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CpeY is a phycoerythrobilin lyase for cysteine 82 of the phycoerythrin I α-subunit in marine *Synechococcus*

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ABSTRACT

Marine *Synechococcus* are widespread in part because they are efficient at harvesting available light using their complex antenna, or phycobilisome, composed of multiple phycobiliproteins and bilin chromophores. Over 40% of *Synechococcus* strains are predicted to perform a type of chromatic acclimation that alters the ratio of two chromophores, green-light–absorbing phycoerythrobilin and blue-light–absorbing phycourobilin, to optimize light capture by phycoerythrin in the phycobilisome. Lyases are enzymes which catalyze the addition of bilin chromophores to specific cysteine residues on phycobiliproteins and are involved in chromatic acclimation. CpeY, a candidate lyase in the model strain *Synechococcus* sp. RS9916, added phycoerythrobilin to cysteine 82 of only the α subunit of phycoerythrin I (CpeA) in the presence or absence of the chaperone-like protein CpeZ in a recombinant protein expression system. These studies demonstrated that recombinant CpeY attaches phycoerythrobilin to as much as 72% of CpeA, making it one of the most efficient phycoerythrin lyases characterized to date. Phycobilisomes from a *cpeY*$^{-}$ mutant showed a near native bilin composition in all light conditions except for a slight replacement of phycoerythrobilin by phycourobilin at CpeA cysteine 82. This demonstrates that CpeY is not involved in any chromatic acclimation-driven chromophore changes and suggests that the chromophore attached at cysteine 82 of CpeA in the *cpeY*$^{-}$ mutant is ligated by an alternative phycoerythrobilin lyase. Although loss of CpeY does not greatly inhibit native phycobilisome assembly in vivo, the highly active recombinant CpeY can be used to generate large amounts of fluorescent CpeA for biotechnological uses.

1. Introduction

Marine cyanobacteria in the genus *Synechococcus* are prolific oxygen-producing prokaryotes distributed broadly in the world's oceans. They are the second most abundant marine phototroph due in large part to their ability to utilize various wavelengths of light for photosynthesis [1,2]. The *Synechococcus* photosynthetic light harvesting antenna, known as the phycobilisome (PBS), is tuned to absorb specific light colors from portions of the visible spectrum in which chlorophyll absorbs poorly [3]. Some isolates of *Synechococcus* are blue light (BL) specialists with an abundance of the BL-absorbing chromophore phycourobilin (PUB) [absorbance maximum (λ_{max}) ~ 495 nm] while others are green light (GL) specialists with an abundance of the GL-absorbing chromophore phycoerythrobilin (PEB) (λ_{max} ~ 545 nm) [3,4]. Additionally, approximately 40% can adjust the bilin chromophore composition of their PBS through a process known as type IV chromatic acclimation (CA4). During CA4, the PUB to PEB ratio (PUB:PEB) is adjusted to be higher in BL and lower in GL in order to optimize light capture in changing light environments of the oceans [2,5–11]. For reviews of CA4, see [2,4,6,9,11,12].

The PBS is a large macromolecular structure in marine *Synechococcus*, measuring between 5 and 20 × 10^6 Da. Each PBS rests against the stromal side of the thylakoid membrane and typically consists of the core phycobiliproteins (PBP) allophycocyanins (AP) and

Abbreviations: BL, blue light; CA4, type IV chromatic acclimation; EICs, extracted ion chromatograms; HPLC, high performance liquid chromatography; HT-, hexahistidine-tagged; LC-MS-MS, liquid chromatography tandem mass spectrometry; MW, molecular weight; Ni-NTA, nickel-nitrilotriacetic acid; NT-, non-tagged; PBS, phycobilisome(s); PE, phycoerythrin; PEB, phycoerythrobilin; PBP, phycobiliprotein(s); PUB, phycourobilin; WT, wild type

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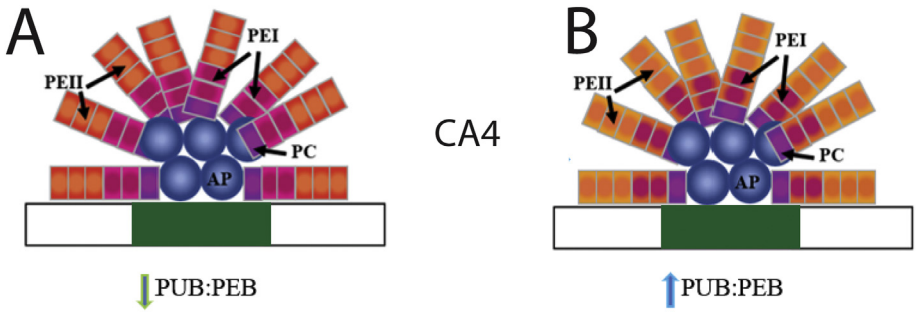

Fig. 1. Model of *Synechococcus* sp. RS9916 PBS depicting CA4 changes.
Model of a phycobilisome containing phycoerythrin I (PEI), phycoerythrin II (PEII), phycocyanin (PC), and an allophycocyanin (AP) core. Color schemes allude to phenotypic changes due to differential bilin chromophore composition during type IV chromatic acclimation (CA4) adapted from [5]. The ratio of phycourobilin (PUB) to phycoerythrobilin (PEB), or PUB:PEB, is adjusted to be lower in green light (A) and higher in blue light (B) in order to optimize light capture. Photosystem reaction centers (green bars) are embedded within the thylakoid membrane (white rectangles).

rod structures that extend from the core and are generally composed of the PBP phycocyanin (PC) and one or more types of phycoerythrin (PE; PEI and PEII, respectively; Fig. 1) [13]. All PBP consist of two similar polypeptide chains (α and β), with each α and β subunit structure being composed of six alpha-helices arranged to form a globular core with two additional extended helices to assist in heterodimer formation (Fig. S1A–B) [14–16]. These αβ heterodimers are arranged in hetero-hexamers $(\alpha\beta)_6$, and with the help of linker proteins, the macro-molecular PBS complex is assembled [17,18]. Each phycobiliprotein subunit always contains one or more bilins that are attached to cysteine (C) residues by the activity of enzymes known as bilin lyases [19]. Once ligated appropriately, each bilin assumes a stretched conformation that is facilitated by interactions with the PBP, allowing energy transfer to occur with nearly ~100% efficiency through the PBS and into the photosynthetic reaction centers within the thylakoid membrane (Fig. 1) [20–22].

Based on sequence similarities, three major groups or clans of bilin lyases have been characterized: CpcS/U type, CpcT type, and CpcE/F type [19,23–27]. Each clan differs from one another in primary amino acid sequence and structure as well as bilin chromophore and attachment site specificity. Solved crystal structures for known members of the distantly related CpcS/U (Protein Gene Bank 3BDR [28–30]) and CpcT [31,32] lyase families show that they adopt a similar antiparallel beta barrel structure. CpcS type lyases are hypothesized to have evolved first because they recognize the central C82-equivalent position in α and β subunits of AP, β-PC, and β-PE [23,24,28,30–34]. CpcS/U type lyases either form homodimers (TeCpcS [28]) or heterodimers (CpcS/CpcU [23]), and members show variability in the type of bilin attached to a C82 equivalent position [23,30,34,35].

CpcE/F type lyases, discussed here, are unrelated in sequence or structure to the other two lyase clans. These contain five to six HEAT-repeat motifs, thought to facilitate protein-protein interactions, coupled with Armadillo repeats, pairs of α-helices forming a globin-like domain with two extended helices (X and Y), which is structural consistent with the enzymic activity demonstrated by members of this clan (Fig. S1C–D) [30,34]. CpcE/F type lyases have a high specificity for a single bilin attached to a single site on a particular phycobiliprotein and are primarily active on α subunits. The CpcE/F clan includes proteins with chaperone-like functions as well as all characterized lyase isomerases, with some members having the capability to remove bilins [11,19,26,35,36]. To date, the crystal structure of only one CpcE/F type lyase has been solved and was found to adopt an alpha helical solenoid shape [37].

Our goal is to characterize the lyases involved in PE biogenesis, especially those involved in the CA4-mediated changes that occur in BL vs GL. In the marine *Synechococcus* sp. RS9916 (hereafter, RS9916), the CpeE/F-type lyase MpeZ was the first lyase-isomerase shown to be part of the CA4 process; it is responsible for attachment of PEB and isomerization to PUB at C83 on the α-subunit of PEII (MpeA) in BL [11]. Shukla et al. hypothesized that another CpcE/F type lyase attached PEB to MpeA-C83 in GL, prompting us to characterize of the role of CpeY in the biosynthesis of PEI and PEII [11]. Sanfilippo et al. recently showed that the lyase MpeY is responsible for ligation of PEB to MpeA-C83 in GL [10]. However, the lyases responsible for the bilin changes that occur at MpeA-C140 and at C139 on the α-subunit of PEI (CpeA) have not yet been identified. Here we show that the *cpeY* gene in RS9916 is orthologous to the *cpeY* lyase gene in the freshwater cyanobacterium *Fremyella diplosiphon* which encodes the lyase FdCpeY that attaches PEB to CpeA-C82 in *F. diplosiphon* [35,38]. Interestingly, FdCpeZ was shown to increase PEB addition activity by FdCpeY up to 40% and act as a chaperone-like protein for CpeB, the β-subunit of PEI [35,38]. The moderate degree of sequence similarity (53%) between the CpeY proteins and a similar genomic context in these two species suggests that this lyase may also play a role in PE biogenesis in RS9916 (Fig. S1) [39]. Although the RS9916 CpeY was unable to attach PEB to MpeA in our recombinant system, it does attach PEB to CpeA in a similar manner to its *F. diplosiphon* homolog but without the requirement of the chaperone-like protein CpeZ. Furthermore, the interruption of *cpeY* in RS9916 only affected the chromophorylation pattern of CpeA-C82 demonstrating that CpeY is not involved in any of the major CA4 changes that occur. The characterization of PBS from *cpeY*$^-$ cells provides insight into compensatory enzyme activity for complete biogenesis of the PBS when mutations affecting critical bilin addition sites occur. A small amount of PUB attached to CpeA-C82 (a critical chromophorylation site) was detected in our *cpeY* mutant analysis, suggesting that addition of PUB is preferential to an inability to add an insufficient amount of the correct bilin PEB to this site. Thus, we demonstrate CpeY functions as a recombinant PEI lyase attaching PEB to CpeA-C82 in RS9916 and is one of the most efficient recombinant phycoerythrin lyases, which may have useful biotechnology applications.

2. Materials and methods

2.1. Construction of plasmids

Primers used to amplify genes by PCR for the construction of expression vectors are listed in Table S1. Amplified fragments were separately cloned into compatible Novagen Duet vectors using corresponding restriction enzymes as listed in Tables S1 and S2. Expression vectors used in this study (Table S2), including two previously described [11,40], were transformed into *E. coli* BL21(DE3) containing bilin synthesis genes (*pebS/hoI*) with all genes under the control of T7 promotors as listed in Table S3. The *mpeA* and *cpeA* sequences were inserted into pCOLA-Duet and pET-Duet (Novagen, Madison, WI), respectively, in frame with the sequence encoding a hexahistidine tag at the amino terminus (Table S1). Clones were sequenced to verify they were free from mutations in the cloning junctions and PCR amplified regions.

2.2. Pull down assays

Pull-down assays were performed using the protocol described previously [23] with a few alterations. Proteins of interest were mixed in an approximately 1:1 ratio (determined by SDS-PAGE) in a small

sample volume (≤1 mL), and allowed to sit on ice for a total of 75 min, with gentle mixing every 25 min. Pull-down mixtures were purified as previously described [38]. All samples were eluted with 250–500 μL elution buffer (20 mM Tris HCL pH 8.0, 100 mM Na/KCL, 200 mM imidazole) and precipitated using 25% TCA.

2.3. Analysis of recombinant protein and bound bilin

In vivo heterologous protein expression was performed using *E. coli* as previously described [33] with the following modifications: a 100 mL starter culture of recombinant protein containing *E. coli* cells were grown at 37 °C overnight then added to a liter of Luria Bertani growth media at 18 °C. Once an OD = 0.6 was achieved, cultures were induced and maintained at 18 °C for 24 h with shaking. Cells were collected by centrifugation at 11,000 ×*g* for 8 min. The wet weight of all cell pellets (ranging from 4.15–4.48 g) was measured and recorded prior to storage at −20 °C. Cell pellets were resuspended at 3.0 mL·g^{-1} complete with mini protease cocktail (Thermo Scientific, Waltham, MA) and 0.01 mg·mL^{-1} lysozyme (Fisher Scientific, Hampton, NH). Table S3 summarizes the combinations of lyases tested. Proteins were purified using Ni-affinity column chromatography (Qiagen, Hilden, Germany) as described previously [33] dialyzed against 20 mM Tris HCL 100 mM Na/KCL (pH 8.0) and 1 mM β-mercaptoethanol at 4 °C. Samples were concentrated by ultrafiltration through an Amicon Ultra centrifugal filter unit (10 kDa cutoff; Novagen/EMD Millipore Corp., Darmstadt, Germany). Total CpeA protein content was quantified using Bradford colorimetric assay (BioRad, Hercules, CA). Prior to spectral scans, samples were diluted to obtain equal concentrations of CpeA across all co-expressions for direct comparison. Absorbance spectroscopy was performed using Perkin Elmer Lambda 35 UV/VIS spectrophotometer followed by fluorescence spectroscopy using a Perkin Elmer LS 55 Fluorescence Spectrometer (Waltham, MA) with excitation at 490 nm (PEB) or 440 nm (PUB) (slit widths were set at 10 nm). Proteins were subsequently resolved by 15% (w/v) polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and ultimately visualized by Coomassie blue staining [23]. To visualize proteins with bound bilin, gels were subjected to zinc-enhanced fluorescence using ChemiDoc MP imaging system (Bio-Rad, Hercules, CA) with excitation at 495 nm (PUB) and 550 nm (PEB).

Using modified Beer-Lambert Law (A = εcl; where l = 1 cm), holo-HT-CpeA concentration was determined using the relative absorbance intensity at 280 nm and the molar extinction coefficient of HTCpeA at 280 nm (ε_{HTCpeA} = 22,140 M^{-1} cm^{-1}). Bio-Rad's ImageLab software was used to calculate relative concentrations of CpeA. To estimate the amount of bound PEB in each sample, purified recombinant CpeA was denatured by dilution 1:9 in 9 M urea, pH 2.0, and the relative absorbance at 550 nm was measured (total bilin content). Absorbance intensity was collected in triplicate as technical replicates and averaged, with standard deviation calculated for each data set (results shown represent two independent biological replicates). The estimated concentration of bound bilin was calculated using the ε_{550} of PEB-peptide (ε = 53.4 mmol^{-1} cm^{-1}) [41]. The concentration of the bilin was divided by the concentration of CpeA and multiplied by 100 to determine the percentage of chromophorylation (Table 1). Independent samples *t*-tests were used to compare means of each sample containing CpeY (equal variances were assumed for co-expressions containing CpeY).

2.4. Native and mutant strains, growth conditions, and collection of PBS

Gene sequences from *Fremyella diplosiphon*, and *Synechococcus* sp. RS9916 were retrieved from GenBank. Amino acid sequences were analyzed using the Clustal W alignment tool from MacVector software V. 12.7.5 (MacVector Inc., Apex, NC) and Phyre^2 prediction system [50]. *Synechococcus* RS9916 cells were obtained from the Roscoff Culture collection as previously described [11]. The *cpeY* interruption mutant was created as previously described [39] using primers listed in Table S1. Clones generated were checked via PCR amplification, sequencing, and subsequently used to interrupt RS9916 *cpeY* via homologous recombination as previously described [11,42]. Mutant strain was verified as depicted in Fig. S2 and described in [39]. Cultures of RS9916 were grown at 22 °C in PCR-S11 media and acclimated for at least 7 days in either BL or GL as previously described [9,10]. PBS were collected and purified as previously described [43].

2.5. HPLC separation of PBS

PBS were purified using methods previously described [4,9,11]. Samples were dialyzed overnight against 5 mM Na phosphate buffer (pH 7.0) and subsequently purified via HPLC using methods outlined in [9,11]. Phycobiliproteins were monitored from 210 nm to 700 nm with specific channels monitoring for total protein (280 nm), PUB (490 nm), and PEB (550 nm). Eluted samples were vacuum-dried and kept at −20 °C prior to digestion for mass spectrometric analysis as previously described [9,11,35].

2.6. Calculating PUB:PEB for native WT and cpeY$^-$ phycobiliproteins

UV/VIS measurements were collected for each HPLC-purified protein using specific channels monitoring for total protein (280 nm), PUB (490 nm), and PEB (550 nm). These spectral readings were used to calculate the ratio of total protein concentration by obtaining peak maxima at 280 nm and extinction coefficients for peptide linked PUB (ε = 94 mmol^{-1} cm^{-1}) and PEB (ε = 53.4 mmol^{-1} cm^{-1}) [41]. CpeA from GL PBS does not contain PUB; thus, data was normalized to account for total protein content at 280 nm. All other data was normalized to PUB content unless otherwise specified.

2.7. Trypsin digestion and liquid chromatography tandem mass spectrometry

Purified proteins were dialyzed against 2 mM sodium phosphate buffer (pH 7.0) and 1 mM β-mercaptoethanol. Only one aliquot of trypsin (dimethylated trypsin from porcine pancreas; Sigma, St. Louis, MO) was added to 2% (w/w) from a 20 μg mL^{-1} stock to the denatured protein mixtures and incubated at 30 °C for 3 h in the dark [11]. The reaction was quenched by adding 30% (v/v) glacial acetic acid. Digested peptides were passed through a pre-equilibrated C8 Sep-Pak cartridge (Waters Corporation, Milford, MA) thereafter the eluted sample was vacuum dried and stored at −20 °C before LC-MS-MS analysis using nano-LC-MS^2 on a Thermo Orbitrap Fusion Lumos instrument and a C_{18} column (75 μm inner diameter). HPLC-UV–VIS-MS-MS data were recorded using an Agilent 1200 Capillary HPLC system with a C_{18} column (0.3 mm × 100 mm; 3 μm Agilent Zorbax-SB300 particles) and the HESI-II source on the Lumos.

3. Results

3.1. Analysis of recombinant proteins

RS9916 genes *cpeA*, *mpeA*, *cpeZ*, *mpeZ*, and *cpeY* (Tables S1 and S2) were expressed using compatible vectors and an *E. coli* heterologous protein expression system in order to test for lyase activity. Purified histidine-tagged (HT) recombinant substrate proteins were analyzed via fluorescence and absorbance spectroscopy (Fig. 2A and E) followed by SDS-PAGE (Fig. 2B–D and F–G). MpeA expressed in the presence of MpeZ, a known lyase isomerase responsible for PUB ligation to C83 on MpeA, was used as a positive control for MpeA (Fig. 2A–D) [11]. MpeA co-expression with CpeZ alone (not shown) mirrored the negative (no-lyase) control. CpeY was unable to ligate PEB to MpeA in this *E. coli* system (Fig. 2A–D).

Evidence of PEB ligation to CpeA was detected for all co-expressions

Table 1
Percentage chromophorylation of CpeA in the presence of CpeY ± CpeZ.

Plasmids[a] used for co-expression	Absorbance λ_{max} (nm) Holoproteins	Concentration of bound PEB (μM)[c]	Concentration of CpeA (μM)[c]	Estimated chromophorylation (%)[e]
pHTCpeA	NA[b]	0.49	4.71	10.3
pHTCpeA + pCpeZ	NA[b]	0.06	3.39	1.7
pHTCpeA + pCpeY	562.3	4.21	5.83	72.3[d]
pHTCpeA + pCpeY + pCpeZ	562.9	3.67	4.90	75.0[d]

[a] Hexa-histidine tagged CpeA (HTCpeA) was expressed in the presence of bilin synthesis genes generating PEB (PebS/Ho1) in addition to non-tagged putative lyases as indicated (See also Table S3).
[b] Not applicable as there was no measurable absorbance at 550 nm.
[c] Concentrations calculated as described in experimental procedures.
[d] Percent difference (−3.72%) and independent sample *t*-test ($t_{10} = -1.87$, $p = 0.05$) were performed comparing CpeA + CpeY and CpeA + CpeY + CpeZ. See also experimental procedures.
[e] The percent chromophorylation was estimated and described in experimental procedures; These results are representative of two independent replicates.

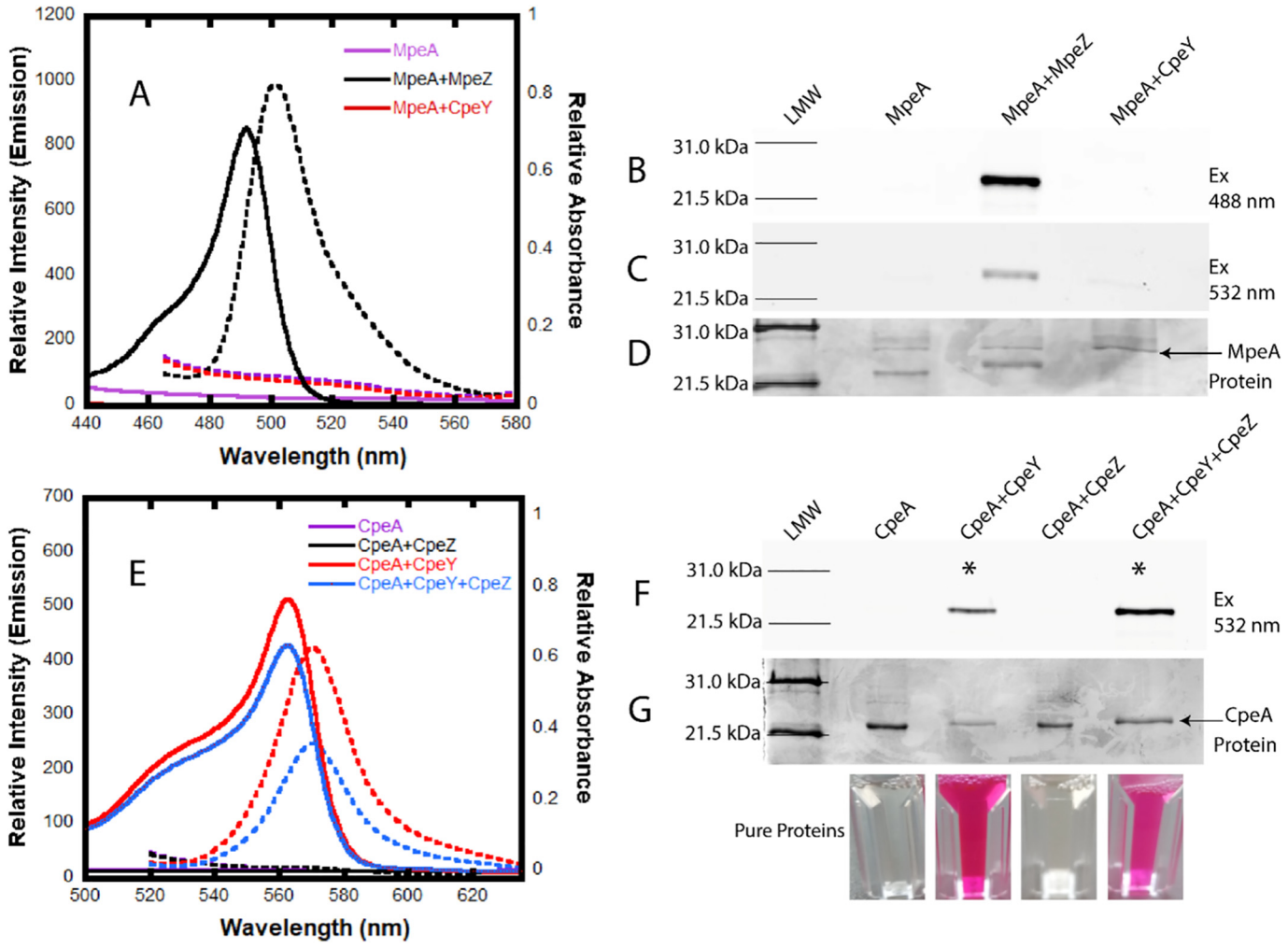

Fig. 2. CpeY from *Synechococcus* sp. RS9916 attaches PEB to CpeA, but not MpeA.
(A) Relative absorbance (solid lines) and fluorescence (dashed lines) representing pure recombinant MpeA expressed without a lyase (purple), with MpeZ (black), CpeY (red), and bilin synthesis genes (present in all samples). Zinc-enhanced fluorescence at 488 nm (excites PUB) (B) and 532 nm (excites PEB) (C) of SDS polyacrylamide gel containing pure protein samples. Coomassie blue stained gel of (B–C) proteins (D) showing LMW (21.5 kDa and 31.0 kDa) and MpeA (~24 kDa). (E) Absorbance (solid lines) and fluorescence (dashed lines) representing pure recombinant CpeA expressed without a lyase (purple, overlaps with black), with CpeY (red), CpeZ (black, overlaps with purple), both CpeY and CpeZ (blue), and bilin synthesis genes (present in a samples). (F) Zinc-enhanced fluorescence at 532 nm of SDS polyacrylamide gel containing pure protein samples. Coomassie blue stained gel of (F) proteins (G) showing LMW and CpeA (~22 kDa) [for abbreviations see Table S3]. *discrepancy in band intensity between lanes in (F) reflects loading amount differences. The 21.3% difference in intensity between bands for CpeA + CpeY and CpeA + CpeY + CpeZ and is not indicative of an increase in bilin addition; compare the amounts of protein loaded in G (See Table 1). These results are representative of three independent replicates.

containing CpeY when analyzed via spectroscopy with absorbance at 559.8 nm and emission at 571.5 nm (Fig. 2E). SDS-PAGE gels showed bilin fluorescence after exciting at 532 nm. We calculate that 72.3% of the purified recombinant CpeA had PEB ligated by CpeY (Fig. 2F–G and Table 1). Purified CpeA expressed with both CpeY and CpeZ showed no enhancement of bilin attachment in spectra compared to CpeY alone (Fig. 2E and Table 1). About 10.3% of purified CpeA had PEB bound in the absence of lyases (non-enzymic addition) and only 1.7% in the presence of CpeZ (Table 1), suggesting CpeZ may inhibit spontaneous chromophorylation. CpeZ did not significantly improve attachment of PEB to CpeA by CpeY (75% chromophorylation; a 3.7% increase) (Fig. 2E–G and Table 1). There was no evidence that HT-CpeZ binds PEB directly (data not shown). CpeA coexpressed in the presence of MpeZ showed a very small amount of bound bilin in the absorbance spectra (489.20 nm) and Zn-enhanced bilin fluorescence of an SDS-PAGE gel (excited at 488 nm) indicative of bound PUB (Fig. S3).

Pull-down assays performed to assess CpeY interaction with CpeA showed an interaction between CpeA and CpeY detected in the absence or presence of PEB (Fig. S4). However, there was no evidence that CpeY binds PEB in this experiment after zinc-enhanced bilin fluorescence (data not shown). Recombinant CpeZ did not interact with either CpeY or CpeA. Thus, it is likely that RS9916 CpeY does not require an interaction with CpeZ or any other lyase to catalyze the robust addition of PEB to CpeA-C82 (Fig. 2E–G).

3.2. LC-MS-MS analysis of recombinant proteins

Recombinant CpeA proteins expressed in the presence of CpeY alone were purified, digested with trypsin, and subjected to liquid chromatography mass spectrometry analysis (LC-MS-MS). Modification of CpeA-C82 resulting from addition of PEB at this site was detected (Figs. 3 and S5). Fig. S5 shows the 490 and 550 nm absorbance traces from this sample as well as EICs for the non-modified $DRAC_{139}APR$ peptide and the bilin modified $VDKC_{82}$*YR peptide. Fig. 3 shows the mass spectrum from the 3.72 min peak (Fig. S5A, red star) and Fig. 3B has the mass spectrum and UV–VIS from the 34.82 min peak (Fig. S5C, blue star). The UV–VIS spectrum clearly demonstrates that PEB is attached to the C82 peptide; no modified version of a C139-containing peptide was observed. No PUB-absorbing peaks were seen on the LC trace (Fig. S5A), and the other two peaks on the 550 nm UV–VIS trace (Fig. S5D) correlated with peptides containing C82 (C*YR and an oxidized version of VDKC*YR peptides). Therefore, CpeY is specific for PEB addition at CpeA-C82.

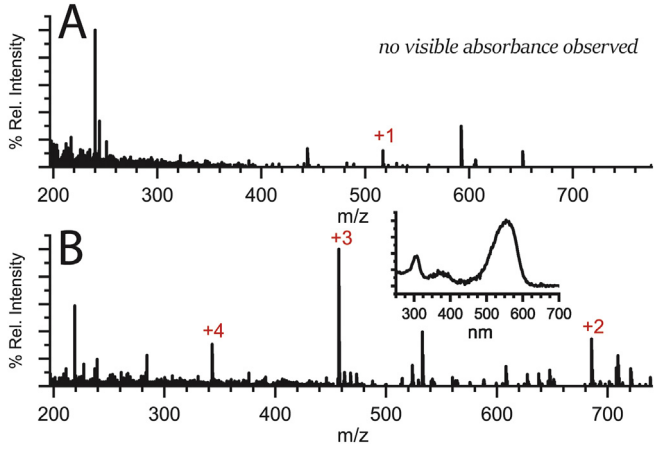

Fig. 3. CpeY from *Synechococcus* sp. RS9916 attaches PEB to CpeA-C82, but not CpeA-C139.
(A) Mass spectrum of peak with retention time of 3.70 min. The red +1 indicates the ion for peptide at the C139 position ($AC_{139}APR + H)^{+}$, *m/z* 517.2558. There is no evidence of bilin attachment at this site. (B) Mass spectrum and UV–VIS spectrum for peak with retention time of 34.82 min. The red +2, +3, and +4 indicate ions for peptides at the C82 position ($VDKC_{82}YR + nH)^{n+}$ modified by PEB (*m/z* 685.3350^{2+}, 457.2253^{3+}, and 343.1706^{4+}, respectively). Absorbance spectrum (upper right corner of B) show a peak at ~545 nm, demonstrating the presence of bound PEB at the C82 position. These results are representative of two independent replicates.

3.3. Analysis of PBS from WT and the *cpeY*$^{-}$ deletion strain

To assess the role of CpeY in RS9916, a *cpeY*$^{-}$ interruption mutant was generated and verified by PCR amplification analyses (Fig. S2) [9,11,39,43]. Wild type (WT) and mutant cells were grown in two separate light conditions: GL for maximum PEB production and BL for maximum PUB production (Fig. 4A–C). Whole cell absorbance and fluorescence spectral data revealed minimal differences between WT and *cpeY*$^{-}$ cells (Fig. 4B–E), with slightly less PUB and PEB absorption, relative to chlorophyll, for the *cpeY* mutant than WT in GL. In order to determine if CpeY has a role in PBS biogenesis, whole PBS were harvested from WT and *cpeY*$^{-}$ cells grown in either GL or BL (Fig. 4A) and separated by high performance liquid chromatography (HPLC; Fig. S6). Relative absorbance and chromatograms were observed using 550 nm (PEB) and 495 nm (PUB) for bilin content (Fig. S6). Among purified PEI and PEII subunits, CpeA from *cpeY*$^{-}$ mutant grown in BL was the main protein that showed a difference compared to the WT subunit (Fig. 5). The slight difference seen for PEB levels in CpeB from the *cpeY*$^{-}$ mutant compared to WT grown in GL (Fig. 5B) was noted, but no discernable differences in chromophore attachment between the WT and the *cpeY*$^{-}$ mutant were detected for CpeB by LC-MS- MS (not shown). For CpeA from GL-grown cells, which has two PEB attached in WT cells, there was no noticeable difference between WT and the *cpeY*$^{-}$ mutant using spectral analysis alone (Fig. 5A). The PUB:PEB ratio of CpeA from BL-acclimated *cpeY*$^{-}$ and WT cells was compared as described in the methods. In PBS harvested from WT cells grown in BL, CpeA binds PUB (at C139) and PEB (at C82) in a 1:1 ratio, or a PUB:PEB of 1. Absorbance spectra for CpeA isolated from the *cpeY*$^{-}$ mutant grown in BL showed a higher PUB:PEB, 1.41, than the WT CpeA PUB:PEB of 0.98 (Fig. 5E). This difference suggests that in BL, there is a decrease in the amount of PEB or an increase in the amount of PUB attached to CpeA in the *cpeY*$^{-}$ mutant relative to WT. Neither MpeA nor MpeB subunits showed differences in the *cpeY*$^{-}$ mutant when compared to the same subunits from WT (Fig. 5A–D, F–H).

3.4. Liquid chromatography tandem mass spectrometry analysis of PBP from the *cpeY*$^{-}$ mutant and WT

To determine which CpeA Cys residue was affected by in the mutant in vivo, each CpeA subunit purified via HPLC was digested with trypsin and subjected to LC-MS-MS. Extracted ion chromatograms (EICs) for peptide $VDKC_{82}YR$ (bilin on C82, sum of *m/z* 457.2252^{3+}) and unmodified $VDKC_{82}YR$ (392.1946^{2+}) from the four light conditions and genotypes are shown in Fig. 6A. Fig. 6B is the UV–VIS and mass spectrum extracted from peak 1 (15.83 min) of the BL *cpeY*$^{-}$ sample and shows $VDKC_{82}YR$ is modified by PUB. Fig. 6C contains the UV–VIS and mass spectrum for peak 2 (17.18 min) from the same sample and indicates PEB modification. Table 2 contains a detailed analysis of the peak areas of EICs for the peptides CYR and VDKCYR with and without bilins attached. Due to the lack of appropriate standards, the ionization efficiencies of all ions were treated as though they were equal. WT sample peak areas are 20% PUB (peak 1), 79% PEB (peak 2), and < 1% unmodified for C82 in BL, and 97% PEB (peak 2), 3% unmodified, and no detectable PUB for C82 in GL. The deletion of *cpeY* increased the percentage of PUB ligated to the CpeA-C82 peptide (38% in BL and 14% in GL) in the mutant while the level of unmodified C82 was about the same as that found in WT. One possibility for the PUB addition in BL to CpeA in WT and *cpeY*$^{-}$ mutant cells is the BL-inducible MpeZ lyase isomerase (see Fig. S3). *mpeZ* RNA is detected in GL but at much lower levels than in BL, where it is highly induced [10].

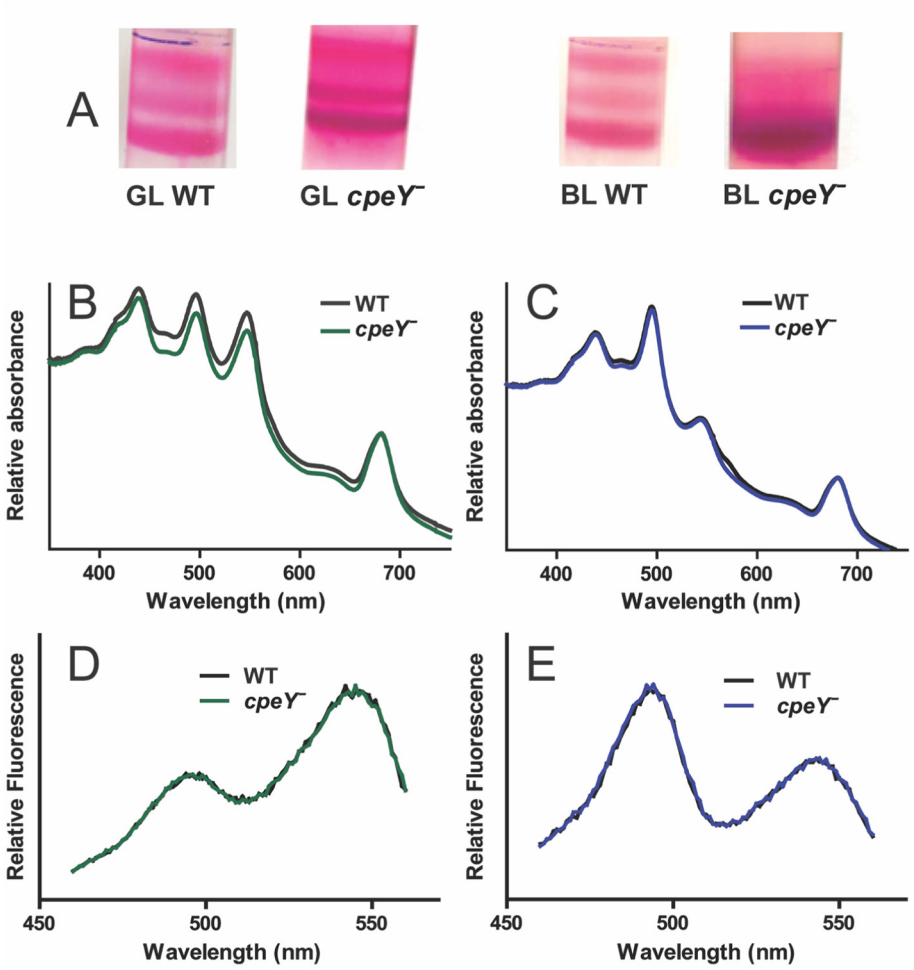

Fig. 4. Comparison of WT and *cpeY*$^{-}$ whole cell PBS collection and analysis.
(A) PBS purified via sucrose gradient from wild type (WT) and *cpeY*$^{-}$ cells grown in blue light (BL) and green light (GL). Average whole cell absorbance scans of two independent replicates for each WT (black lines) and *cpeY*$^{-}$ in GL (green line; B) and BL (blue line; C). Relative fluorescence scans of *cpeY*$^{-}$ grown in GL (green line; E) and BL (blue line; E) compared to WT cells (black lines) with PUB peak present at 495 nm and PEB peak at 545 nm.
Figure adapted from [39].

4. Discussion and conclusions

Photosynthetic marine microorganisms in the genus *Synechococcus* are picocyanobacteria that have adapted to utilize a wide range of light niches contributing to their ubiquity and high rates of global primary production [1–3]. Due to a wide diversity in photosynthetic pigments, different strains of *Synechococcus* have evolved to be blue-light specialists, green light specialists, or blue-green light generalists [2–4,9]. The blue-green generalists undergo CA4, involving differential attachment of PEB (λ_{max} ~ 545 nm) and PUB (λ_{max} ~ 495 nm) within the CpeA and MpeA proteins [2,4–6,9,12,44]. Lyase and lyase isomerase attachment of PEB and PUB, respectively, via post-translational modification of PEI and PEII is essential for efficient acquisition of blue/green light [10,11,35,38,40]. In RS9916, current research detailing the biosynthesis of the PBS, particularly the proteins involved in CA4, is emerging [2,4–6,9,10,12,43]. Shukla et al. characterized the CA4 process in RS9916 and showed that only 3 out of 11 bilins attached to PEI and PEII change when cells are shifted from GL to BL [11]. They also showed that under blue light saturated conditions, the bilin lyase isomerase MpeZ ligates PUB at one of these sites that change: C83 on MpeA. Since then, there have been additional searches for genes that might encode the lyases and lyase isomerases responsible for the other chromophore changes that occur during CA4 in BL and GL [11]. Although initially, RS9916 CpeY was suggested as the PEB lyase for MpeA-C83 in GL [11], Sanfilippo et al. recently showed that MpeY is the PEB lyase for this site on MpeA in GL and that transcript levels of *mpeZ* vs *mpeY* determine which enzyme is produced more abundantly and therefore which chromophore is attached at C83 [10]. The equivalent position in CpeA, C82, is not involved in CA4 and has a constitutively attached PEB, and here we show that CpeY is the lyase responsible for PEB ligation at this position [6,44,45].

Analysis of PBS collected from *cpeY*$^{-}$ cells shows an increase in PUB ligated to CpeA-C82 peptides in BL when compared to WT rather than the lack of a chromophore at this position [9,39]. Initially we were surprised to detect small amounts of PUB at CpeA-C82 in BL which we had not observed before [11]; however, the MS equipment used to acquire the current data is much more sensitive than that used to acquire the data in a previous manuscript [11]. We hypothesize that MpeZ, whose RNA level is 40-fold upregulated in BL, may compensate for CpeY in some cases, attaching PUB at CpeA-C82 when CpeY is absent. We have also determined that small amounts of PUB are attached by MpeZ to CpeA in our recombinant system, albeit very inefficiently. In the absence of *cpeY*, there is probably strong selection pressure to ligate any bilin possible at this position on CpeA, and the most likely enzymes to do this in vivo are MpeY, which is constitutively expressed at the RNA level and normally ligates PEB at MpeA-C83, or MpeZ, whose transcripts are highly upregulated by BL, but are present in GL also, albeit at much lower levels [10].

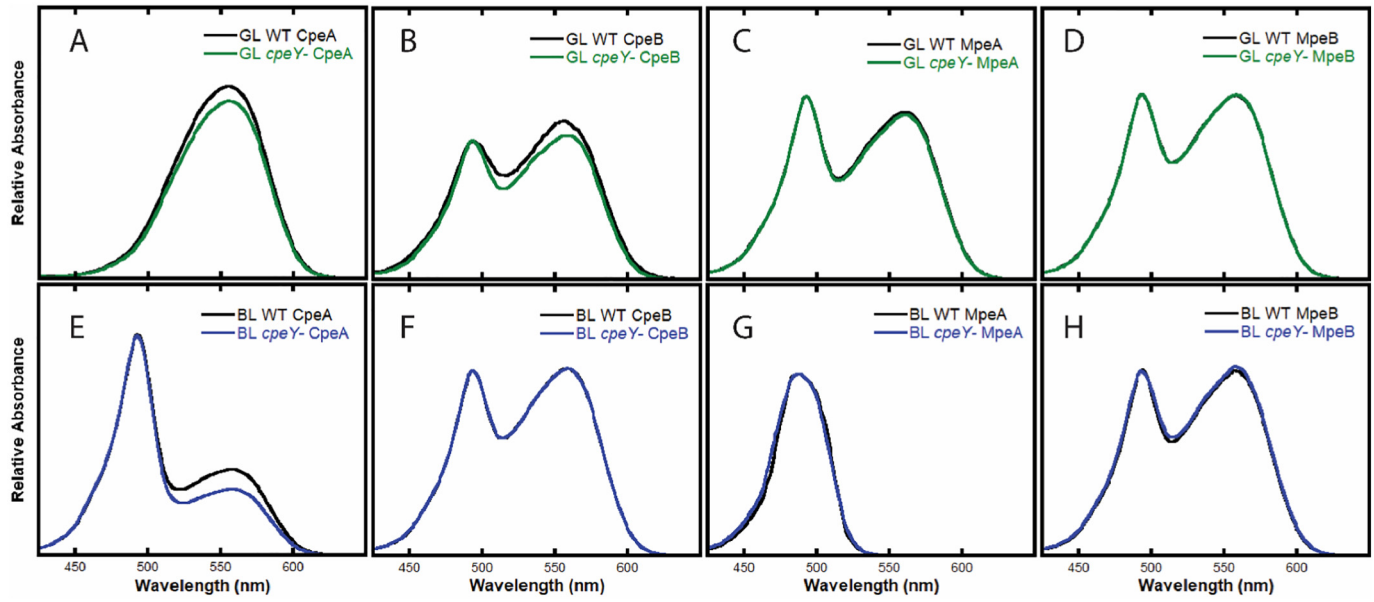

Fig. 5. Spectral comparisons for PEI and PEII proteins from WT and the *cpeY*$^{-}$ as analyzed via HPLC.
Absorbance spectra of phycoerythrin I (PEI) and II (PEII) phycobiliprotein subunits separated by HPLC from wild type (WT; black lines) and *cpeY*$^{-}$ cells grown in green light (green lines) and blue light (blue lines). Individual phycobiliprotein subunits are denoted as follows: α-subunit of PEI (CpeA; A & E), β-subunit of PEI (CpeB; B & F), α-subunit of PEII (MpeA; C & G), β-subunit of PEII (MpeB; D & H). These results are representative of two independent replicates.

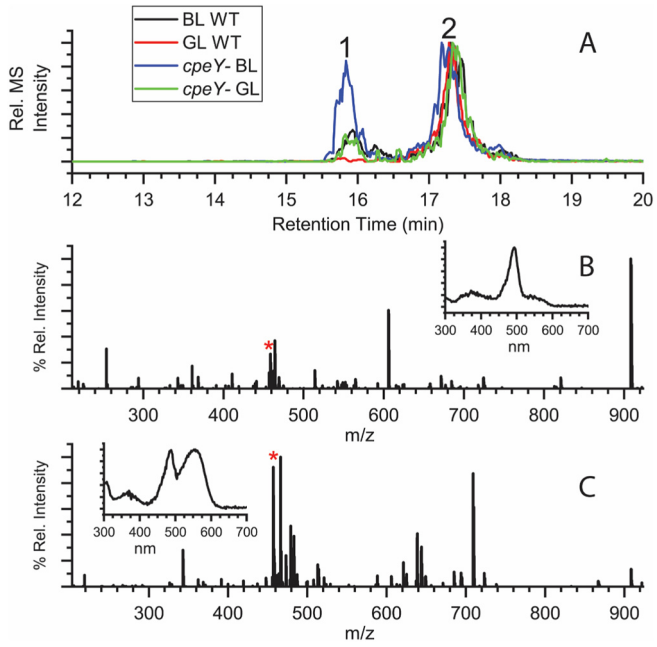

Fig. 6. Liquid chromatography tandem mass spectrometry (LC-MS-MS) analysis of HPLC purified phycobiliproteins CpeA from WT and *cpeY*$^{-}$ grown under two different light conditions.
(A) Combined extracted ion chromatograms (EICs) for the peptide CpeA $VDKC_{82}YR$ for four samples WT and *cpeY*$^{-}$ isolated from BL and GL at *m/z* 457.2252^{3+} or 392.1946^{2+} modified by PEB. Numbers 1 and 2 indicate 2 versions of the peptide observed. Representative mass spectra and UV–VIS spectra from peaks with retention time of 15.83 min (B) and 17.18 min (C) from *cpeY*$^{-}$ cells grown in BL. Absorbance spectra (inset in B–C) show a peak at 490 nm for PUB and/or 550 nm for PEB.

RS9916 CpeY (440 amino acids) and MpeY (398 amino acids) are 41% similar. The *mpeY* gene is present in all PEII-containing *Synechococcus* strains, whereas homologous *cpeY* genes can be found in other species of cyanobacteria possessing genes for the expression of PEI but lacking homologues of *mpeY* and PEII genes [3]. Grébert describes a diversity of alleles for the *mpeY* gene in marine *Synechococcus* species that undergo two different types of CA4; furthermore, the specific subtype of CA4 corresponds with the specific allele of *mpeY* that each strain possesses [2,46]. The need for specific alleles of *mpeY* in addition to the lack of *mpeY* in species also lacking PEII, suggests that *mpeY* evolved for specific function on PEII. The *cpeY* gene in RS9916 is orthologous to the characterized *cpeY* lyase gene in the freshwater species *F. diplosiphon* [35,38]. An *F. diplosiphon cpeY* mutant showed a two-fold decrease in the amount of PEB bound to FdCpeA when compared to WT [35,38]. This dramatic decrease suggests that *cpeY* encodes a protein with a critical function on PEI in *F. diplosiphon*, a species that lacks PEII. Since RS9916 possess PEI and PEII in addition to *cpeY* and *mpeY* homologues, it is possible that MpeY is acting upon CpeA-C82 to compensate for the loss of the CpeY lyase for this system; however, we were unable to demonstrate activity of MpeY on CpeA in our recombinant system because MpeY is not as soluble in *E. coli* as CpeY or MpeZ (data not shown).

Analysis of the RS9916 *cpeY*$^{-}$ mutant shows that this lesion has little effect on the biosynthesis of the PBS, and data presented here also demonstrate that CpeY is not involved in any other changes that occur during CA4 at positions MpeA-C140 or CpeA-C139. However, we clearly demonstrate that RS9916 CpeY is one of the most active recombinant PEB lyases characterized to date. Moreover, CpeY can be used to help characterize other potential lyases for the C139 position since the attachment of one or more bilins, particularly at the central C82 equivalent position, better enables PBPs to fold and remain soluble in *E. coli* [17,23,33,40,47,48]. Localized environmental changes, such as bilin addition to a single cysteine residue, may be necessary for proper protein conformation and folding. In the future, this will allow other lyases to be tested in *E. coli* for their ability to attach an additional bilin on the same subunit. In particular, characterization of CpeY as the PEB lyase for CpeA-C82 opens up testing of other putative lyases/lyase isomerases for activity at C139, a position involved in CA4 for which the lyase/lyase isomerase has not yet been characterized [17,49].

Recombinant protein expression analyses show that CpeY alone is capable of robust lyase activity on CpeA subunits. Utilizing an *E. coli* expression system, we demonstrated the ability of CpeY to attach PEB to as much as 72.3% of all CpeA present, making it one of the most efficient single-subunit lyases when compared to the CpcE/CpcF CpcA-

Table 2
Summary of abundance of bilin on Cysteine 82 residues from WT and *cpeY*$^{-}$ PEI α-subunit (CpeA) as measured using LC-MS-MS.

Sample	Available light condition[a]	Bilin attached[b]	Percentage PEB[c]	Percentage PUB[c]	Percentage unmodified[c]
WT CpeA	GL	PEB	97.1	0	2.9
cpeY^{-} CpeA	GL	PEB	83.2	14.0	2.8
WT CpeA	BL	PEB	79.3	19.9	0.8
cpeY^{-} CpeA	BL	PEB	37.6	62.0	0.4

[a] Cells grown in the presence of green light (GL) or blue light (BL) for at least a week prior to collection [4,10].
[b] Bilin identified using spectral analysis of eluted fractions with paired fragments.
[c] Percentages calculated by taking the sums of the relevant ions (m/z 457.2252^{3+} and 343.1707^{4+} for modified VDKCYR, m/z 514.2390^{2+} and 343.1617^{3+} for modified CYR, m/z 392.1946^{2+} and 267.7989^{2+} for unmodified VDKCYR, and m/z 441.1915^{1+} and 221.0994^{2+} for unmodified CYR) at the appropriate retention times (split peak @ 3.0 and 4.1 min for the unmodified CYR and VDKCYR, 15.9 min for PUB modified CYR and VDKCYR, 17.00 min for PEB-modified CYR, and 17.32 min for PEB-modified VDKCYR) and dividing it by the total area for all 4 EICs.

C84 phycocyanobilin lyase (33% chromophorylation [50]) or the PecE/PecF recombinant PecA-C84 phycoviolobilin lyase (66% chromophorylation [51]). CpcS/U type lyases characterized to date have shown a chromophorylation range of 17–72% for allophycocyanin (α and β subunits) and phycocyanin β-subunits [33]. CpcT lyases have shown 17% chromophorylation of CpcB at C-153 in *E. coli* [33]. RS9916 CpeY activity is not dependent on CpeZ in *E. coli*, unlike the important chaperone-like role of CpeZ in enhancing CpeY activity in the distantly related freshwater species *F. diplosiphon* [35,38]. However, we cannot rule out a role for CpeZ as a chaperone-like protein for CpeB folding and chromophore ligation, nor for enhancing the chromophore ligation at C139 (by an as-yet uncharacterized lyase) on CpeA.

The unique spectral properties and high fluorescence quantum yields of these phycobiliproteins make them valuable resources for biotechnological applications [45,52]. Fluorescent probes engineered from PBP are widely used in fluorescence microscopy, fluorescence activated cell sorting, immunoassays, flow cytometry, histochemistry assays, and detection of reactive oxygen species [45]. Understanding the biosynthetic pathways of these proteins is invaluable as they make excellent photon receptors in their native state [45,53]. As we make progress towards characterization of all enzymes required for the post-translational modification of these proteins, we hope to harness some of these bilin lyases for the creation of recombinant fluorescent bio-imaging probes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2020.148215.

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Supporting Information

CpeY is a phycoerythrobilin lyase for cysteine 82 of the phycoerythrin I α-subunit in marine *Synechococcus*

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Fig. S1: *cpeBA* operon and predicted structure of phycobiliproteins and the CpeY lyase and CpeZ chaperone-like protein in *Synechococcus* sp. RS9916
Fig. S2: *Synechococcus* sp. RS9916 *cpeY* interruption construct and PCR amplification agarose gel confirmation
Fig. S3: Recombinant protein co-expression of *Synechococcus* sp. RS9916 CpeA to test for MpeZ activity for CpeA-C82.
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Table S1: Oligonucleotide primers used in this study
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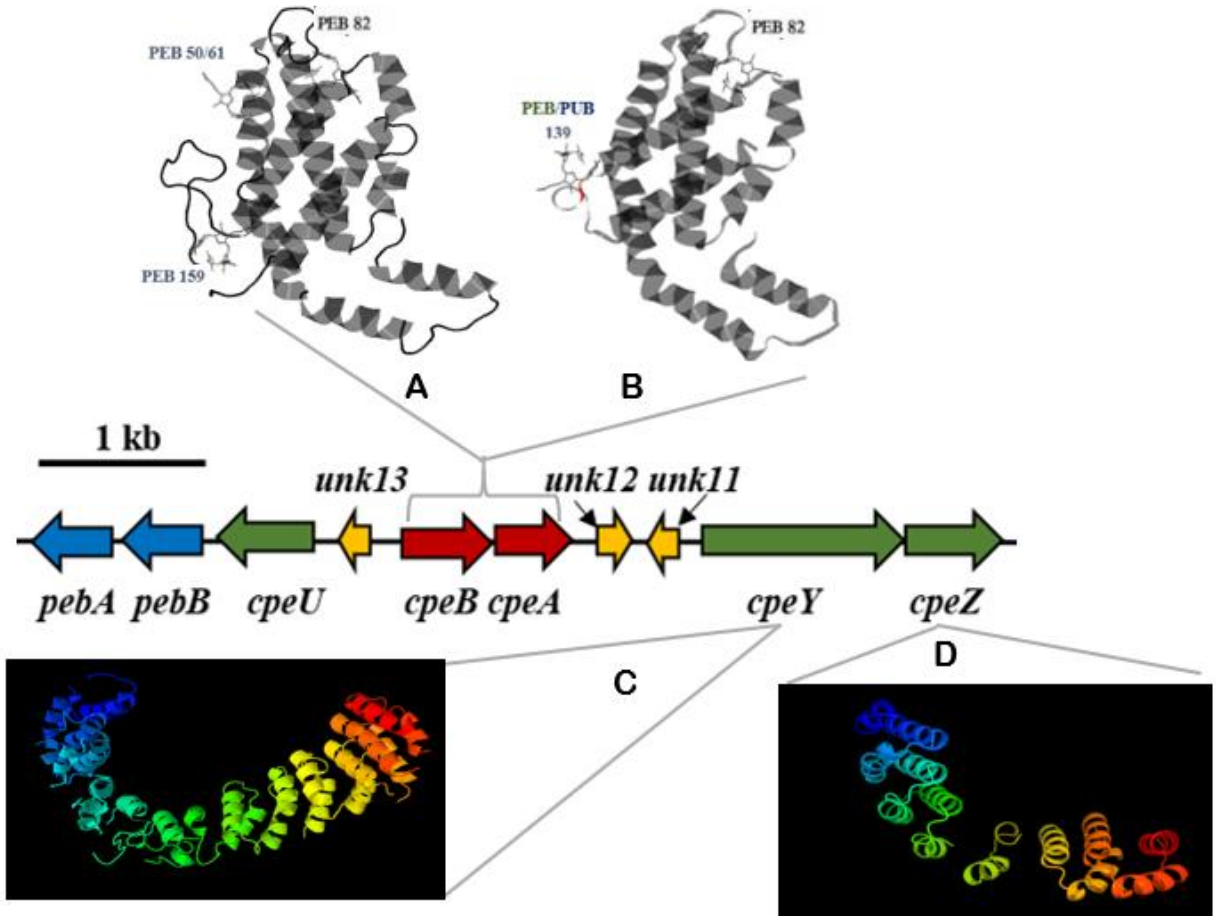

Fig. S1: *cpeBA* operon and predicted structure of phycobiliproteins and the CpeY lyase and CpeZ chaperone-like protein in *Synechococcus* sp. RS9916

Directional map of *Synechococcus* RS9916 phycoerythrin I (PEI) structural genes (red arrows) depicting PEB synthesis genes (blue arrows), putative lyase genes (green arrows), and genes of unknown function (yellow arrows). The ribbon diagram represents the β subunit (**A**) and α subunit (**B**) predicted structure using Phyre 2 [1]. Cysteine attachment sites from PEI in *Synechococcus* sp. RS9916 are labeled for PEB/PUB attachment. Expanded images depict predicted 3D structures of putative bilin lyase CpeY (**C**) and chaperone-like protein CpeZ (**D**) using Phyre2 prediction system [1].

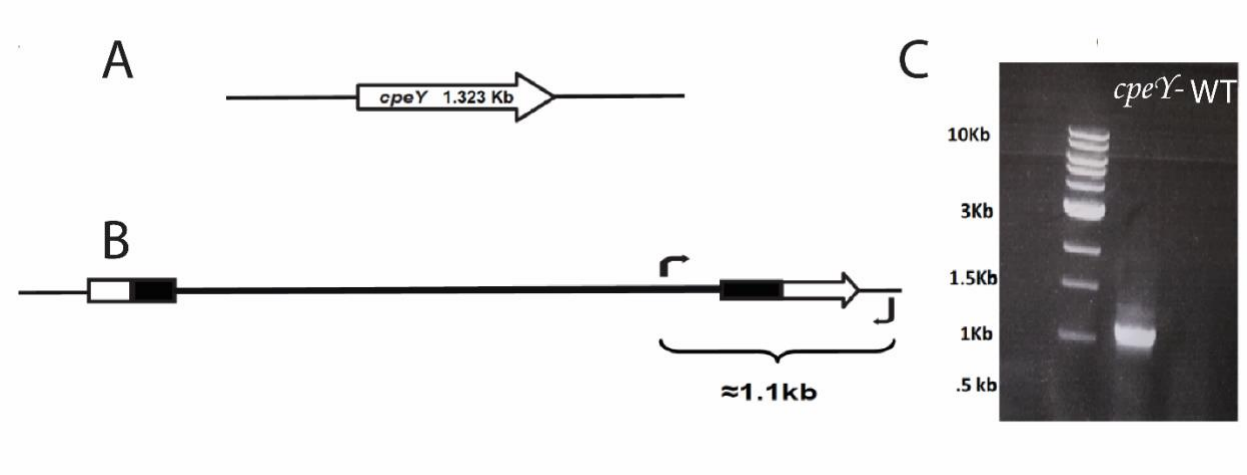

Fig. S2: *Synechococcus* sp. RS9916 *cpeY* interruption construct and PCR amplification agarose gel confirmation

(A) Depiction of the wild type RS9916 *cpeY* gene and **(B)** the *cpeY* interruption mutant. Thick black line represents plasmid sequence and dark boxes represent *cpeY* DNA regions introduced by the mutation. Bent arrows indicate primer specific regions for PCR amplification. **(C)** Image of agarose gel resolving PCR amplified fragments for RS9916 *cpeY* interruption mutant (*cpeY*-) and wild type (WT) cells. Figure adapted from [2].

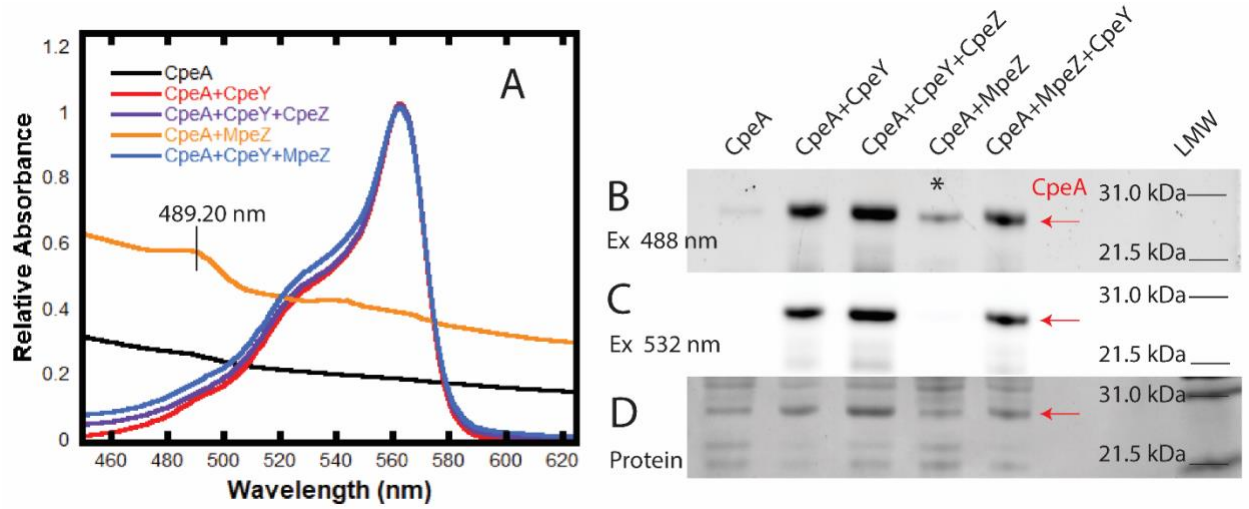

Fig. S3: Recombinant protein co-expression of *Synechococcus* sp. RS9916 CpeA to test for MpeZ activity for CpeA-C82.

Relative absorbance (**A**) for co-expression of RS9916 substrate CpeA in the presence of C82 PEB lyase CpeY, hypothesized chaperone-like protein CpeZ, and lyase isomerase MpeZ (attaches PEB and isomerizes to PUB on MpeA) and bilin synthesis genes shows ligated PEB (λ_{max} ~545 nm) with peaks at ~561 nm. Absorbance at 489.20 nm for co-expression of CpeA+MpeZ is indicative of bound PUB (λ_{max} ~495 nm). SDS-PAGE gels (**B-D**) depicting ~2 ug of CpeA (~24.0 kDa) per lane for each co-expression with 10 mM $ZnSO_4$ enhanced fluorescence followed by excitation at 488 nm (**B**; excitation of bound PUB), 532 nm (**C**; excitation of bound PEB), and Coomassie Blue total protein stain (**D**). * indicates Zn-enhanced fluorescence of gel excited at 488 nm (indicative of bound PUB) for co-expression of CpeA+MpeZ. Note, excitation at 488 nm excites both PEB and PUB; an abundance of PEB bound to CpeA is present in all lanes containing proteins expressed in the presence of CpeY. These results are representative of two independent replicates.

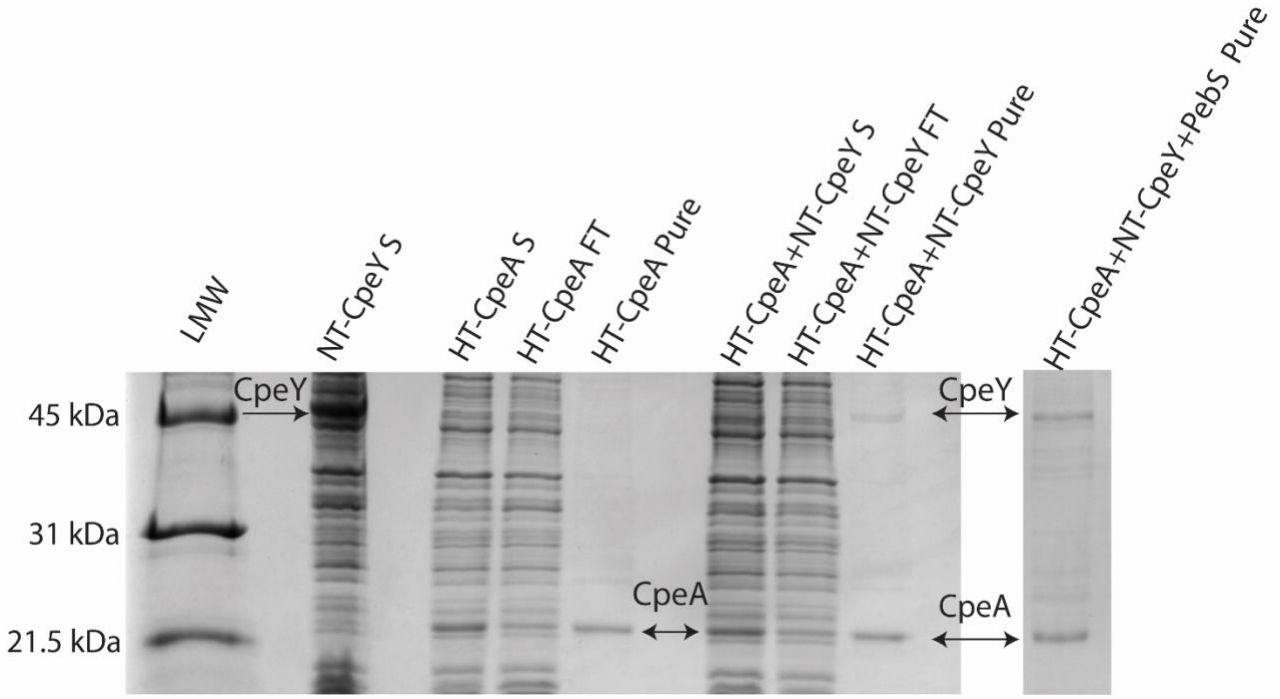

Fig. S4: Pull down assay for CpeA and CpeY co-expression

Coomassie stained gel containing protein samples resolved via SDS-PAGE from pull down assay [3] demonstrating soluble protein interaction between hexahistidine tagged CpeA (HT-CpeA) and non-tagged CpeY (NT-CpeY). Resolved fractions (left to right) for soluble NT-CpeY (~46.2 kDa) supernatant (S) and nickel chromatography purified HT-CpeA (~22.15 kDa) and interaction of HT-CpeA+NT-CpeY fractions for supernatant (S), flow through (FT), and imidazole eluent (pure). All expressions performed in the absence of bilin, showing co-purification of CpeY with its potential substrate CpeA (HT-CpeA+NT-CpeY pure). Far right lane shows Coomassie-stained gel containing purified CpeA proteins when expressed in the presence of CpeY and bilin synthesis genes (PebS/Ho1) depicting co-purification of CpeY with CpeA in the presence of PEB. These results are representative of two independent replicates.

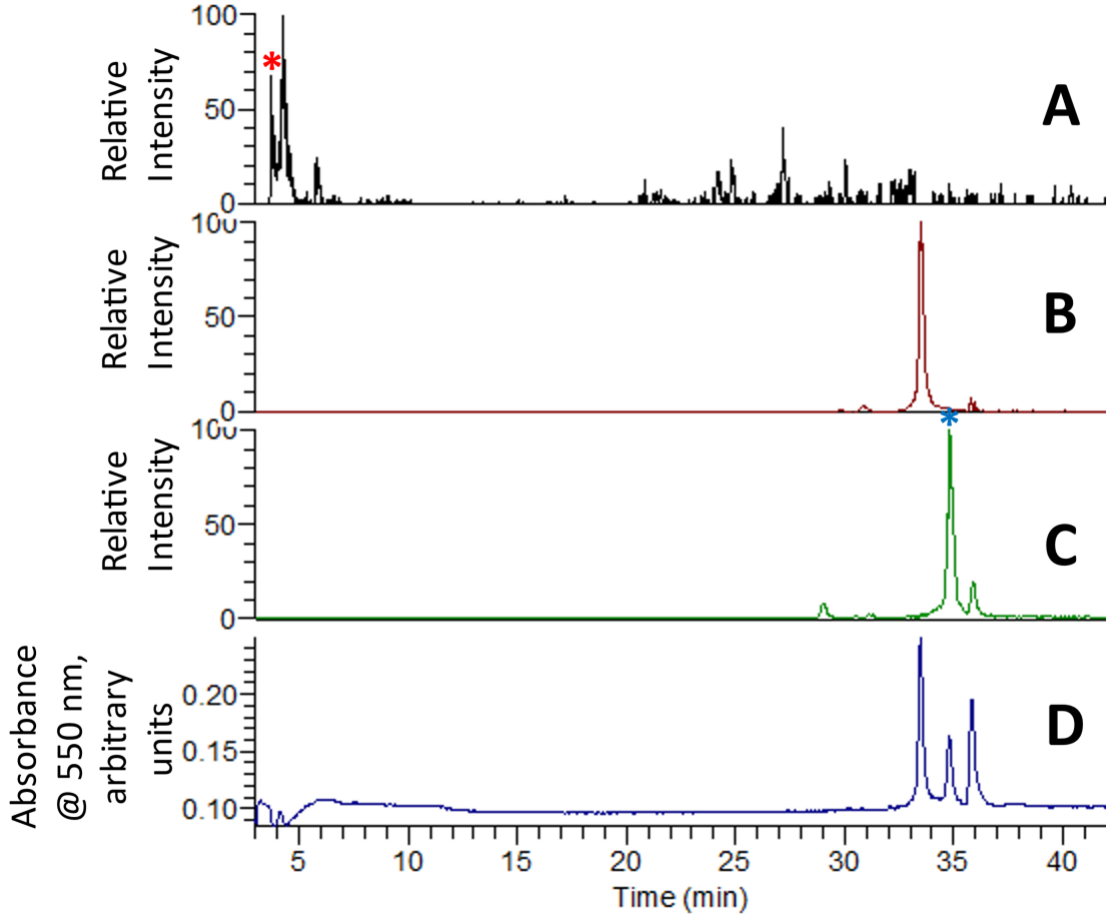

Fig. S5: Liquid chromatography tandem mass spectrometry (LC-MS-MS) analysis of trypsin digested recombinant protein CpeA+CpeY+PEB

Extracted ion chromatograms (EICs) and 550 nm chromatograms for **(A)** *m/z* 517.2551$_{1+}$ (AC$_{139}$APR+H)$_{1+}$, **(B)** *m/z* 514.2390$_{2+}$ (C$_{82}$*YR+2H)$_{2+}$, **(C)** *m/z* 457.2252$_{3+}$ (VDKC$_{82}$*YR+3H)$_{3+}$, and **(D)** Absorbance at 550 nm from a tryptic digest of recombinant protein CpeA expressed with CpeY and PEB. Cysteine 82 is modified with PEB in traces **B** and **C**. * indicates the location of the ACAPR peak in the EIC. Note, the 35.83 min peak in trace **D** corresponds to peptide ((C$_{82}$YR+PEB-2H)+2H)$_{2+}$, *m/z* 513.2313$_{2+}$. These results are representative of two independent replicates.

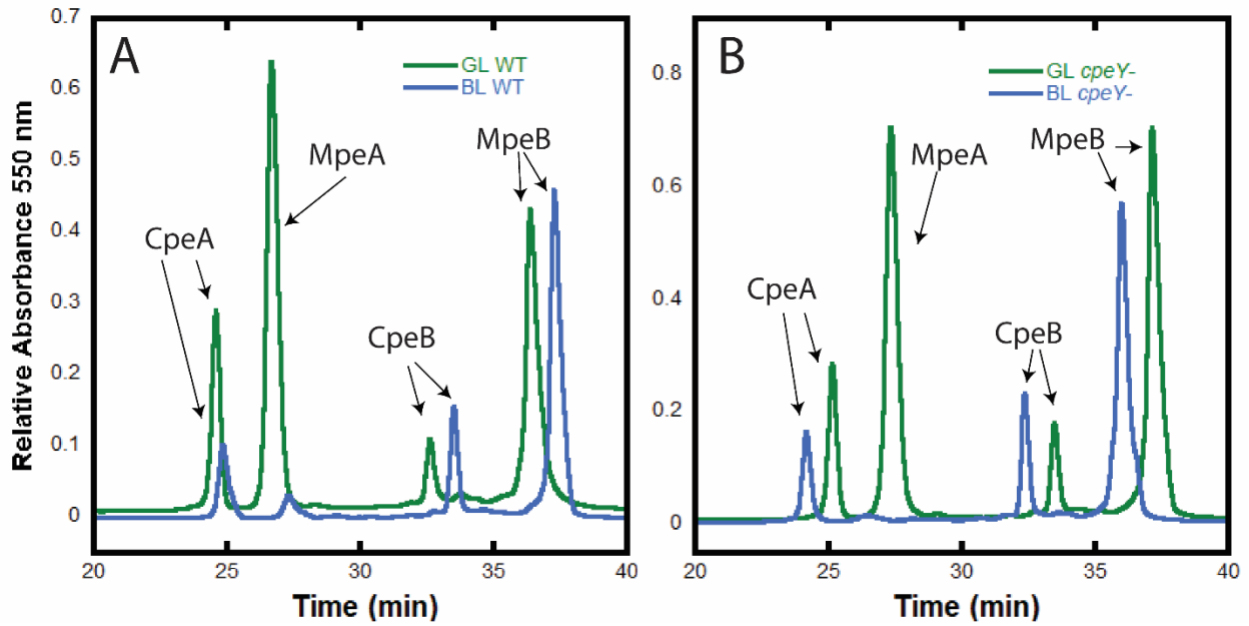

Fig. S6: Chromatograms and spectral comparisons of CpeA proteins from WT and *cpeY-* as analyzed via HPLC

Chromatograms extracted for relative absorbance at 550 nm (PEB) for *Synechococcus* sp. RS9916 phycobiliproteins isolated from cells grown in and abundance of blue light (BL) and green light (GL) for wild type (WT; panel **A**) and *cpeY-* (**B**). In GL saturated conditions WT CpeA binds 2 PEB bilins shown by single spectral peak at 550 nm (**A,** green line); whereas, in BL saturated conditions WT CpeA binds one PUB and one PEB (PUB:PEB = 0.96) (**A**). Compared to WT, *cpeY-* CpeA shows an increased retention time (~1 min) in GL and a reduced retention time (~1 min) in BL (**B**) with an increase in PUB:PEB (1.41) in BL in *cpeY-* cells. These results are representative of two independent replicates.

Table S1: Oligonucleotide primers used in this study

Primer name	Sequence* (listed 5' to 3')
cpeY.F.NdeI	5'-AAACATATGCCCATGAACTGGAGACC -3'
cpeY.R.EcoRV	5'-AGCCGGATATCTTCTAGCAATTGTGG -3'
cpeZ.F.BglII	5'-GCATAGATCTGATGGACTCTTCTGTGCAATCG-3'
cpeZ.R.XhoI	5'-GCATCTCGAGTGAGACTTTCTCGACGC-3'
HTcpeA.F.BamHI	5'-GCATGGATCCGATGAAGTCTGTCGTGACCAC-3'
HTcpeA.R.HindIII	5'-GCATAAGCTTTCAAGAAAGAGCGTTGATCA-3'
HTMpeA.F.BamHI^	5'-TCTCCCTTATGCGACTCCTGCATT- 3'
HTMpeA.R.EcoRI^	5'-TGCGGCCGTGTACAATACGATTAC-3'
BamHI-cpeY-internal for+	5'-ATAGGATCCACTTATATATGGCGGCTGCGCACT-3'
BamHI-cpeY-internal-rev+	5'-ATAGGATCCTGAATCGAACCACGAAGGAGCTGA-3'

* Engineered restriction enzyme sites are underlined
^See also [4]
+Primers used for generation of the interruption mutant *cpeY-* [2]

Table S2: Plasmids used in this study

Plasmid name[a]	*Recombinant proteins produced*[b]	*Parent vector*	*Antibiotic*[c]	*Reference*
pHTCpeA	*Synechococcus* sp. RS9916 HT-CpeA	pETDuet-1	Ap	This study
pCpeZ	*Synechococcus* sp. RS9916 NT CpeZ	pCDFDuet-1	Sp	This study
pCpeY	*Synechococcus* sp. RS9916 NT CpeY	pCOLADuet-1	Km	This study
pHTMpeA	*Synechococcus* sp. RS9916 HT-MpeA	pETDuet-1	Ap	This study
pHTMpeA-pCOLA	*Synechococcus* sp. RS9916 HT-MpeA	pCOLADuet-1	Km	This study
pMpeZ	*Synechococcus* sp. RS9916 NT MpeZ	pCDFDuet-1	Sp	[4]
pPebS	Myovirus Ho1 and NT-PebS	pACYCDuet-1	Cm	[5, 6]

[a] Genes encoding hexahistidine-tags fused to protein product are indicated as "HT-"
[b] "NT-" is an abbreviation for "Non-Tagged" referring specifically to hexahistidine-tags
[c] Antibiotic resistance used to select for the presence of the plasmid (Ap: ampicillin; Cm: chloramphenicol; Km: kanamycin; Sp: spectinomycin)

Table S3: Recombinant protein co-expressions and abbreviations

PE subunit[a]	Lyase[b]	Recombinant co-expression abbreviations[c]
HTMpeA	--	MpeA
HTMpeA	MpeZ	MpeA+MpeZ
HTMpeA	CpeY	MpeA+CpeY
HTCpeA	--	CpeA
HTCpeA	CpeY	CpeA+CpeY
HTCpeA	CpeZ	CpeA+CpeZ
HTCpeA	CpeY+CpeZ	CpeA+CpeY+CpeZ

[a]HT indicates the presence of a hexa-histidine tag fused to protein product
[b]Lyase enzymes expressed from clones of RS9916 genes of same name
[c]Expressed with PebS/HoI to generate PEB from *E. coli*'s heme

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