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Characterization of green mutants in *Fremyella diplosiphon* provides insight into the impact of phycoerythrin deficiency and linker function on complementary chromatic adaptation

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ABSTRACT

Functions of phycobiliprotein (PBP) linkers are less well studied than other PBP polypeptides that are structural components or required for the synthesis of the light-harvesting phycobilisome (PBS) complexes. Linkers serve both structural and functional roles in PBSs. Here, we report the isolation of a phycoerythrin (PE) rod-linker mutant and a novel PE-deficient mutant in *Fremyella diplosiphon*. We describe their phenotypic characterization, including light-dependent photosynthetic pigment accumulation and photoregulation of cellular morphology. PE-linker protein CpeE and a novel protein impact PE accumulation, and thus PBS function, primarily under green light conditions.

1. Introduction

Photosynthetic organisms depend upon light absorption for the production of chemical energy and reductant during photosynthesis, as well as to direct optimal growth and development in a process known as photomorphogenesis. Most photosynthetic organisms, including many cyanobacteria, exhibit limited mobility in their environments and thus fine-tuning their growth and developmental patterns to match ambient light conditions is critical for the maximization of photosynthetic efficiency, growth, and development. To accomplish this feat, these organisms possess sensory photoreceptors that serve to monitor the ambient photoenvironment and communicate variations in light availability and quality to the organism [1]. A classic example of a well-developed ability of cyanobacteria to respond and adapt to changes in the photoenvironment is the process of complementary chromatic adaptation (CCA). CCA is classically defined by the ability of a distinct group of cyanobacteria to alter their physiology and metabolism in response to changes in the prevalent wavelengths of ambient light [2]. This process has been most thoroughly studied in the freshwater, filamentous cyanobacterium *Fremyella diplosiphon*, which exhibits light-dependent changes in the accumulation of photosynthetic pigments and in cell and filament morphologies in response to changes in red light (RL) and green light (GL) (reviewed by Refs. [3,4]).

During CCA, the pigmentation changes that occur are due to changes in the protein content in the phycobilisomes (PBSs). PBSs contain chromophore-bound proteins, i.e., phycobiliproteins (PBPs), and linkers. PBSs in *F. diplosiphon* contain three PBPs, which include allophycocyanin (AP), phycocyanin (PC) and phycoerythrin (PE) (reviewed by Ref. [5]). Light-absorbing chromophores are covalently attached to PBPs via the enzymatic action of lyase proteins (reviewed by Ref. [6]). Chromophorylated PBPs (α and β subunits) assemble into disk-shaped trimers, two of which are stacked together by PBP-associated linkers to produce a hexamer that is incorporated either in the PBS core or to produce the outward-radiating rods of PBSs (reviewed by Ref. [7]).

PBS linkers serve structural roles in the synthesis of PBSs, but also serve important functional roles in energy transfer from the outward part of the PBS into the core where energy is ultimately transferred to the photosynthetic reaction center chlorophylls (Chl; reviewed by Ref. [7]). Despite the central roles these polypeptides serve in light sensing and the conversion of light energy to chemical energy, relatively little is known about their structures or specific functions. During CCA, in addition to the synthesis and accumulation of differential PBPs under distinct light conditions,

Abbreviations: AP, allophycocyanin; CCA, complementary chromatic adaptation; CLSM, confocal laser scanning microscopy; GL, green light; PBP, phycobiliprotein; PBS, phycobilisome; PC, phycocyanin; PCi, inducible PC; PE, phycoerythrin; RL, red light; WT, wild type.

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Table 1
Primer sequences, PCR conditions, product size and insertional mutant isolation for genes analysed in PE-deficient mutants.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Ann. Temp.[a] (°C)	WT product (bp)	Insert
cpeC	ATGCCATTTGGACCAGC	GAATTGTCTGGGATGCCAT	55	861	No
cpeD	ATGGCATCCCAGACAATTC	GGTTTCTATCCAAAGTGCCAT	55	750	No
cpeE	ATGGCACTTTGGATAGAAACC	TTTTAGCTATTTGCTTTGGGATAG	55	759	FdG101
cpeS	ATGGAAACCAAAGTG	CTAGGCACCAGTGTT	44	727	No
cpeT	ATGACCTCTTCACTA	TCATAGGATATTTGGG	44	625	No
cpeR	ATGAAATCCCAAATA	GATCGCCATTAGTGG	44	363	No
cpeB	GTTCTATATATTGTCTTGTTCAGGG	AAACTGTGTTGGATGTCAATCTG	48	555	No
cpeA	ATGAAATCAGTTGTT	CTAGGAGAGAGAGTT	48	495	No
cpeY	ATGGATAAGCGCTTTTTT	TTAGGCTGTGATTTCTTG	48	1290	No
cpeZ	ATGCCGACAACAGAAGAA	TTATTTTTCTCCCCGCTG	50	618	No
cpeF	TCTAGAACCTCAGCT	CCAACATCTTGGGAG	48	1089	No
pebA	TTAGAGTTGACCGTA	CTGATAGAGAGTCAA	40	1027	No
pebB	ATTCGGAGCGAAGCG	TTATTTGATAGCTGA	40	768	No
cotAB	CGCGGATCCTTGTGCCTGAGTCACTTCGC	CGCGGATCCTTTACCATTGATGTGGCACTGA	64	1955	No
rcaE	ATGAATATTGCTGCTTGTGAT	TCATTGGGATATTGGCGT	50	1968	No

[a] Annealing temperature.

the expression of genes encoding light color-specific linkers is also regulated [3,7]. Under RL conditions, PC linkers are upregulated in the *cpcB2A2*-containing inducible PC (PCi) operon, which also encodes the PCi apoproteins [8]. Under GL conditions, the expression of PE linkers is increased through regulation of the *cpeCDE*-containing PE operons [3,9]. Two operons containing PE linkers, i.e., *cpeCD* and *cpeCDE*, were initially identified in *F. diplosiphon*. The *cpeCD* operon was reported to accumulate to higher levels than *cpeCDE* under GL conditions [10,11]. Recently, a more extensive *cpeE*-containing operon, i.e., *cpeCDESTR*, has been reported [9]. The functions of the additional genes in the operon—i.e., *cpeS*, *cpeT*, and *cpeR*—are still under investigation, although *cpeR* is required for the expression of the PE apoprotein-encoding *cpeBA* operon [9,12].

Notably, the identification and transcriptional regulation of the PE-linker gene *cpeE* was reported first in the early 1990s [10], and *cpeE* from *F. diplosiphon* was one of the first to be reported and its expression characterized [10,13]. Related proteins have been reported in other systems, including marine species *Synechococcus* sp. Strain WH8102 [14]. Recent genomic analyses identified a large number of putative PBP linker genes in a number of cyanobacterial species [15]. Yet, there are still relatively few examples of *in vivo* functional characterization of these proteins. Much of what has been discovered about the functions of PBS-class genes in cyanobacteria has been learned from the isolation and characterization of pigmentation mutants. Here, we report the isolation of two new green (FdG) pigmentation mutants in *F. diplosiphon*, i.e., mutants that are constitutively green in color independent of the light conditions under which the strains are grown. One of these green mutants is a *cpeE* mutant and its phenotypic characterization provides novel insight into the function of a cyanobacterial linker. The second green mutant harbors a novel mutation impacting PE accumulation.

2. Materials and methods

2.1. Strains and growth conditions

F. diplosiphon strain SF33, a shortened-filament mutant strain that displays wild-type (WT) pigmentation responses in GL and RL [16], was used as WT parental strain. *F. diplosiphon* cultures were grown in BG-11 medium [17] buffered with 20 mM HEPES at pH 8.0 (hereafter BG-11/HEPES) at 28 °C as detailed [18]. Cultures in liquid media were grown with shaking at 175 rpm, whereas cultures grown on plates were maintained on BG-11/HEPES media solidified with 1.5% (w/v) agar as previously described [18,19]. GL and RL sources at ~10–20 μmol m^{-2} s^{-1} were previously described [18]. Irradiance was measured using a Li-Cor quantum Sensor (model LI-190SA, Li-Cor, Lincoln, NE) connected to a Li-Cor light meter (model LI-250).

2.2. Cell absorption spectrum measurements

Whole-cell spectral scans were obtained from 400 to 800 nm for cells grown in liquid culture that were adjusted to an absorbance at 800 nm (A_{800}) of ~0.1.

2.3. Mutagenesis

SF33 cells were heat-shocked at 45 °C to induce mutagenesis and to facilitate the isolation of pigment mutants as described previously [20].

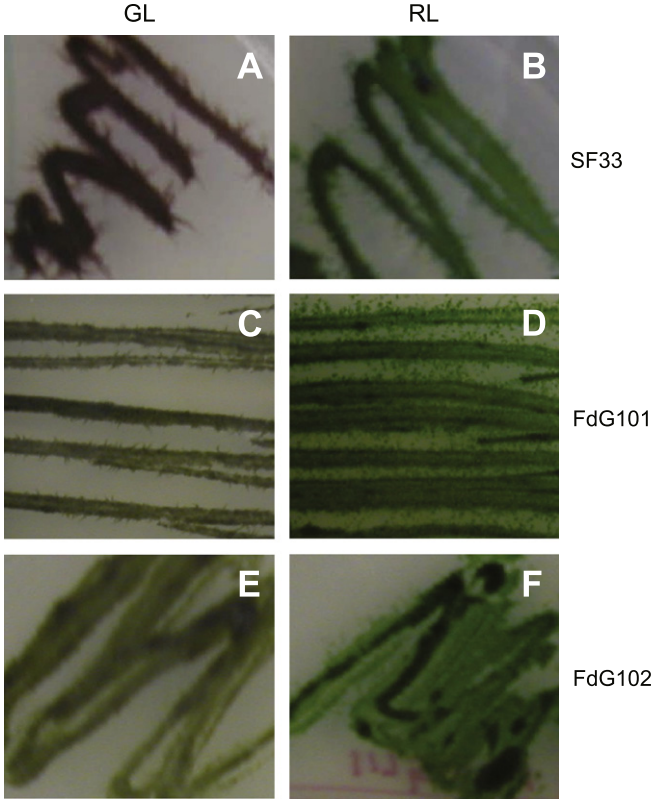

Fig. 1. Color phenotypes of SF33 wild-type and green pigmentation mutants (FdG) grown for ~13 days in either green light (GL) or red light (RL): (A, B) SF33, (C, D) FdG101, and (E, F) FdG102.

2.4. Isolation of F. diplosiphon genomic DNA

We isolated genomic DNA from *F. diplosiphon* cells using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research Corporation, Orange, CA) according to the manufacturer's instructions.

2.5. PCR amplification

PCR analyses of *F. diplosiphon* genes were conducted via standard PCR procedures using isolated genomic DNA as a template [21]. PCR reactions used gene-specific primers as indicated (Table 1).

2.6. Pigment extraction and quantification

Photosynthetic pigments, including Chl*a* and PBPs, were extracted from *F. diplosiphon* cells growing in liquid culture; pigment content was quantified as previously described [18].

2.7. Confocal laser scanning microscopy

We prepared slides of live *F. diplosiphon* cells and analysed them by confocal laser scanning microscope (CLSM) using both differential interference contrast (DIC) optics and fluorescence excitation/emission filters as previously detailed [18,22].

3. Results

We isolated two green mutants, FdG101 and FdG102, from heat-shock mutagenesis of SF33 cells. These mutants were green under GL illumination, conditions under which SF33 cells are brick red (Fig. 1A, C and E). The FdG mutants were indistinguishable in color from SF33 on agar plates under RL conditions (Fig. 1B, D and F). In whole-cell spectral scans of liquid cultures, FdG101 and FdG102 exhibited severely diminished PE peaks relative to SF33 cells when grown in GL (Fig. 2). Under RL conditions, both FdG strains exhibit a strong peak for PC (Fig. 2). FdG101 appears to have a higher PC peak relative to chlorophyll *a* (Chl*a*) than SF33, whereas FdG102 has a slightly lower peak relative to Chl*a* than SF33.

We extracted and quantified PBPs and Chl*a* in all of the strains. We quantified light-dependent differences in pigment levels and compared them with those observed in spectral scans (Table 2). We observed reduced levels of PE relative to Chl*a* or AP under both GL and RL conditions for both FdG101 and FdG102 compared to SF33, but PE/AP levels were most severely reduced under GL for both mutants. This observation of reduced PE levels is best represented by PE/PC ratios, which were 1.22 for FdG101 and 2.39 for FdG102, compared to 5.66 for SF33 under GL (Table 2), rather than values for PE relative to Chl*a*, as chlorophyll levels were reduced by up to 67% in the FdG mutants relative to SF33. PC accumulation was also impacted under RL for FdG102. This finding supports the aforementioned lower PC peak relative to Chl*a* observed for this line (Fig. 2C). The PC/AP level under RL for FdG102 was ~60% of levels measured for SF33 under identical growth conditions (Table 2).

As PE values under GL were the PBP levels most severely impacted for both green mutants as compared to SF33, we conducted PCR analyses of a number of PE-associated genes to determine the genetic mutation associated with the observed phenotypes. PCR analyses demonstrated that for amplification of the *cpeE* gene (759 bp), the FdG101 mutant contains a product that is ~1.4 kb larger than that for SF33 (Fig. 3A). DNA sequencing analyses showed that the FdG101 mutant contains a single *IS701* insertion in reverse orientation in the *cpeE* gene sequence at base pair 652 (Fig. 3B).

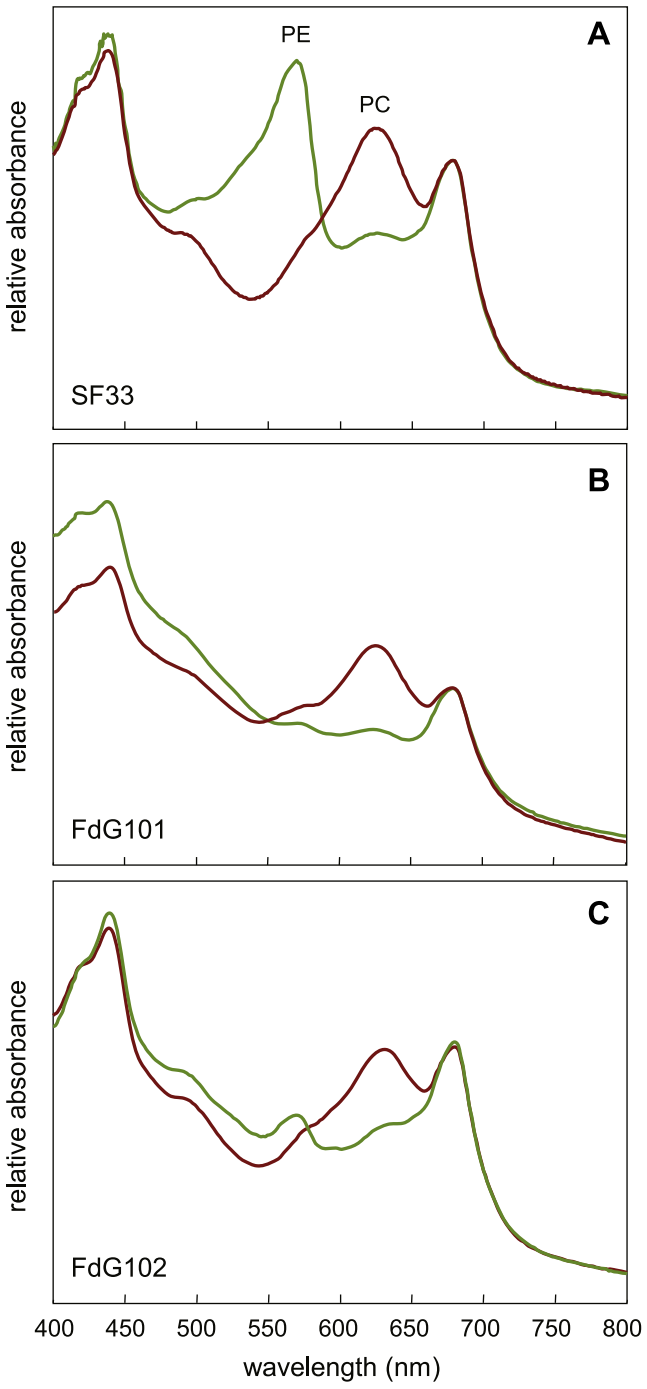

Fig. 2. Whole-cell absorbance spectral scans of SF33 wild-type and FdG mutant strains. Representative whole-cell spectral scans of (A) SF33, (B) FdG101, and (C) FdG102 mutant strains. Cells were grown in GL (green line) or RL (red line) conditions. Absorption maxima for phycoerythrin (PE, ~565 nm) and phycocyanin (PC, ~620 nm) are indicated. The left and right-most peaks (~430 and 680 nm, respectively) are chlorophyll absorption peaks. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

Notably, *IS701* is ~1.4 kb in length [23]. Thus, the FdG101 strain is referred to as Δ*cpeE*::*IS701* mutant, hereafter. The *cpeE* gene encodes a PE rod linker (PE $L_R^{27.6}$) in *F. diplosiphon* [10]. The FdG102 mutant does not have an insertion in the *cpeE* gene, or in any other known gene related to PE synthesis or regulation, including *cpeC* [11], *cpeD* [11], *cpeS* [9], *cpeT* [9], *cpeR* [12], *cpeB* [24], *cpeA* [24], *cpeY* [25], *cpeZ* [25], *cpeF* (Genbank Accession number AF170279), *pebA* [20], *pebB* [20], or *cotAB* [26] (Table 1). The FdG102 mutant thus likely represents a novel class of green

Table 2
Phycobiliprotein quantification and ratios.

Strain	Light	Phycobiliprotein levels (μg/μg Chl*a*) [a]			Phycobiliprotein ratios[a]		
		PE	PC	AP	PE/AP	PC/AP	PE/PC
SF33 WT	GL	4.31 (±2.24)	0.86 (±0.54)	1.12 (±0.75)	4.51 (±1.39)	0.79 (±0.12)	5.66 (±1.38)
	RL	1.13 (±0.93)	4.49 (±3.88)	2.55 (±1.79)	0.45 (±0.07)	2.27 (±1.17)	0.22 (±0.06)
Δ*cpeE*::*IS701* (FdG101)	GL	3.21 (±0.55)	2.65 (±0.46)	2.16 (±0.53)	1.51 (±0.14)	1.24 (±0.10)	1.22 (±0.01)
	RL	0.57 (±0.08)	4.29 (±0.36)	1.51 (±0.23)	0.38 (±0.01)	2.87 (±0.14)	0.13 (±0.01)
FdG102	GL	2.02 (±1.99)	0.78 (±0.74)	1.59 (±1.56)	1.37 (±0.20)	0.58 (±0.12)	2.39 (±0.29)
	RL	0.85 (±0.23)	2.65 (±0.74)	2.12 (±0.67)	0.42 (±0.10)	1.29 (±0.18)	0.33 (±0.10)
Δ*cpeE* vs. SF33[b]	GL	74.5	308.1[c]	192.9[c]	33.5	156.9	21.6
	RL	50.4	95.5	59.2	84.4	126.4	59
FdG102 vs. SF33[b]	GL	46.9	91.7	142.0	30.4	73.4	42.2
	RL	75.2	59.0	83.1	93.3	56.8	150.0

[a] Numbers indicate means (±standard deviations) for at least three independent experiments.
[b] % of value for Δ*cpeE* or FdG102 strain relative to value for SF33 strain.
[c] Numbers are relative to chlorophyll values, which are 33–67% lower in Δ*cpeE* than SF33.

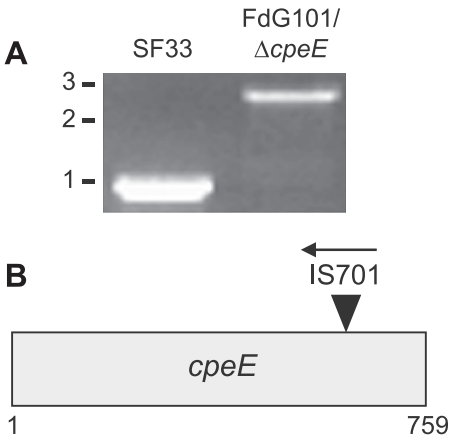

Fig. 3. Analyses of *cpeE* gene structure in SF33 wild-type and FdG101/Δ*cpeE*::IS701 strains. (A) PCR amplification of the *cpeE* gene in the SF33 and FdG101 (Δ*cpeE*::IS701 mutant) strains. Numbers to the left represent sizes in kilobases. (B) Map of the position of the IS701 insertion at base pair 652 in the *cpeE* gene (full length 759 bp).

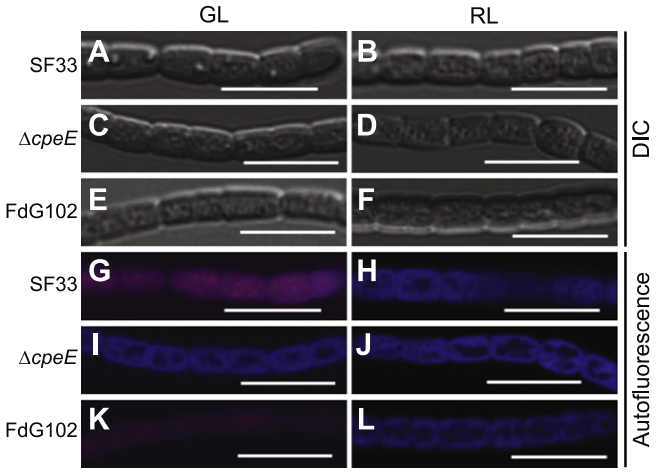

Fig. 4. Confocal microscopy analyses of cellular morphology in SF33 wild-type, Δ*cpeE*::IS701 and FdG102 mutant strains. Cultures were maintained in constant green (GL) or red (RL) light. Representative slices from a Z-series of differential interference contrast (DIC) images: (A, B) SF33, (C, D) Δ*cpeE*::IS701, and (E, F) FdG102. Maximum projection phycobiliprotein autofluorescence images: (G, H) SF33, (I, J) Δ*cpeE*::IS701, and (K, L) FdG102. All images captured using 40× oil objective with 2× zoom. Bars: 10 μm. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

mutant, though it is nearly identical in color phenotype to the Δ*cpeE*::*IS701* mutant (Fig. 1). As the FdG102 mutant also has somewhat reduced PC levels in RL, in addition to its severe reduction of PE in GL, we assessed whether CCA regulatory gene *rcaE*, which encodes a sensory protein that impacts light-dependent PE and PC accumulation [27,28], was disrupted in this line. Using PCR analyses, we found no apparent insert in the *rcaE* gene of this line (Table 1).

We conducted confocal laser scanning microscopy (CLSM) of all three strains to gain additional insight into the relative light-dependent pigmentation profiles and cellular morphology of these lines. Our CLSM profiles confirmed the differential phenotypes of Δ*cpeE*::*IS701* and FdG102 relative to SF33 (Fig. 4). In agreement with the assessment of spectral scans (Fig. 2) and quantification of pigments (Table 2), our cell biology analyses show that Δ*cpeE*::*IS701* has extremely limited PE accumulation under GL conditions (Fig. 4I), as indicated by the nearly complete lack of pink coloring in the autofluorescent CLSM images. By comparison, FdG102 shows severely depleted, but visible, PE fluorescence relative to SF33 (compare Fig. 4G and K). There were no apparent differences observed in PBP autofluorescence for the mutants relative to SF33 for cultures grown under RL (Fig. 4H, J and L).

The mutants display some degree of differences in cellular morphology relative to SF33 under either GL or RL (Fig. 4A–F). As minor differences were apparent, we quantified cell lengths of Δ*cpeE*::*IS701* and FdG102 relative to those of SF33 under both GL and RL (Table 3). Whereas SF33 cells grown under GL were longer than cognate cells grown under RL as previously determined [18,19,29], cells of the Δ*cpeE* mutant were only marginally significantly different in length ($p < 0.05$) under GL conditions compared to RL conditions. Notably, both GL- and RL-grown Δ*cpeE*::*IS701* cells were more similar in length to SF33 cells grown under GL (Table 3), i.e., cells of the Δ*cpeE*::*IS701* strain are significantly elongated under RL ($p < 0.001$). FdG102 cells were also longer under GL than RL ($p < 0.05$); however, the difference between FdG102 cells grown in GL vs. RL was less significant than those measured for SF33 under identical conditions, which were highly significantly

Table 3
Cell length measurements.

	Cell length (μm)[a]	
	GL	RL
SF33 WT	5.21 (±0.60)	3.95 (±0.53)
Δ*cpeE*::*IS701*	4.67 (±0.71)	5.01 (±0.57)
FdG102	5.21 (±0.74)	4.88 (±0.69)

[a] Numbers indicate medians (±standard errors) for 100 cells measured for each strain under each light condition.

different ($p < 0.001$). This difference was due to FdG102 cells being highly significantly longer under RL than RL-grown SF33 cells ($p < 0.001$), whereas no significant difference was observed in the lengths of FdG102 cells grown under GL relative to the lengths of GL-grown SF33 cells ($p \geqslant 0.05$).

4. Discussion

The Δ*cpeE*::*IS701* mutant, though very similar in its color phenotype to the FdG102 mutant when grown on agar plates, is distinct in the underlying genetic cause of its phenotype. The Δ*cpeE*::*IS701* mutant has subtle phenotypic differences relative to FdG102, which has lower PC levels in RL and slightly higher PE levels in GL than does the Δ*cpeE*::*IS701* mutant. These observations are observable in spectral scans (Fig. 2C), in quantification of PBP levels (Table 2), and in CLSM analysis (Fig. 4K).

Although FdG102 displays a severe defect in PE accumulation in GL and slightly reduced PC accumulation in RL, the mutant does not contain an insertional mutation in a regulatory gene encoding the photosensory receptor RcaE. Whereas FdG102 is most severely impacted by a lack of a GL-dependent response for the induction of PE, it is largely still very responsive to RL in the induction of PCi accumulation, though overall levels do not reach that of the SF33 parental strain (Fig. 2 and Table 2). Thus, we hypothesize that the FdG102 mutant possesses a novel mutation primarily impacting the GL-dependent induction of PE. We anticipate that the characterization of this novel mutation that impacts GL-dependent PE accumulation will yield novel insight into the regulation of PE and thus PBS regulation in *F. diplosiphon*.

Although the PBP components of PBS have been studied in great detail, comprehensive studies of linker peptides are much less common. Thus, this work represents one of only a few such analyses of linker peptide function *in vivo*. This study explores the role of CpeE, which impacts PE accumulation under GL and has an impact on overall photosynthetic pigment levels as evidenced by reduced chlorophyll accumulation in the mutant, particularly under GL. Our analyses of cellular morphology of these two green mutants determined that severe limitations of PBP and Chl*a* levels have a minor impact or no significant impact on cellular morphology under GL. Although significant differences were observed in the cell lengths of both the Δ*cpeE*::*IS701* and FdG102 mutants under RL conditions, i.e., cells are longer than RL-grown SF33 cells, these are not the growth conditions under which severe reductions in PBP content are observed. Thus, these results largely support prior observations that the regulation of cellular morphology occurs mostly independently of the photoregulation of pigment accumulation [18,19,30].

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