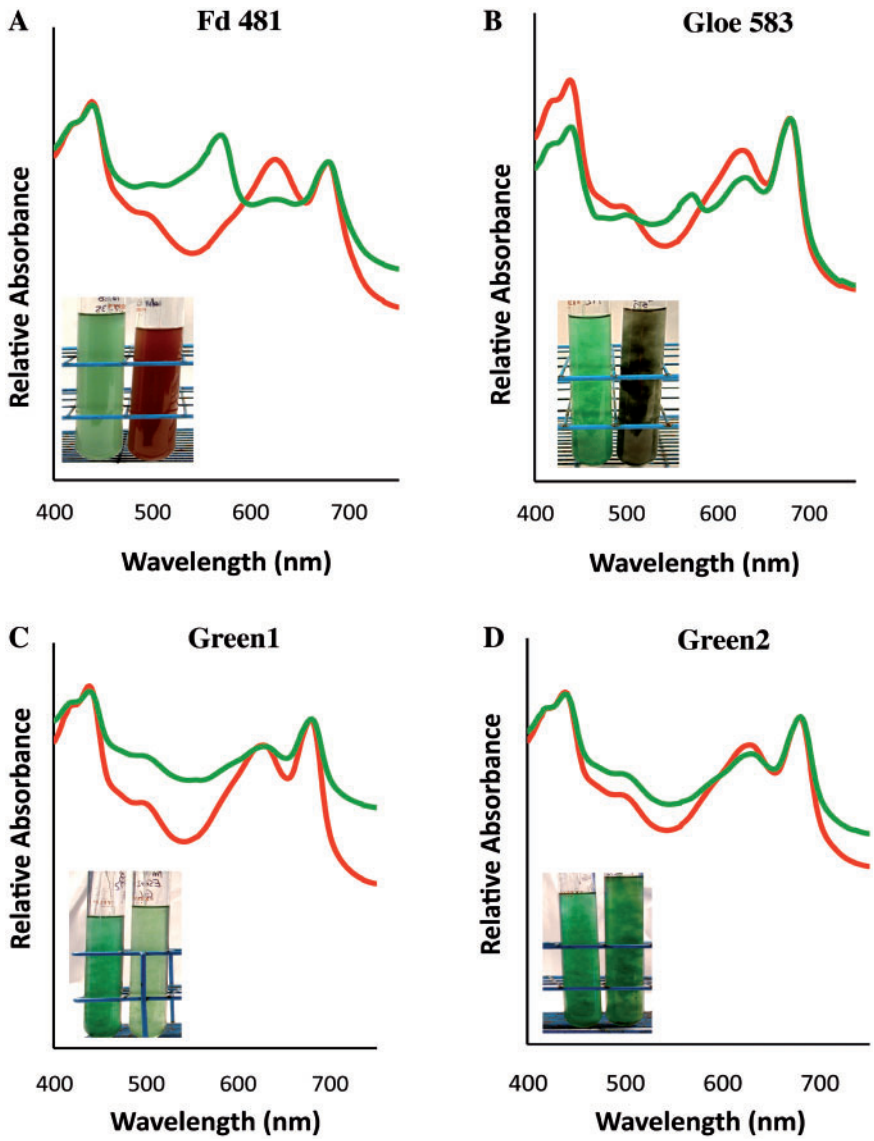

Fig. 1 Whole-cell absorption spectra of cyanobacteria grown in liquid BG-11 in 15 µmol m^{-2} s^{-1} of RL (red line) or GL (green line). Representative scans of (A) *F. diplosiphon* UTEX 481 strain SF33; (B) *Gloeotrichia* UTEX 583, wild type; (C) *Gloeotrichia* mutant GREEN1; (D) *Gloeotrichia* mutant GREEN2. The *x*-axis is the wavelength from 400 to 750 nm and the *y*-axis is the relative absorbance. PC peak is found at ~620 nm, PE peak at ~570 nm, chlorophyll peaks are at ~430 nm and 680 nm. Inset pictures represent fully adapted liquid cultures, RL-grown cells on left and GL-grown cells on right.

Gloeotrichia 583 and also known as *Calothrix* sp. PCC 7103) have previously been identified as group III chromatic adapters that produce roughly three times as much PC in RL as in GL and twice as much PE in GL as in RL (Tandeau de Marsac 1977). We purchased *Gloeotrichia* 583 from the University of Texas culture collection and using the cya106 and cya781a primers (Nubel et al. 1997) amplified the 16S rRNA gene to compare with the published 16S rRNA sequence of *Calothrix* PCC 7103. BLASTN analysis indicated that the 16S rRNA sequence from *Gloeotrichia* 583 is 99% identical, at the nucleotide level, to that deposited for *Calothrix* PCC 7103 (AM230700.1) (Altschul et al. 1997). Current conventions of bacterial taxonomy suggest naming two strains as different species when the 16S rRNA genes differ by >3% (Wayne et al. 1987). Under this system, *Gloeotrichia* 583 and *Calothrix* PCC 7103 are strains of the same species and thus *Gloeotrichia* 583 should be expected to chromatically adapt in a manner similar to that reported in 1977 for *Calothrix* PCC 7103.

We used *F. diplosiphon*, a model organism used to study CCA for >40 years, as a comparison point for CCA in *Gloeotrichia* 583. In *F. diplosiphon* the classic group III type of chromatic adaptation can easily be seen by eye as changes in culture color or by measuring the absorbance between 400 and 800 nm of cells in liquid culture (**Fig. 1A**). However, *Gloeotrichia* 583 did not exhibit the classic group III response described for the strain *Calothrix* PCC 7103 by Tandeau de Marsac (1977).

Table 1 Average phycobiliprotein content of cyanobacterial strains grown in RL and GL

	AP ($\mu g\,ml^{-1}$)	PC ($\mu g\,ml^{-1}$)	PE ($\mu g\,ml^{-1}$)	PC/AP	PE/AP	PC RL/PC GL	PE GL/PE RL
Fd 481 RL	89 (16)	218 (3)	97 (8)	2.45	1.09	3.3	5.77
Fd 481 GL	73 (13)	54 (7)	459 (36)	0.74	6.29		
Gloe 583 RL	50 (9)	118 (16)	50 (4)	2.36	1.0	1.32	3.51
Gloe 583 GL	39 (7)	70 (9)	137 (11)	1.79	3.51		
GREEN1 RL	54 (10)	126 (17)	46 (0.004)	2.33	0.85	0.65	1.08
GREEN1 GL	38 (7)	135 (18)	35 (3)	3.55	0.92		
GREEN2 RL	45 (8)	145 (20)	37 (3)	3.22	0.82	1.12	0.98
GREEN2 GL	30 (12)	86 (12)	25 (2)	2.87	0.83		

Fd 481 is SF33, the short filament strain of *F. diplosiphon*. Gloe 583 is *Gloeotrichia* sp. UTEX 583. Values in parentheses are standard deviation of the mean.

In the *Gloeotrichia* 583 culture, PC production remained much higher in GL compared with *F. diplosiphon* and much higher than predicted based on Tandeau de Marsac's original study. Based on whole-cell scans, PE was synthesized in GL and not synthesized in RL, but PC is highly abundant in both RL and GL (**Fig. 1B**). Thus *Gloeotrichia* 583 cultures have a bright blue–green appearance in RL as expected, but in GL they have a blackish-green appearance rather than the brick red color seen in *F. diplosiphon* (**Fig. 1A, b**).

Measurement of phycobiliprotein levels mirrored the data from the whole-cell scans (**Table 1**). *F. diplosiphon* exhibited a 3.3-fold induction of PC in RL and a 5.77-fold induction of PE in GL. In *Gloeotrichia* 583 we observed a 3.5-fold induction of PE in GL, but only a slight 1.32-fold difference in PC in RL compared with GL. This fold induction of PC in RL for *Gloeotrichia* 583 places it at the borderline used to define group II and group III chromatic adapters (Tandeua de Marsac 1977). In that study the author decided that PC levels in RL did not vary by >20% from the levels of PC in GL. In *Gloeotrichia* 583, there is >20% less PC in GL than in RL. Therefore, PBS levels alone cannot be used to classify *Gloeotrichia* 583 as a group II or a group III chromatic adapter.

One genomic difference between group III adapter *F. diplosiphon* and group II adapter *N. punctiforme* is the number of PC-encoding operons. *F. diplosiphon* has three PC operons. PC1 is constitutively expressed, PC2 is induced in RL and repressed in GL and PC3 is induced in sulfur-limiting conditions (Conley et al. 1988, Mazel et al. 1988, Kehoe and Gutu 2006, Alvey et al. 2007). In contrast, *N. punctiforme* contains a single operon containing the structural genes for PC. We used conserved primers to amplify the *cpcBA* intergenic region in *Gloeotrichia* 583 (Neilan et al. 1995). While the *cpcBA* intergenic region of *Calothrix* PCC 7103 has not been deposited in GenBank, the sequence for *Gloeotrichia* 583 was 96% and 98% identical to *Calothrix* sp. UAM 372 (EU009166) at the nucleotide and amino acid level, respectively. The sequence is also 78% identical at the nucleotide level to *cpcB1A1*, the operon encoding the constitutive form of PC, of *F. diplosiphon*. The *Gloeotrichia* 583 *cpcBA* intergenic region sequence has been deposited to GenBank as accession number HM852510. Southern analysis using this *cpcBA* intergenic region as a probe and moderate stringency indicated that *Gloeotrichia* 583 may only contain a single PC operon (data not shown) as opposed to the multiple, differentially regulated PC operons found in *F. diplosiphon* (Bryant 1981, Conley et al. 1988, Kehoe and Gutu 2006). Thus, while the slight reduction in PC production seen in GL may allow *Gloeotrichia* 583 to be classified as a group III adapter, it is possible that the genomic structure and/or the regulatory mechanisms for CCA in this species differ from those found in *F. diplosiphon* and may more closely resemble the group II response seen in *N. punctiforme* (Hirose et al. 2010).

Analysis of additional morphological traits associated with CCA

As addressed in Bennett and Bogorad's seminal work on CCA (Bennett and Bogorad 1973), several morphological traits characterize CCA, in addition to the canonical pigmentation changes. Cell shape, filament length changes and hormogonia, heterocyst, gas vesicle and necridia production are different between RL- and GL-grown *F. diplosiphon* cultures. While several of these traits are regulated by the redox potential of the photosynthetic pathway (Campbell et al. 1993), filament length and cell shape are regulated by RcaE, the photoreceptor that mediates a significant portion of the pigment changes in *F. diplosiphon* (Bordowitz and Montgomery 2008). Therefore, changes in cell shape and filament length in response to light color were investigated in *Gloeotrichia* 583. In *F. diplosiphon*, cells from RL-grown cultures tend to be round, while cells from GL-grown cultures tend to be rectangular (Bennett and Bogorad 1973). In *Gloeotrichia* 583, cells grown in GL tended to be slightly longer and thinner than cells grown in RL (**Fig. 2**). Additionally, the GL-grown cells appeared to contain inclusions of unknown function that were absent from the RL-grown cells. However, light color did not alter cell shape to the extreme seen in *F. diplosiphon*, and the slight differences observed were not statistically significant (data not shown).

In addition to difference in cell shape, *F. diplosiphon* also has marked differences in filament length between RL-grown cultures and GL-grown cultures. Bennett and Bogorad (1973) observed that the RL-grown cultures have shorter filaments

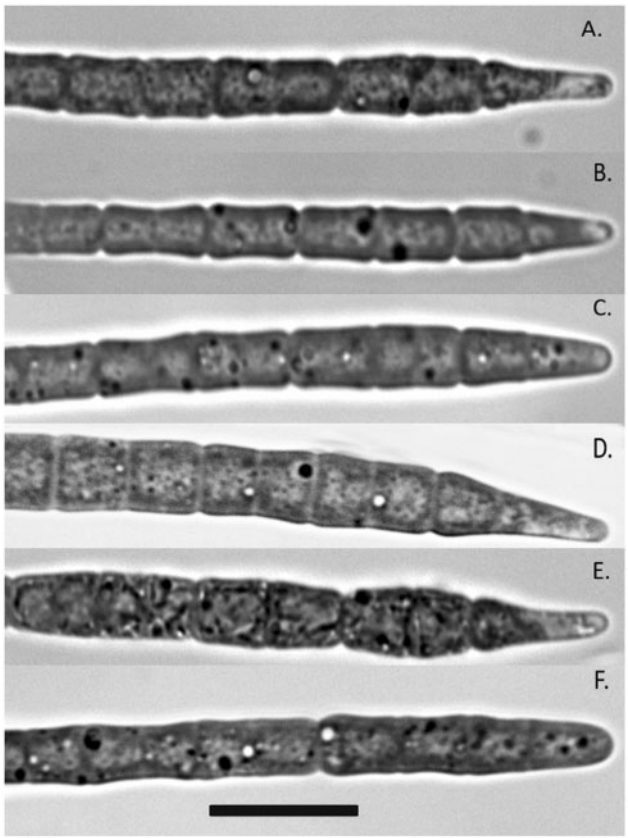

Fig. 2 Cell shape of *Gloeotrichia* 583 wild type and GREEN mutants is not affected by light quality. Cells were grown in liquid BG-11 in 15 µmol m^{-2} s^{-1} RL or GL. Images were taken at ×1000 magnification under oil. Scale bar 10 µm. (A) Wild-type GL; (B) wild-type RL; (C) GREEN1 GL; (D) GREEN1 RL; (E) GREEN2 GL; (F) GREEN2 RL.

due to an increase in necridia formation and cell lysis. In wild-type *Gloeotrichia* 583, filaments from RL-grown cultures were somewhat shorter than filaments from GL-grown cultures, but there was no statistical difference between mean filament length in RL-grown cultures and GL-grown cultures (two tailed *t*-test, $P > 0.5$). Additionally, both cultures had similar ranges of filament length (GL, 32.5–2497.8 µm; RL, 43.4–2128.6 µm). In general, the *Gloeotrichia* 583 filaments were very long, and 20–60% of the filaments, depending on the culture, were longer than the diameter of the field of view even at low magnification (×40 total magnification, data not shown).

Identification of a putative CCA regulatory gene in *Gloeotrichia* 583

Given the observed differences between the chromatic adaptation responses of *F. diplosiphon* and *Gloeotrichia* 583, we wanted to investigate the presence of known CCA regulatory genes in *Gloeotrichia* 583. Using the available genomic information for *N. punctiforme*, we amplified the *ccaS* gene by PCR and used that as a probe to detect a *ccaS* homolog in *Gloeotrichia* 583 by Southern blot analysis. In moderately low-stringency conditions we were not able to detect a homolog of *rcaE* using a *F. diplosiphon rcaE* probe (**Fig. 3A**). Using identical hybridization conditions we were able to detect a presumed homolog of *ccaS* (**Fig. 3B**). This is additional evidence that *Gloeotrichia* 583 has a CCA response more similar to the group II response of *N. punctiforme* than of the group III response of *F. diplosiphon*.

Isolation of green mutants of *Gloeotrichia* UTEX 583

Two *Gloeotrichia* 583 mutants that are unable to produce PE and are constitutively green were isolated as spontaneous pigmentation mutants growing on a plate of wild-type *Gloeotrichia* 583 cells. These strains were named GREEN1 and GREEN2. Whole-cell absorbance of these strains indicated that neither strain produced PE in GL because the absorbance profiles lacked identifiable PE peaks at 565 nm (**Fig. 1c, d**). Phycobiliprotein measurements indicated extremely low levels of PE in any light condition for both mutant strains (**Table 1**). Cell shape and filament length was not significantly different in the mutant strains compared with wild type (**Fig. 2** and data not shown).

Identification of the genetic lesion in GREEN1 and GREEN2

While it was possible that the mutant strains had a lesion in a regulatory gene, it was equally likely that they contained a mutation in *cpeBA* or *cpeCDE*. In *F. diplosiphon*, many spontaneous mutants are caused by novel insertions of a native transposable element (e.g. see Seib and Kehoe 2002, Whitaker et al. 2009). We conducted Southern hybridizations using *F. diplosiphon cpeBA* and *cpeCDE* as probes to identify whether GREEN1 and GREEN2 carried insertion mutations in these structural genes. There was no difference in restriction pattern between wild-type *Gloeotrichia* 583, GREEN1 and GREEN2 when *cpeCDE* was used as a probe (data not shown). However, when *cpeBA* was used as a probe a non-wild-type restriction pattern was seen in GREEN1 and GREEN2 (**Fig. 4**). The presence of a larger band in the mutants could indicate either a transposon insertion into the *cpeBA* operon or a base change that resulted in the loss of a restriction site. To determine the nature of the lesion in *cpeBA*, we used a series of degenerate primers (**Table 2**) to PCR amplify the *cpeBA* region in wild-type *Gloeotrichia* 583 (deposited to GenBank as Accession Number HM51857). When the same primers were used to amplify this region from GREEN1 and GREEN2 cells, the predominant product was ~1 kb larger than the wild-type product with an additional band that was slightly smaller than the wild-type band (**Fig. 5A**). Sequencing of the mutant PCR products indicated that the larger product in the mutant strains contained an insertion of 1085 bp located between nucleotides 347 and 348 of *cpeA* (**Fig. 5B**). A hypothetical translation of the mutant sequence indicated that the insertion would result in the loss of 43 C-terminal amino acids. The largest open reading

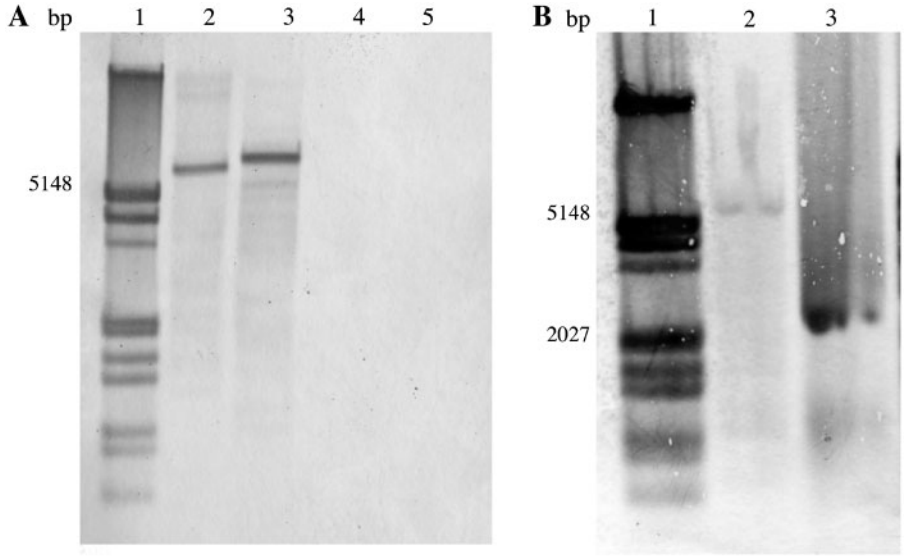

Fig. 3 *Gloeotrichia* 583 contains a homolog of *ccaS* but not *rcaE*. Southern blot analysis of RcaE (A) and CcaS (B) in *Gloeotrichia* 583 indicates the presence of *ccaS* but not r*caE*. (A) *F. diplosiphon rcaE* was used to probe digested *F. diplosiphon* genomic DNA [lanes 2 (*Xba*I) and 3 (*Nci*I)] and digested *Gloeotrichia* 583 DNA [lanes 3 (*Xba*I) and 4 (*Nci*I)]. Lane 1, Roche DIG-labeled DNA molecular weight marker III. (B) *N. punctiforme ccaS* was used to probe *Xmn*I-digested *Gleotrichia* 583 DNA (lane 2) and the purified *ccaS* PCR product from *N. punctiforme* (lane 3). Lane 1, Roche DIG-labeled DNA molecular weight marker III.

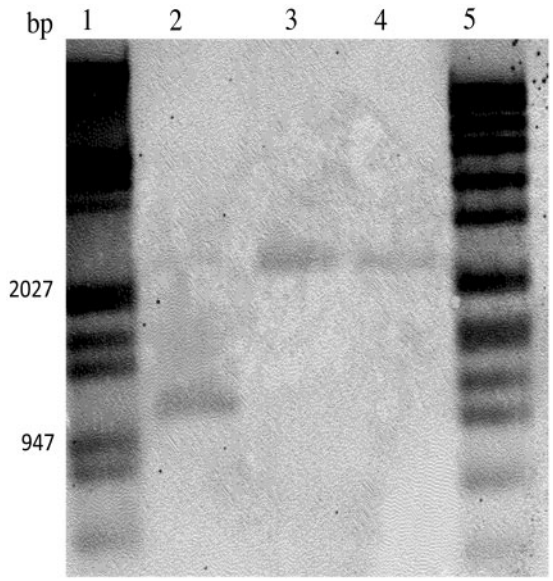

Fig. 4 *Gloeotrichia* GREEN mutants contain an insertion in *cpeBA*. Southern blot analysis of *cpeBA* in *Gloeotrichia* wild type and GREEN mutants indicates an insertion of approximately 1 kb in both GREEN1 and GREEN2. Lane 1, Roche DIG-labeled DNA molecular weight marker III; lane 2, *Hin*CII-digested wild-type *Gloeotrichia* 583 DNA; lane 3, *Hin*CII-digested GREEN1 *Gloeotrichia* 583 DNA; lane 4, *Hin*CII-digested GREEN2 *Gloeotrichia* 583 DNA; lane 5, Roche DIG-labeled DNA molecular weight marker VII.

frame (996 bp) within the insertion sequence was named G1 (GenBank accession number HM751858). This sequence was analyzed via BLASTX (Altschul et al. 1997), and was 52% identical to two hypothetical proteins in *Acaryochloris marina* MBIC 11017 (gene identifiers: AM1 2664 and AM1 6286) and 50% identical to a putative transposase from *Cyanothece* sp. PCC 8801 (gene identifier: PCC8801_4459). All three of these sequences contain COG3415, which is classified as a transposase, and inactivated transposase derivatives (Tatusov et al. 1997, 2003). The *A. marina* and *Cyanothece* sequences were identified by genome sequencing projects and do not appear to be inserted into genes. AM1 6286 is located next to an identified IS4 family transposase, but AM1 2664 and PCC8801_4459 are both surrounded by hypothetical proteins. The smaller fragment did not contain this insertion sequence but carried a deletion of 10 bases beginning at base 351 that prematurely truncated CpeA. Thus, even though a PCR product that appeared to be wild type in length was amplified from the mutant strains, these strains did not retain a fully functional copy of *cpeA*.

Discussion

CCA is a fairly widespread acclimation response of cyanobacteria to changes in wavelength availability within a niche. In her 1977 study Tandeau de Marsac reported chromatic adaptation in four orders of cyanobacteria representing 15 genera. CCA has best been studied in members of Nostocales (*F. diplosiphon* and *N. punctiforme*). However, even within this order different types of adaptation and regulatory mechanisms have been seen. Our study organism, *Gloeotrichia* 583, is also a member of Nostocales, but of a different family (Rivulariaceae) than either *N. punctiforme* (Nostocaceae) or *F. diplosiphon* (Microchaetaceae). We hope to better understand the

Table 2 Primers used for PCR amplification of *cpeBA* in this study

Primer	Sequence	Location	Purpose	Reference
SynB3R	TCRCGCAGGCAAGCAGCCAT	Within *cpeB*	Amplify *cpeBA* in cyanobacteria	Everroad and Wood 2006
cpeBFP	ATGCCACTTGACGCTTTTTC	Nt 1 of *cpeBA*	Amplify *cpeBA* in *Gloeotrichia* 583	This study
GlocpeBF40	GATGCTAGCACCGCTTGTATT	40 nt from ATG of *cpeB*	Amplify *cpeBA* in *Gloeotrichia* 583	This study
cpeAR7	AGGAGAGAGAGTTGATTGCGT	Nt 1066 of *cpeBA*	Amplify *cpeBA* in *Gloeotrichia* 583	This study
PE-PCR2	TGACCAATGACAAATGACCAATGA	150 bp downstream of *cpeA*	Amplify *cpeBA* in *F. diplosiphon*	Seib and Kehoe 2002
PE-PCR5	AGCCTGCTCCTTTCTCTAATGG	360 bp upstream of *cpeB*	Amplify *cpeBA* in *F. diplosiphon*	Seib and Kehoe 2002
Cya106F	CGG ACG GGT GAG TAA CGC GTG A	16S rRNA	To amplify a portion of the 16S rRNA gene	Nubel et al. 1997
Cya781Ra	GAC TAC TGG GGT ATC TAA TCC CAT T	16S rRNA	To amplify a portion of the 16S rRNA gene	Nubel et al. 1997
cpeBF	GGCTGCTTGTTTACGCGACA	*cpcBA* intergenic region	To amplify the intergenic region between *cpcB* and *cpcA*	Neilan et al. 1995
cpeAR	CCAGTACCACCAGCAACTAA	*cpcBA* intergenic region	To amplify the intergenic region between cpcB and cpcA	Neilan et al. 1995

Nt/nt, nucleotide(s).

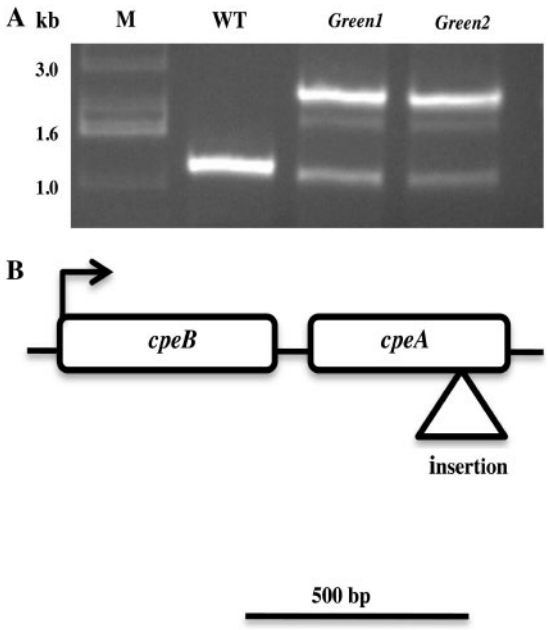

Fig. 5 Analyses of *cpeBA* operon structure in wild-type and GREEN mutant strains. (A) PCR amplification of the *cpeBA* genomic region in the wild-type (WT) and GREEN mutant strains. Numbers to the left represent sizes in kb. (B) Map position of the insertion in the *cpeA* gene. Arrow indicates the direction of transcription within the *cpeBA* operon.

regulatory mechanisms of CCA by expanding the number of species whose response has been studied in detail.

In this study we examined the CCA response of *Gloeotrichia* UTEX 583, a filamentous freshwater cyanobacterium. Unexpectedly, our analysis indicated that *Gloeotrichia* 583 did not display the chromatic response recorded for *Calothrix* PCC 7103 by Tandeau de Marsac (1977). Our analysis of phycobiliprotein levels showed an ~3-fold increase in PC in RL for *F. diplosiphon*, as expected, but only a slight 1.35-fold induction of PC in *Gloeotrichia* 583. In 1977 Tandeau de Marsac classified group II adapters as those that had a <1.2-fold increase in PC RL compared with GL. In the strictest sense then, *Gloeotrichia* 583 should be placed in group III. However, the response of *Gloeotrichia* 583 is strikingly different than the extreme regulation of both PC and PE seen in *F. diplosiphon*, the model organism used for analysis of group III chromatic adaptation (Bennett and Bogorad 1973, Kehoe and Gutu 2006). In *Gloeotrichia* 583 and *N. punctiforme* PE is produced only in GL (Hirose et al. 2010). While there is no decrease in PC production in GL in *N. punctiforme*, there is a slight reduction in *Gloeotrichia* 583. Thus, instead of alternating between red cells (grown in GL) and blue–green cells (grown in RL) as seen in *F. diplosiphon*, *Gloeotrichia* 583 and *N. punctiforme* alternate between blackish-green cells and blue–green cells (**Fig. 1B** and Hirose et al. 2010). The observed reduction in PC production may be sufficient criteria to place *Gloeotrichia* in group III, based on Tandeau de Marsac's classification system (1977). However, future investigations of PBS gene transcript accumulation and identification of regulatory components may show that *Gloeotrichia* 583 will be better placed in group II.

The chromatic response of *Gloeotrichia* 583 differed from that of *F. diplosiphon* in control of morphological traits as well. No significant differences in cell shape or filament length were seen between RL- and GL-grown cultures. *F. diplosiphon* cells are rod shaped in GL and more coccoid in RL; this difference in shape is regulated by RcaE (Bordowitz and Montgomery 2008). However, the requirement for active RcaF and RcaC differs

between RL- and GL-grown cultures. In RL, both RcaF and RcaC are required for the coccoid cell shape to develop, and in the absence of either protein, cells are constitutively rod shaped. However, the absence of RcaE results in a coccoid-shaped cell in GL, indicating a requirement for RcaE in GL (Bordowitz et al. 2010). This difference may indicate that RcaE acts through a different signaling system in GL to control cell shape. Additionally, we found no significant difference in mean filament length between cultures grown in RL and GL in *Gloeotrichia* 583, though in Bennett and Bogorad's original study, filaments from GL-grown cultures were nine times longer than filaments from RL-grown cultures (Bennett and Bogorad 1973). *Gloeotrichia* 583 filaments were much longer (RL median length 760 μm and GL median length 847 μm) than those recently reported for *F. diplosiphon* (RL median length 94.6 μm, GL median length 130 μm; Bordowitz and Montgomery 2008). While RcaE has been shown to be involved in filament length regulation in response to RL and GL, the involvement of RcaC and RcaF has not yet been determined (Bordowitz and Montgomery 2008).

Given the difference between the CCA responses of *F. diplosiphon* and *Gloeotrichia* 583, it is reasonable to hypothesize that there are differences in the CCA regulatory mechanisms in these species. An *rcaE* homolog was not identified in the *Gloeotrichia* genome via Southern analysis and low-stringency conditions (**Fig. 3A**). However, we have been able to detect a possible *ccaS* homolog in the *Gloeotrichia* genome using the same hybridization conditions and *N. punctiforme ccaS* as a probe (**Fig. 3B**). This result may indicate either that *Gloeotrichia* 583 uses an as-yet-unidentified mechanism to regulate CCA or that despite moderate regulation of PC production, uses CcaS in a manner similar but not identical to the CcaS-regulated CCA in *N. punctiforme*. We are working to identify and sequence *ccaS*, *ccaR* in the *Gloeotrichia* 583 genome and to determine whether *Gloeotrichia* contains homologs to other members of the RcaE signaling pathway (RcaC or RcaF) or CpeR, which has been identified as an important regulator of *cpeBA* gene expression in both *F. diplosiphon* and *N. punctiforme* (Seib and Kehoe 2001, Hirose et al. 2010).

In addition, we isolated two non-PE-producing mutants, GREEN1 and GREEN2. Though isolated from different colonies, both GREEN1 and GREEN2 contained the same insertion in *cpeA*. Interestingly, PCR amplification of *cpeA* from the mutants indicated that both mutants contained two alleles of the *cpeA* gene. One form contains the insertion, while the other contains a frameshift mutation that prematurely truncates CpeA. This observation indicated that *Gloeotrichia* 583, like many cyanobacteria, contained cells that are polyploid and that these cultures are not uni-genomic. It is possible that the frameshift allele was the result of removal of a transposon-like insertion from *cpeA*. No wild-type copies of *cpeA* have been identified in the mutant strains. Without the CpeA subunit, no functional PE can be produced, regardless of light conditions. The insertion sequence contained a hypothetical protein similar to proteins in COG 3415, which are classified as transposon and inactivated transposon derivatives. The G1 insertion sequence is not similar to previously identified cyanobacterial transposons such as IS4 found in *F. diplosiphon* (Whitaker et al. 2009), but the characteristic of the insertion sequence suggests that *Gloeotrichia* 583 may contain transposons that could be harnessed for mutational studies similar to those conducted in *F. diplosiphon*.

The recent explosion of genomic information in cyanobacteria has indicated that while the RcaE-based regulatory mechanism identified in *F. diplosiphon* may be found in other species, it is not the only regulatory mechanism used by chromatic adapters (Li et al. 2008, Hirose et al. 2010). Interestingly, *F. diplosiphon* contains two regulatory mechanisms—the RcaE-based system and the Cgi system (Kehoe and Gutu 2006, Li et al. 2008), but the CcaS/CcaR system from *N. punctiforme* has not been identified in this species. Hirose et al. (2010) analyzed the genome of *Synechococcus* PCC 7335, the only group III chromatic adapter with a published genome, and found homologs of RcaE/F/C but not homologs of CcaS or CcaR, leaving open the possibility that the unidentified Cgi system is not CcaS/CcaR in group III adapters. Even within the two RcaE-containing species, location of the L-box, which is bound by RcaC, is not identical (Li et al. 2008) and is found regulating different genes. In *F. diplosiphon*, RcaC regulates *cpcB2A2*, *cpeCDE* and *pcyA* operons, while in *Synechococcus* PCC 7335 the L-box is located in the promoters of *cpc2*, *pcyA* and *pebAB* but not in the promoter of *cpeC* (Li et al. 2008). This collection of observations demonstrates that CCA regulation is variable and that a full understanding of CCA requires that a variety of species and multiple examples of group II and group III adapters must be studied. With its intermediate CCA response and potential CcaS homolog, *Gloeotrichia* 583 may provide interesting insights into the regulation of CCA. Our laboratory is currently working to identify structural PBS genes and homologs of known regulatory genes in the *Gloeotrichia* 583 genome. This work may lead us to better understand the many ways in which cyanobacteria are able to acclimate to changes in their light environment.

Materials and Methods

Cell growth conditions

Gloeotrichia UTEX 583 (also called *Calothrix* sp. PCC 7103 and *Calothrix* sp. ATCC 27905) was used as wild-type strain. *F. diplosiphon* strain SF33 was used as a comparison with *Gloeotrichia* UTEX 583 (Cobley et al. 1993). The *Gloeotrichia* 583 GREEN mutants were isolated as spontaneous mutants during routine cell culture maintenance. All cultures were completely acclimated to light conditions by growing from low cell density in 15 μmol m^{-2} s^{-1} continuous RL (GE F20T12/R/24/CVG, λ_{max} 618–622 nm) or GL (GE F20T12/G/89/CVG, λ_{max} ~540 nm), for a minimum of 5 d at 27°C. All cells were grown in BG-11 plus 10 mM HEPES (pH 8.0) and bubbled with air. As noted in the text, *Gloeotrichia* UTEX 583 does

not have a CCA response identical to that described for *Calothrix* PCC 7103 (Tandeau de Marsac 1977). It should also be noted that the strain available from the American Type Culture Collection (*Calothrix* ATCC 27905), while having a 16S rRNA sequence identical to that of *Gloeotrichia* UTEX 583 and 99% identical to that of *Calothrix* PCC 7103 (data not shown), has several growth characteristics that differ from *Gloeotrichia* UTEX 583. *Calothrix* ATCC 27905 did not grow suspended in liquid culture, but formed mats along the sides of the culture tube. Additionally, when grown on solid media, the filaments of *Calothrix* ATCC 27905 grew in spirals and were difficult to remove from the medium surface. Since the cells of *Calothrix* ATCC 27905 did not grow suspended in liquid media, we were not able to complete whole-cell scans, but visual inspection of cultures indicate that the CCA response of *Calothrix* ATCC 27905 is more similar to that of *Gloeotrichia* UTEX 583 than *F. diplosiphon*. Since we have not analyzed *Calothrix* PCC 7103 CCA response, it is possible that the differences between the reported response (Tandeau de Marsac 1977) and the observed CCA response for *Gloeotrichia* 583 represent differences between strains. Phycobiliprotein composition was assessed via whole-cell scans of fully adapted cultures brought to equivilent concentration by A_{750} measurement on a Beckman DU640 spectrophotometer. Phycobiliprotein production was measured using the technique described by Tandeau de Marsac and Houmard (1988), though cells were disrupted using sonication of chilled samples. We found the cells of *Gloeotrichia* 583 to be much more resistant to lysozyme disruption than those of *F. diplosiphon*.

Cell and filament measurements

For cell and filament measurements, cultures were grown as above for a minimum of 7 d to be fully adapted. Cell density was standardized for all cultures (A_{750} ~1) and cells were bubbled for an additional 24 h. Both cell and filament measurements had a sample size of 100. Filaments were visualized at ×40 total magnification and photographed with a micrometer in view. Photographs were printed out and filaments were measured using a Scalex Plan Wheel SA2 (similar to the process described in Bennett and Bogorad 1973). Cells were visualized at ×1000 total magnification and were measured using the ocular micrometer. Differences between growth condition (RL/GL) and genetic composition (wild type/GREEN1/GREEN2) were tested using the two tailed Student's *t*-test.

DNA isolation and Southern blotting

Genomic DNA was isolated from *Gloeotrichia* 583 using the Fungal/Bacterial DNA kit from Zymo Research. Standard Southern blotting techniques were used to detect *cpeBA* in the *Gloeotrichia* 583 genome (Shui et al. 2009). The *cpeBA* gene from *F. diplosiphon* was PCR amplified (**Table 2**) and used as a probe to detect the *cpeBA* gene in *Gloeotrichia* UTEX 583 and mutant strains GREEN1 and GREEN2. Low-stringency hybridizations using *N. punctiforme ccaS* or *F. diplosiphon rcaE* as probes were conducted as described above but at 30°C. The DIG High Prime DNA Labeling and Detection Kit (Roche cat # 1 745 832) was used for non-isotopic detection of probes for Southern hybridization.

PCR amplification of *Gloeotrichia* 583 genes

All amplifications were carried out using the Failsafe PCR system from Epicentre and amplification conditions as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, X°C for 40 s, 65°C for 2 min and a final extension at 65°C for 4 min. The melting temperatures of the primer pairs (**Table 2**) used in any particular reaction determined the annealing temperature (X) of the reaction. PCR products that were to be sequenced were cleaned using the Zymo DNA Clean and Concentrator kit. All sequencing was done using an ABI Hitachi 3730XL DNA Analyzer and BigDye fluorescent terminator chemistry at the Genomics Core Facility (Pennsylvania State University, University Park, PA, USA).

The CpeBA sequences from several cyanobacteria were retrieved from NCBI and aligned using ClustalW. Degenerate PCR primers were generated from conserved regions in the protein sequences. Additional *cpeBA* primers generated by other studies were also used (Everroad and Wood 2006). From the sequence of an original 350 bp fragment of *cpeB* amplified using SynB3R (Everroad and Wood 2006) and cpeBFP, non-degenerate primers were made to amplify the remainder of the operon, i.e. cpeBGloF40. The entire *cpeBA* operon in both wild type and GREEN mutants was amplified using cpeBFP and cpeAR7 and the resulting PCR fragments were cloned using Invitrogen's TOPO TA cloning system and sequenced.

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