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Joseph E Sanfilippo, Adam A Nguyen, Laurence Garczarek, Jonathan A Karty, Suman Pokhrel, et al.. Interplay between differentially expressed enzymes contributes to light color acclimation in marine *Synechococcus*. *Proceedings of the National Academy of Sciences of the United States of America*, 2019, 116 (13), pp.6457-6462. 10.1073/pnas.1810491116 . hal-02322866

**HAL Id: hal-02322866**

**<https://hal.science/hal-02322866>**

Submitted on 21 Oct 2019

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**Interplay between differentially expressed enzymes contributes to light color acclimation in marine *Synechococcus***

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## Abstract

Marine *Synechococcus*, a globally important group of cyanobacteria, thrives in various light niches in part due to its varied photosynthetic light harvesting pigments. Many *Synechococcus* strains use a process known as chromatic acclimation to optimize the ratio of two chromophores, green-light absorbing phycoerythrobilin (PEB) and blue-light absorbing phycourobilin (PUB), within their light harvesting complexes. A full mechanistic understanding of how *Synechococcus* cells tune their PEB to PUB ratio during chromatic acclimation has not yet been obtained. Here, we show that interplay between two enzymes named MpeY and MpeZ controls differential PEB and PUB covalent attachment to the same cysteine residue. MpeY attaches PEB to the light harvesting protein phycoerythrin-II in green light, while MpeZ attaches PUB to phycoerythrin-II in blue light. We demonstrate that the ratio of *mpeY* to *mpeZ* mRNA determines if PEB or PUB is attached. Additionally, strains encoding only MpeY or MpeZ do not acclimate. Examination of strains of *Synechococcus* isolated from across the globe indicates that the interplay between MpeY and MpeZ uncovered here is a critical feature of chromatic acclimation for marine *Synechococcus* worldwide.

**Significance Statement**

Marine phytoplankton are responsible for nearly half of the primary productivity on Earth, and the picocyanobacterium *Synechococcus* contributes up to 16% of this total. *Synechococcus* strains optimize their fitness by using different chromophores for photosynthetic light harvesting in different light color niches. Many *Synechococcus* strains use chromatic acclimation to adjust chromophore composition to ambient blue to green light ratios. We determined that interplay between two chromophore attachment enzymes, MpeY and MpeZ, plays a role in chromatic acclimation at the level of gene expression. The unique ability of these two enzymes to attach different chromophores to the same residue makes them potentially useful for differential labeling of that residue for fluorescence imaging technologies.

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## Introduction

Members of the cyanobacterial genus *Synechococcus* are distributed throughout the marine environment, constituting a global population of approximately  $7 \times 10^{26}$  cells (1). The ubiquity of this genus is partially due to the wide diversity of its photosynthetic pigments, which allows for niche occupancy of a large variety of light environments (2, 3). There are three main pigment types of *Synechococcus* strains adapted to distinct spectral niches in the marine environment: blue light specialists, green light specialists, and blue-green generalists (2, 4).

These three pigment types differ by the composition of their light harvesting complexes, known as phycobilisomes (PBS). PBS are typically fan-shaped, with a central core that is appressed to the photosynthetic or thylakoid membranes, and multiple rods which extend from the core and provide increased surface area for photon capture. PBS consist of phycobiliproteins, apoproteins containing one to three covalently attached chromophores that absorb specific wavelengths of light (5), and linker proteins, which have structural and energy transfer roles (6). Although there are several kinds of phycobiliproteins, all are / heterodimers. In marine *Synechococcus*, rods always contain the phycobiliprotein phycocyanin and usually contain one or two different forms of phycoerythrin called phycoerythrin-I (PE-I) and phycoerythrin-II (PE-II) (2, 3, 6). The  $\alpha$  and  $\beta$  subunits of PE-I are encoded by the *cpeBA* operon and are called CpeA and CpeB, while the  $\alpha$  and  $\beta$  subunits of PE-II are encoded by the *mpeBA* operon and are called MpeA and MpeB. These

heterodimers are located in different regions of the rods, with CpeA and CpeB in the core-proximal regions and MpeA and MpeB in the core distal regions.

Phycoerythrin in marine *Synechococcus* strains contain two different types of chromophores, blue-light absorbing phycoerythrin (PUB) [maximum absorbance ( $Abs_{max}$ ) ~495 nm] and green-light absorbing phycoerythrobilin (PEB) ( $Abs_{max}$  ~550 nm) (2, 7). The total PUB:PEB ratio in all of the rods of the PBS determines if a strain is a blue or green light specialist. Blue light specialists have a high PUB:PEB ratio, while green light specialists have a low PUB:PEB ratio. Blue-green generalists are capable of modifying their PUB:PEB ratio in response to the ambient blue-green ratio through a process known as type IV chromatic acclimation or CA4 (8, 9). In the CA-4 capable strain *Synechococcus* sp. RS9916, each CpeA/CpeB dimer contains four PEB and one PUB in green light, and three PEB and two PUB in blue light. Each MpeA/MpeB dimer has four PEB and two PUB in green light, and two PEB and four PUB in blue light (10).

Enzymes known as phycobilin lyases attach light harvesting chromophores on phycobiliproteins to conserved cysteine residues, forming thioether bonds (11, 12). Most lyases have high specificity for both particular chromophores and individual cysteine residues (12-14). While much is known about lyases capable of attaching phycocyanobilin ( $Abs_{max}$  ~660 nm) (11, 15-21), less is known about the enzymes involved in attaching PEB (22, 23) and PUB (10, 24, 25). Despite the rich chromophore diversity found in marine *Synechococcus*, only two phycobilin lyases, RpcG and MpeZ, have been characterized so far in this group (10, 24). RpcG attaches PUB to cysteine 83 (C83) of the phycocyanin -

subunit (24) and MpeZ attaches PUB to MpeA-C83 (10). These are dual function enzymes that have both lyase and isomerase activity, the latter allowing them to convert PEB into PUB. MpeU is required for attachment of PUB (25), but its precise function remains to be characterized. The attachment and isomerization of PEB to PUB to phycobiliproteins by lyase-isomerases is critical since these cyanobacteria have no known pathway that directly synthesizes PUB.

Blue-green generalists capable of CA4 comprise the most abundant pigment type, making up approximately 40% of the global *Synechococcus* population of the oceans (26). By differentially producing PEB and PUB in response to changes in ambient light color, blue-green generalist strains can optimize light harvesting in a variety of light color environments. Interestingly, CA4 has evolved twice, through acquisition of related, but distinct, genomic islands. CA4-A strains contain a genomic island encoding the PUB lyase-isomerase MpeZ, while CA4-B strains possess a genomic island encoding MpeW, an as yet uncharacterized member of the CpeY-MpeY-MpeZ family (7). Both CA4-A and CA4-B genomic islands also encode two master regulators, FciA and FciB. In the model CA4-A strain *Synechococcus* sp. RS9916, these proteins inversely control the expression of *mpeZ* and two other genes located in the same genomic island, which are more highly expressed in blue light than green light (4, 10). During CA4-A, MpeA-C83, MpeA-C140, and CpeA-C139 bind PUB in blue light and PEB in green light (10). Of these three changes, MpeZ catalyzes the attachment of PUB to MpeA-C83 in blue light. However, the lyase responsible for attaching PEB to MpeA-C83 in green light is unknown.

Here, we investigate the role of a previously uncharacterized, putative PEB lyase named MpeY in CA4. We demonstrate that in the model CA4-A strain *Synechococcus* sp. RS9916, MpeY attaches PEB to MpeA-C83 in green light. Additionally, we show that the expression ratio of *mpeY* and *mpeZ* mRNA determines if PEB or PUB is attached to MpeA-C83 in green and blue light. By analyzing environmental isolates from across the world's oceans, we provide evidence that interplay between MpeY and MpeZ is globally conserved and that strains encoding only MpeY or MpeZ do not acclimate. Thus, the selective use of MpeY and MpeZ for the attachment of different chromophores at a single site within a phycobiliprotein is an evolutionary innovation that contributes to the process of CA4-A in marine *Synechococcus*.

## Results

### MpeY controls attachment of PEB in green light

The *Synechococcus* sp. RS9916 genomic region implicated in MpeA and MpeB biosynthesis contains the *mpeBA* operon and two putative phycobilin lyases, MpeY and MpeU (Fig 1A)(2, 25). While MpeU is not closely related to MpeZ, MpeY and MpeZ sequences from this strain share 47% amino acid sequence identity overall (Fig. S1A), showing that these proteins are paralogs. Based on the genomic context of the *mpeY* gene and the relatedness of its encoded protein to MpeZ, we hypothesized that MpeY has a chromophore attachment role for either MpeA or MpeB.

Fluorescence spectroscopy was used to determine the relative excitation at 495 and 550 nm, which provides an estimate of the ratio of PUB to PEB in an *mpeY* interruption mutant (Fig. S1B) and control cells (Fig. 1B and C). In blue light, the PUB:PEB ratio of the *mpeY* mutant was indistinguishable from control cells, but in green light, PEB fluorescence decreased, relative to PUB fluorescence, in *mpeY* mutant cells. Therefore, MpeY is likely required for PEB attachment in green light. The possibility of polar effects was tested by cloning the *mpeY* gene and its promoter into an autonomously replicating plasmid and transforming it into the *mpeY* mutant. In green light, this plasmid restored the normal PUB:PEB ratio to the *mpeY* mutant (Fig. S2A), demonstrating that *mpeY* is necessary for PEB attachment in green light.

Whole cell absorbance spectroscopy showed that in blue light, *mpeY* mutant cells were indistinguishable from control cells but that in green light, *mpeY* mutant cells had lower levels of both PUB and PEB absorbance relative to chlorophyll levels (Fig. 1D and E). The effect on PEB was greater than the effect on PUB. Growth measurements demonstrated that *mpeY* mutant and control cells grew at the same rate in blue light but that in green light, *mpeY* mutant cells grew more slowly (Fig. S2B). This may be due to less efficient MpeA and MpeB biosynthesis in green light, resulting in a decrease in the number of complete PBS per cell and thus the overall amount of PUB and PEB in *mpeY* mutant cells. Together, these results demonstrate that MpeY is involved in PEB attachment in green light.

### **MpeY attaches PEB to MpeA-C83**

The function of MpeY was further examined by using HPLC to separate the phycoerythrin subunits MpeA, MpeB, CpeA, and CpeB from PBS that had been purified from *mpeY* mutant cells grown in blue or green light. UV-VIS absorption spectroscopy of these proteins revealed that MpeA isolated from blue-light-grown *mpeY* mutant and control cells had identical profiles, while the PUB:PEB absorbance ratio for MpeA was higher in the *mpeY* mutant than in control cells after growth in green light (Fig. 2A and B). In addition, the absorption profiles of MpeB, CpeA, and CpeB isolated from *mpeY* mutant cells were indistinguishable from control cells after growth in either blue or green light (Fig. S3A-F). We therefore conclude that among the four PE subunits and two light color conditions, MpeY is specifically required for PEB attachment to MpeA in green light.

In *Synechococcus* sp. RS9916, chromophores are attached to MpeA at C75, C83, and C140. To determine which of these residues were targeted by MpeY, we used tandem mass spectrometry combined with UV-VIS spectroscopy to examine peptides containing these residues from *mpeY* mutant and control cells (Fig. S4). Cells grown in blue light had PUB attached to all three residues from both *mpeY* mutant and control cells (Fig. S5). In addition, for cells grown in green light, PUB was attached to C75 (Fig. 2C and D) and PEB was attached to C140 (Fig. 2G and H) from both cell types. The only observable difference in chromophorylation was at MpeA-C83 from cells grown in green light, where PEB was attached in control cells (Fig. 2E) and PUB was present in *mpeY* mutant cells (Fig. 2F). Collectively, these data show that out of the 11 cysteines that are chromophorylated in the four PE subunits (10), MpeY is required specifically for PEB attachment to MpeA-C83, and that this attachment can only be detected in green light.

The lyase activity of recombinant MpeY was examined using a heterologous plasmid coexpression system in *Escherichia coli*. Three different hexahistidine-tagged (HT-) versions of MpeA were used as substrates: wild type, HT-MpeA; a mutant with C83 replaced by alanine (A), HT-MpeA(C83A); and a mutant with C75 and C140 each replaced with A, HT-MpeA(C75A, C140A). We also expressed NusA-tagged (Nus) MpeY along with enzymes required for PEB synthesis in *E. coli*. After coexpression and cell lysis, HT-MpeA and mutant derivatives were purified using Ni-NTA affinity chromatography and analyzed using SDS-PAGE (Fig. 2I). Zinc-enhanced fluorescence was used to show that MpeY is sufficient to covalently attach PEB to C83 of MpeA (Fig. 2I) and thus is a PEB lyase.

#### ***mpeY*<sup>-</sup> suppressor mutations increase *mpeZ* expression**

MpeY attaches PEB to MpeA-C83 in green but not blue light, indicating that it plays a role in the chromophore changes at that residue during CA4. The mechanism through which MpeY operates was further characterized by selecting for spontaneous suppressor mutants with higher growth rates than the *mpeY* mutant. Passaging of three independent *mpeY* mutant cultures for approximately 100 generations selected for apparent suppressor mutations in the CA4 genomic island (Fig. 3A) that increased growth rate relative to the *mpeY* mutant (Fig. S6A) yet maintained their initial interruptions of *mpeY* (Fig. S6B). Fluorescence spectroscopy analysis of each putative suppressor mutant revealed that all had an increased PUB:PEB ratio during growth in white light, compared to the original

*mpeY* mutant and control cells (Fig. 3B), and were indistinguishable from one another, suggesting they could be caused by similar mutations.

A similarly high PUB:PEB ratio had been previously observed for an interruption mutant of *fciB* in all light conditions examined (4) and the suppressor mutants grew at a rate that was very similar to the *fciB* mutant (Fig. S6A). In addition, all of the suppressor mutants had a higher level of PUB absorbance than the original *mpeY* mutant and were phenotypically indistinguishable from the *fciB* mutant (Fig. S6C). These data collectively suggested that the suppressor mutations interrupted the normal expression of *fciB*. Sequencing revealed a unique *fciB* mutation site in each of the three suppressor mutants examined, while no *fciB* mutation was present in control cells that were also passaged for 100 generations (Fig. 3A). Two of the suppressor mutants contained frameshift mutations in the genomic region encoding the N-terminal portion of FciB. One was due to a 5' TC 3' insertion (Suppressor 1) and the other to a 5' TC 3' deletion (Suppressor 3) in a region containing six 5' TC 3' repeats. Suppressor 2 contained a glycine to arginine missense mutation at residue 251 in the C-terminal portion of FciB, which is predicted to encode a helix-turn-helix DNA binding motif (7). Fluorescence excitation spectroscopy showed that the original *mpeY* mutant phenotype was restored after transformation of each suppressor strain with *fciA* and *fciB*, which previously complemented an *fciB* interruption mutant (4) (Fig. S7A), demonstrating that these *fciB* mutations caused the suppressor phenotypes.

Based on previous results showing that *mpeZ* transcript levels were constitutively high in both blue and green light in a *fciB* mutant (4), we tested the *mpeY*<sup>-</sup> suppressor mutants for

elevated *mpeZ* transcript levels during growth in white light. RNA-Seq data demonstrated that absolute *mpeZ* RNA abundance was much higher than *mpeY* RNA abundance in blue light but much lower in green light, and that neither was ever completely absent (Fig. S7B). RNA blot analyses showed that *mpeY* mutant cells had low levels of *mpeZ* transcripts, while both *mpeY* suppressor and *fciB* mutant cells had approximately 50-fold higher *mpeZ* transcript levels (Fig. 3C). Therefore, the *mpeY* suppressor mutants appear to modify the *mpeY* mutant growth phenotype in white light by inactivating *fciB*, leading to the elevation of the expression of *mpeZ*. Because MpeZ is known to attach PUB to MpeA-C83 (10), the phenotypes of these suppressor mutants strongly suggest that the relative expression of *mpeY* and *mpeZ* controls the attachment of PEB versus PUB.

### **Interplay between MpeZ and MpeY controls chromophorylation of the same cysteine during CA4**

Our discovery that *mpeY* suppressor mutants increased *mpeZ* expression led us to hypothesize that the MpeZ:MpeY ratio, driven by the relative expression levels of *mpeZ* and *mpeY*, controls whether PUB or PEB is attached at MpeA-C83 during CA4. We tested this by examining existing RNA-Seq data from *fciA* and *fciB* interruption mutants. As previously shown (4), the former is locked in CA4 green light phenotype and always has PEB at MpeA-C83, while the latter is locked in the CA4 blue light phenotype and always has PUB at MpeA-C83 (Fig. 4A, top). In addition, the *mpeZ*:*mpeY* expression ratio differed from the control in the *fciA* and *fciB* mutants, being high in the *fciB* mutant and low in the *fciA* mutant in both blue and green light (Fig. 4A, bottom). Thus, the *mpeZ*:*mpeY* mRNA expression ratio is correlated with the type of chromophore attached to MpeA-C83,

suggesting that this ratio has a major role in determining whether PUB or PEB is attached to MpeA-C83 during CA4.

This was directly tested by introducing an autonomously replicating plasmid expressing *mpeY* from its native promoter into wild-type cells. The *mpeY* RNA levels in this transformed line were 8-fold higher in green light and 14-fold higher in blue light than in wild type cells transformed with the empty vector (Fig. S7 C and D). Fluorescence emission spectra obtained from green-light-grown cells showed that PUB and PEB fluorescence spectra for the *mpeY* overexpression strain was indistinguishable from the empty vector control strain. However, for cells grown in blue light the *mpeY* overexpression strain exhibited a higher PEB to PUB fluorescence ratio than the empty vector control strain (Fig. 4, B and C). The simplest and most reasonable explanation for this result is that overproduced MpeY was outperforming MpeZ in blue light, leading to the addition of PEB instead of PUB at MpeA-C83. Therefore, we conclude that interplay between MpeZ and MpeY controls which of these two chromophores are attached at this residue during CA4.

### **Interplay between MpeY and MpeZ is widespread, suggesting a paradigm for pigment diversification in the marine environment**

Our data show that in *Synechococcus* sp. RS9916, a strain isolated from the Gulf of Aqaba in the Red Sea, MpeY and MpeZ both act on the same residue as part of CA4. To explore if interplay between MpeY and MpeZ is widespread, we examined 20 strains isolated from

seven regions around the globe that encode MpeY and/or MpeZ (Table S1), analyzing the phylogenetic relatedness of these enzymes (Fig. 5, *right*). Nine of these isolates encoded only MpeY, one isolate encoded only MpeZ, and 10 isolates encoded both MpeY and MpeZ (Fig. 5, *left*). While the single isolate encoding only MpeZ, BIOS-E4-1, was a blue light specialist (PUB rich), all nine isolates that only encode MpeY were green light specialists (PEB rich). Nine of the 10 strains, isolated from six different oceanic regions, encoded both MpeY and MpeZ and were blue-green generalists capable of CA4. The single exception, MVIR-18-1, was previously found to lack significant blue-green regulation of its *mpeZ* gene (7). From an evolutionary point of view, the phylogenetic analysis of these two enzymes (Fig. 5, *right*) shows that they derived from a common lyase ancestor and that after duplication, these proteins diverged structurally and functionally in a way that maintained attachment site specificity while diverging in the type of chromophore that each attached. Taken together, these findings support the widespread ability of two paralogous enzymes, the lyase MpeY and the lyase-isomerase MpeZ, to act in a concerted fashion on the same residue, thereby contributing to the pigment diversity observed in marine *Synechococcus*.

## Discussion

The successful expansion of *Synechococcus* throughout the lit zone of the world's oceans likely relied in part on the diversification of its photosynthetic light harvesting abilities. Blue light specialists, green light specialists, and blue-green generalists have evolved to incorporate an optimal combination of chromophores in their PBS to efficiently utilize

solar energy in all light color niches encountered in the underwater environment. Specific energy absorption and transfer characteristics of each type of PBS are determined by which chromophore is added at each cysteine attachment site throughout the light-harvesting structure. These additions are catalyzed by phycobilin lyases and lyase-isomerases, making this a pivotal group of enzymes for successful light color adaptation of *Synechococcus* in the marine environment. Marine *Synechococcus* strains have up to 14 genes per genome that are predicted to encode phycobilin lyases that attach chromophores to distinct cysteine residues within the apoproteins of the PBS, although it is yet to be determined how specificity is achieved between each lyase and its cognate residue (10, 13, 14). Our finding that MpeY and MpeZ attach different chromophores to the same residue increases our understanding of lyase residue specificity and provides additional insights into how different chromophores are attached. The MpeY overexpression experiment, which resulted in an increase of PEB:PUB ratio (Fig 4B), strongly suggests that the relative proportion of the MpeY and MpeZ enzymes in 9916 cells determines which chromophore, PEB or PUB, is bound to MpeA-C83. This site binds PUB in green-light grown *mpeY* mutant (Fig 2F), demonstrating that MpeZ is present under these conditions. Therefore, in wild-type cells grown in green light, even though the *mpeZ* transcript level is 6-times lower than in blue light (Shukla et al. 2012), MpeZ must be present, but outcompeted by MpeY. The *mpeY* and *mpeZ* genes co-occur in many *Synechococcus* strains and, in most case, this results into the ability for these cells to perform CA4 (Fig. 5). The only isolate (MVIR-18-1) that has both *mpeY* and *mpeZ* but is not a blue-green specialist provides additional support of our model because it has been shown that there is little to no blue-green light regulation of *mpeZ* gene expression in MVIR-18-1, making this strain unable to carry out

normal CA4 (7). The only strain (BIOS-E4-1) that has *mpeZ* but not *mpeY* is a blue light specialist and also does not differentially express its *mpeZ* gene (7). As recently suggested (26), BIOS-E4-1 is likely to have previously possessed both *mpeZ* and *mpeY*, then lost *mpeY* and the light color regulation of *mpeZ*, leading to a blue light specialist phenotype. Interestingly, the BIOS-E4-1 genotype is similar to the mutants that arose in our *mpeY* suppressor screen, where inactivating *mpeY* led to more rapidly growing mutants (Fig. S6) that lost CA4 by expressing *mpeZ* at blue light levels even in green light conditions (Fig. 3C). BIOS-E4-1 also lacks *fciA* and *fciB*. The loss of these CA4 regulators might have been the next evolutionary step after the inactivation of *mpeY* and the generation of a *fciB* suppressor mutant. The inactivation of *fciB* would have made FciA useless, leading to eventual loss of *mpeY*, *fciB*, and *fciA* from the genome of these mutants. Thus, our inactivation of *mpeY* may have initiated a series of genetic changes that parallel an evolutionary process that is occurring in the oceans. In the natural environment, such BIOS-E4-1-like genotypes seem to predominate in some permanently iron-poor areas such as the tropical South Pacific Ocean (26).

The evolution of a light-color-regulated process in which two enzymes vie for the attachment of two different chromophores to the same site is an innovative, efficient way to add flexibility to light harvesting capability in photosynthetic organisms. This system, which involved the duplication of the common ancestor of MpeY and MpeZ, the divergence of their expression and functions, but the retention of their site of action (Fig. 5), may serve as a paradigm for how phenotypic plasticity of pigmentation is widely generated with paralogous genes in marine *Synechococcus*. Because CA4 has evolved

more than once and appears in approximately 40% of all *Synechococcus* cells in the ocean (26), the capacity to alter light harvesting clearly provides strains with a distinct evolutionary advantage.

The interplay between MpeY and MpeZ is an outstanding example of how horizontally acquired gene products interact with components encoded in the core genome to confer a novel trait, such as CA4. Although it has been previously established that horizontal acquisition of genomic islands can drive the evolution of novel traits in marine *Synechococcus* (28-30), precisely how these events influence the subsequent phenotype of the organism is still not well understood (31).

In summary, we have discovered that interplay between the lyase MpeY and lyase-isomerase MpeZ controls the attachment of two different light harvesting chromophores, each absorbing a different wavelength, to the same residue of a light harvesting protein in marine *Synechococcus*. Despite their evolutionary relatedness, MpeY attaches PEB and MpeZ attaches PUB, and in strains containing both *mpeY* and *mpeZ*, the *mpeY*: *mpeZ* expression ratio determines if PEB or PUB is attached. Additionally, isolates with only *mpeY* or *mpeZ* are incapable of CA4. We conclude that the interplay between MpeY and MpeZ is important for CA4 in marine *Synechococcus* and hypothesize that other forms of this interplay occur between other paralogous lyase/lyase-isomerase pairs in this important group. In addition to furthering our basic understanding of a globally important marine picophytoplankton, the previously unidentified ability of two enzymes to vie for

attachment of spectrally different chromophores to the same cysteine residue will provide new tools for further development of multi-color fluorescence labeling technology.

## **Materials and methods**

**Strains and growth conditions.** Growth conditions were as previously described (10). Details are provided in Supporting Information. Wild type cells were genetically unaltered *Synechococcus* sp. RS9916 cells (32) and control cells were wild type cells containing a mini-Tn5 transposon insertion (33). This insertion did not affect CA4 and provided comparable kanamycin resistance to the *mpeY* interruption mutant used in the study. A Biotek Synergy-MX spectrofluorometer was used to generate fluorescence spectra as described (10).

***In vivo* heterologous expression and purification of recombinant proteins.** Plasmid constructs are provided in Supporting Information. Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) as previously described (16, 23). Details are provided in Supporting Information. Cell lysis, centrifugation and protein purification were conducted as previously described (23).

**Protein analysis by spectroscopy and gel-electrophoresis.** Most polypeptide samples were resolved by polyacrylamide gel electrophoresis (PAGE, 15% w/v) in the presence of sodium dodecyl sulfate (SDS) and visualized as previously described (23).

***mpeY* disruption/complementation/overexpression.** Transformation of *Synechococcus* sp. RS9916 by conjugation was performed as previously described (4, 10, 34). Details are provided in Supporting Information.

**HPLC and MS-MS analysis of phycobiliproteins.** Phycobilisomes were purified as described (10, 35). HPLC was used to separate each phycobiliprotein subunit and samples were digested with trypsin as described previously (10, 23). HPLC separated and trypsin-digested phycobiliprotein subunits were analyzed by UV-VIS and MS/MS as described (10).

**Suppressor analyses.** To select for *mpeY* mutant suppressors, three independent *mpeY* mutant cell lines were grown in white light for approximately 100 generations. Details are provided in Supporting Information.

**RNA analyses.** RNA sequencing data were further analyzed from previous experiments (4), except that instead of values for the blue light control being set to 100 and values normalized across each replicate separately, the raw values were averaged. RNA blots were performed as previously described (4, 36). Values were normalized as previously described (4, 36). Details are provided in Supporting Information.

**Comparative genomics and phylogenetic analyses.** Data on *mpeY* and *mpeZ* gene content were retrieved from the *Synechococcus* genomes described in Table S1 using the Cyanorak v2 database ([www.sb-roscoff.fr/cyanorak](http://www.sb-roscoff.fr/cyanorak)). Phylogenetic reconstructions were

performed using Maximum likelihood (ML; PhyML v3.0), Neighbor Joining (NJ, Phylip 3.69), and Bayesian inference (BI; MrBayes v3.1.2) as previously described (7). The ML tree with all bootstrap or posterior probability values from the different methods was then manually refined using Archaeopteryx Version 0.9901 (37).

## Acknowledgments

We thank members of the Kehoe laboratory, especially Lisa Wiltbank, as well as Andrian Gutu, Jeff Palmer, and Jake McKinlay for helpful discussions and comments. This research was supported by National Institutes of Health Training Grant to J.E.S. (T32-GM007757), National Science Foundation Grants to D.M.K. (MCB-1029414 and MCB-1818187) and to W.M.S. (MCB-1244339), and by the Office of the Vice Provost for Research at Indiana University, Bloomington, through its Bridge Funding Program to D.M.K. and by the French Agence Nationale de la Recherche Programs SAMOSA (ANR-13-ADAP-0010) for F.P. and L.G.

## Figure Legends

**Fig 1.** MpeY controls PEB attachment in green light. (A) *mpeY* is located in the PE-II (*mpeBA*) genomic region. Fluorescence excitation spectra, with emission set at 580 nm and normalized to PUB at 495 nm, of control and *mpeY* mutant cells grown in (B) blue or (C) green light. Whole cell absorbance spectra of control and *mpeY* mutant cells grown in (D) blue or (E) green light. Control cell results shown by black lines and *mpeY* mutant cell

results shown by blue or green lines, which were all normalized to the chlorophyll absorbance peaks at 440 and 680 nm. Fluorescence and absorbance spectra are averages of three independent replicates.

**Fig 2.** MpeY attaches PEB to MpeA-C83. Absorbance spectra of purified MpeA protein from control (black lines) and *mpeY* mutant cells (blue or green lines) grown in (A) blue or (B) green light. MpeA peptides containing C75 (C and D), C83 (E and F), or C140 (G and H) from control (C, E, and G) or *mpeY* mutant (D, F, and H) cells grown in green light. Red italic: chromophore difference between control and *mpeY* mutant. Spectra are representative of three independent replicates. (I) Zinc-enhanced fluorescent gel showing PEB or PUB covalently bound to HT-MpeA from *E. coli* under various coexpression conditions (shown on bottom). Representative of three replicates.

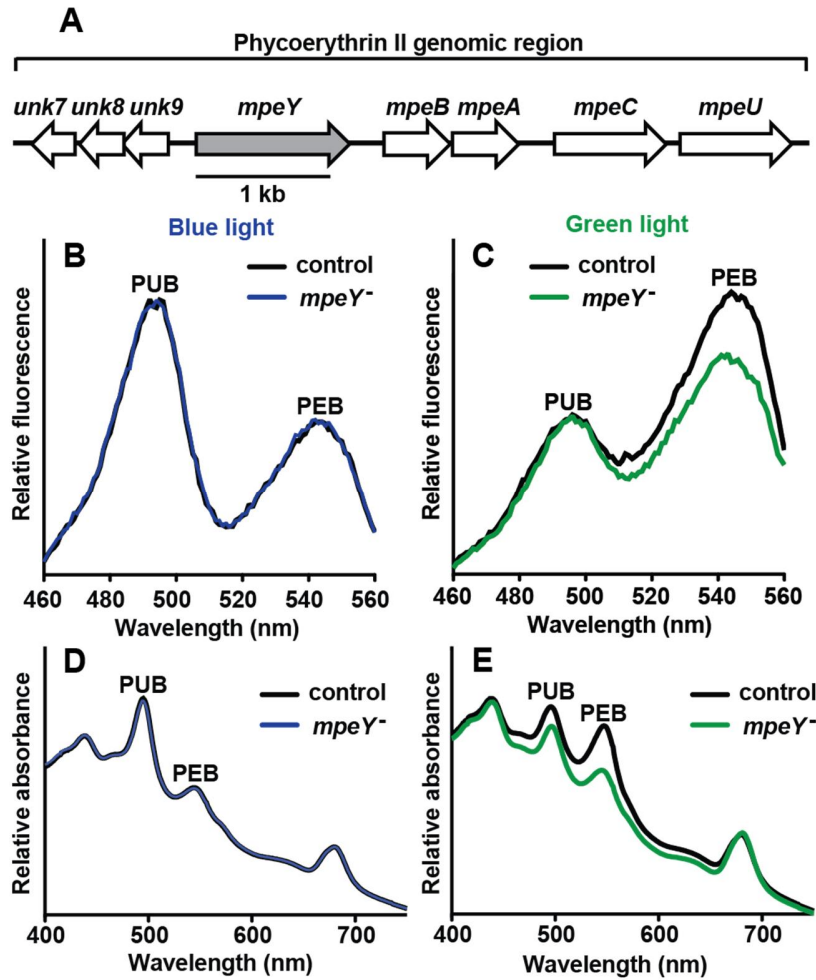
**Fig 3.** *mpeY*<sup>-</sup> suppressor mutations increase *mpeZ* expression. (A) *mpeY*<sup>-</sup> suppressor mutations (S1-S3) are located in *fciB*, within the CA4 genomic island. S1 is a deletion of two base pairs and S3 is an insertion of two base pairs, which both are frameshift mutations. S2 is a missense mutation changing glycine to arginine at residue 251. (B) Fluorescence excitation spectra, with emission set at 580 nm, of control (gray line), *mpeY* mutant (black line), *mpeY* mutant suppressor mutants S1-S3 (purple lines), and *fciB* mutant (orange lines) cells grown in white light, in which cells have the same pigmentation phenotype as when grown in green light. Fluorescence spectra are an average of three independent replicates. (C) Mean *mpeZ* transcript levels from *mpeY* mutant, *mpeY*<sup>-</sup> suppressor mutants S1-S3, and *fciB* mutant cells grown in white light. Values from *mpeY* mutant cells were set to 1 after

rRNA normalization. Transcript levels for the *mpeY* and *fciB* mutants are averages of three independent replicates with error bars showing the SEM. Transcript levels for the *mpeY* mutant suppressor mutants S1-S3 are the combined average of one independent replicate each of S1, S2, and S3.

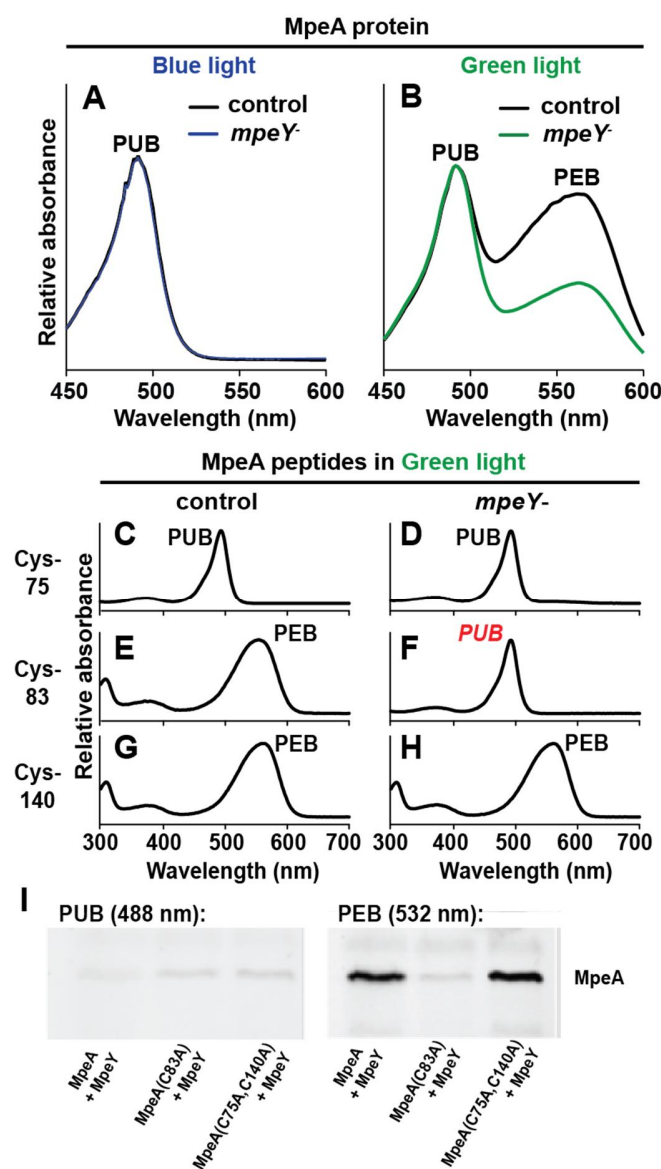
**Fig 4.** The ratio of transcripts encoding MpeY and MpeZ determines the chromophorylation state of MpeA-C83. (A) Summary of bilins attached to MpeA-C83 in different light colors and genetic backgrounds (4). Transcript level ratios from RNA-sequencing of *mpeZ* and *mpeY* in control, *fciA* mutant, and *fciB* mutant cells grown in blue (blue bars) or green (green bars) light. Error bars show the SEM of three independent replicates for each strain and light condition. Fluorescence excitation spectra, with emission set at 580 nm and normalized to PUB at 495 nm, of wild type cells with either empty vector (black lines) or a vector overexpressing *mpeY* (indicated by *mpeY*<sup>++</sup> with blue or green lines) grown in (B) blue or (C) green light. Fluorescence spectra are averages of three independent replicates.

**Fig 5.** Interplay between MpeY and MpeZ is conserved throughout *Synechococcus* isolates capable of CA4. (Left) Phycobilisome pigment phenotype of 20 *Synechococcus* isolates and corresponding presence of *mpeY* (red) and/or *mpeZ* (yellow) in their genomes. (Right) Maximum Likelihood (ML) analysis of MpeY and MpeZ using the distantly related phycobilin lyase CpeY as a root (23). Colored circles indicate the pigment phenotype (see insert). Series of three numbers at nodes correspond to bootstrap values for ML and Neighbor-Joining analyses and Bayesian posterior probabilities (PP, ranging between 0

and 1). Only values higher than 60% for bootstrap values and 0.60 for PP are shown on the phylogenetic tree. Underline denotes *Synechococcus* sp. RS9916, the strain used throughout this study.

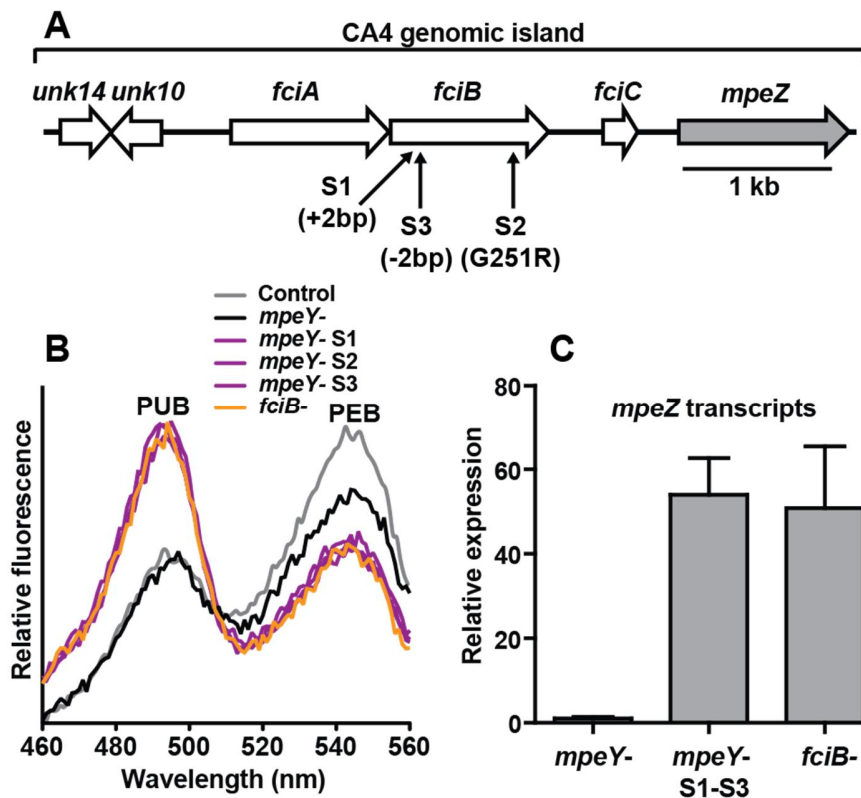


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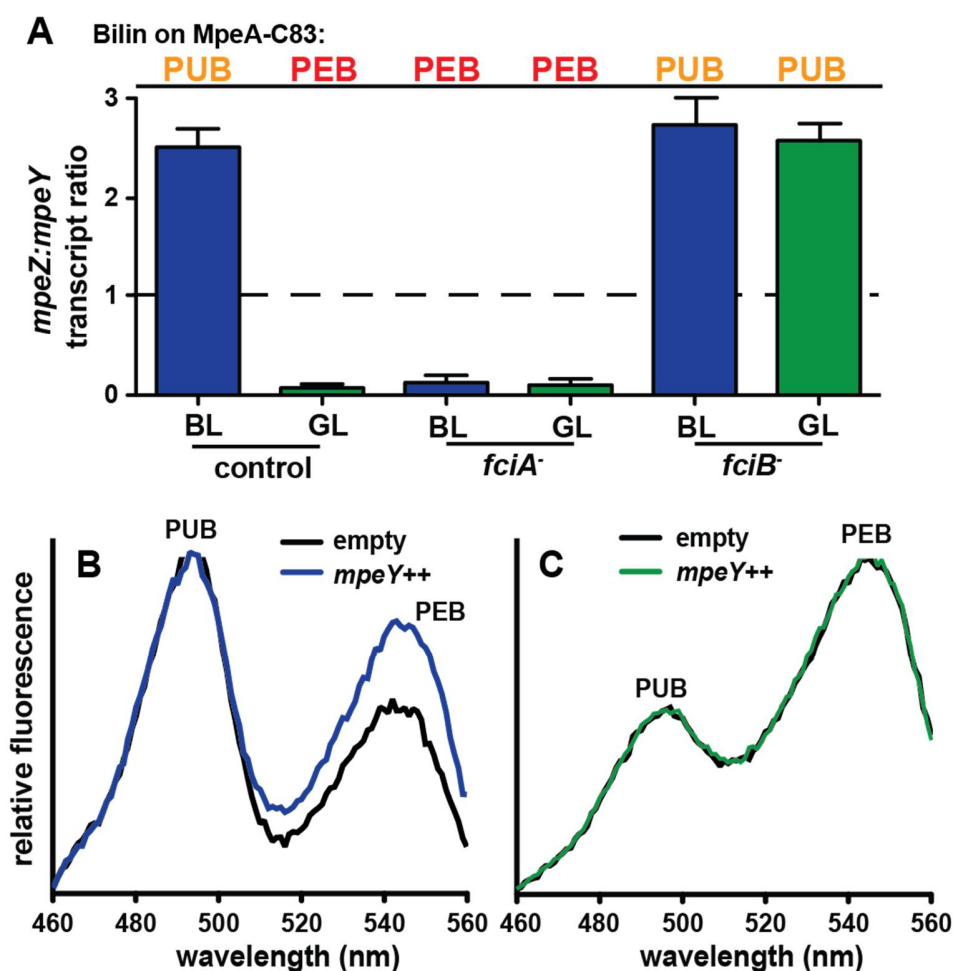


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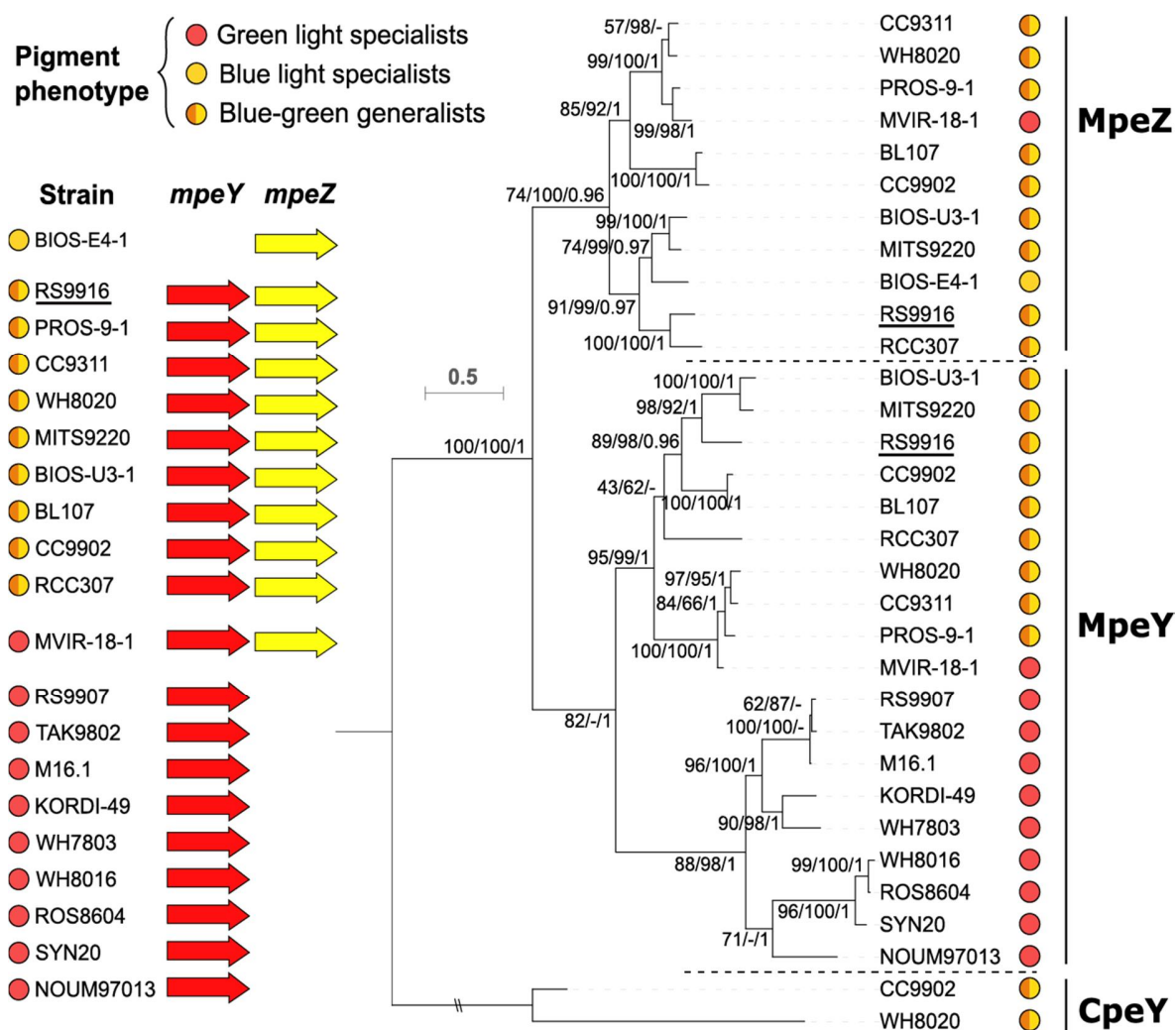
conditions (shown on bottom). Representative of three replicates.



**Fig 3.** *mpeY*- suppressor mutations increase *mpeZ* expression. (A) *mpeY*- suppressor mutations (S1-S3) are located in *fciB*, within the CA4 genomic island. S1 is a deletion of two base pairs and S3 is an insertion of two base pairs, which both are frameshift mutations. S2 is a missense mutation changing glycine to arginine at residue 251. (B) Fluorescence excitation spectra, with emission set at 580 nm, of control (gray line), *mpeY*- mutant (black line), *mpeY*- mutant suppressor mutants S1-S3 (purple lines), and *fciB*- mutant (orange lines) cells grown in white light. Fluorescence spectra are an average of three independent replicates. (C) Mean *mpeZ* transcript levels from *mpeY*- mutant, *mpeY*- suppressor mutants S1-S3, and *fciB*- mutant cells grown in white light. Values from *mpeY*- mutant cells were set to 1 after rRNA normalization. Transcript levels for the *mpeY*- and *fciB*- mutants are averages of three independent replicates with error bars showing the SEM. Transcript levels for the *mpeY*- mutant suppressor mutants S1-S3 are the combined average of one independent replicate each of S1, S2, and S3.



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PP are shown on the phylogenetic tree. Underline denotes *Synechococcus* sp. RS9916, the strain used throughout this study.

## Supporting Information

### Supporting Methods

**Strains and Growth Conditions.** Semi-continuous cultures were grown in polycarbonate culture flasks at 22°C in PCR-S11 media in constant light at an irradiance of 10  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  from fluorescent white light bulbs unless noted (Chroma 75 T12; General Electric). Filters (LE716 Mikkell Blue and LE738 Jas Green; LEE Filters) were used to generate blue and green light. When necessary, 50  $\mu\text{g mL}^{-1}$  kanamycin and/or 30  $\mu\text{g mL}^{-1}$  spectinomycin was added to cultures. Absorbance spectra were produced using a Beckman DU640B spectrophotometer.

**Plasmid construction.** The plasmids and primers used are listed in Table S2. pJASmpeY was made by PCR amplification and cloning of a 750 bp internal region of *mpeY* into the BamHI site of pMUT100. pJS9 was made by PCR amplification and cloning of the promoter and ribosome-binding site of *mpeY* into the BamHI and AseI sites of pJS1. For the creation of expression constructs used in this study, each gene was amplified by PCR amplification from *Synechococcus* sp. RS9916 DNA using the primers listed in Table S2. All amplified DNA and cloning junctions were checked by sequencing.

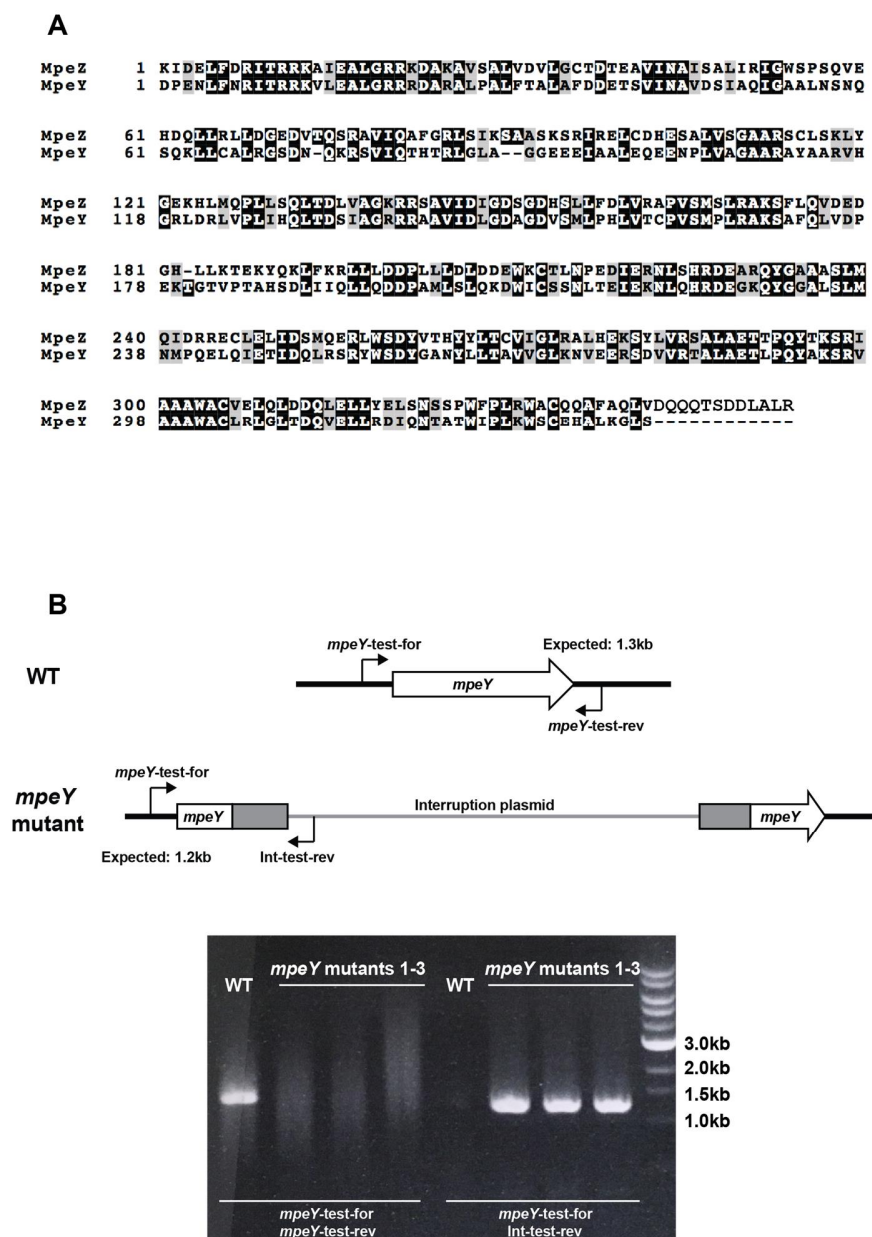
***In vivo* heterologous expression of recombinant proteins.** The induced cultures expressing PEB synthesis proteins were placed at 18°C for approximately 24 h at 220 rpm. Cells were harvested by centrifugation at 11,000  $\times g$  for 10 min and moved to storage at -20°C. Cell pellets were resuspended in Buffer O (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl), 0.1x complete mini protease inhibitor cocktail and 0.01  $\text{mg mL}^{-1}$  lysozyme

in volumes based on the relative mass of the pellets. The largest pellet was resuspended in a maximum volume of 15 mL of buffer.

***mpeY* disruption/complementation/overexpression.** Individual colonies were picked from plates and tested by PCR amplification and DNA sequencing. All transformed cells were maintained with 50  $\mu\text{g L}^{-1}$  kanamycin and/or 30  $\mu\text{g L}^{-1}$  spectinomycin when grown in liquid culture. *mpeY* disruption was generated by transformation of pJASmpeY and *mpeY* complementation and overexpression lines were generated by transformation of pJS9. A list of strains is provided in Table S2.

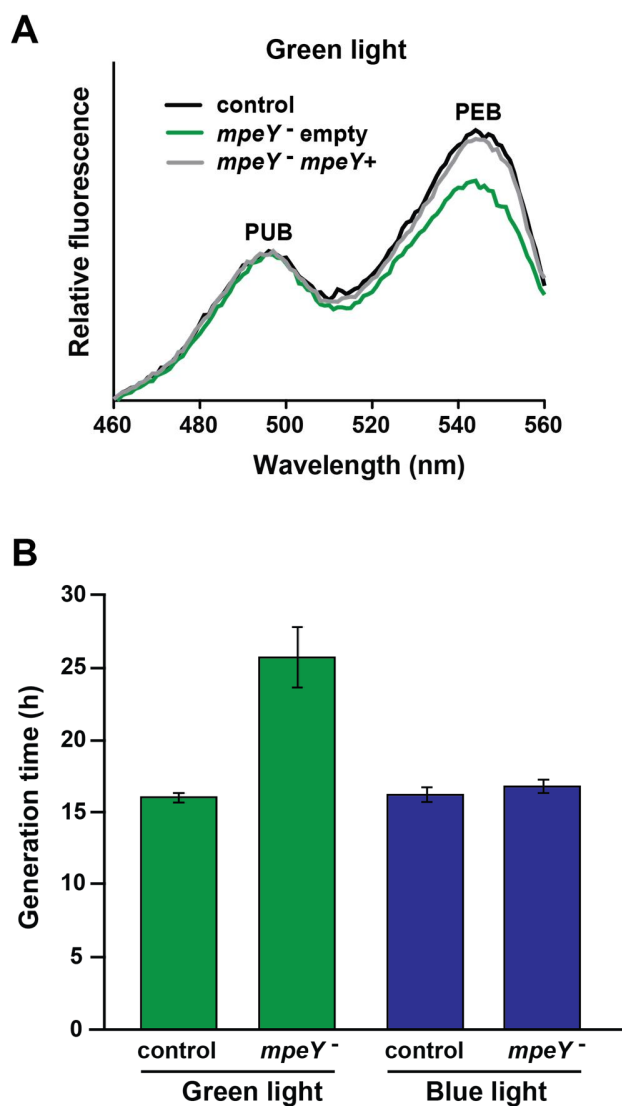
**Suppressor analyses.** Cultures were plated and individual colonies were picked and tested by PCR amplification to confirm interruption of *mpeY*. PCR amplification and sequencing were used to identify spontaneous mutations in *fciB*. pJS3 was transformed into suppressor mutant lines to complement the *fciB* mutations. Generation time was calculated as the  $\log_2$  of the change in whole cell absorbance at 750 nm over time during the linear range of growth.

**RNA Analyses.** Cells were acclimated to blue or green light for at least 7 days, RNA was purified, and libraries were generated using an Epicentre (Illumina) ScriptSeq complete bacteria kit. Normalized reads for *mpeZ* and *mpeY* were calculated using custom Perl scripts. For RNA blots, 10  $\mu\text{g}$  of RNA was added per lane. Blots were probed with DNA probes to *mpeY* or *mpeZ* generated by PCR amplification and radiolabeling, using primers mpeY-probe-for and rev and mpeZ-probe-for and rev (shown in Table S2).

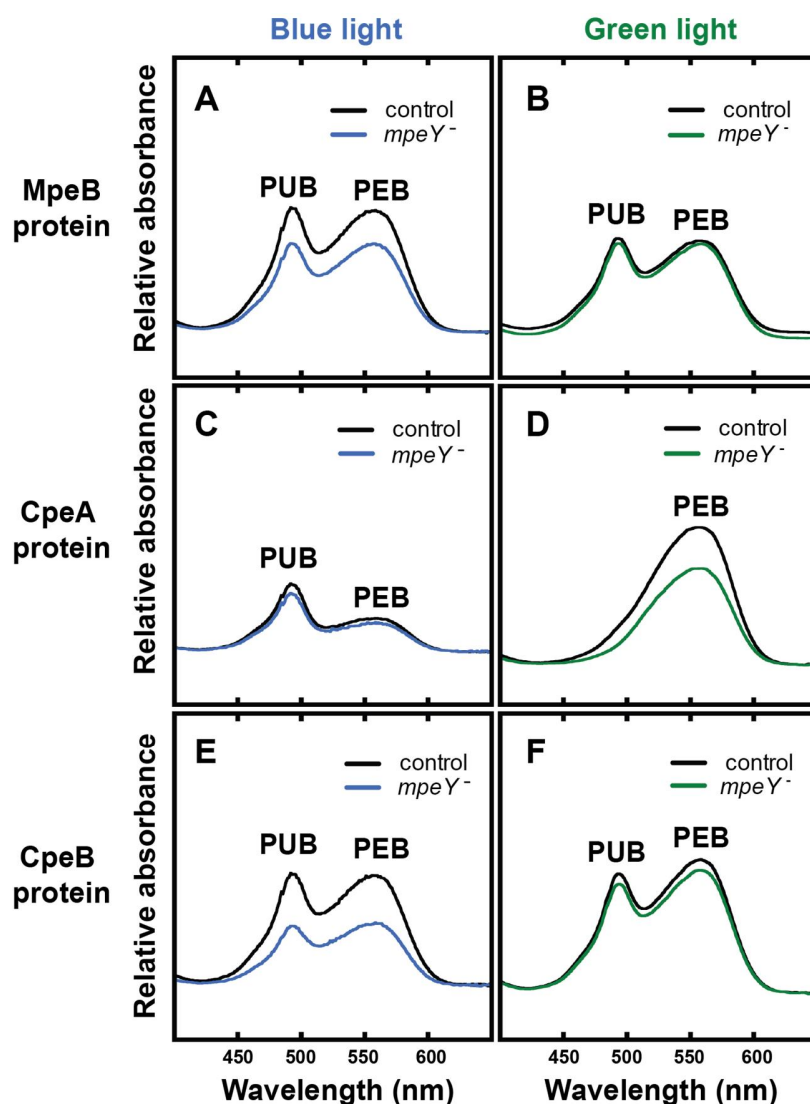


**Fig S1.** Sequence comparison of MpeY and verification of the *mpeY* mutant. (A) Alignment of MpeY and MpeZ sequences from *Synechococcus* sp. RS9916 using Clustal Omega (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3261699/>). Identical residues are labeled in black and similar residues are labeled in gray. (B) Schematic of *mpeY* interruption and PCR verification of three independent *mpeY* mutants. Bent arrows mark the sites and

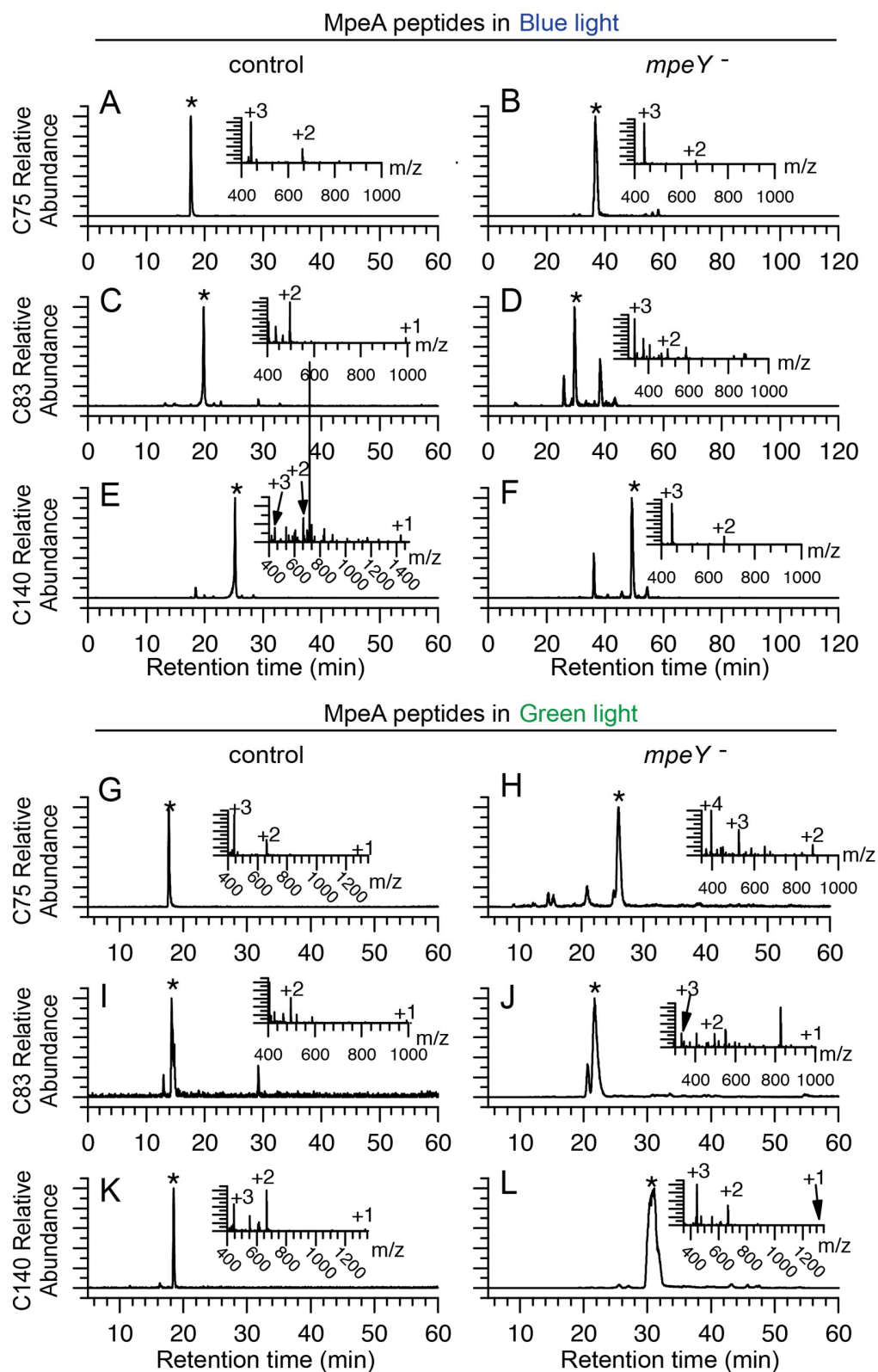
directions of priming for PCR amplification reactions.



**Fig S2.** Complementation and growth rate analysis of the *mpeY* mutant. (A) Fluorescence excitation spectra, with emission set at 580 nm, of control cells (black line), *mpeY* mutant cells with empty vector (green line), and *mpeY* mutant cells with vector containing *mpeY* (gray line) grown in green light. Fluorescence spectra are averages of three independent replicates. (B) Generation time, in hours, for control and *mpeY* mutant cells grown in green or blue light. Generation times are averages of three independent replicates with error bars showing the SEM. The difference between the mean values for green light grown cells was significant (P value < 0.05).

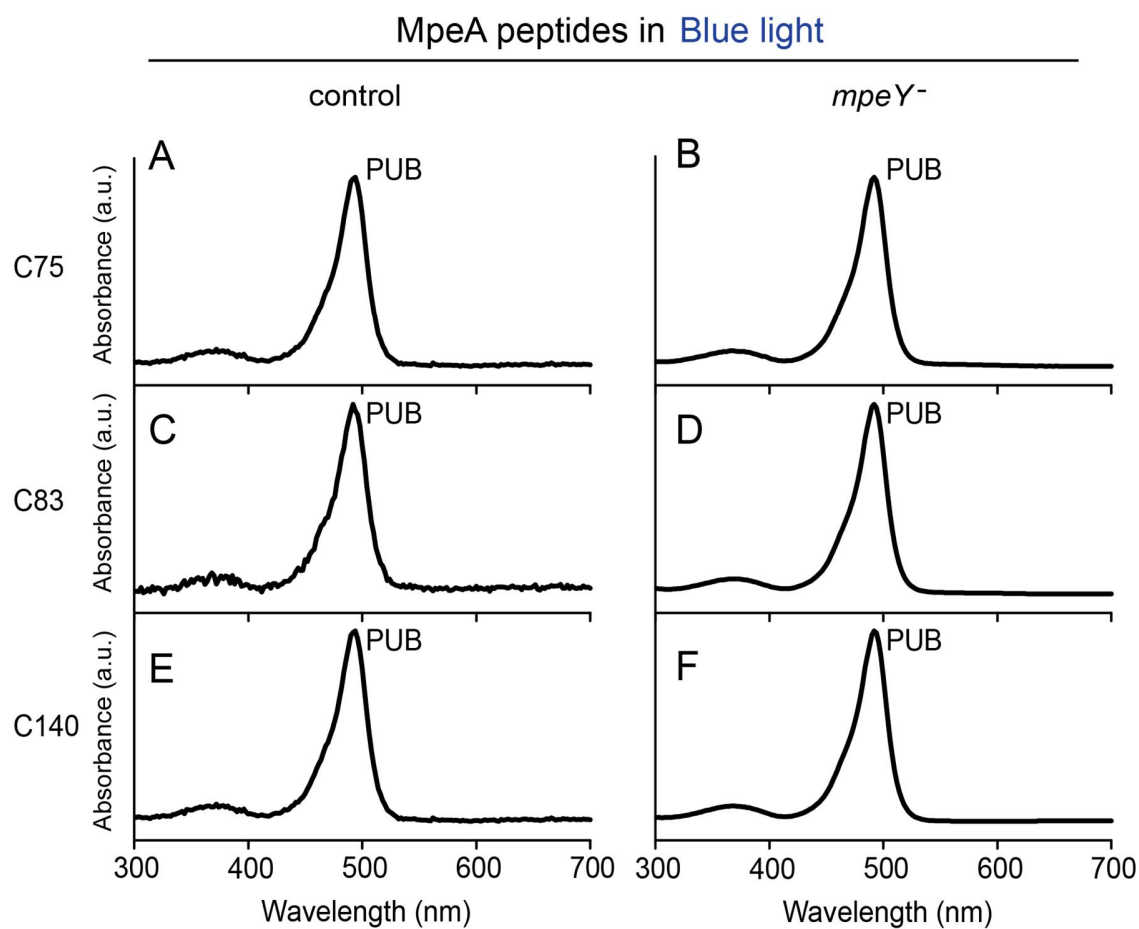


**Fig S3.** MpeY does not affect MpeB, CpeA, or CpeB chromophorylation. Absorbance spectra of (A, B) MpeB, (C, D) CpeA, or (E, F) CpeB purified from control cells (black lines) and *mpeY* mutant cells (blue or green lines) grown in (A, C, and E) blue or (B, D, and F) green light. PUB absorbs at ~495 nm and PEB absorbs at ~550 nm. Spectra are representative of three independent replicates. The amplitudes of absorbance values of independent spectra cannot be compared quantitatively. Only the presence/absence of any particular chromophore and the relative absorbance ratios within a spectrum can be quantitatively compared with another spectrum.

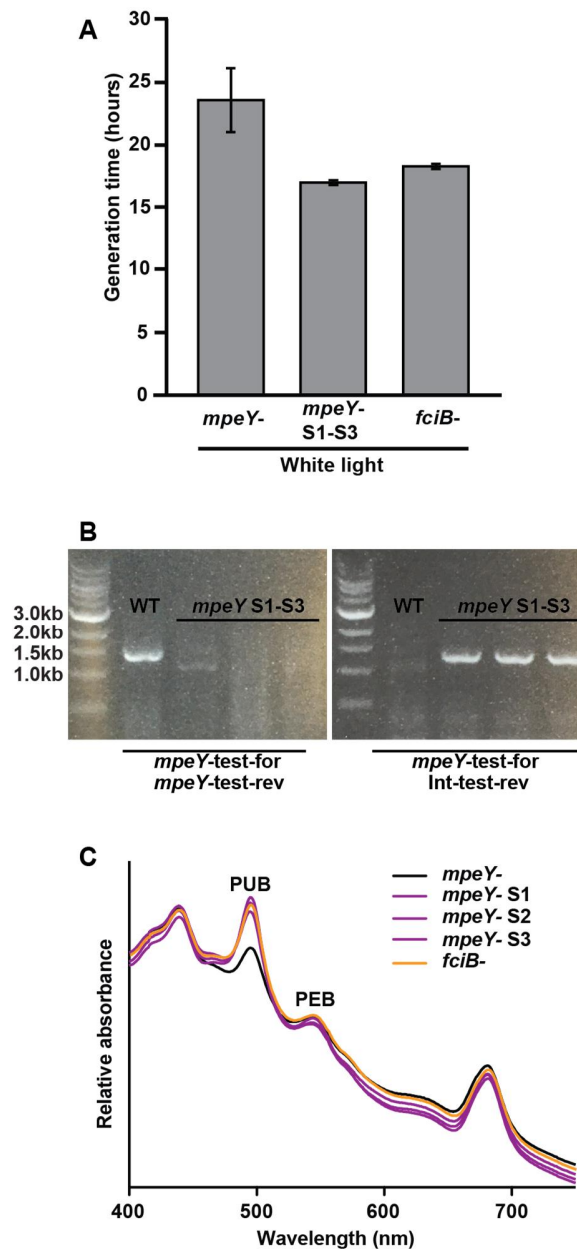


**Fig S4.** HPLC-MS data from tryptic digests of MpeA indicating the chromophore

attached to (A, C, E, G, I, and K) C75, C83, and C140 from control and (B, D, F, H, J, and L) *mpeY* mutant cells grown in (A-F) blue and (G-L) green light. Table S3 contains the exact retention times, m/z ratios, and visible absorption maxima for each peptide.

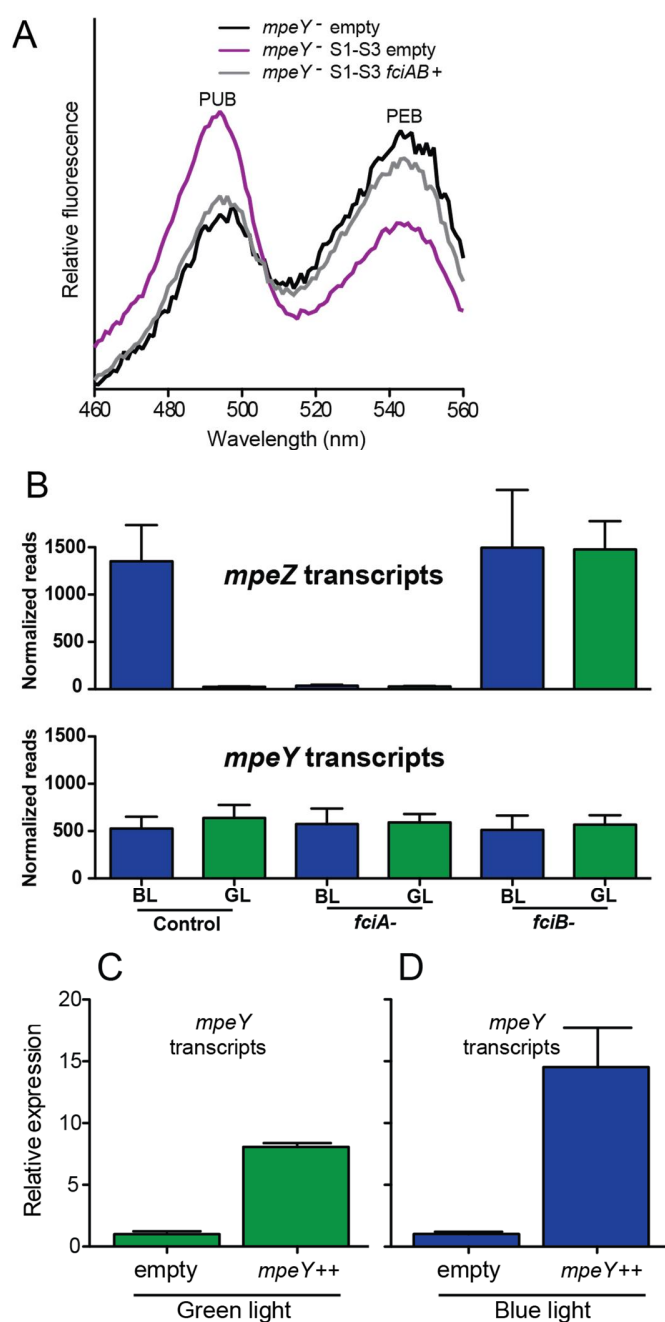


**Fig S5.** MpeY does not affect MpeA chromophorylation in blue light. Absorbance spectra of MpeA peptides containing (A and B) C75, (C and D) C83, or (E and F) C140 from (A, C, and E) control or (B, D, and F) *mpeY*<sup>-</sup> cells grown in blue light.



**Fig S6.** *mpeY*<sup>-</sup> suppressor mutant analyses. (A) Generation time for *mpeY* mutant, *mpeY*<sup>-</sup> suppressor mutants S1-S3, and *fciB* mutant cells grown in white light. Generation times are averages of three independent replicates with error bars showing the SEM, except for the generation time for S1-S3, which is the average of three independent replicates each of S1, S2, and S3. The growth rates of the suppressor mutants were indistinguishable from one another. *mpeY*<sup>-</sup> and *mpeY*<sup>-</sup> S1-S3 values are statistically different with a P-value

$< 0.05$ . (B) PCR amplification check of the three independent *mpeY*<sup>-</sup> suppressor mutants for continued interruption of *mpeY*. (C) Whole cell absorbance spectra of the *mpeY*<sup>-</sup> mutant (black line), three *mpeY*<sup>-</sup> suppressor mutants (purple lines), and *fciB* mutant (orange line) cells after growth in white light. Each absorbance spectrum is an average of three independent replicates.



**Fig S7.** Complementation of *mpeY*<sup>-</sup> suppressor mutants and quantitation of *mpeY*<sup>++</sup> overexpression. (A) Fluorescence excitation spectra of *mpeY*<sup>-</sup> mutant cells with empty vector (black line), *mpeY*<sup>-</sup> suppressor mutants S1-S3 with empty vector (purple line) and *mpeY*<sup>-</sup> suppressor mutants S1-S3 containing a vector expressing *fciA* and *fciB* (gray line)

grown in white light. (B) Mean transcript levels, measured by RNA-Seq, of *mpeZ* and *mpeY* in blue and green light in wild type control cells and the *fciA* and *fciB* mutants.

Mean transcript levels, measured by RNA blot analysis, of *mpeY* in wild type cells transformed with either an empty vector (empty) or a vector containing *mpeY* (*mpeY*++) grown in either (C) green or (D) blue light. Values from cells containing the empty vector were set to 1 after rRNA normalization. Error bars are the SEM from three independent replicates.

**Table S1.** Description of strains used in Figure 5

Strain_Name	RCC*	Subcluster	Clade	Pigment_Type	Sea or Ocean	Region	References
KORDI-49	-	5.1	WPC1	green-light specialist	North Pacific Ocean	East China Sea	(26)
M16.1	791	5.1	II	green-light specialist	North Atlantic Ocean	Gulf of Mexico	(26)
NOUM97013	2433	5.1	VII	green-light specialist	South Pacific Ocean	-	(26)
ROS8604	2380	5.1	I	green-light specialist	North Atlantic Ocean	English Channel	(26)
RS9907	2382	5.1	II	green-light specialist	Red Sea	Gulf of Aqaba	(26)
SYN20	2035	5.1	I	green-light specialist	North Sea	-	(26)
TAK9802	2528	5.1	II	green-light specialist	South Pacific Ocean	Takapoto atoll	(2)
WH7803	28	5.1	V	green-light specialist	North Atlantic Ocean	Sargasso Sea	(2)
MVIR-18-1	2385	5.1	I	green-light specialist	North Sea	-	(7)
WH8016	2535	5.1	I	CA4-A	North Atlantic Ocean	Woods Hole	(7)
BIOS-U3-1	2533	5.1	CRD1	CA4-A	South Pacific Ocean	Chile upwelling	(7)
BL107	515	5.1	IV	CA4-A	Mediterranean Sea	Spanish coast	(7)
CC9311	1086	5.1	I	CA4-A	North Pacific Ocean	California Current	(7)
CC9902	2673	5.1	IV	CA4-A	North Pacific Ocean	California Current	(7)
						Equatorial Pacific Ocean	
MIT9220	2571	5.1	CRD1	CA4-A	Pacific Ocean		(7)
PROS-9-1	328	5.1	Ib	CA4-A	Mediterranean Sea	-	(7)
RS9916	555	5.1	IX	CA4-A	Red Sea	Gulf of Aqaba	(7)
WH8020	2437	5.1	I	CA4-A	North Atlantic Ocean	-	(7)
RCC307	307	5.3		CA4-A**	Mediterranean Sea	-	(7)
BIOS-E4-1	2534	5.1	CRD1	blue-light specialist	South Pacific Ocean	South East Pacific	(7)

, Roscoff Culture Collection Number

\*\*This strain does perform CA4 but the amplitude of variation of the PUB:PEB is lower than in other CA4-A strains (7)

**Table S2.** List of strains, plasmids, and primers used

Strain	Description
WT	<i>Synechococcus</i> sp. RS9916, isolated from the Red Sea
Control	Kan <sup>R</sup> , mini-Tn5 insertion in uncharacterized gene RS9916_32112
WT pJS1	Spec <sup>R</sup> , contains autonomously replicating vector
WT pJS9	Spec <sup>R</sup> , contains vector expressing <i>mpeY</i>
<i>mpeY</i>	Kan <sup>R</sup> , plasmid insertion disrupting <i>mpeY</i>
<i>mpeY</i> pJS1	Kan <sup>R</sup> , Spec <sup>R</sup> , contains autonomously replicating vector
<i>mpeY</i> pJS9	Kan <sup>R</sup> , Spec <sup>R</sup> , contains vector expressing <i>mpeY</i>
<i>mpeY</i> S1	Kan <sup>R</sup> , TC deletion in 5' end of <i>fciB</i>
<i>mpeY</i> S2	Kan <sup>R</sup> , G251R missense mutation in <i>fciB</i>
<i>mpeY</i> S3	Kan <sup>R</sup> , TC insertion in 5' end of <i>fciB</i>
<i>mpeY</i> S1 pJS1	Kan <sup>R</sup> , Spec <sup>R</sup> , contains autonomously replicating vector
<i>mpeY</i> S2 pJS1	Kan <sup>R</sup> , Spec <sup>R</sup> , contains autonomously replicating vector
<i>mpeY</i> S3 pJS1	Kan <sup>R</sup> , Spec <sup>R</sup> , contains autonomously replicating vector
<i>mpeY</i> S1 pJS3	Kan <sup>R</sup> , Spec <sup>R</sup> , contains vector expressing <i>fciA</i> and <i>fciB</i>
<i>mpeY</i> S2 pJS3	Kan <sup>R</sup> , Spec <sup>R</sup> , contains vector expressing <i>fciA</i> and <i>fciB</i>
<i>mpeY</i> S3 pJS3	Kan <sup>R</sup> , Spec <sup>R</sup> , contains vector expressing <i>fciA</i> and <i>fciB</i>
Plasmid	Description
pMUT100	Kan <sup>R</sup> suicide vector used for homologous recombination
pRL528	Helper plasmid, carries <i>mob</i>
pRK24	Conjugal plasmid, RK2 derivative
pJASmpeY	pMUT100 derivative, for disruption of <i>mpeY</i>
pJS1	Spec <sup>R</sup> , autonomously replicating in RS9916
pJS3	pJS1 derivative, for expression of <i>fciA</i> and <i>fciB</i>
pJS9	pJS1 derivative, for expression of <i>mpeY</i>
pMpeA	pCOLA Duet; produces 9916 HT-MpeA
pMpeZ	pCDF Duet; produces 9916 NT-MpeZ
pMpeA:C83A	pCOLADuet; produces 9916 HT-MpeA(C83A)
pMpeA:C75,140A	pCOLADuet; produces 9916 HT-MpeA(C75,140A)
pMpeY	pET(44b); produces 9916 Nus-MpeY
pPcyA	pACYCDuet-1; produces Ho1 and HT-PcyA
pPebS	pACYCDuet-1; produces Ho1 and NT-PebS
Primer	Sequence (5' to 3')
Mut-BamHI- <i>mpeY</i> -for	TGCTGGAAGCACTGGGGAG
Mut-BamHI- <i>mpeY</i> -rev	ATCGGATCCCAACGTTCTTCAGCCCAACAACCTGC
<i>mpeZ</i> -probe-for	TTTTGGGCTGCACCGATACT
<i>mpeZ</i> -probe-rev	ACGATGGCTCAGATTTTCGCT
<i>mpeY</i> -probe-for	GCCAGAAGCTTCTTTGTGCACTCC

<i>mpeY</i> -probe-rev	GAAGTGAAAGCATTGCGGGGTCAT
Exp-BamHI- <i>mpeY</i> -for	ACTGGATCCGTTGCCTGCTCCAAAGGGATCACA
Exp-AseI- <i>mpeY</i> -rev	CACATTAATTTATGACAAGCCTTTAAGGGCGTGTTT
<i>fciB</i> -seq1	CCTTAATTCAGACGCAAAATCACTGAGAGAG
<i>fciB</i> -seq2	AGGTGTCTCAATTGCTGAAATCAAGGCTTC
<i>fciB</i> -seq3	CCTGACCTGCATCATCATCTCCATGATT
<i>fciB</i> -seq4	CTACTTAACTCGCTTATCGATTATCTCAATCAAATGAG
<i>mpeY</i> -test-for	GATGCAGAGCTTGTTCCACCTCAG
<i>mpeY</i> -test-rev	GAACCGGAATGTGAGGACACACAA
Int-test-rev	ACTCCTGCATTAGGAAGCAGCCCAGT
9916 Nus MpeY;F BamHI	GCAGGGATCCGGCGAACGATTTCGATATTT
9916 Nus MpeY-HT;R EcoRI	GCCGGAATTCGCTGACAAGCCTTTAAGG

**Table S3.** List of retention times, m/z ratios, and visible absorption maxima that indicate the bilins attached to cysteines 75, 83, and 140 in MpeA under various light conditions and genotypes. The letters in the graph column refer to the data presented in Figure S4.

Graph	Light Color	Genotype	Peptide Sequence	Retention Time (min)	Ion A m/z	Ion B m/z	Ion C m/z	VIS Peak, nm	Chromophore
A	Green	WT	KC* <sub>75</sub> AT EGK	17.66 <sup>a</sup>	441.545 <sup>3+</sup>	661.821 <sup>2+</sup>	1322.637 <sup>1+</sup>	490	PUB
B	Green	<i>mpeY</i> -	KC* <sub>75</sub> AT EGK	36.63 <sup>b</sup>	441.539 <sup>3+</sup>	661.813 <sup>2+</sup>	n/a	490	PUB
C	Green	WT	C* <sub>83</sub> KR	19.84 <sup>a</sup>	496.750 <sup>2+</sup>	992.509 <sup>1+</sup>	n/a	550	PEB
D	Green	<i>mpeY</i> -	C* <sub>83</sub> KR	29.54 <sup>b</sup>	331.498 <sup>3+</sup>	496.748 <sup>2+</sup>	n/a	490	PUB
E	Green	WT	NDGC* <sub>14</sub> <sub>0</sub> SPR	25.24 <sup>a</sup>	667.786 <sup>2+</sup>	445.526 <sup>3+</sup>	1334.583 <sup>1+</sup>	550	PEB
F	Green	<i>mpeY</i> -	NDGC* <sub>14</sub> <sub>0</sub> SPR	49.22 <sup>b</sup>	445.519 <sup>2+</sup>	667.778 <sup>2+</sup>	n/a	550	PEB
G	Blue	WT	KC* <sub>75</sub> AT EGK	17.71 <sup>a</sup>	441.546 <sup>3+</sup>	661.818 <sup>2+</sup>	1322.634 <sup>1+</sup>	490	PUB
H	Blue	<i>mpeY</i> -	KC* <sub>75</sub> AT EGKEK	25.99 <sup>c</sup>	395.688 <sup>4+</sup>	527.252 <sup>3+</sup>	790.384 <sup>2+</sup>	490	PUB
I	Blue	WT	C* <sub>83</sub> KR	14.34 <sup>a</sup>	496.749 <sup>2+</sup>	992.499 <sup>1+</sup>	n/a	490	PUB
J	Blue	<i>mpeY</i> -	C* <sub>83</sub> KR	21.71 <sup>c</sup>	331.493 <sup>3+</sup>	496.744 <sup>2+</sup>	992.506 <sup>1+</sup>	490	PUB
K	Blue	WT	NDGC* <sub>14</sub> <sub>0</sub> SPR	18.49 <sup>a</sup>	667.786 <sup>2+</sup>	445.522 <sup>3+</sup>	1334.603 <sup>1+</sup>	490	PUB
L	Blue	<i>mpeY</i> --	NDGC* <sub>14</sub> <sub>0</sub> SPR	31.06 <sup>c</sup>	445.516 <sup>3+</sup>	667.781 <sup>2+</sup>	1334.588 <sup>1+</sup>	490	PUB

Notes:

- a. 60 minute LC gradient
- b. 120 minute LC gradient
- c. 105 minute LC gradient

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